Physiological and proteomic analyses reveals that brassinosteroids application improves the chilling stress tolerance of pepper seedlings

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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 the 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, carbohydrate metabolism, energy metabolism, protein biosynthesis, amino acid synthesis, redox and stress defence (ascorbate peroxidase, glutathione peroxidase and superoxide dismutase). Up-regulated DEPs were associated with the 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.

Keywords Pepper · Brassinosteroids · Chilling stress · Ultrastructure · Proteome

Introduction
Chilling is an abiotic stress which impairs plant development, fruit yield, and quality (Zhang et al. 2021). Chilling stress often affects open-field crops in the Northern hemisphere, and the crops are grown in a solar-greenhouse system during the winter to early spring season (Wani et al. 2016). Chilling stress directly damages the photosynthetic apparatus or indirectly disrupts the process of photosynthesis by affecting the carbon–oxygen cycle, photosynthetic pigment synthesis, and CO₂ supply (Allen and Ort 2001). Plants subjected to chilling stress undergo dramatic alternations at the molecular level, such as gene transcription, proteins synthesis and metabolism (Ma et al. 2013; Dou et al. 2021).

Brassinosteroids (BRs) are phytohormones that share similar structures to those 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 help to 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 protein profiles under stress conditions, providing reliable information to understand the signaling and metabolic processes related to various unfavourable environmental constraints (Hossain et al. 2012). The iTRAQ-based proteomic analysis provides technical advantages over traditional 2D gel electrophoresis analysis (Owiti et al. 2011). Furthermore, the combination of iTRAQ and MS/MS techniques allows for 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 their 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-induced chilling stress tolerance of pepper seedlings, and this can be useful for plant biologist interested in studying the mechanisms of BRs to improve stress tolerance under other kinds of abiotic stresses. 

Materials and methods

Plant material and growing conditions

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

According to our previous work, 0.1 μM 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 0.1 μM as pretreatment and then exposed to low temperature. The seedlings were pretreated with ddH2O or 0.1 μM EBR for 24 h and then placed at 25/15 °C (day/night) with 300 μmol m⁻² s⁻¹ light intensity, or at low-temperature treatment (15/5 °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 °C until further use.

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 °C for 15 min and later, the temperature was reduced to 85 °C until the samples attained a constant weight.

Gas exchange parameters and Rubisco activity determination

Photosynthetic parameters such as stomatal conductance (Gs), transpiration rate (Tr) and intercellular CO₂ concentration (Ci) were recorded by the CIRAS-2 System (PP Systems, Haverhill, MA, USA). The chlorophyll fluorescence in pepper leaves was measured by a fluorimeter (IMAG-PAM, Heinz Waltz, Germany). Rubisco activity was measured according to the Nie et al. (1993) method.

Anatomical studies

After being exposed 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).
Proteomics extraction and iTRAQ labelling

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 °C. BSA was used as a standard to measure proteins (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 °C for 16 h. 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 112, 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.

Peptide separation and LC–MS/MS

Strong cation exchange (SCX) fractionation of the combined peptide mixture was re-suspended in 4 mL buffer A (25 mmol NaH2PO4 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 NaH2PO4 and 1 mol KCl in 25% ACN, pH2.7) for 7 min, followed by a linear gradient in buffer B from 5 to 60% for 20 min, finally within 2 min to keep the buffer B ratio raised to 100% for 1 min, and then back to 5% for 10 min. The components were re-dissolved in the Buffer A (5% acetonitrile, 0.1% formic acid) to about 0.5 μg μl⁻¹, and centrifuged at 30,000 X g for 15 min at 4 °C. BSA was used as a standard to measure proteins (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 °C for 16 h. 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.

Database search and quantification

The data was processed by Proteome Discoverer v.1.2 (Thermo Scientific) and searched with in-house Mascot software (version 2.3.02; Matrix Science, London, U.K.) as mentioned by Shi et al. (2021) against the pepper database (35,101 sequences). In the process of protein identification, the parameters were selected as follows: fragment mass tolerance values: 0.1 Da; peptide 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 (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 change 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.

**Real-time quantitative PCR analysis**

Sixteen highly expressed 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. (2016a).

**Statistical analysis**

Data on growth and physiological parameters was analyzed using SPSS software (IBM SPSS 22.0, IBM Corporation, New York, USA). Means were compared using Duncan’s multiple range test at $P<0.05$. MicroCal Origin 8.0 Professional (OriginLab Corporation, Northampton, USA) was used to prepare charts. The Pearson test was used to calculate correlations at protein and transcription level using Lan et al. (2012) method to reveal weak correlation between proteome results in the study and transcriptome results obtained previously in the same time samples (Li et al. 2016a).

