A PROTEOMICS STUDY OF THE MUNG BEAN EPICOTYL REGULATED BY BRASSINOSTEROIDS UNDER CONDITIONS OF CHILLING STRESS

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Abstract: Mung bean *CYP90A2* is a putative brassinosteroid (BR) synthetic gene that shares 77% identity with the *Arabidopsis CPD* gene. It was strongly suppressed by chilling stress. This implies that exogenous treatment with BR could allow the plant to recover from the inhibited growth caused by chilling. In this study, we used proteomics to investigate whether the mung bean epicotyl can be regulated by brassinosteroids under conditions of chilling stress. Mung bean epicotyls whose growth was initially suppressed by chilling partly recovered their ability to elongate after treatment with 24-epibrassinolde; 17 proteins down-regulated by this chilling were re-up-regulated. These up-regulated proteins are involved in methionine assimilation, ATP synthesis, cell wall construction and the stress response. This is consistent with the re-up-regulation of methionine synthase and S-adenosyl-L-methionine synthetase, since chilling-inhibited mung bean epicotyl elongation could be partially recovered by exogenous treatment with DL-methionine. This is the first proteome established for the mung bean species. The regulatory relationship between brassinosteroids and chilling conditions was investigated, and possible mechanisms are discussed herein.

Key words: Proteomics, Chilling, Brassinosteroid, Mung bean, Epicotyl

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INTRODUCTION

A plant chilling injury means an injury that is caused by a temperature drop to below 15°C but above the freezing point [1]. Mung bean (*Vigna radiata* L.) is a chilling-sensitive plant that has been the subject of chilling studies for decades [2, 3]. In a previous study, mung bean *CYP90A2* (GenBank: AF279252) cDNA, which shares 77% identity with the *Arabidopsis CPD* (constitutive photomorphogenesis and dwarfism) cDNA, was found to be strongly suppressed by chilling [4]. The *Arabidopsis CPD* gene has been confirmed to be responsible for brassinosteroid synthesis, and its mutant showed a strong dwarf phenotype [5]. This implied that under chilling conditions, brassinosteroid (BR) synthesis failure can inhibit the growth of mung bean seedlings, and that exogenous treatment with BR could allow the plant to recover from this growth inhibition.

BRs were discovered in the 1970s, and subsequent genetic analysis has shown that they are essential phytohormones required for cell growth, vascular differentiation, the stress response, male fertility, pigment biosynthesis, and other developmental responses [6]. Increasing attention has been drawn to their role in the response to chilling [7]. An improvement in the growth rate under chilling conditions was observed in cucumber upon BR treatment [8].

Proteomics is a powerful tool that is increasingly applied in the study of almost any aspect of protein expression or function. However, little proteomics-based research has been done on BR-promoted cell growth [9]. Moreover, the correlation between BR and the stress response is not yet fully understood. This study aimed to establish the expression profile of mung bean epicotyl proteins regulated by BR under chilling conditions; this might contribute in part to the disclosure of the molecular mechanism behind this phenomenon.

MATERIALS AND METHODS

Plant material and growth conditions

Mung bean (*Vigna radiata* L., cv 2937) seedlings were germinated on moist vermiculite for 5 days in a growth chamber (28ºC). The growth conditions were set at 70% RH, with a photoperiod of 16 h light, 8 h dark, and a light intensity of 200 $\mu molm^{-2}s^{-1}$. Another growth chamber was set at 10ºC, and the light intensity was set at a lower level of 100 $\mu molm^{-2}s^{-1}$ to avoid photooxidation. Each pot contained ten unique 5-day old seedlings, and underwent the subsequent treatments in this study. The stocks of chemicals were from Sigma, MO, USA: abscisic acid (ABA, 100 mM/NaOH), benzyladenine (BA, 5 mM/NaOH), 24-epibrassinolide (EBL, 3 mM/DMSO), indole-3-acetic acid (IAA, 10 mM/NaOH), gibberellic acid (GA$_3$, 30 mM/EtOH), putrescine (10 mM/H$_2$O), DL-methionine (Met, 100 mM/NaOH) and Brz220 (10 mM/DMSO). The seedlings were sprayed with two milliliters of the diluted chemicals containing 0.01% (v/v) tween 20 once before treatments. The optimal concentration of chemicals applied was determined by analyzing the epicotyl elongation rate. After the treatments, the epicotyls were cut and used for Northern, Western and 2-DE analysis.

