Physiological And Proteomic Analyses Reveals That Brassinosteroids Application Improves The Chilling Stress Tolerance of Pepper Seedlings

Jie Li (gsau23@126.com)
Honghe University https://orcid.org/0000-0002-7395-8207
Hamza Sohail
Huazhong Agricultural University: Huazhong Agriculture University
Muhammad Azher Nawaz
University of Sargodha
Chaowei Liu
China West Normal University
Ping Yang
Honghe University

Research Article

Keywords: pepper, brassinosteroids, chilling stress, ultrastructure, proteome

Posted Date: July 13th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-524724/v1

Version of Record: A version of this preprint was published at Plant Growth Regulation on December 1st, 2021. See the published version at https://doi.org/10.1007/s10725-021-00778-6.

Abstract

Brassinosteroids (BRs) are important in plant resistance to chilling stress. However, limited information is available regarding the specific mechanisms involved at proteomic level. We utilized iTRAQ proteomic approach, physiological assays and information obtained from cellular ultrastructure to clarify the underlying molecular mechanism of BRs to alleviate chilling stress in pepper (*Capsicum annuum* L.). Foliar application of 24-epibrassinolide (EBR) improved photosynthesis and improved cell structure by presenting a distinct mesophyll cell and chloroplast with well-developed thylakoid membranes in the leaves of pepper seedlings. We identified 346 differentially expressed proteins (DEPs), including 217 up-regulated proteins and 129 down-regulated proteins in plants under chilling (Chill) and Chill + EBR treated plants. Most of the DEPs were related to multiple pathways, including photosynthesis, carbohydrates metabolism, energy metabolism, protein biosynthesis, amino acids synthesis, redox and stress defence (ascorbate peroxidase, glutathione peroxidase, and superoxide dismutase). Up-regulated DEPs were associated with photosynthetic electron transfer chain, oxidative phosphorylation, GSH metabolism pathway, Calvin cycle and signaling pathway. The physiochemical analysis showed that EBR treatment improved the tolerance of pepper seedlings to chilling stress.

Introduction

Chilling is an abiotic stress which impairs plant development, fruit yield, and quality (Zhang et al. 2021). The chilling stress often affects open-field crops in the Northern hemisphere, and the crops are grown in a solar-greenhouse system during winter to early spring season (Wani et al. 2016). Chilling stress directly damages the photosynthetic apparatus or indirectly disrupts the process of photosynthesis. For instance, carbon-oxygen cycle, photosynthetic pigment synthesis, and CO_2 supply are affected by the chilling stress via stomatal limitations (Allen and Ort 2001). Plants subjected to chilling stress undergo dramatic alternations at the molecular level, such as gene transcription and proteins synthesis and metabolism (Ma et al. 2013; Dou et al. 2021).

Brassinosteroids (BRs) are phytohormones that shares similar structure found in animal and insect steroids (Divi and Krishna 2009). BRs have high bioactivity that influences a large number of physiological and developmental processes in plants, for instance, pollen tube growth, cell elongation, induction of polarization of cell membrane, cell death, and nucleic acid and protein synthesis (Fang et al. 2019; Ahammed et al. 2020). Therefore, BRs play a vital role in reducing the impact of biotic and abiotic stresses (Arora et al. 2008; Ogweno et al. 2008; Kanwar et al. 2012; Rajewska et al. 2016; Xia et al. 2018), and helps improve the yield of crops under stress conditions (Wu et al. 2008). Some previous studies related to BRs-induced chilling stress amelioration highlights the role of BRs at the physiological, biochemical and genetic levels (Liu et al. 2011; Gao et al. 2015). Moreover, in our previous study, we also reported that BRs application could alleviate the damage caused by excessive light and decreased ROS accumulation in pepper seedlings under low-temperature stress (Li et al. 2015). Although these findings enhance our understanding of BRs responses and chilling-induced stress in plants, some questions remain unanswered, such as plants proteomic responses to chilling stress.

Proteomics analysis is an overview of a complete set of a plant's protein profiles under stress condition, providing reliable information to understand the signaling and metabolic processes related to various unfavourable environmental conditions (Hossain et al. 2012). The iTRAQ-based proteomic analysis provides technical advantages for profiling protein expression changes compared with traditional 2D gel electrophoresis analysis (Owiti et al. 2011). iTRAQ combined with MS/MS provides an accurate quantitation of DEPs, particularly for low abundant proteins (Yang et al. 2011).

In our previous studies, we observed the role of BRs in inducing tolerance against chilling stress at the physiological and transcription level in pepper (Li et al. 2016a) and cucumber (Yang et al. 2019). The transcriptome analyses revealed a specific gene expression profile of low-temperature resistance induced by BRs. However, mRNA expression changes are not always well correlated with protein changes because there are many other factors involved in it regulation (Pradet-Balade et al. 2001; Yan et al. 2006). We hypothesize that proteomic analysis can provide insights into BRs-induced chilling stress responses at the protein level. Therefore, we utilized iTRAQ-based quantitative proteomic analysis, combined with physiological assays and cellular ultrastructure observations, to explore an underlying molecular mechanism of BRs induced chilling stress tolerance in pepper seedlings. This study provides valuable information regarding the mechanism of BRs to improve chilling stress tolerance of pepper seedlings, and this can be useful for the plant biologist interested to study the mechanisms of BRs to improve stress tolerance under other kinds of abiotic stresses.

Materials And Methods

2.1. Plant material and growing conditions

The pepper seeds of cultivar Xiangyan-16 were placed in an incubator at 28^{o}C for 72 h under dark conditions for germination. The specific conditions for planting and growing are described by Li et al. (2016). After germination and early establishment, seedlings were fertilized twice a week with ½-strength Hoagland solution.

According to our previous work, 0.1μM EBR was an optimum concentration (Jie et al. 2015). Fifty days after sowing, uniform pepper seedlings were shifted to a controlled growth chamber. The seedlings were subjected to three treatments: (a) Control: sprayed with double distilled water pretreatment under normal temperature; (b) Chill: sprayed with double distilled water as pretreatment and then exposed to low temperature; (c) Chill + EBR: sprayed with EBR at 0.l μM as pretreatment and then exposed to low temperature. The seedlings were pretreated with ddH_2O or 0.l μM EBR for 24 h and then placed at 25/15^{o}C (day/night) with 300 μmol m^{-2} s^{-1} light intensity, or at low-temperature treatment (15/5^{o}C), and light intensity of 100 μmol m^{-2} s^{-1}. The other growth conditions were as follows: 12-h light period and 80% relative humidity. Leaves were harvested after seven days of low-temperature treatment and stored at -80^{o}C until further use.

2.2. Morphology and biomass determination

On the 7th day after low-temperature treatment, plant height and main-root length were measured. The fresh plant weight was measured using an electronic balance. To measure the dry biomass, samples were placed in an oven at 105^{o}C for 15 minutes and later, the temperature was reduced to 85^{o}C until the samples attained a constant weight.

2.3. Gas exchange parameters determination

Photosynthetic parameters stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO_2 concentration (Ci) were recorded by CIRAS-2 System (PP Systems, USA). The chlorophyll fluorescence in pepper leaf was measured by fluorimeter (IMAG-PAM, Heinz Waltz, Germany).

2.4. Anatomical studies

After exposure to chilling stress for seven days, leaf specimens were fixed in FAA solution for 48 h according to the method described by (Willey 1971). The ultrastructure of the mesophyll cell was detected by a transmission electron microscope (JEOL TEM-100CX, Japan) at an accelerating voltage of 75 kV as described by Zeng et al. (2016) and Nawaz et al. (2018).

2.5. Proteomics extraction and iTRAQ labelling

Pepper leaf proteins from three biological replicates in Chill and Chill + EBR samples were extracted with the Lysis buffer method (Wang et al. 2016). The homogenate was centrifuged at 30,000 X g for 15 min at 4^{o}C. Proteins were measure using BSA as a standard as described by (Bradford 1976).

The lyophilized protein powder (100 µg) from each sample was digested with Trypsin Gold (Promega, Madison, WI, USA) with an enzyme to substrate ratio of 1:30 sequencing-grade trypsin at 37^{o}C for 16 hours. The digested peptides were labeled using iTRAQ reagent according to the manufacturer's instructions (Applied Biosystems, Inc., Foster city, CA). The samples were labeled with the respective tags as follows: three biological replicates of alone chilling treated samples (Chill) were labeled with the reporter tags 118 and 121, respectively; and three biological replicates of chilling with EBR treated samples (Chill + EBR) were labeled with the reporter tags 114 and 116, respectively, and then were multiplexed and vacuum-dried.

2.6. Peptide Seperation and LC-MS/MS

Strong cation exchange (SCX) fractionation of the combined peptide mixture was re-suspended in 4mL buffer A (25 mmol NaH_2PO_4 in 25% ACN, pH2.7). In the column for gradient elution at a rate of 1 mL min^{-1}: firstly, the elution with 5% buffer B (25 mmol NaH_2PO_4 and 1 mol KCl in 25% ACN, pH2.7) for 7 min, followed by a linear gradient in buffer B from 5–60% for 20 min, finally within 2 min to keep buffer B ratio raised up to 100% for 1 min, and then back to the 5% as balance for 10 min. The components were re-dissolved in the Buffer A [5% acetonitrile (CAN), 0.1% formic acid (FA)] to about 0.5 µg µl^{-1}, and centrifuged at 20,000 X g for 10 min to remove the insoluble substance. Then, we used the LC-20AB HPLC Pump system (ATM SCX column, 250 X 4.6 mm) with the Trap columns (C18-CL-3 um 120A, 350 µm X 5mm, Eksigent) and analytical columns (C18-3µm 120A, 75umX 18 cm, Welch) to separate. For

separation: firstly, the sample was loaded to the Trap column at a flow rate of 8 μl min^{-1} within 4 min, followed by a total flow rate of 300 nl min^{-1}, the samples was taken into the analytical column and separated and transmitted to the mass spectrometry system. Firstly, with 5% Buffer B [95% acetonitrile (CAN), 0.1% formic acid (FA)] to elute for 5 min, followed by a linear gradient in buffer B from 35–35% for 35 min, then in up to 60% within 5 minutes, and then buffer B was increased up to 80% and kept for 2 min, finally back to 5% within 1 min as balance for 10 min.