**Results**

**Morphological parameters and anatomical changes**

Exposure of pepper seedlings to chilling stress influences morphological, cell microstructural and ultrastructural traits (Figs. 1, 2, 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 improved the epidermis, fibre tissue, phloem tissue, xylem tissue and parenchymatous area of the hollow pith under chilling stress conditions (Fig. 2), suggesting that the EBR treatment increased the plant height and root length observed under chilling stress conditions. Chilling stress caused remarkable changes in ultrastructure of mesophyll cells. Specifically, chilling caused chloroplast swelling, loss of polarity, chloroplast degradation, blurred starch granules, and the thylakoid substrate layer to disappear. 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).

**Fig. 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. 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. Each histogram represents a mean value of nine independent experiments and the vertical bars indicate SE (n = 9). Columns marked with the same lowercase letters are not significantly different and different lowercase letters indicate significant differences between treatments by the Duncan’s multiple range test at $P<0.05$. Springer
Photosynthetic parameters and Rubisco activity

The stomatal conductance (Gs) and transpiration rate (Tr) were decreased after seven days of chilling treatment (Table 1). EBR application reduced the inhibitory effect of chilling stress on photosynthesis, and improved Gs and Tr in contrast with non-EBR treated plants. Furthermore, preapplication of EBR counteracted chilling-induced decreases in Rubisco activity in the leaves of the pepper seedlings.

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 4661 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-fold change 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 temperatures.

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 Fig. 5 and Table S6. The 346 DEPs were categorized into 22 biological processes, 15 cellular components, and 7 molecular functions (Fig. 4). In the biological processes, most of 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).
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 the energy production and conversion category. All the DEPs in the amino acid transport and metabolism category were up-regulated proteins. In the 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 far as translation is concerned, ribosomal structure and biogenesis category, there were 11 up-regulated and 19 down-regulated DEPs in the EBR + Chill treatment compared with the Chill treatment. All the DEPs in the cell wall/membrane/envelope biogenesis category were up-regulated proteins. In the 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 the stress defense 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).

Changes in RNA transcription

Correlation analysis between iTRAQ-seq results and the expression of corresponding DEPs is presented in Fig. S1. We selected ten up-regulated DEPs and six down-regulated DEPs with high expression for transcript level 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 (Fig. S1).

Table 1 Effect of EBR on net photosynthetic rate ($P_n$), stomatal conductance ($G_s$), intercellular $CO_2$ concentration ($C_i$), transpiration rate ($T_r$) and Rubisco activity of pepper under control, chilling and chilling + EBR treatments

| Treatment      | $P_n$ [μmol(CO$_2$) m$^{-2}$ s$^{-1}$] | $C_i$ [μmol mol$^{-1}$] | $G_s$ [mmol(H$_2$O) m$^{-2}$ s$^{-1}$] | $T_r$ (mmol m$^{-2}$ s$^{-1}$) | Rubisco activity [μmol m$^{-2}$ s$^{-1}$] |
|----------------|----------------------------------------|--------------------------|---------------------------------------|-------------------------------|------------------------------------------|
| Control        | $4.7 \pm 0.10a$                        | $212.3 \pm 9.5b$         | $54.3 \pm 4.6a$                       | $1.33 \pm 0.15a$              | $3.68 \pm 0.27a$                        |
| Chill          | $2.1 \pm 0.13c$                        | $273.0 \pm 28.7a$        | $21.7 \pm 3.1c$                       | $0.70 \pm 0.07c$              | $1.82 \pm 0.13c$                        |
| Chill + EBR    | $4.2 \pm 0.22b$                        | $255.7 \pm 26.3ab$       | $33.3 \pm 5.9b$                       | $1.02 \pm 0.08b$              | $2.76 \pm 0.08b$                        |

Data are the means of three replicates ($\pm$ SE, n = 3). The means followed by lowercase same letter do not differ significantly and different lowercase letters indicate significant differences between treatments according to Duncan’s multiple range test ($P < 0.05$)
Fig. 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.