Preparation of CYP90A2 polyclonal antibody
The cDNA sequence of the hydrophilic region (~20 kDa) of the CYP90A2 protein was amplified by 5'ATTCCCGGGGGTTCAGCCGGCGGAAGTTTCGG and 3'CCGCTCGAGGAGAGTGAAGAAGCCTTCAATG primers, which contained an *Sma* I site and *Xho* I site at each primer end. The DNA fragment was ligated into pGEX-4T-1 plasmid and transformed into *E. coli* BL21. IPTG (0.1 mM) was added into the culture medium to induce the expression of the CYP90A2/GST fusion protein under culture conditions at 20ºC. The extracted CYP90A2 fusion protein was cleaved by 10 × cleavage buffer (THROMBIN Clean Cleave™ Kit, Sigma) and was separated from GST protein by SDS-PAGE. The coomassie blue-stained CYP90A2 protein was eluted by Model 422 electro-eluter (Bio-Rad, CA, USA) and the antibody was prepared according to Harlow and Lane [10].

Northern hybridization
Mung bean epicotyls were ground in liquid nitrogen and the total RNA was extracted using Trizol reagent (Invitrogene, CA, USA) according to the manufacturer's instructions. Twenty micrograms of RNA was loaded and electrophoresed in a 0.8% formaldehyde-denatured agarose gel. The blotted Hybond-N$^+$ membranes (Amersham Pharmacia Biotech, Buckinghamshire, England) were hybridized with a [^{32}P]-dCTP radioisotope-labeled full-length *CYP90A2* cDNA probe (Random Prime Labelling System, Amersham Pharmacia Biotech). The membranes were prehybridized (42ºC), hybridized (42ºC), washed (65ºC), and subjected to autoradiography using the standard procedure [11].

Protein extraction
Mung bean epicotyls were ground in liquid nitrogen and suspended with an extraction buffer: 50 mM Tris-HCl (pH 8.0), 3 mM phenylmethylsulfonyl fluoride (PMSF), 1% (v/v) 2-mercaptoethanol, 0.2% (v/v) Triton X100, 10% (w/v) sucrose and 10 mM ascorbic acid with a 1:5 tissue/buffer ratio. After centrifugation at 12000 g for 10 min, the supernatants were precipitated with acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.3% (w/v) dithiothreitol (DTT) at -20ºC overnight. The protein pellets were washed with acetone twice and air-dried.

Western hybridization
The dried protein pellets from the chilling treatments (0 d~4 d) were dissolved in SDS sample buffer: 62.5 mM Tris-HCl (pH 6.8), 3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. Forty micrograms of proteins were separated by SDS-PAGE and blotted onto Hybond-P PVDF membrane (Amersham Pharmacia Biotech) with the Semi-phor system (Hoefer Pharmacia Biotech, CA, USA). The blotted membrane was blocked in Gelatin-blocking buffer, and hybridized with CYP90A2 polyclonal antibody (1:1500) and Goat anti-rabbit IgG antibody (1:3000) with alkaline phosphatase conjugate (Sigma) according to the standard methods [11]. The expression level of CYP90A2 protein was calculated from three repeats with a Phosphorimager destimeter (Amersham Pharmacia Biotech).