The mass spectrometry system was performed with a TripleTOF 5600 mass spectrometer (AB SCIEX, Concord, ON, Canada) coupled with online micro flow HPLC system (Shimadzu, Japan), and the ion source was Nanospray IIIsource. When the data was collected, the parameters of TripleTOF 5600 mass spectrometer were set as follows: ion spray voltage 2500 V, nitrogen pressure was 30 psi, spray pressure 15 psi, and the scanning mode was reflected mode with a distinguishability of greater than or equal to 30,000 FWHM for TOF MS scans. For information dependent acquisition (IDA), accumulate 250 ms in the primary mass spectrum and only scan the ions with a charge state of 2^+ to 5^+ was exceeded, and selected the top 30 production scans with over 120 CPS intensity, and 3.3 s for a cycle. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

2.7. Database search and quantification

The data was processed by Proteome Discoverer 1.2 (PD 1.2, Thermo Fisher Scientific) and searched with in-house Mascot software 2.3.02 (Matrix Science, U.K.) against the pepper database (35101 sequences). In the process of protein identification, the parameters were selected as follows: fragment mass tolerance values: 0.1 Da; peptide the mass tolerance values: 0.05 Da; the enzyme: trypsin with a maximum of one missed cleavage; variable modifications: Gln->pyro-Glu (N-termQ), Oxidation (M), iTRAQ8plex (Y); and the fixed modifications were Carbamidomethyl (C), iTRAQ8plex (N-term), iTRAQ8plex (K) Carbamidomethyl (C), iTRAQ (N-term) and iTRAQ (K). The charge states of the peptide were set to 2^+ and 3^+. Specifically, it was an automatic decoding database that selected a random database in Mascot by selecting the decoy checkbox, and tested the raw spectra and the real database. By a Mascot probability analysis, only peptides at the 95% confidence interval were counted as being successfully identified so that reducing the probability of false peptide identification. Each of the positive protein identification contained at least one unique peptide. For protein quantitation it was required that a protein contains at least two unique spectra. The ratios of protein to protein were weighted and normalized by median proportions in Mascot. We used only the ratio of $P < 0.05$, and only a fold changes greater than 1.2 or less than 0.833 was considered as significant. Strategy for aggregation of peptide reporter ion ratios to generate a protein ratio: first, the ionic strength of the peptide was normalized; the selected peptide was a unique peptide to calculate the peptide ratio; and the protein ratio was represented by the median (median, removal extremum) of the peptide ratio. Handling of shared peptides: the reliability of the protein depends on whether it contains a unique peptide, and the reliably protein contains at least one unique peptide. The proteins given in the data are all reliably proteins. Normalization: the median value of peptide abundance was normalized. Statistical testing: When the two samples were compared, the ratio of protein abundance is more than 1.2 times that of the difference. When the P value was less than 0.05, the protein

was considered as the difference protein between different samples. And the Mascot was not clouded treatment of missing values.

The proteins were annotated according to PepperGDB database release 2.0 (http://peppersequence.genomics.cn/page/species/download.jsp). Functional annotations of the proteins were conducted using Blast2GO program (Bioinformatics Department, CIPF, Valencia, Spain) against the non-redundant protein database (NR; NCBI. The KEGG database (http://www.genome.jp/kegg/) was used to classify and group these identified proteins. Gene Ontology (GO) is an international standardization of gene function classification system. We used the clusters of orthologous groups (COG) database to classify and group the identified proteins.

2.8. Real-time quantitative PCR analysis

Sixteen transcripts genes involved in DEPs were selected for qRT-PCR assay, and the genes and gene-specific primers were prepared (Table S7). qRT-PCR was performed using the method described in our previous report Li et al. (2016).

2.9. Statistical analysis

Data was analyzed using SPSS software (IBM SPSS 22.0, IBM Corporation, New York, USA). Means were compared using Duncan's multiple range test at $P < 0.05$. Origin 8.0 (Origin Lab Corporation) was used to prepare charts.

Result

3.1. Morphological parameters and anatomical changes

Exposure of pepper seedlings to chilling stress influences morphological, cell microstructural and ultrastructural traits (Figs. 1–3). Exogenous EBR application alleviated the inhibited growth of pepper seedlings under chilling stress conditions (Fig. 1). The phenotype changes were confirmed by quantitative analysis that revealed a marked increase in plant height, main-root length, fresh weight, and dry weight for EBR-treated pepper seedlings compared with non-EBR treated seedlings exposed to chilling stress (Fig. 1). Leaf structure showed that cells became plump and EBR foliar application increased the thickness of both midrib and lamina in the leaf compared with non-EBR treated leaves. In EBR treated leaves, the center of the main vascular bundle was well developed in midrib region. Beneath the two layers of epidermal cells, palisade parenchyma tissue is composed of elongated cells. The EBR treatment under chilling stress conditions improved the epidermis, fibre tissue, phloem tissue, xylem tissue and parenchymatous area of the hollow pith (Fig. 2). Hence, it could be the reason for the shorter seedlings and roots observed under chilling stress conditions. Chilling stress caused remarkable changes in ultrastructure of mesophyll cell. Specifically, chilling caused chloroplast swelling, loss of polarity, chloroplast degradation, blurred starch granules, and thylakoid substrate layer were disappeared. However, the application of EBR under chilling stress alleviated the damage to chloroplast and thylakoid and improved the cell membrane's stability under chilling stress conditions (Fig. 3).

3.2. Photosynthetic parameters

The stomatal conductance (*G*s) and transpiration rate (*T*r) was decreased after seven days of chilling treatment (Table 1). EBR application reduced the inhibitory effect of chilling stress on photosynthesis and improved *G*s and *T*r in contrast with non-EBR treated plants.

Table 1
Effect of EBR on net photosynthetic rate (P_n), stomatal conductance (*G*s), intercellular CO_2 concentration (*C*i) and transpiration rate (*T*r) of pepper under chilling stress conditions.

Treatment	P_N [μmol(CO_2) m^{-2} s^{-1}]	*C*i (μmol mol^{-1})	*G*s [mmol(H_2O) m^2 s^{-1}]	Tr (mmol m^{-2} s^{-1})
Control	4.7 ± 0.10a	212.3 ± 9.5b	54.3 ± 4.6a	1.33 ± 0.15a
Chill	2.1 ± 0.13c	273.0 ± 28.7a	21.7 ± 3.1c	0.70 ± 0.07c
Chill + EBR	4.2 ± 0.22b	255.7 ± 26.3ab	33.3 ± 5.9b	1.02 ± 0.08b

The means followed by similar letter do not differ significantly according to Duncan's multiple range test ($P \leq 0.05$).

3.3. Protein identification and quantification using iTRAQ

To determine the proteomic changes of pepper seedlings caused by EBR application under low-temperature stress, the leaf proteomes of chilling and chilling + 0.1 μM EBR-treatment for seven days were generated using the iTRAQ method. 17,913 peptides were identified from trypsin-digested proteins in chill-stressed pepper seedlings. Using the Mascot software, 51,136 spectra discriminated to known spectra, 44,081 spectra were uniquely mapped, and 17,913 peptides, 16,350 unique peptides, and 4,661 proteins were identified. All proteins identified by MS/MS, and their peptides of proteins are provided in Table S1. Over 72% of proteins included at least two peptides. Other proteins were identified for a single high-confidence peptide assignment with a 95% confidence level ($P < 0.05$) and a 1.2-foldchange as up or 0.833-fold as down-regulation. In the leaves of chill-stressed pepper seedlings treated with EBR, 346 differentially expressed proteins (DEPs) were identified, including 217 up-regulated DEPs (Table S2) and 129 down-regulated DEPs (Table S3), compared with non-EBR treated plants exposed to low temperature.

3.4. GO and COG analyses of DEPs

The functions of the DEPs in leaves were annotated by their GO terms, including the biological process, cellular component categories and molecular functions (Fig. 4 and Table S4, S5); and the clusters of COG function are summarized in Figs. 5 and Table S6. The 346 DEPs were categorized into 22 biological processes, 15 cellular component and 7 molecular functions (Fig. 4). In the biological process, mostly the DEPs were related to metabolic processes (20.83%), cellular processes (18.28%), and response to a stimulus (9.85%). In the cellular component, most DEPs were involved in the cell (22.55%), cell part (22.55%), and organelle (18.55%). In molecular functions, most of the DEPs were involved in catalytic

activity (47.73%) and binding (36.62%). To further examine the different proteins in Chill + EBR treatment compared with Chill treatment, the DEPs were classified into 22 clusters of COG function. The most functional categories were carbohydrate transport (23), post-translational modification (26), energy production and conversion (20), translation ribosomal structure and biogenesis (29), and general function prediction (41).

3.5. Functional classification of differentially expressed proteins regulated by EBR under chilling stress conditions

The functions of the identified DEPs between Chill and Chill + EBR treatment were classified into 8 categories based on functional and metabolic features, including energy production and conversion (36), amino acid transport and metabolism (12), carbohydrate transport and metabolism (19), translation, ribosomal structure and biogenesis (30), cell wall/membrane/envelope biogenesis (2), post-translational modification, protein turnover, chaperones (15), signal transduction mechanisms (7), and stress defence (21; Table S6). Among them, there were 18 up-regulated and 18 down-regulated DEPs in energy production and conversion category. All the DEPs in the amino acid transport and metabolism category were up-regulated proteins. In carbohydrate transport and metabolism category, 12 DEPs in the EBR-treated group were up regulated, and 7 DEPs were down-regulated compared with Chill treatment. As for as translation is concerned, ribosomal structure and biogenesis category, there were 11 up-regulated and 19 down-regulated DEPs in EBR + Chill treatment compared with the Chill treatment. All the DEPs in the cell wall/membrane/envelope biogenesis category were up-regulated proteins. In post-translational category, 12 DEPs in the EBR-treated group were up regulated, and only 3 DEPs were down-regulated compared with Chill treatment. There were 2 up-regulated and 5 down-regulated DEPs under EBR + Chill treatment in the signal transduction mechanisms category compared with Chill treatment. As for stress defence category, 14 DEPs in the EBR-treated group were up regulated, and 7 DEPs were down-regulated compared with the Chill treatment (Table 2, Fig. 6).

Table 2
Functional classifications of identified proteins significantly expressed in the EBR-treated leaves of pepper compared with non-EBR treated leaves exposed to chilling stress, determined by Mascot probability analysis.

Accession	Protein name	Species	Cov	[a] Fold Change
Energy production and conversion				
Capana08g001794	Cytochrome c	Solanum tuberosum	20.5	1.44**
Capana01g000364	PGR5-like protein 1A	Arabidopsis thaliana	19.8	1.15*
Capana01g002586	Thylakoid lumenal 19 kDa protein	Arabidopsis thaliana	9.8	1.26**
Capana10g000690	Thylakoid membrane phosphoprotein 14 kDa	Arabidopsis thaliana	11.2	1.24*
Capana06g000226	Adrenodoxin-like protein	Bos taurus	15.5	1.53*
Capana12g000375	UDP-glycosyltransferase 74E2	Arabidopsis thaliana	17.5	1.11*
Capana01g003224	Citrate synthase	Solanum tuberosum	17.7	1.20**
Capana07g001847	Phosphoenolpyruvate carboxylase 1	Arabidopsis thaliana	56.0	1.23**
Capana07g001846	Phosphoenolpyruvate carboxylase 2	Flaveria trinervia	14.2	1.29*
Capana10g001751	NADP-dependent malic enzyme	Phaseolus vulgaris	38.2	1.30*
Capana07g000354	Reticuline oxidase-like protein	Arabidopsis thaliana	12.0	1.17*
Capana11g000426	Aldehyde oxidase 4	Arabidopsis thaliana	9.8	1.14*
Capana02g000901	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3	Solanum tuberosum	20.7	1.14*
Capana03g001736	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6	Arabidopsis thaliana	14.2	1.27*
Capana03g003147	Calcium sensing receptor	Arabidopsis thaliana	11.3	1.18*
Capana08g000763	Non-specific lipid-transfer protein	Helianthus annuus	9.2	1.22**
Capana06g001609	Non-specific lipid-transfer protein 2	Nicotiana tabacum	46.5	1.29**
Capana08g002006	Non-specific lipid-transfer protein-like protein At2g13820	Arabidopsis thaliana	6.0	1.14*
Capana12g002920	2-oxoisovalerate dehydrogenase subunit alpha	Dictyostelium discoideum	6.9	0.86*