Fig. 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.
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 | Ratio |
|-----------|--------------|---------|-----|-------|
| **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 luminal 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* |
| Capana12g000901 | 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* |
| 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 | Pismu sativum | 27.6 | 0.77** |
| Capana01g000647 | Chlorophyll a-b binding protein P4 | Pismu sativum | 23.1 | 0.64* |
| Capana07g000048 | Photosystem I P700 chlorophyll a apoprotein A1 | Nicotiana tomentosiformis | 6.7 | 0.65* |
| Capana05g001698 | 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 wallchii | 6.4 | 0.73* |

**Carbohydrate transport and metabolism**

| Accession | Protein name | Species | Cov | Ratio |
|-----------|--------------|---------|-----|-------|
| 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** |
| Capana04g000404 | 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* |
Table 2 (continued)

| Accession     | Protein name                                             | Species       | Cov  | *Ratio  |
|---------------|----------------------------------------------------------|---------------|------|---------|
| 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**  |
| Capana01g00250  | 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

| Accession     | Protein name                                             | Species       | Cov  | *Ratio  |
|---------------|----------------------------------------------------------|---------------|------|---------|
| 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**  |
| Capana05g00144 | 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

| Accession     | Protein name                                             | Species       | Cov  | *Ratio  |
|---------------|----------------------------------------------------------|---------------|------|---------|
| 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 lycopersicum | 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*   |
| Capana11g000467 | 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*   |
| Capana09g004669 | 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**  |
| Capana06g000058 | 50S ribosomal protein L17                                  | Nicotiana tabacum | 30.2 | 0.82*   |
| Capana09g001418 | 50S ribosomal protein L2                                   | Solanum lycopersicum | 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*   |
| Accession   | Protein name                          | Species                          | Cov  | Ratio  |
|-------------|--------------------------------------|----------------------------------|------|--------|
| Capana08g001184 | Proline–tRNA ligase                   | Thermus thermophilus             | 19.3 | 0.83*  |
| Capana03g002188 | Putative rRNA 2'-O-methyltransferase fibrillin 3 | Arabidopsis thaliana             | 12.0 | 0.86*  |
| Capana00g003378 | UDP-glucose 4-epimerase GEP48         | Cyanopsis 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*  |
| 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*  |
| Capana05g001818 | ATP-dependent zinc metalloprotease FTSH 4 | Arabidopsis thaliana         | 20.5 | 0.82*  |
| 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*  |
| Capana02g003105 | Zeaxanthin epoxidase                  | Capsicum anuum                   | 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** |
| Capana09g003105 | 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*  |
| Capana02g003509 | Probable monodehydroascorbate reductase | Arabidopsis thaliana         | 10.4 | 1.17*  |
| Capana02g002017 | Quinone oxidoreductase IIG3           | Homo sapiens                     | 31.4 | 1.15*  |
| Capana02g002207 | Probable S'-adenyllysulfate reductase 1 | Oryza sativa                   | 27.8 | 0.76*  |
| Capana10g000190 | Heat shock protein 82 (Fragment)      | Nicotiana tabacum                 | 31.4 | 0.87*  |
| Capana02g00952  | Probable glutathione S-transferase    | Nicotiana tabacum                 | 24.6 | 0.83*  |
| Capana05g001394 | Ferritin-3                            | Glycine max                      | 22.7 | 0.78*  |
Integration of proteomic and transcriptomic profiles

The variable transcriptome and proteome datasets are depicted in Fig. 7. Due to the complex relationship between the mRNA and protein levels of associated genes, we performed a correlation analysis between all DEGs (Li et al. 2016a) and DEPs. Overall, most of the DEGs and DEPs showed no corresponding correlation. In addition, we used the correlation coefficient to quantify the correlation between the transcriptome and the proteome. We have observed 22 genes with positive (Quadrant 3 and 7) and 26 genes with negative correlation (Quadrant 1 and 9) between the transcriptome and the proteome; 469 genes were different only at the protein level (Quadrant 4 and 6), and 101 genes were different only at the transcriptome level. There were 2533 genes (Quadrant 5) with no difference in protein or transcription levels (Table S8).

**Table 2** (continued)

| Accession     | Protein name                        | Species                      | Cov  | Ratio |
|---------------|-------------------------------------|------------------------------|------|-------|
| 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* |

*Ratio between intensities of identified protein species in Chill vs Chill + EBR plants
The ratios that were statistically significant (p < 0.05 and 0.01) were indicated with “*” and “**”, respectively. Ratio changes in expression level of at least 1.2-fold up-regulated or 0.883-fold down-regulated

**Fig. 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, the red histogram shows the number of up-regulated proteins, and the green histogram represents the number of down-regulated proteins.