IEF assay and second dimension SDS-PAGE

The dried protein pellets from four treatments – seedlings grown at 28ºC sprayed with 2 ml of 10 µM EBL (28/EBL), their control (28/Control), seedlings grown at 10ºC sprayed with 2 ml of 10 µM EBL (10/EBL), and their control (10/Control) – were dissolved in a sample buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 60 mM DTT and 2% (v/v) pH 4-7 IPG buffer [12]. The epicotyl proteins (300 µg) were mixed with DeStreak Rehydration solution and soaked into 18 cm DryStrip (Amersham Pharmacia Biotech) for rehydration on the Ettan IPGphor system. The voltage was set to 32 kVhr for DryStrip pH 4-7. After IEF analysis, the stripped gels were subjected to reduction with 1% (w/v) DTT in an equilibration buffer: 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8), 6 M urea and 30% (v/v) glycerol, and followed by alkylation with 2.5% (w/v) iodoacetamide (IAA) in the same buffer. The equilibrated gels were attached with 0.5% agarose to the top of a vertical 12.5% SDS-PAGE system modified from Laemmli [13].

Image analysis and In-gel protein digestion

The 2-D electrophoresis gels were stained with SYPRO-Ruby (Molecular Probes, OR, USA) and digitally scanned using a Typhoon 9400 fluorescence image scanner (Amersham Bioscience, NJ, USA). Protein spots were automatically detected and analyzed using ImageMaster software (Amersham Bioscience). The experiments were repeated three times, and only the proteins that corresponded to epicotyl elongations under four treatments were excised and subjected to the in-gel digestion process according to the guidelines given in the In-Gel Tryptic Digestion Kit (Pierce, IL, USA) and research articles [14].

MALDI-quadrupole (Q)-TOF MS/MS analysis

The lyophilized samples were resuspended in 0.1% trifluoroacetic acid (TFA). The matrix, alpha-cyano-4-hydroxycinnamic acid (CHCA), was premixed in 30% acetonitrile (ACN)/0.1% TFA solution (w/v). The sample and CHCA mixture were loaded onto the MALDI plate (PerSeptive Biosystems, CA, USA) via the dried droplet method [15]. MALDI-quadrupole (Q)-TOF-MS was performed on a Voyager-DE PRO (PerSeptive Biosystems) operating in reflector mode. The instrument was equipped with a 337-nm nitrogen laser source operating at a frequency of 3-20 Hz. The spectra were recorded in reflector mode at an acceleration voltage of 20 kV, 70% grid voltage, 0% guide wire voltage, 100 ns delay and a low mass gate of 500 Da. Fifty laser shots were averaged for a typical spectrum. The mass calibration of the spectra was obtained by using mixtures of three reference peptides (human angiotensin I, ACTH clip 1-17 and ACTH clip 18-39) covering the m/z 500-3500 range. The most intense ions in the TOF-MS spectrum were selected for optimized MS/MS analysis. The MS/MS data was searched against the NCBInr database of all green plants using the MASCOT search program (http://www.matrixscience.com/) and presented according to the published guidelines [16].