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana05g001264	Pyruvate dehydrogenase E1 component subunit alpha	Solanum tuberosum	18.1	0.91*
Capana08g001599	Dihydrolipoyl dehydrogenase	Synechocystis	22.1	0.82**
Capana03g003212	Chlorophyll a-b binding protein 13	Solanum lycopersicum	41.1	0.73*
Capana00g002793	Chlorophyll a-b binding protein 1A	Solanum lycopersicum	57.0	0.69**
Capana00g002801	Chlorophyll a-b binding protein 1C	Solanum lycopersicum	57.0	0.74*
Capana00g002802	Chlorophyll a-b binding protein 1D (Fragment)	Solanum lycopersicum	58.5	0.69*
Capana07g001245	Chlorophyll a-b binding protein 5	Solanum lycopersicum	38.5	0.45**
Capana09g000473	Chlorophyll a-b binding protein 5	Solanum lycopersicum	35.5	0.49**
Capana05g002549	Chlorophyll a-b binding protein 6A	Solanum lycopersicum	26.5	0.85*
Capana08g000250	Chlorophyll a-b binding protein 7	Solanum lycopersicum	27.4	0.75*
Capana09g001520	Chlorophyll a-b binding protein CP29.1	Arabidopsis thaliana	29.8	0.74*
Capana03g000797	Chlorophyll a-b binding protein P4	Pisum sativum	27.6	0.77**
Capana01g000647	Chlorophyll a-b binding protein P4	Pisum sativum	23.1	0.64*
Capana07g000048	Photosystem I P700 chlorophyll a apoprotein A1	Nicotiana tomentosiformis	6.7	0.65*
Capana00g001698	Photosystem II CP43 chlorophyll apoprotein	Solanum tuberosum	48.3	0.84**
Capana01g001866	Photosystem II D2 protein		12.4	0.77**
Capana00g001211	Photosystem Q(B) protein	Crucihimalaya wallichii	6.4	0.73*
Carbohydrate transport and metabolism				

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana03g002552	Acid beta-fructofuranosidase AIV-18	Capsicum annuum	18.5	1.27**
Capana10g002008	Beta-fructofuranosidase, insoluble isoenzyme 1	Daucus carota	5.7	1.17*
Capana03g004318	Beta-galactosidase 9	Arabidopsis thaliana	12.5	1.14*
Capana06g000368	Beta-glucosidase B	Emericella nidulans	15.9	1.26**
Capana01g002323	Glucan endo-1,3-beta-glucosidase	Nicotiana tabacum	38.7	1.46**
Capana07g002216	Probable inactive beta-glucosidase 14	Oryza sativa	5.1	1.29*
Capana06g000586	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	Bacillus cereus	23.7	1.16**
Capana03g000942	Strictosidine synthase 1	Arabidopsis thaliana	34.0	1.23**
Capana04g000041	Bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase	Malus domestica	30.6	1.20*
Capana05g000670	Pyruvate kinase, cytosolic isozyme	Solanum tuberosum	20.4	1.26**
Capana10g001802	42 kDa endochitinase	Trichoderma harzianum	10.3	1.40*
Capana03g001381	Glyceraldehyde-3-phosphate dehydrogenase (Fragment)	Cavia porcellus	41.5	1.11*
Capana08g002333	Anthocyanin 3'-O-beta-glucosyltransferase	Gentiana triflora	8.9	0.88*
Capana07g000871	Putative glucose-6-phosphate 1-epimerase	Cenchrus ciliaris	25.0	0.87*
Capana11g000082	Probable aquaporin PIP2-8	Arabidopsis thaliana	11.4	0.87*
Capana01g000250	Probable aquaporin PIP-type pTOM75	Solanum lycopersicum	8.8	0.87*
Capana06g000557	Probable aquaporin TIP1-1	Oryza sativa	4.4	0.82*
Capana02g003365	Granule-bound starch synthase 2	Solanum tuberosum	15.4	0.79**
Capana03g004113	Brassinosteroid-regulated protein BRU1	Glycine max	13.5	0.70*
Amino acid transport and metabolism				

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana02g002837	2-hydroxyacyl-CoA lyase	Arabidopsis thaliana	26.8	1.20**
Capana08g002049	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	Solanum lycopersicum	18.0	1.19*
Capana10g001347	Pyruvate decarboxylase isozyme 1 (Fragment)	Nicotiana tabacum	4.0	1.40*
Capana06g001561	Glycine cleavage system H protein	Flaveria anomala	39.5	1.27**
Capana00g000453	Glycine cleavage system H protein	Flaveria pubescens	35.8	1.18*
Capana02g002229	Aspartic proteinase	Cucurbita pepo	22.8	1.19*
Capana00g003499	Phenylalanine ammonia-lyase	Nicotiana tabacum	21.2	1.18*
Capana08g002409	Putative serine carboxypeptidase-like 53	Arabidopsis thaliana	19.2	1.22**
Capana07g000176	Serine carboxypeptidase 24	Arabidopsis thaliana	9.6	1.21**
Capana05g000144	Serine carboxypeptidase-like 27	Arabidopsis thaliana	18.4	1.13*
Capana09g002146	Serine carboxypeptidase-like 50	Arabidopsis thaliana	4.0	1.13*
Capana11g000199	Serine carboxypeptidase-like 51	Arabidopsis thaliana	10.7	1.20*
Translation, ribosomal structure and biogenesis				
Capana02g001443	40S ribosomal protein S4	Solanum tuberosum	30.1	1.25*
Capana03g001371	60S ribosomal protein L17-2	Arabidopsis thaliana	16.8	1.12*
Capana07g000070	Ribonuclease S-2	Nicotiana alata	26.6	1.31**
Capana07g000069	Ribonuclease S-2	Solanum tuberosum	25.9	1.42*
Capana08g002514	Tyrosine–tRNA ligase	Acanthamoeba polyphaga mimivirus	18.4	1.21*
Capana10g000821	DEAD-box ATP-dependent RNA helicase 12	Oryza sativa	7.8	1.22*
Capana10g001534	DEAD-box ATP-dependent RNA helicase 38	Oryza sativa	23.1	1.15*
Capana06g002575	Elongation factor 1-alpha	Solanum lycopersicum	29.2	1.11*

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana11g000046	Elongation factor 1-alpha	Solanum lycopersicum	29.2	1.35**
Capana06g000271	Eukaryotic translation initiation factor 2 subunit beta	Triticum aestivum	10.6	1.11*
Capana03g000761	Outer envelope protein 64	Arabidopsis thaliana	12.6	1.27*
Capana06g001824	40S ribosomal protein S15a-1	Arabidopsis thaliana	37.7	0.86*
Capana00g004669	40S ribosomal protein S7	Avicennia marina	18.4	0.87*
Capana11g001672	40S ribosomal protein S9 (Fragment)	Nicotiana tabacum	37.6	0.84*
Capana09g000692	50S ribosomal protein L13	Spinacia oleracea	15.1	0.75*
Capana00g005041	50S ribosomal protein L14	Nicotiana sylvestris	45.9	0.84**
Capana06g000258	50S ribosomal protein L17	Nicotiana tabacum	30.2	0.82*
Capana00g001418	50S ribosomal protein L2	Solanum tuberosum	23.4	0.70*
Capana06g000813	50S ribosomal protein L29	Arabidopsis thaliana	17.7	0.80*
Capana08g000356	50S ribosomal protein L3	Nicotiana tabacum	22.0	0.76**
Capana07g002013	50S ribosomal protein L4	Nicotiana tabacum	20.0	0.77**
Capana03g001207	60S ribosomal protein L27a-3	Arabidopsis thaliana	14.9	0.88*
Capana02g003372	60S ribosomal protein L34	Nicotiana tabacum	16.7	0.85*
Capana03g002586	60S ribosomal protein L35a-2	Arabidopsis thaliana	24.1	0.87*
Capana02g001384	60S acidic ribosomal protein P0	Chenopodium rubrum	24.6	0.78*
Capana06g000591	60S acidic ribosomal protein P2	Parthenium argentatum	54.5	0.90*
Capana03g002890	DEAD-box ATP-dependent RNA helicase 52B	Oryza sativa	18.4	0.86*
Capana01g004415	Exosome complex exonuclease RRP42	Mus musculus	12.5	0.86*
Capana08g001184	Proline–tRNA ligase	Thermus thermophilus	19.3	0.83*

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana03g002188	Putative rRNA 2'-O-methyltransferase fibrillarin 3	Arabidopsis thaliana	12.0	0.86*
Cell wall/membrane/envelope biogenesis				
Capana00g003378	UDP-glucose 4-epimerase GEPI48	Cyamopsis tetragonoloba	23.1	1.51**
Capana08g000827	Tyramine N-feruloyltransferase 4/11	Nicotiana tabacum	4.8	1.26**
Capana00g002001	Outer membrane lipoprotein blc	Citrobacter freundii	25.4	1.23**
Capana03g000177	Protein ROOT HAIR DEFECTIVE 3	Arabidopsis thaliana	13.6	1.19*
Posttranslational modification, protein turnover, chaperones				
Capana04g000276	Cysteine proteinase (Fragment)	Carica papaya	25.6	1.34*
Capana03g003545	Protease Do-like 2	Arabidopsis thaliana	5.6	1.32*
Capana02g000903	Protease Do-like 8	Arabidopsis thaliana	9.4	1.33*
Capana10g002505	Proteasome subunit alpha type-3	Spinacia oleracea	25.6	1.23*
Capana12g000252	UBX domain-containing protein 1	Bos taurus	4.7	1.31**
Capana03g003580	Uncharacterized protein yyaO	Bacillus subtilis (strain 168)	10.2	1.35*
Capana03g001261	Protein-L-isoaspartate O-methyltransferase	Arabidopsis thaliana	13.0	1.12*
Capana01g002650	Subtilisin-like protease	Arabidopsis thaliana	12.6	1.13*
Capana03g002474	Subtilisin-like protease	Arabidopsis thaliana	6.6	1.21*
Capana07g000993	Subtilisin-like protease	Arabidopsis thaliana	4.1	1.21*
Capana05g002292	26S proteasome non-ATPase regulatory subunit 1	Gallus gallus	10.7	1.38**
Capana03g001145	Glutaredoxin	Ricinus communis	43.2	1.24*
Capana03g003773	Proteasome subunit beta type-2-A	Arabidopsis thaliana	43.6	0.86*
Capana05g001834	Peptide methionine sulfoxide reductase B3	Oryza sativa	23.9	0.90*

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana05g001818	ATP-dependent zinc metalloprotease FTSH 4	Arabidopsis thaliana	20.5	0.82*
Signal transduction mechanisms				
Capana04g001485	Probable protein phosphatase 2C 11	Arabidopsis thaliana	10.1	1.14*
Capana03g001979	Probable protein phosphatase 2C 5	Oryza sativa	8.4	1.19*
Capana04g001050	14-3-3 protein 7	Solanum lycopersicum	33.2	0.83**
Capana12g002700	14-3-3-like protein D	Nicotiana tabacum	36.3	0.87*
Capana02g000552	14-3-3-like protein E	Nicotiana tabacum	26.2	0.85**
Capana03g001590	Calmodulin	Solanum lycopersicum	45.0	0.84*
Capana08g002036	Probable protein phosphatase 2C 10	Arabidopsis thaliana	20.7	0.87*
Stress defense				
Capana02g003105	Zeaxanthin epoxidase	Capsicum annuum	10.6	1.20*
Capana03g000109	Aldehyde dehydrogenase family 7 member B4	Arabidopsis thaliana	27.1	1.19*
Capana03g004052	Glutathione reductase	Spinacia oleracea	13.2	1.15*
Capana02g001146	Thioredoxin O2	Arabidopsis thaliana	8.9	1.22*
Capana01g001681	Cinnamoyl-CoA reductase 1	Arabidopsis thaliana	19.4	1.46**
Capana03g004565	Glutathione S-transferase U9	Arabidopsis thaliana	18.7	1.13*
Capana07g000817	Peroxiredoxin Q	Triticum aestivum	35.1	1.22**
Capana00g003105	Probable glutathione S-transferase	Nicotiana tabacum	28.1	1.17*
Capana07g002003	Probable glutathione S-transferase	Nicotiana tabacum	18.2	1.21**
Capana07g002010	Probable glutathione S-transferase	Nicotiana tabacum	19.1	1.18*
Capana04g002111	Probable L-ascorbate peroxidase 6	Oryza sativa	32.6	1.34**
Capana06g002374	Superoxide dismutase [Fe]	Nicotiana plumbaginifolia	47.1	1.18*