**Fig. 7** Nine-quadrant diagram analysis between transcriptomic and proteomic data of the EBR-treated and non-EBR treated plants exposed to chilling stress. The red dots (Quadrant 1, 3, 7 and 9) indicated that the proteome and transcriptome were all different genes; the blue dots (Quadrant 4 and 6) indicated that the genes were different only at protein level; the green dots (Quadrant 2 and 8) indicated that the genes were different only at transcriptome level; the black dots (Quadrant 5) indicated that the proteome and transcriptome was not differentially expressed; Dotted line indicated threshold line of differentially expressed genes. The values of ordinate and abscissa stand for the log2 value regarding the fold change of DEGs and DEPs, respectively. DEPs referred to stress responsive with the fold change > 1.2-fold for up-accumulated proteins or < 1.2-fold for down-accumulated proteins in abundance (*P < 0.05). DEGs were assigned with an adjusted p-value less than 0.05 found by DESeq

**Discussion**

**Morphological response of pepper to EBR under chilling stress**

In our work, improved plant height, main-root length, and biomass accumulation were observed in EBR-treated...
pepper plants 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 the ultrastructure of leaf mesophyll cells. 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.

**Energy and carbohydrate metabolism related proteins**

Energy loss and disturbance in carbohydrate metabolism is the main symptoms triggered in response to chilling. In our experiment, predominant changes were observed associated with photosynthesis and carbohydrate metabolism (Table 2; Fig. 6). The EBR application improved the synthesis of carbohydrates that promote plant growth. Using iTRAQ quantification, we found that up-regulated PGR5-like protein 1A involved in cyclic electron flow around photosystem 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 promoting Rubisco activation and gene expression related to photosynthesis (Yu et al. 2004; Xia et al. 2009a). Moreover, our data on Rubisco enzyme activity also proved that EBR could accelerate CO$_2$ fixation and improve photosynthesis of pepper leaf. The assimilation of CO$_2$ may be associated with stomatal or non-stomatal factors or both. Here, the application of EBR improved the Gs and Tr but had a non-significant decrease in Ci 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). Rubisco activity, regeneration capacity, chlorophyll and Rubisco carboxylation are all possible reasons for the non-stomatal factor affecting photosynthesis (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 into chlorophyll apoprotein and the reaction-center subunit of the photosystem, chlorophyll a/b binding proteins are used for harvesting of light and energy transfer. There were 18 up-regulated and 18 down-regulated DEPs in the energy production and conversion category between the Chill and Chill + EBR treatment in this study. The down-regulated DEPs include chlorophyll a-b binding protein, 2-oxoisovalerate dehydrogenase subunit alpha, pyruvate dehydrogenase E1 component subunit alpha, dihydrolipoyl dehydrogenase, photosystem I P700 chlorophyll apoprotein A1, photosystem II CP43 chlorophyll apoprotein, photosystem II D2 protein, and photosystem Q(B) protein; all these proteins are Calvin cycle-specific enzymes and photosystem-related (Table 2; Fig. 6). Therefore, according to our study, chilling stress inhibits 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 temperatures.

**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 induces 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 signal transduction, cell proliferation, and anabolism that are responsible for resistance to abiotic stress in plants. 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, 14–3-3-like protein D, 14–3-3-like protein E 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 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 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 conditions 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 were up-regulated 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$_2$O$_2$) to H$_2$O, responsible for the oxidation of specific substances such as ascorbic acid in abiotic stress tolerance, and these are encoded by iso-enzymes that belong 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] and probable monodehydroascorbate reductase were up-regulated by EBR application under chilling stress conditions, and these proteins play a vital role in plant resistance to 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 a part of plant hormone biosynthesis (Bouvier et al. 1996). Zeaxanthin epoxidase protein was up-regulated by EBR application 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 proteins, new peptide subunit assembly, protein degradation, and repair of nucleolus damage. They have an automatic adjustment function and adjust their transcription and translation (DeMaio 1999). Interestingly, heat shock protein 82 was down-regulated 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.

Taken together, the EBR-modulated chilling stress signalling is received by a few receptor proteins 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. 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 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 the photosynthetic electron transfer chain, oxidative phosphorylation, GSH metabolism pathway, energy metabolism, and the Calvin cycle and ribosomes. Our results conclude 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 cell damage, leading to 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 the chilling tolerance of peppers, as 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-regulating the expression of proteins associated with photosynthesis, amino acid, and carbohydrate metabolism, signal transduction, and the maintenance of antioxidant and stress defense. Based on our study results, exogenous EBR application can improve growth and development of the pepper under chilling stress conditions. Moreover, the gene and protein information provided in this report can be utilized by plant biologists in pepper crop improvement programs.

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Author contributions JL conceived and designed the experiments. YP performed the experiments and collected the data. MAN and HS helped to perform the analysis and write up of this report. CL and PY contributed reagents/materials/analysis tools and overall supervised this study. All authors have read and approved the final manuscript.
Declarations

Conflict of interest The authors declare no competing financial interest.

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