RESULTS AND DISCUSSION

Feedback regulation and the chilling response of mung bean *CYP90A2*
Feedback inhibition of biosynthetic genes by end products is a classical mechanism in hormonal homeostasis [17]. In previously reported research, all of the BR biosynthetic genes cloned from *Arabidopsis* were down-regulated in the presence of BR [18]. In this study, we also found that the transcription of the mung bean *CYP90A2* gene was down-regulated by BR and up-regulated by the BR synthesis inhibitor, Brz220 (Fig. 1A). With its high sequence homology (77%) to the *Arabidopsis CPD/CYP90A1* gene and the feedback regulation in its gene transcription, *CYP90A2* was postulated to be involved in BR biosynthesis in mung bean, though further experiments should be conducted to confirm this. In the temperature response, we found that the expression of the *CYP90A2* gene was strongly suppressed by chilling within a day, and that the protein level started to decrease after three days (Fig. 1B, C, and D). This implied that exogenous treatment with BR could allow a plant to recover from the growth inhibition caused by chilling.
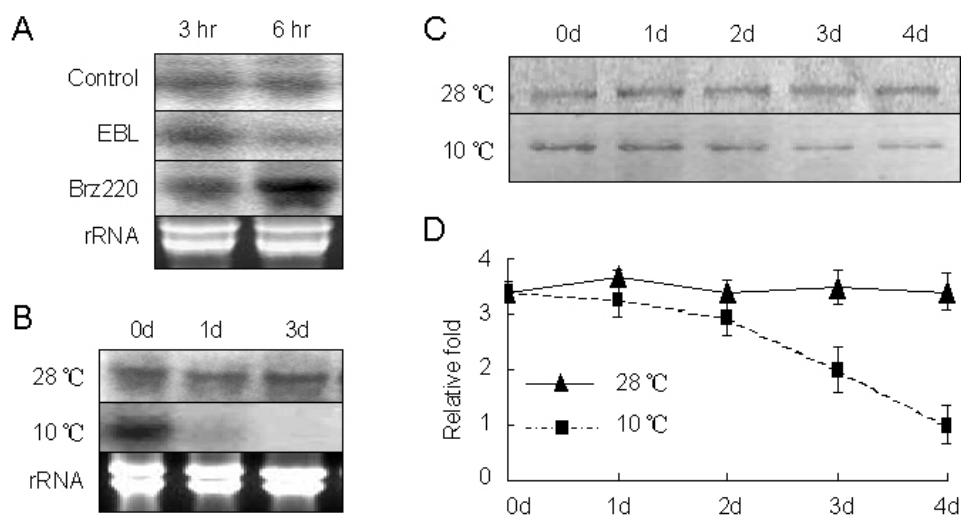

Fig. 1. The feedback regulation and chilling suppression of mung bean *CYP90A2*. After spraying with 2 ml of 10 μM EBL, 10 μM Brz220 and an equal amount of solvent, the 5-day old seedlings were kept in a 28ºC growth chamber and harvested at 3-h intervals (A). In the chilling response, 5-day old seedlings without pretreatment were transferred to a 28ºC or 10ºC growth chamber for 3 days (B) or 5 days (C). The blotted membranes from A and B were hybridized with radioisotope-labeled full-length *CYP90A2* cDNA. EtBr-stained rRNA was used as loading control. The blotted membrane from C was hybridized with CYP90A2 polyclonal antibody. The expression level of CYP90A2 protein was statistically calculated from three repeats (D).

BR-promoted epicotyl elongation and re-up-regulation of proteins affected by chilling conditions