The asterisk “*” means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk “**” means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

Accession	Protein name	Species	Cov	[a] Fold Change
Capana02g003509	Probable monodehydroascorbate reductase	Arabidopsis thaliana	10.4	1.17*
Capana02g002017	Quinone oxidoreductase PIG3	Homo sapiens	31.4	1.15*
Capana02g002207	Probable 5'-adenylylsulfate reductase 1	Oryza sativa	27.8	0.76*
Capana10g000190	Heat shock protein 82 (Fragment)	Nicotiana tabacum	31.4	0.87*
Capana02g000952	Probable glutathione S-transferase	Nicotiana tabacum	24.6	0.83*
Capana05g001394	Ferritin-3	Glycine max	22.7	0.78*
Capana05g002051	Ferritin-4	Glycine max	18.4	0.81**
Capana01g000496	FAM10 family protein At4g22670	Solanum lycopersicum	32.1	0.86**
Capana02g001978	Haloalkane dehalogenase	Xanthobacter autotrophicus	16.8	0.90*

The asterisk "*" means the significant change for Chill_114 vs. Chill + EBR_121or Chill_118 vs. Chill + EBR_116, and the asterisk "**" means the significant change for Chill_114 vs. Chill + EBR_121 and Chill_118 vs. Chill + EBR_116 by Mascot a probability analysis based on iTRAQ.

3.6. Changes in RNA transcription

Correlation analysis between iTRAQ-seq result and expression of corresponding DEPs is presented in Figure S1. We selected ten up-regulated DEPs and six down-regulated DEPs for transcript levels RT-PCR analysis. The result showed that the protein expression identified by iTRAQ had a strong positive correlation with the RT-PCR (R^2 = 0.702), suggesting that the data obtained from iTRAQ-seq was reliable (Figure S1).

Discussion

4.1. Morphological response of pepper to EBR under chilling stress

Chilling stress inhibits plant growth and biomass accumulation (Bajguz 2009; Fan et al. 2014). BRs have high bioactivity that influences diverse physiological and developmental processes and helps plants to tolerate various stresses (Liu et al. 2009; Ahammed et al. 2020). According to some reports, BRs positively affect tolerance against low temperature for cucumber (Hu et al. 2010), grapevines (Xi et al. 2013) and Chinese cabbage (Fan et al. 2014). Application of BRs improved the resistance against chilling condition and enhanced the root length, plant height, total biomass and root biomass in maize (Singh et al. 2012). In our work, improved plant height, main-root length and biomass accumulation were observed

in EBR-treated pepper plant exposed to chilling conditions (Fig. 1) compared with non-EBR treated plants. Meanwhile, microstructural cell size became bigger in EBR + Chill treatment compared with Chill treatment (Fig. 2). Chilling stress caused remarkable changes in ultrastructure of leaf mesophyll cell. However, the application of EBR under chilling stress improved restoration of chloroplast morphology because matrix plate-like cells appeared to form a formal grana ultrastructure (Fig. 3). Morphology and ultrastructure observations suggest that EBR-treated cells trigger defense mechanisms under chilling stress conditions.

4.2. Energy and carbohydrate metabolism related proteins

Energy loss and disturbance in carbohydrate metabolism is the main factor triggered in response to chilling. In our experiment, predominant changes were observed associated with photosynthesis and carbohydrate metabolism (Table 2; Fig. 6). EBR application improved the synthesis of carbohydrates that promotes plant growth. Using iTRAQ quantification, we found that up-regulated (1.15-fold) PGR5-like protein 1A (Capana01g000364) related to cyclic electron flow in PS I.

Photosynthesis is the physical and chemical process of converting light energy into chemical energy utilized for the growth and development of plants (Yang et al. 2014). Our previous reports showed that the EBR application caused a significant increase in the P_n of pepper seedlings (Li et al. 2016a). Moreover, this phenomenon may result in the activation and/or induction of enzymes in chloroplasts by EBR. According to a report, EBR enhances the Calvin cycle's capacity by improving CO_2 assimilation and promotes Rubisco activation and genes expression related to photosynthetic (Yu et al. 2004; Xia et al. 2009a). The assimilation of CO_2 may be associated with stomatal or non-stomatal factors or both. Here, the application of EBR improved the *G*s and *T*r but had a non-significant decrease in *C*i under chilling stress conditions. Consequently, non-stomatal factors might be ascribed to the promotion of photosynthetic rate caused by EBR application under chilling stress conditions (Table 2). The possible reasons for the non-stomatal factor affecting photosynthesis include RuBP regeneration capacity, chlorophyll and carotenoids retrogradation and Rubp carboxylation (Pezeshki 1994).

Plants need to maintain a balance between the generation of energy and carbohydrate metabolism during chilling (Merewitz et al. 2011). During energy production and conversion translated in chlorophyll apoprotein and the reaction-center subunit of the photosystem, chlorophyll a/b binding proteins (Capana03g003212, Capana00g002793, Capana00g002801, Capana00g002802, Capana07g001245, Capana09g000473, Capana05g002549, Capana08g000250, Capana09g001520, Capana03g000797 and Capana01g000647) are used for harvesting of light and energy transfer. There were 18 up-regulated and 18 down-regulated DEPs in energy production and conversion category between the Chill and Chill + EBR treatment in this study. The down-regulated DEPs includes chlorophyll a-b binding protein, 2-oxoisovalerate dehydrogenase subunit alpha (Capana12g002920), pyruvate dehydrogenase E1 component subunit alpha (Capana05g001264), dihydrolipoyl dehydrogenase (Capana08g001599), photosystem I P700 chlorophyll an apoprotein A1 (Capana07g000048), photosystem II CP43 chlorophyll apoprotein (Capana00g001698), photosystem II D2 protein and photosystem Q(B) protein

(Capana00g001211); all these proteins are Calvin cycle-specific enzymes and photosynthesis-related (Table 2; Fig. 6). So according to our study, chilling stress inhibited photosynthesis in pepper. However, EBR could improve energy conversion and light harvesting capacity during cold stress. Thus, it could be a reason that seedlings pretreatment with EBR maintained a higher P_n than non-EBR treated seedlings when exposed to low temperature.

4.3. Proteins involved in signal transduction and stress defence

It is essential to understand the involvement of proteins responsible for signal transduction in EBR-mediated growth and chilling tolerance. It is considered that ABA-induced moisture deficiency, stomatal closure, and plant resistance during leaf senescence can be mediated by phosphatase 2C family protein phosphatase in *Arabidopsis* (Singh et al. 2015). In this study, protein phosphatase 2C 11 and 2C 5 associated with signal transduction were up regulated in EBR pretreated plants. Wang et al. (2008) found that 14-3-3 protein induce primary metabolism, enzyme activities, and ion transport through the effect of direct protein-protein interactions in cellular organization; for instance, H^+-ATPase, GTPase, and apoptosis induce signaling transduction, cell proliferation and anabolism that is responsible for resistance against abiotic stress in plant. According to another report, Chelysheva et al. (1999) found that in suspension-cultured sugar beet cells, 14-3-3 protein interacts with H+-ATPase to increase chilling stress tolerance through ATPase/14-3-3 expression, and improved ATPase activity. In this study, we observed that 14-3-3 protein 7 (Capana04g001050), 14-3-3-like protein D (Capana12g002700), 14-3-3-like protein E (Capana02g000552) were substantially down-regulated by the application of EBR, indicating their involvement mainly in EBR-induced growth and likely decreased interaction between plasma membrane H^+-ATPase and a dimer of 14-3-3 protein. The calmodulin-Ca^{2+} complex can stimulate some enzymes, ion channels and other proteins that are mostly protein kinases and phosphatases. Our data suggested that Capana03g001590 was down-regulated (0.84-fold) by EBR application under chilling stress conditions, indicating that calmodulin mediates a large number of enzymes stimulated by EBR.

Remarkable changes were observed for up-regulated (1.22-fold) proteins identified in stress defence in EBR-treated plants exposed to chilling stress compared with non-EBR treated plants. Up-regulated thioredoxin O2 (Capana02g001146) belongs to sulphur alcohols-two sulfide oxidation that may be involved in various redox reactions reduced by thioredoxin reductases NTRA and NTRB (Laloi et al. 2001). During stress condition in plants, the GSH conjugation is catalyzed to natural cytotoxic and xenobiotic compounds with a reactive electrophilic centre by glutathione S-transferase (Vijayakumar et al. 2016). In this study, three glutathione S-transferases (Capana00g003105, Capana07g002003, and Capana07g002010) were up-regulated (1.17, 1.21 and 1.18-fold, respectively) in EBR-treated seedlings exposed to chilling stress compared with non-EBR treated seedlings (Table 2). Ascorbate peroxidases are supposed to oxidize hydrogen peroxide (H_2O_2) to H_2O, responsible for the oxidation of specific substances such as ascorbic acid in abiotic stress tolerance, and these are encoded by isoenzymes that belonged to a large multigene family (Schulz et al. 2002). The protein superoxide dismutase catalyzes the destruction of the O_2 that is normally produced in the cells and is toxic to biological systems (Li et al.

2016b). The superoxide dismutase [Fe] (Capana06g002374) and probable monodehydroascorbate reductase (Capana02g003509) were up-regulated (1.18 and 1.17-fold) by EBR application under chilling stress conditions, and these proteins play a vital role in plant resistance various environmental constraints (Table 2). The protein Capana02g003509 was found to catalyze MDA conversion to ascorbate and oxidise NADH in the defence process (Table 2). Zeaxanthin epoxidase protein (Capana02g003105) is involved in abscisic acid biosynthesis, and this is the part of plant hormone biosynthesis (Bouvier et al. 1996). Zeaxanthin epoxidase protein (Capana02g003105) was up-regulated by EBR application under chilling stress conditions compared with non-EBR treated plants.

Heat shock proteins (HSP) are not only induced under heat stress, but these are also induced under chilling stress conditions. HSPs are involved in the protection of cell organelles. HSPs cause the folding of protein new peptide subunit assembly, protein degradation and repair of nucleolus damage. They have automatic adjustment function and adjust their transcription and translation (DeMaio 1999). Interestingly, heat shock protein 82 (Capana10g000190) was down-regulated (0.87-fold) by EBR application. According to a report, heat shock protein 82 undergoes a functional cycle linked to its ATPase activity (Gambill et al. 1993). Taken together, EBR application is involved in signal transduction under chilling stress conditions; it improves the antioxidant defence system that inhibits oxidative damage creating suitable growth conditions for seedling growth and development.

4.4. Proteins involved in other biological processes

Huge number of DEPs were observed linked with different biological processes, when plants were exposed to Chill and EBR + Chill treatment. The proteins associated with the regulation of amino acid transport and metabolism, translation, ribosomal structure and biology, post modification, protein turnover, chaperones, and cell wall/membrane/envelope biogenesis were affected by chilling EBR application. According to a report, protein synthesis was restricted in cold conditions by some pathways in petunia seedlings placed at 2^{o}C for five days (Zhang et al. 2016). In this study, the 12 proteins associated with amino acid transport and synthesis were up-regulated by the EBR application under chilling stress conditions (Table 2). It can be assumed, that increased endogenous EBR regulates the expression of different proteins responsible for chilling tolerance in pepper.

Ribosomes are responsible for protein biosynthesis. Ribosomes are complex molecular machines that serve as the site of biological protein translation and synthesis through reading the RNA, and linking amino acids (Poole et al. 2016). The 40S/60S ribosomal protein, an essential component of the proteasome, is an independent ATP and ubiquitin protease that plays a crucial role in various cell physiological pathways; for instance, in gene transcription, cellular cycle regulation and protein degradation. In this study, 2 ribosomal proteins (40S S4 and 60S L17-2) significantly up-regulated by EBR application (Table 2), indicating that pepper's metabolism was increased and protein degradation was improved by EBR application. In our study, 15 ribosomal proteins were markedly down-regulated. It was observed that many ribosomal proteins are involved in functional structure and translation. Therefore, it was presumed that the ribosomal proteins played an important role by regulating the translation of related pepper proteins. The 2 down-regulated glycine cleavage system H proteins in the present study

were reported to catalyze glycine degradation (Timm et al. 2012). Glycine cleavage system H proteins are localized in mitochondria in plant and respond to glycine production in the peroxisome to convert serine. This also takes place in photorespiratory cycle in mitochondria. In our study, glycine cleavage system H proteins (Capana06g001561, 1.27; Capana00g000453, 1.18) were down-regulated by EBR treatment. This showed that the leaves absorb EBR under chilling stress conditions. Therefore, it was presumed that glycine metabolism might be improved by decreasing the abundance of protein due to EBR availability. It has been supported that glycosyltransferase protein protects tobacco against salt damage by changing the cell's redox equilibrium state under salt stress condition (Sun et al. 2013).

In this report, UDP-glucose 4-epimerase GEPI48 was up-regulated by EBR application under chilling stress conditions. Serine protease is associated with the degradation of damaged proteins in the plant, which can degrade beta-casein in vivo (Zienkiewicz et al. 2012). The proteasome is characterized via its own ability to cleave peptides in a multicatalytic proteinase complex, which contains an ATP-dependent proteolytic activity. Subtilisin-like protease can induce accumulation and activation of cell wall-regulated enzymes necessary to regulate the relaxation of the cell wall and the swelling of cell mucus (Rautengarten et al. 2008). In the present study, expression of cysteine proteinase, subtilisin-like protease and glutaredoxin (Capana04g000276, Capana01g002650, Capana03g002474, and Capana07g000993) were up-regulated (1.34, 1.13, 1.21 and 1.21-fold, respectively) by EBR + Chill treatment (Table 2) compared with only Chill treatment. Our study also provides evidence that the application of EBR can penetrate in vivo through leaves. These findings indicated that EBR-regulated ribosomes and chaperones might be involved in synthesis or maintenance of other stress-related DEPs under chilling conditions.

We used the iTRAQ to analyze the DEPs that are mainly involved in signal transduction pathways for enhancing photosynthesis, redox, and chill-tolerance. BR's signal transduction and the reactive oxygen species (ROS) produced by its application can play a synergistic role induces the expression of respiratory burst oxidase homolog (RBOH) gene. At the same time, NADPH oxidase also induces ROS accumulation and activates MAPKs to increase plants' resistance (Hao et al. 2013). Taken together, the EBR-modulated chilling stress signalling is received by a few receptors proteins (Capana03g000177and Capana08g000827) at the cell membrane. According to a previous study, BR regulates stress tolerance in *cucumis sativus* via brassinosteroid receptors triggering the active state of plasma membrane-induced RBOH (Xia et al. 2009b). In addition, EBR induced electron transfer to sense the signal conduction for chilling stress and triggered the expression of proteins involved in photosynthesis and energy metabolism to maintain a regular supply of energy to the cells (Fig. 7). We found that EBR could down-regulate the protein associated with the cell wall's ductility for sustaining cell integrity under chilling stress conditions. EBR application also up regulated the proteins associated with antioxidant enzymes in order to maintain redox homeostasis in cells. Most of the up-regulated DEPs are associated with photosynthetic electron transfer chain, oxidative phosphorylation, GSH metabolism pathway, energy metabolism, and Calvin cycle and ribosomes. Our results concludes that EBR can accelerate the metabolism of pepper seedlings under chilling stress by increasing energy supply, enhancing the scavenging capacity of active oxygen species and reducing the cell damage leading towards improved chilling tolerance of pepper seedlings.

Conclusions

Chilling stress decreased the seedling growth, cell size, and internal lamellae of the stroma thylakoids. Exogenous application of EBR improved chilling tolerance of pepper reflected by higher membrane stability and recovery of the chloroplast morphology in the stroma. Using iTRAQ, we identified 346 DEPs, including 217 up-regulated proteins and 129 down-regulated proteins in Chill and Chill + EBR treatment. The chilling tolerance triggered by EBR application contributes to up-regulate the expression of proteins associated with photosynthesis, amino acids, and carbohydrates metabolism, signal transduction, and the maintenance of antioxidant and stress defense. Based on our study's results, exogenous EBR application can improve pepper plants' growth and development under chilling stress conditions. Moreover, the genes and proteins information provided in this report can be utilized by plant biologist in pepper crop improvement programs.

Declarations

Conflicts of Interest: The authors declare no competing financial interest.

Author contributions: Jie Li conceived and designed the experiments. Yang Ping performed the experiments and collected the data. Muhammad Azher Nawaz and Hamza Sohail helped to perform the analysis and write up of this report. Chaowei Liu and Ping Yang contributed reagents/materials/analysis tools and overall supervised this study. All authors have read and approved the final manuscript..

Acknowledgments: We are grateful to the staff of Experiment Station of Gansu Agricultural University for providing assistance in experimental work. The financial support for this study was provided by National Natural Science Foundation of China (NSFC) (31760592), Special Scientific Research Fund for Ph.D of Honghe University (XJ17B07; XJ17B08), and Yunnan Province Applied Basic Research Plan of China (2018FD087).

References

1. Ahammed GJ, Li X, Liu A, Chen S (2020) Brassinosteroids in Plant Tolerance to Abiotic Stress. J Plant Growth Regul 39:1451–1464. https://doi.org/10.1007/s00344-020-10098-0
2. Allen DJ, Ort DR (2001) Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci 6:36–42. https://doi.org/10.1016/S1360-1385(00)01808-2
3. Arora N, Bhardwaj R, Sharma P, Arora HK (2008) Effects of 28-homobrassinolide on growth, lipid peroxidation and antioxidative enzyme activities in seedlings of Zea mays L. under salinity stress. Acta Physiol Plant 30:833–839. https://doi.org/10.1007/s11738-008-0188-9
4. Bajguz A (2009) Brassinosteroid enhanced the level of abscisic acid in Chlorella vulgaris subjected to short-term heat stress. J Plant Physiol 166:882–886. https://doi.org/10.1016/j.jplph.2008.10.004
5. Bouvier F, D'Harlingue A, Hugueney P et al (1996) Xanthophyll biosynthesis: Cloning, expression, functional reconstitution, and regulation of β-cyclohexenyl carotenoid epoxidase from pepper

(Capsicum annuum). J Biol Chem 271:28861–28867. https://doi.org/10.1074/jbc.271.46.28861

6. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
7. Chelysheva VV, Smolenskaya IN, Trofimova MC et al (1999) Role of the 14-3-3 proteins in the regulation of H+-ATPase activity in the plasma membrane of suspension-cultured sugar beet cells under cold stress. FEBS Lett 456:22–26. https://doi.org/10.1016/S0014-5793(99)00923-0
8. DeMaio A (1999) Letters to the Editor-Heat Shock Proteins. Facts, thoughts, and dreams-Reply. Shock 12:323–324
9. Divi UK, Krishna P (2009) Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance. N Biotechnol 26:131–136. https://doi.org/10.1016/j.nbt.2009.07.006
10. Dou L, Sun Y, Li S et al (2021) Transcriptomic analyses show that 24-epibrassinolide (EBR) promotes cold tolerance in cotton seedlings. PLoS One 16:1–21. https://doi.org/10.1371/journal.pone.0245070
11. Fan H, Du C, Xu Y, Wu X (2014) Exogenous nitric oxide improves chilling tolerance of Chinese cabbage seedlings by affecting antioxidant enzymes in leaves. Hortic Environ Biotechnol 55:159–165. https://doi.org/10.1007/s13580-014-0161-z
12. Fang P, Yan M, Chi C et al (2019) Brassinosteroids act as a positive regulator of photoprotection in response to chilling stress. Plant Physiol 180:2061–2076. https://doi.org/10.1104/pp.19.00088
13. Gambill BD, Voos W, Pil Jung, Kang et al (1993) A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. J Cell Biol 123:109–117. https://doi.org/10.1083/jcb.123.1.109
14. Gao H, Kang LN, Liu Q et al (2015) Effect of 24-epibrassinolide treatment on the metabolism of eggplant fruits in relation to development of pulp browning under chilling stress. J Food Sci Technol 52:3394–3401. https://doi.org/10.1007/s13197-014-1402-y
15. Hao J, Yin Y, Fei S (2013) Brassinosteroid signaling network: Implications on yield and stress tolerance. Plant Cell Rep 32:1017–1030. https://doi.org/10.1007/s00299-013-1438-x
16. Hossain Z, Nouri MZ, Komatsu S (2012) Plant cell organelle proteomics in response to abiotic stress. J Proteome Res 11:37–48. https://doi.org/10.1021/pr200863r
17. Hu WH, Wu Y, Zeng JZ et al (2010) Chill-induced inhibition of photosynthesis was alleviated by 24-epibrassinolide pretreatment in cucumber during chilling and subsequent recovery. Photosynthetica 48:537–544. https://doi.org/10.1007/s11099-010-0071-y
18. Jie LI, Ping Y, Jianming XIE, Jihua YU (2015) Effects of 24-epibrassinolide on growth and antioxidant enzymes system in pepper roots under chilling stress. J Nucl Agric Sci 29:1001–1008
19. Kanwar MK, Bhardwaj R, Arora P et al (2012) Plant steroid hormones produced under Ni stress are involved in the regulation of metal uptake and oxidative stress in Brassica juncea L. Chemosphere 86:41–49. https://doi.org/10.1016/j.chemosphere.2011.08.048