The growth-promoted effect of BRs on plants under chilling conditions was discussed in Krishna [7]. Brassinolide, 28-homobrassinolide and EBL are the three biologically active BRs that have been widely used in physiological studies [19]. In this study, pretreatment with 10 µM EBL not only significantly
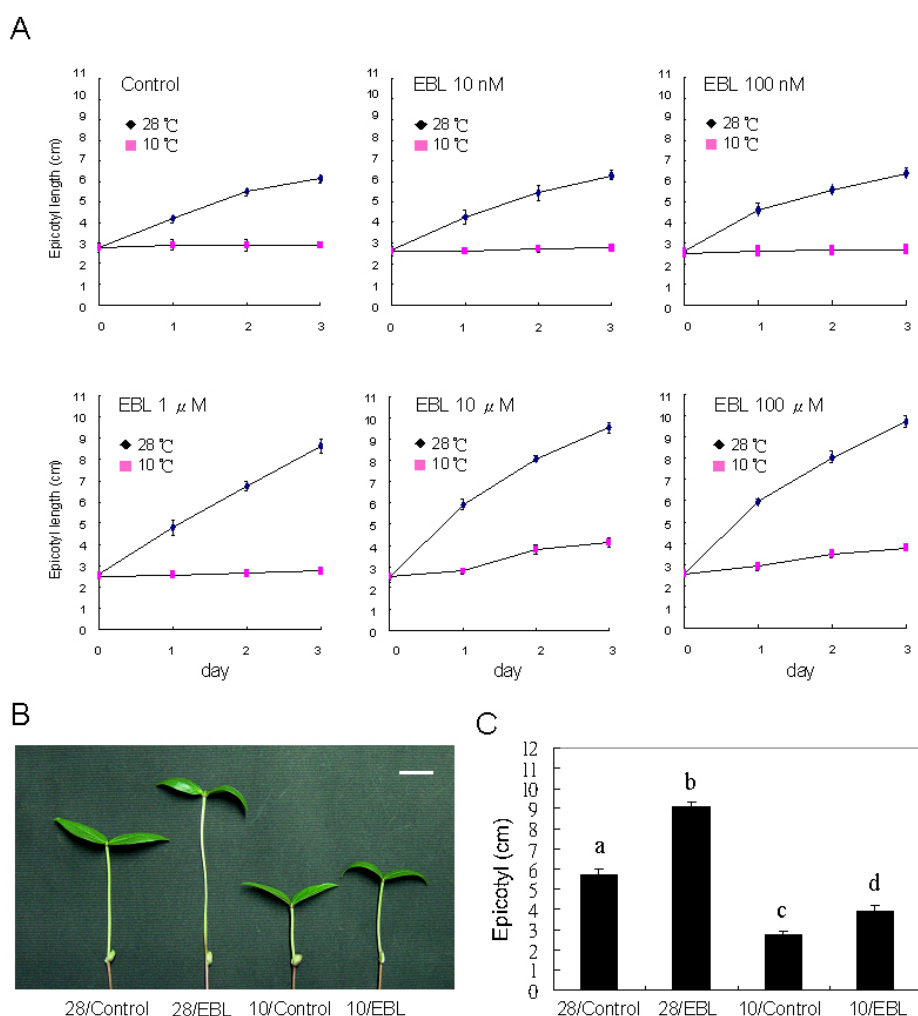

Fig. 2. BR-promoted seedling growth. After spraying with 2 ml of a serial dilution of EBL or a control treatment, the 5-day old seedlings were transferred to a 28ºC or 10ºC growth chamber for three days (A). The closer comparison (B) and the statistical data (C) of epicotyl length promoted by pre-treatment with 10 µM EBL were shown. Statistic bars marked with a different letter were significantly different using Fisher's LSD ($p < 0.05$).
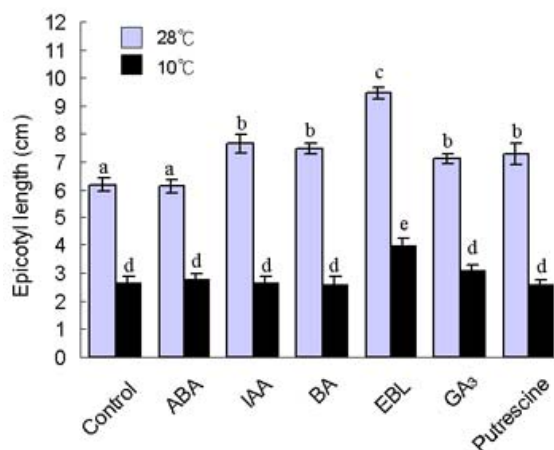
Fig. 3. A comparison of the elongation effect between phytohormones. After spraying with 2 ml of ABA (1 μM), IAA (10 μM), BA (1 μM), EBL (10 μM), GA$_3$ (1 μM), and putrescine (1 μM), the 5-day old seedlings were transferred to a 28ºC or 10ºC growth chamber for three days. Statistic bars of epicotyl length marked with a different letter were significantly different using Fisher's LSD ($p < 0.05$).

promoted epicotyl elongation, but also allowed the plant to partially recover from the growth-inhibiting effects of chilling (Fig. 2). Comparing this elongation effect to that of other phytohormones, such as ABA, BA, IAA, GA$_3$ and putrescine, we found that EBL showed the most active effect in epicotyl elongation (Fig. 3). In order to gain insight into BR-regulated proteins, epicotyls from four treatments (28/Control, 28/EBL, 10/Control, and 10/EBL) were subjected to proteomic analysis. In the 2-D electrophoresis assay, more than 622 epicotyl proteins were detected on SYPRO-Ruby stained gels (Fig. 4A). As shown in the enlarged insets in Fig. 4B, from a comparison of the 28/Control and 10/Control treatments, 24 proteins were down-regulated and 5 were up-regulated under chilling conditions. Furthermore, 18 proteins were up-regulated and 5 were down-regulated by BR in the 28ºC control and treatment. By integrating the 2-DE of the four treatments (28/Control, 28/EBL, 10/Control, and 10/EBL), seventeen proteins down-regulated by chilling were re-up-regulated upon EBL treatment; protein No. 14 was down-regulated under both chilling conditions and upon EBL treatment.

Protein identifications and putative functions in the BR-mediated recovery of growth in seedlings affected by chilling conditions
In the MALDI-TOF MS/MS analysis, ten of the re-up-regulated proteins and one down-regulated protein were successful identified with significant hits (P < 0.05) in MASCOT probability analysis (Tab. 1). A closer comparison of these proteins and the fold change were represented individually, as shown in Fig. 5. According to the reported functions, these proteins were predicted to be involved in cell growth, wall formation, ATP production, the stress response, and methionine assimilation.

Hemicellulose is a major component of primary plant cell walls, and many of the glycosyl residues found in hemicellulose are derived from the sugar precursor UDP-glucuronic acid (UDP-GlcUA). UDP-glucose dehydrogenase (UGD), which controls the biosynthesis of UDP-GlcUA, shows a characteristic increased activity under diverse conditions during growth, differentiation, stress responses and pathogenic attack [20]. Though the involvement of UDG in BR-related studies has scarcely been discussed, we believe that relief of the cell wall limitation is the primary step in BR-mediated cell elongation.

Microtubules constructed from two closely related 55 KDa proteins called alpha and beta tubulin have an important role in directing cell enlargement. After BR treatment of *Arabidopsis bul1* and *dim1* mutants, microtubules reorganized and became correctly oriented, suggesting the involvement of BR in microtubule organization and cell elongation [21]. In rice proteomics, tubulin content also exhibited an evident increase upon BR treatment [22]. Therefore, the re-up-regulation of alpha tubulin was considered to correspond highly to the BR-mediated growth observed in this study.

It is well known that a growing cell requires a synchronized increase in ATP levels. It has been reported that the activities of enolase and ATP synthase, the two key enzymes responsible for glycolysis and electron-drive ATP synthesis, decline under chilling conditions [23]. Resembling our prediction that BR-mediated cell growth recovery post-exposure to chilling conditions was accompanied by ATP production, enolase and F1 ATPase were re-up-regulated in our study. By contrast to ATP synthase, which generally functions to synthesize ATP, vacuole ATP synthase (V-ATPase) is responsible for the acidification of intracellular compartments and generation of an electrochemical gradient by hydrolyzing ATP. The importance of V-ATPase in cell growth has been proved in the *Arabidopsis det3* mutant, in which the large C-subunit of the V-ATPase is encoded, and which exhibits a dwarf phenotype [24]. The significance of V-ATPase for the adaptation to stressful growth conditions was also discussed. Under stress conditions such as high salinity, drought, cold, acid stress, anoxia, and excess heavy metals in the soil, survival of the cells strongly depends on maintaining or adjusting the activity of the V-ATPase [25]. Therefore, the involvement of BR-enhanced V-ATPase in mung bean epicotyl elongation affected by chilling temperatures could be considered, and further analysis could be conducted on this in the future.