20. Laloi C, Rayapuram N, Chartier Y et al (2001) Identification and characterization of a mitochondrial thioredoxin system in plants. Proc Natl Acad Sci U S A 98:14144–14149. https://doi.org/10.1073/pnas.241340898
21. Li J, Yang P, Gan Y et al (2015) Brassinosteroid alleviates chilling-induced oxidative stress in pepper by enhancing antioxidation systems and maintenance of photosystem II. Acta Physiol Plant 37:1–11. https://doi.org/10.1007/s11738-015-1966-9
22. Li J, Yang P, Kang J et al (2016a) Transcriptome analysis of pepper (Capsicum annuum) revealed a role of 24-epibrassinolide in response to chilling. Front Plant Sci 7:1–17. https://doi.org/10.3389/fpls.2016.01281
23. Li Z, Zhang Y, Xu Y et al (2016b) Physiological and iTRAQ-based proteomic analyses reveal the function of spermidine on improving drought tolerance in white clover. J Proteome Res 15:1563–1579. https://doi.org/10.1021/acs.jproteome.6b00027
24. Liu Y, Jiang H, Zhao Z, An L (2011) Abscisic acid is involved in brassinosteroids-induced chilling tolerance in the suspension cultured cells from Chorispora bungeana. J Plant Physiol 168:853–862. https://doi.org/10.1016/j.jplph.2010.09.020
25. Liu Y, Zhao Z, Si J et al (2009) Brassinosteroids alleviate chilling-induced oxidative damage by enhancing antioxidant defense system in suspension cultured cells of Chorispora bungeana. Plant Growth Regul 59:207–214. https://doi.org/10.1007/s10725-009-9405-9
26. Ma NN, Zuo YQ, Liang XQ et al (2013) The multiple stress-responsive transcription factor SlNAC1 improves the chilling tolerance of tomato. Physiol Plant 149:474–486. https://doi.org/10.1111/ppl.12049
27. Merewitz EB, Gianfagna T, Huang B (2011) Protein accumulation in leaves and roots associated with improved drought tolerance in creeping bentgrass expressing an ipt gene for cytokinin synthesis. J Exp Bot 62:5311–5333. https://doi.org/10.1093/jxb/err166
28. Nawaz MA, Chen C, Shireen F et al (2018) Improving vanadium stress tolerance of watermelon by grafting onto bottle gourd and pumpkin rootstock. Plant Growth Regul 85:41–56. https://doi.org/10.1007/s10725-018-0372-x
29. Ogweno JO, Song XS, Shi K et al (2008) Brassinosteroids alleviate heat-induced inhibition of photosynthesis by increasing carboxylation efficiency and enhancing antioxidant systems in Lycopersicon esculentum. J Plant Growth Regul 27:49–57. https://doi.org/10.1007/s00344-007-9030-7
30. Owiti J, Grossmann J, Gehrig P et al (2011) ITRAQ-based analysis of changes in the cassava root proteome reveals pathways associated with post-harvest physiological deterioration. Plant J 67:145–156. https://doi.org/10.1111/j.1365-313X.2011.04582.x
31. Pezeshki SR (1994) Response of baldcypress (Taxodium distichum) seedlings to hypoxia: Leaf protein content, ribulose-1, 5-bisphosphate carboxy-lase/oxygenase activity and photosynthesis. Photosynthetica 30:59–68

32. Poole AM, Jeffares DC, Hoeppner MP, Penny D (2016) Does the Ribosome Challenge our Understanding of the RNA World? J Mol Evol 82:1–4. https://doi.org/10.1007/s00239-015-9720-x
33. Pradet-Balade B, Boulmé F, Beug H et al (2001) Translation control: Bridging the gap between genomics and proteomics? Trends Biochem Sci 26:225–229. https://doi.org/10.1016/S0968-0004(00)01776-X
34. Rajewska I, Talarek M, Bajguz A (2016) Brassinosteroids and Response of Plants to Heavy Metals Action. Front Plant Sci 7:1–5. https://doi.org/10.3389/fpls.2016.00629
35. Rautengarten C, Usadel B, Neumetzler L et al (2008) A subtilisin-like serine protease essential for mucilage release from Arabidopsis seed coats. Plant J 54:466–480. https://doi.org/10.1111/j.1365-313X.2008.03437.x
36. Schulz PA, Rivera PH, Studart N (2002) Renormalization approach to quantum-dot structures under strong alternating fields. Phys Rev B - Condens Matter Mater Phys 66:1–9. https://doi.org/10.1103/PhysRevB.66.195310
37. Singh A, Jha SK, Bagri J, Pandey GK (2015) ABA inducible rice protein phosphatase 2C confers ABA insensitivity and abiotic stress tolerance in arabidopsis. PLoS One 10:1–24. https://doi.org/10.1371/journal.pone.0125168
38. Singh I, Kumar U, Singh SK et al (2012) Physiological and biochemical effect of 24-epibrassinoslide on cold tolerance in maize seedlings. Physiol Mol Biol Plants 18:229–236. https://doi.org/10.1007/s12298-012-0122-x
39. Sun YG, Wang B, Jin SH et al (2013) Ectopic Expression of Arabidopsis Glycosyltransferase UGT85A5 Enhances Salt Stress Tolerance in Tobacco. PLoS One 8:. https://doi.org/10.1371/journal.pone.0059924
40. Timm S, Florian A, Arrivault S et al (2012) Glycine decarboxylase controls photosynthesis and plant growth. FEBS Lett 586:3692–3697. https://doi.org/10.1016/j.febslet.2012.08.027
41. Vijayakumar H, Thamilarasan SK, Shanmugam A et al (2016) Glutathione transferases superfamily: Cold-inducible expression of distinct GST genes in Brassica oleracea. Int J Mol Sci 17:. https://doi.org/10.3390/ijms17081211
42. Wang C, Ma QH, Lin ZB et al (2008) Cloning and characterization of a cDNA encoding 14-3-3 protein with leaf and stem-specific expression from wheat. DNA Seq - J DNA Seq Mapp 19:130–136. https://doi.org/10.1080/10425170701447515
43. Wang J, Yao L, Li B et al (2016) Comparative proteomic analysis of cultured suspension cells of the halophyte halogeton glomeratus by iTRAQ provides insights into response mechanisms to salt stress. Front Plant Sci 7:1–12. https://doi.org/10.3389/fpls.2016.00110
44. Wani SH, Sah SK, Sanghera G et al (2016) Genetic Engineering for Cold Stress Tolerance in Crop Plants. Adv Genome Sci 173–201. https://doi.org/10.2174/9781681081731116040010
45. Willey RL (1971) Microtechniques: a laboratory guide. Macmillan New York
46. Wu CY, Trieu A, Radhakrishnan P et al (2008) Brassinosteroids regulate grain filling in rice. Plant Cell 20:2130–2145. https://doi.org/10.1105/tpc.107.055087

47. Xi Z, Wang Z, Fang Y et al (2013) Effects of 24-epibrassinolide on antioxidation defense and osmoregulation systems of young grapevines (V. vinifera L.) under chilling stress. Plant Growth Regul 71:57–65. https://doi.org/10.1007/s10725-013-9809-4
48. Xia XJ, Fang PP, Guo X et al (2018) Brassinosteroid-mediated apoplastic H2O2-glutaredoxin 12/14 cascade regulates antioxidant capacity in response to chilling in tomato. Plant Cell Environ 41:1052–1064. https://doi.org/10.1111/pce.13052
49. Xia XJ, Huang LF, Zhou YH et al (2009a) Brassinosteroids promote photosynthesis and growth by enhancing activation of Rubisco and expression of photosynthetic genes in Cucumis sativus. Planta 230:1185–1196. https://doi.org/10.1007/s00425-009-1016-1
50. Xia XJ, Wang YJ, Zhou YH et al (2009b) Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol 150:801–814. https://doi.org/10.1104/pp.109.138230
51. Yan SP, Zhang QY, Tang ZC et al (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol Cell Proteomics 5:484–496. https://doi.org/10.1074/mcp.M500251-MCP200
52. Yang P, Nawaz MA, Li F et al (2019) Brassinosteroids regulate antioxidant system and protect chloroplast ultrastructure of autotoxicity-stressed cucumber (Cucumis Sativus L.) seedlings. Agronomy 9:. https://doi.org/10.3390/agronomy9050265
53. Yang Y, Qiang X, Owsiany K et al (2011) Evaluation of different multidimensional LC-MS/MS pipelines for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis of potato tubers in response to cold storage. J Proteome Res 10:4647–4660. https://doi.org/10.1021/pr200455s
54. Yang Y, Tang M, Sulpice R et al (2014) Arbuscular Mycorrhizal Fungi Alter Fractal Dimension Characteristics of Robinia pseudoacacia L. Seedlings Through Regulating Plant Growth, Leaf Water Status, Photosynthesis, and Nutrient Concentration Under Drought Stress. J Plant Growth Regul 33:612–625. https://doi.org/10.1007/s00344-013-9410-0
55. Yu JQ, Huang LF, Hu WH et al (2004) A role for brassinosteroids in the regulation of photosynthesis in Cucumis sativus. J Exp Bot 55:1135–1143. https://doi.org/10.1093/jxb/erh124
56. Zeng YH, Zahng YP, Xiang J et al (2016) Effects of chilling tolerance induced by spermidine pretreatment on antioxidative activity, endogenous hormones and ultrastructure of indica-japonica hybrid rice seedlings. J Integr Agric 15:295–308. https://doi.org/10.1016/S2095-3119(15)61051-6
57. Zhang L, Guo X, Zhang Z et al (2021) Cold-regulated gene LeCOR413PM2 confers cold stress tolerance in tomato plants. Gene 764:145097. https://doi.org/10.1016/j.gene.2020.145097
58. Zhang W, Zhang H, Ning L et al (2016) Quantitative proteomic analysis provides novel insights into cold stress responses in petunia seedlings. Front Plant Sci 7:1–13. https://doi.org/10.3389/fpls.2016.00136
59. Zienkiewicz M, Ferenc A, Wasilewska W, Romanowska E (2012) High light stimulates Deg1-dependent cleavage of the minor LHCII antenna proteins CP26 and CP29 and the PsbS protein in

Arabidopsis thaliana. Planta 235:279–288. https://doi.org/10.1007/s00425-011-1505-x

Figures

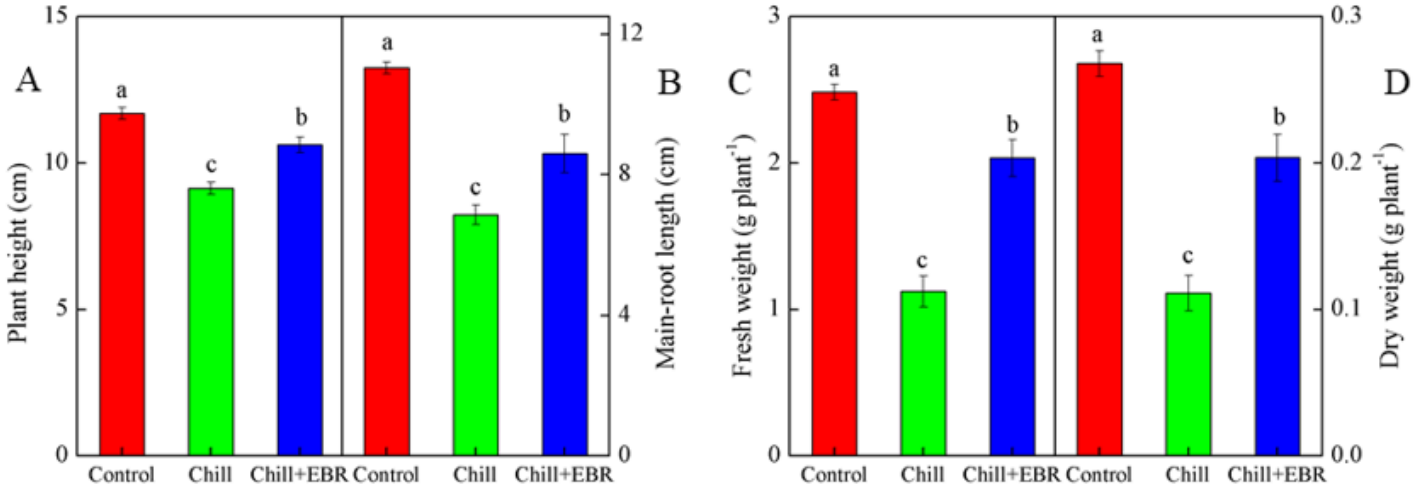

Figure 1

Plant height (A), main-root length (B), fresh and dry weight (C, D) in control condition and chill-stressed pepper seedlings with or without exogenous application of EBR. Different letters indicated significant differences at P < 0.05. Control, foliar spray of DDH2O under normal condition; Chill, foliar spray of DDH2O under chilling stress; Chill+EBR, foliar sprayed of 0.1 μM EBR under chilling stress.