Fructose-1,6-bisphosphate aldolase (FBP aldolase) is a constituent of both the glycolytic and gluconeogenic pathways in plants. Therefore, an increase and/or activation of aldolase appears to be implicated in growth mainly through the promotion of ATP synthesis in the glycolytic pathway. The involvement of FBP aldolase in cell growth was identified in rice roots treated with gibberellin [26]. In this study, FBP aldolase showed down-regulation under either chilling conditions or upon BR treatments (Fig. 5). Concerning this opposing result that a growing cell requires ATP in bulk, further experiments should be conducted to

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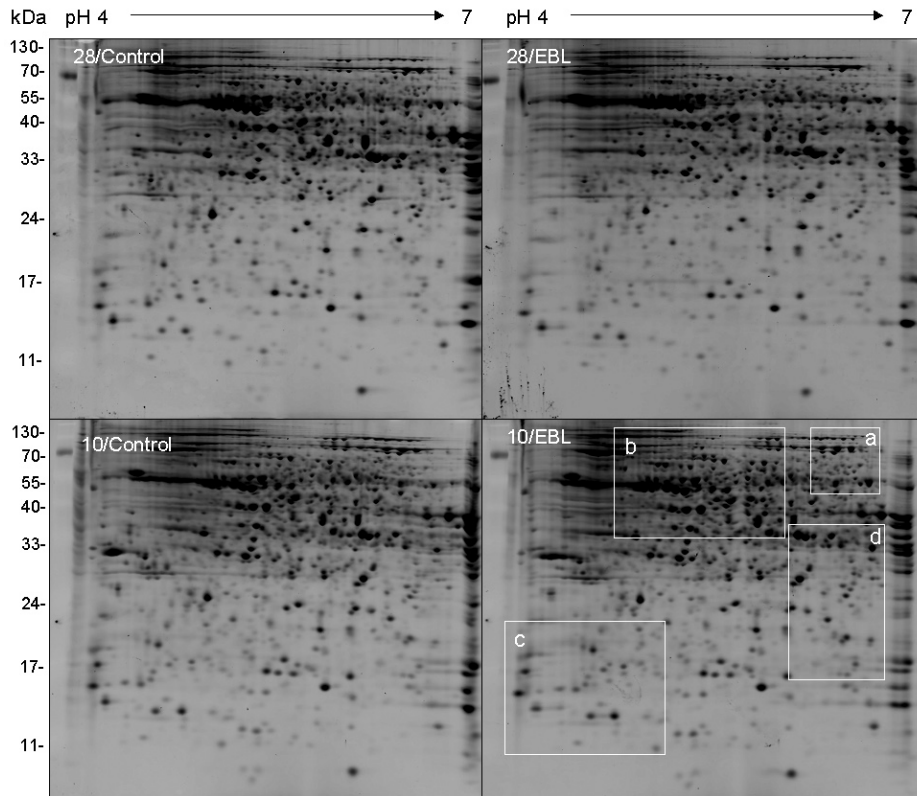
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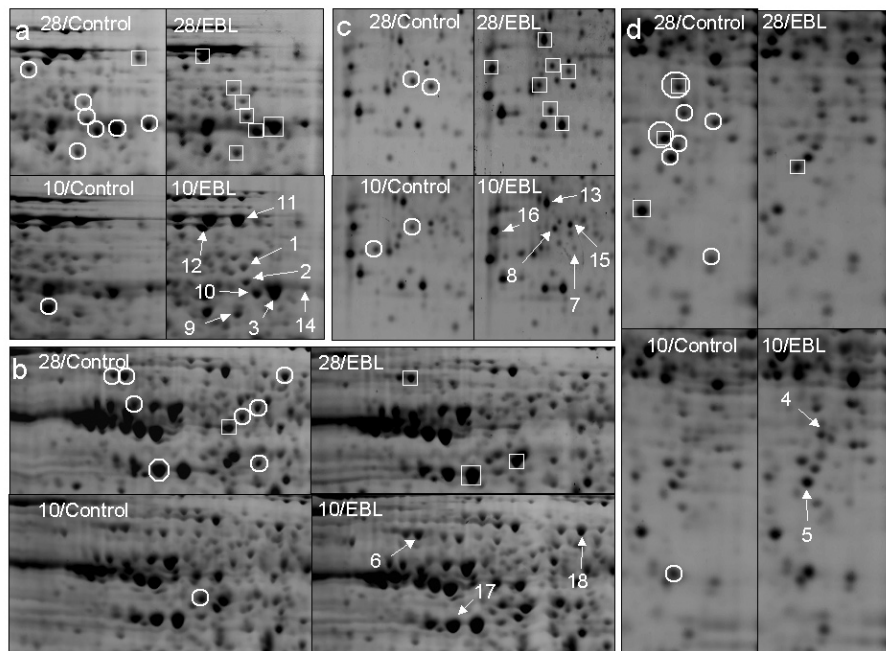
Tab. 1. Differentially expressed proteins identified by MS/MS.