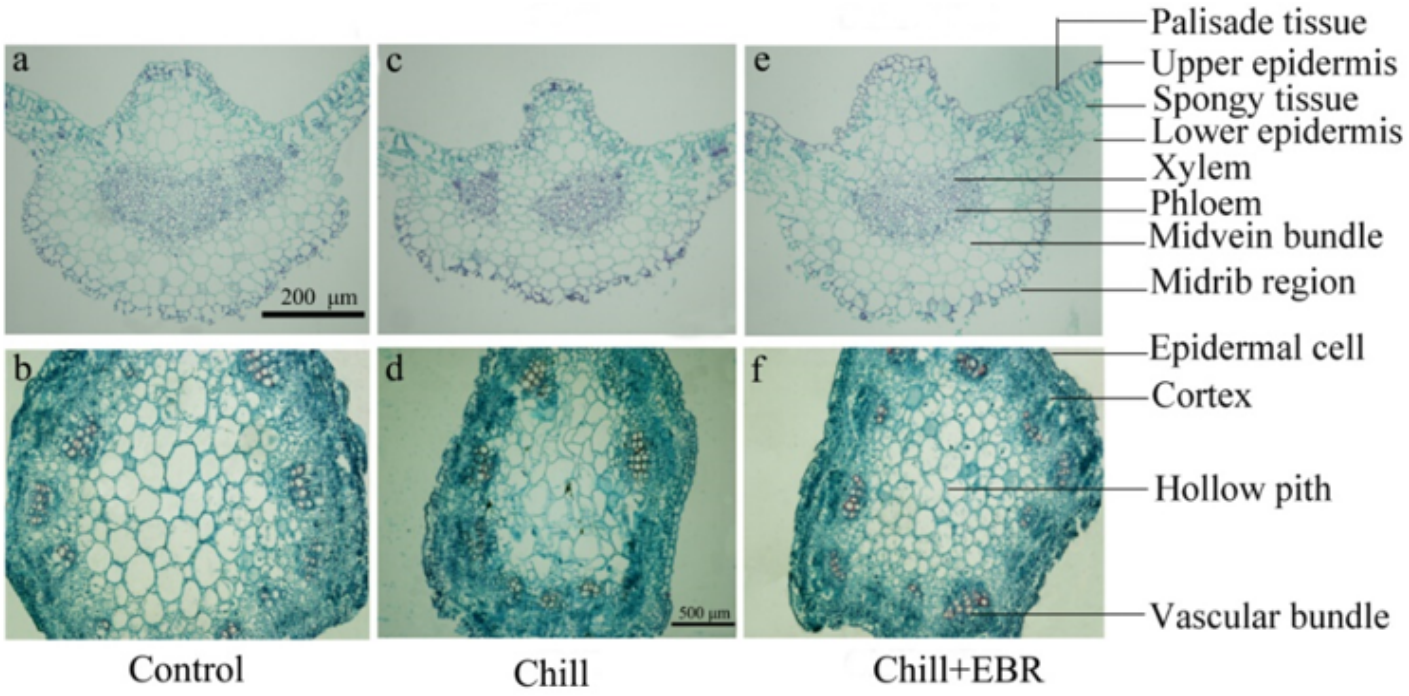

Figure 2

Transverse section of leaf (A) and stem (B) in control condition (a, b), chill-stressed treatment (c, d), and chill-stress with EBR (e, f) of pepper seedlings. Control, foliar spray of DDH2O under normal condition; Chill, foliar spray of DDH2O under chilling stress; Chill+EBR, foliar spray of 0.1 μM EBR under chilling stress.

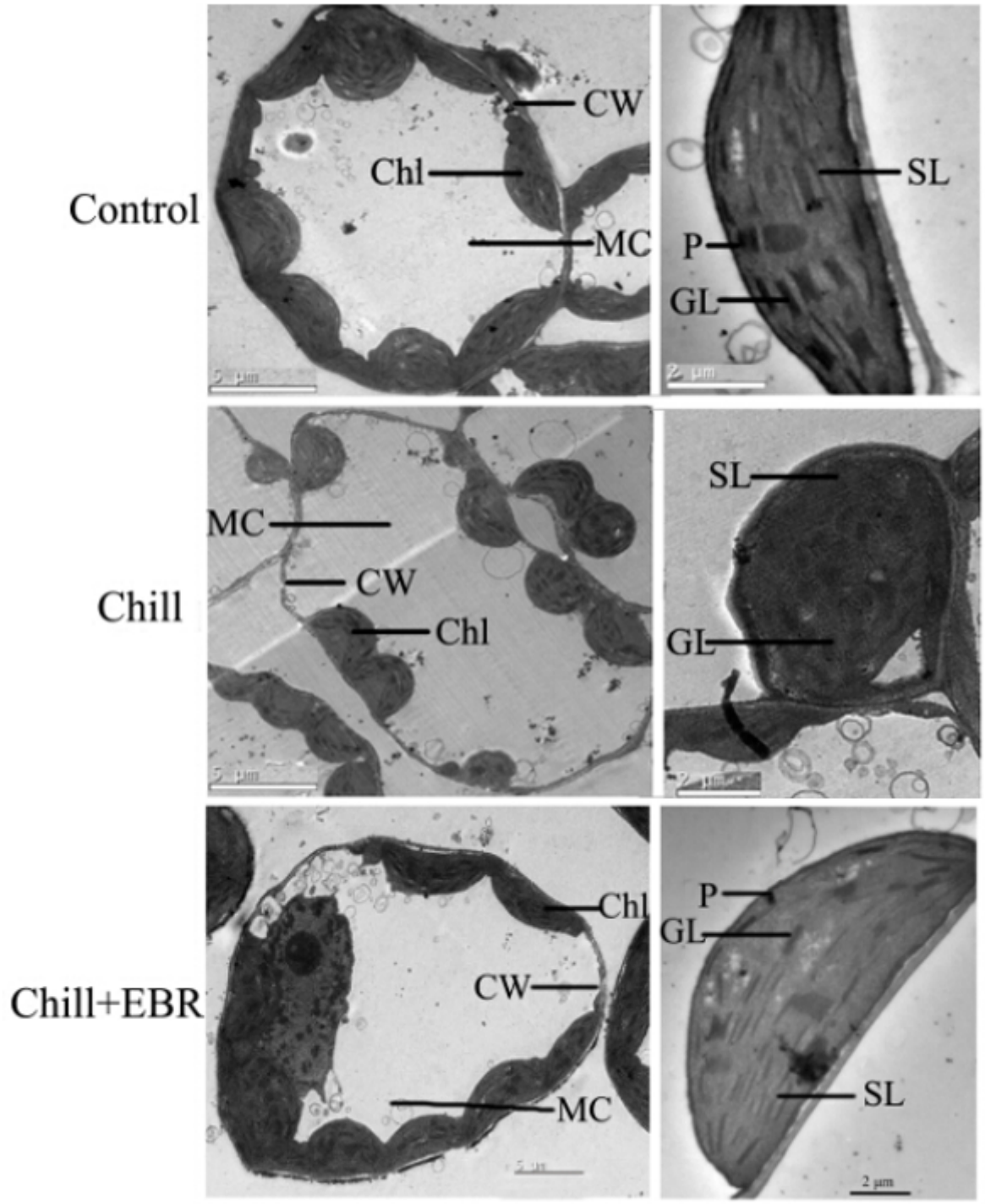

Figure 3

Mesophyll cell and chloroplast in control condition, chill-stressed treatment, and chill-stress with EBR application of pepper seedlings. MC, Mesophyll cell; Chl, chloroplast; CW, cell wall; GL, grana lamellae; SL,

stroma lamellae; P, plastoglobules. Control, foliar spray of DDH2O under normal condition; Chill, foliar spray of DDH2O under chilling stress; Chill+EBR, foliar spray of 0.1 μM EBR under chilling stress.

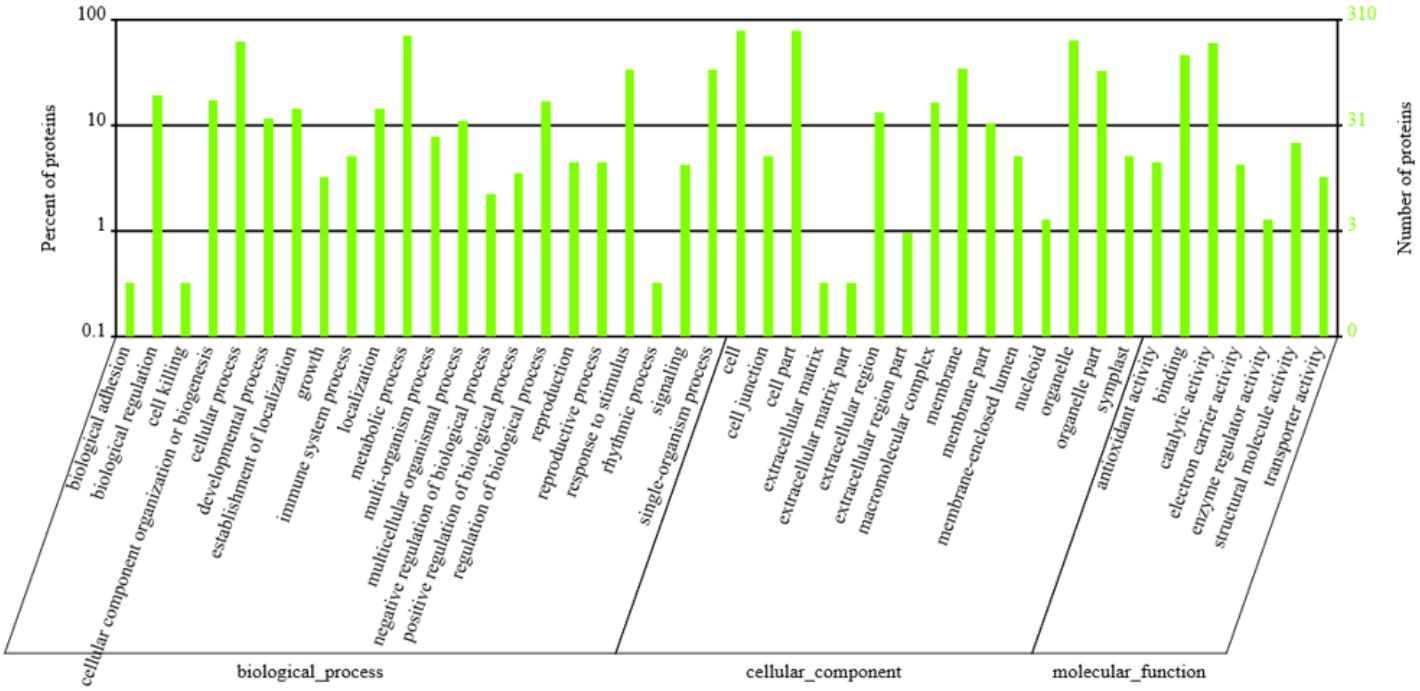

Figure 4

Gene ontology (GO) analysis of differentially expressed proteins (DEPs) in leaves of pepper seedling between Chill and Chill+EBR treatment. We selected all 346 DEPs for GO annotating to 3 terms including the biological process, cellular component categories and molecular function.

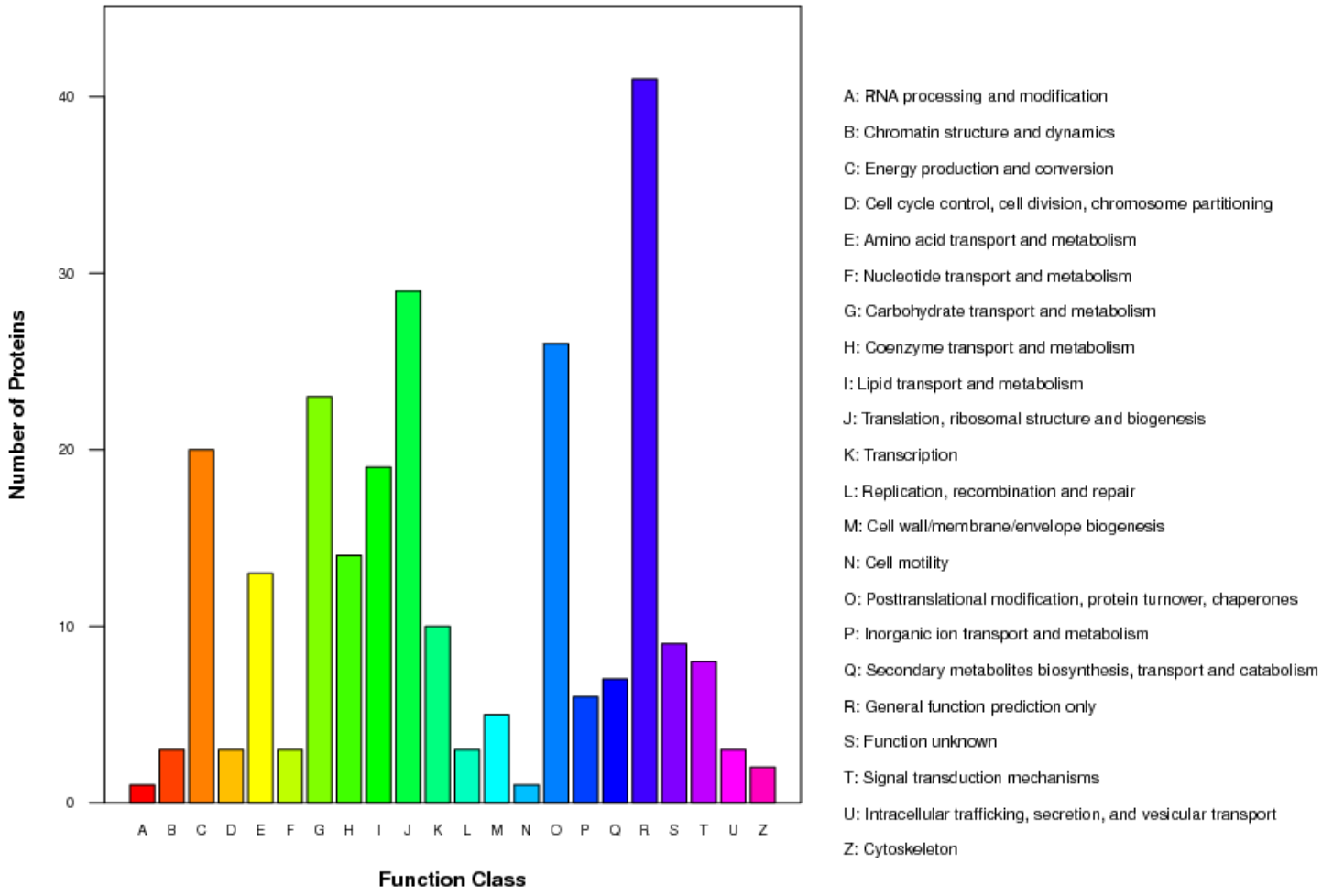

Figure 5

The clusters of COG function of differentially expressed proteins (DEPs) in leaves of pepper seedling between Chill and Chill+EBR treatment. We selected all 346 DEPs for functional classification by cluster of orthologous groups in iTRAQ data.

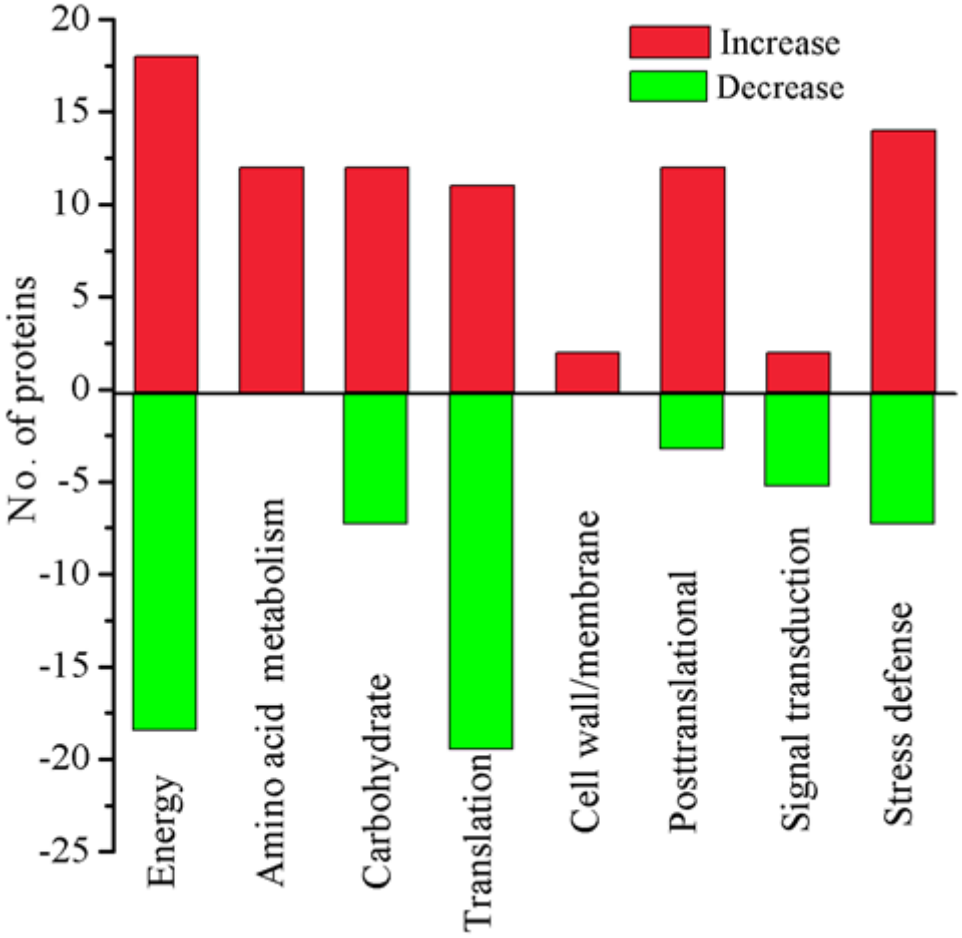

Figure 6

The differentially expressed proteins within each functional category in the EBR-treated and non-EBR treated plants exposed to chilling stress. In the same functional classification for 346 DEPs, the red histogram shows the number of up-regulated proteins, and the green histogram represents the number of down-regulated proteins.

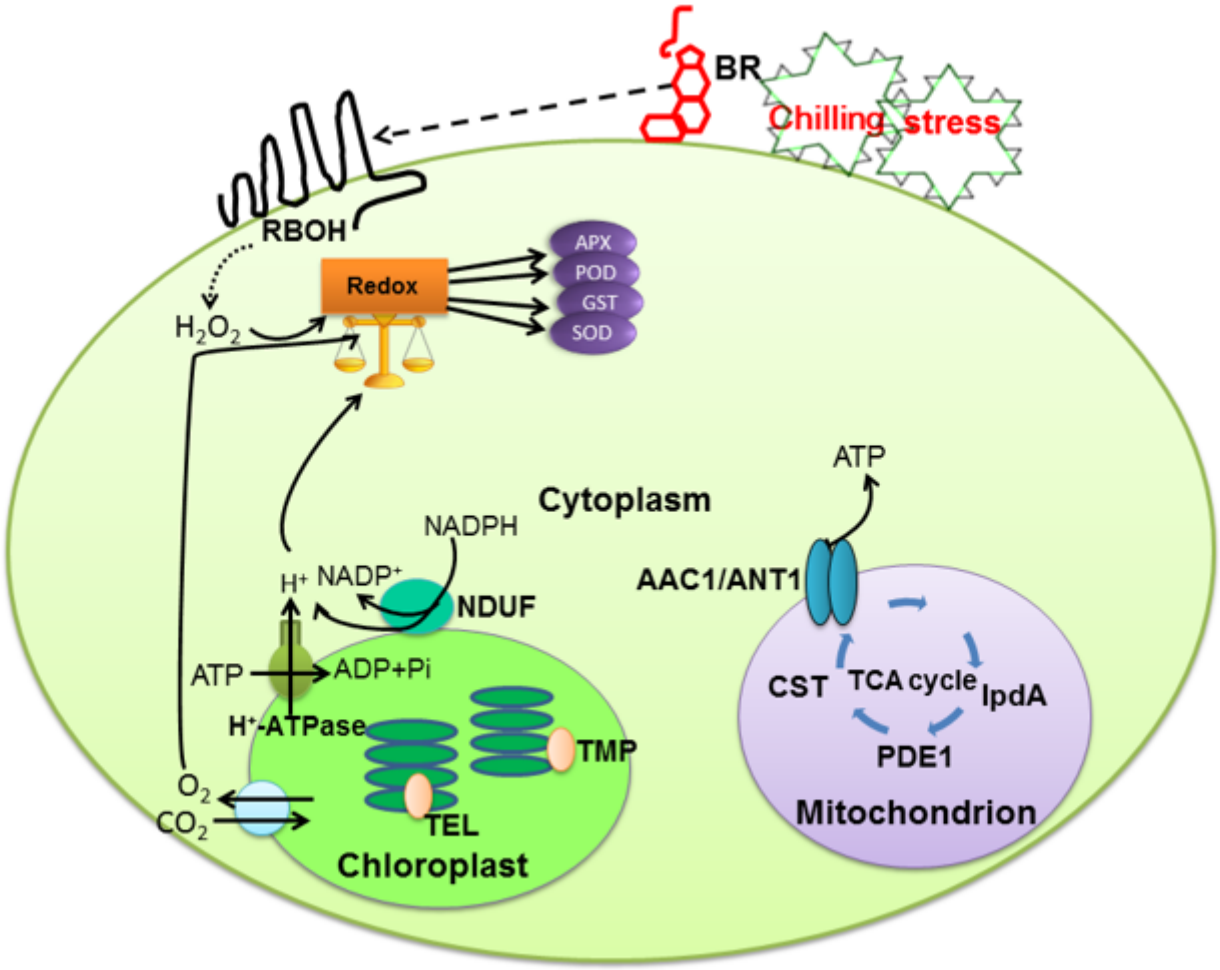

Figure 7

Model depicting BR-mediated chilling tolerance via key transporters, redox, photosynthetic electron transfer chain, Calvin cycle and signaling pathway in leaf cell of pepper seedling. RBOH, respiratory burst oxidase homolog; NDUF, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9; TMP, thylakoid membrane phosphoprotein 14 kDa, chloroplastic; TEL, thylakoid lumenal 19 kDa protein, chloroplastic; AAC1/ANT1, ADP,ATP carrier protein 1, mitochondrial; CST, citrate synthase, mitochondrial; lpdA, dihydrolipoyl dehydrogenase; PDE1, Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial.

Supplementary Files

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

- SupportingInformation.rar