Spot No.	Protein	AC[a]	Exp.Mr (kDa)/pI	Theo.Mr (kDa)/pI	Score	Sequence[b]
1	Aspartate-tRNA ligase	7269540	61.2/ 6.6	57.1/ 6.0	75	YGAPPHGGFGVGLER
2	UDP-glucose dehydrogenase	11994517	56.3/ 6.6	53.1/ 5.7	110	AADLTYWESAAR LSIYDPQVTEDQIQR
3	F1-ATPase alpha subunit	34539443	46.3/ 6.8	44.6/ 6.6	115	VVDALGVPIDGR RVEVQAPGIIER EAFPGDVFYLHSR
4	Mung bean seed albumin	1000708	28.6/ 6.6	30.2/ 6.8	47	NTVFADSIDSAFR
6	Vacuolar ATP synthase	67952	67.3/ 5.1	68.8/ 5.3	82	NIIHFYNLANQAVER
9	Enolase	42521309	45.4/ 4.7	47.7/ 5.3	315	VNQIGSVTESIEAVR AAVPSGASTGIYEALELR IEEELGAEAVYAGANFR AAVPSGASTGIYEALELRDGGSDYLGK
10	SAM synthetase	1655578	46.2/ 6.7	43.0/ 5.5	212	AQVDYEKIVR YLDEKTIFHLNPSGR TIFHLNPSGR FVIGGPHGDAGLTGR FVIGGPHGDAGLTGRK
11	Methionine synthase	17017263	88.5/ 6.5	84.4/ 5.7	132	YLFAGVVDGR YGAGIGPGVYDIHSPR TLTSLNGVTAYGFDLVR
12	Methionine synthase	17017263	88.4/ 6.4	84.4/ 5.7	177	YLFAGVVDGR YGAGIGPGVYDIHSPR ALAGQKDEAYFAANAAAQASR
14	Cytosolic FBP aldolase	3021338	53.2/ 6.9	38.5/ 6.2	68	VAPEVVAEHTVR GILAADESTGTIGKR
17	Alpha tubulin	296494	44.6/ 5.2	49.8/ 4.9	103	AVFLDLEPTVIDEVR QLFHPEQLISGKEDAANNFAR

a) Accession number, b) The sequence of matched peptides

Fig. 4. Proteins regulated by EBL under both normal and chilling conditions. Three hundred micrograms of epicotyl proteins from four treatments (28/Control, 28/EBL, 10/Control, and 10/EBL) were analyzed using 2-D gel electrophoresis and ImageMaster software. The proteins showing consistency from the three repeats are in windows a, b, c, and d (A). In the enlarged panels (B), the open circles indicated in the control treatment (28/Control) represent proteins down-regulated by chilling, while they represent the proteins up-regulated in the chilling treatment (10/Control). The open rectangles indicated in the control treatment (28/Control) represent the BR down-regulated proteins, while they represent the BR up-regulated proteins in the EBL treatment (28/EBL). The numbers indicated by arrows in the 10/EBL treatment represent proteins that are specifically regulated by BR under chilling conditions.
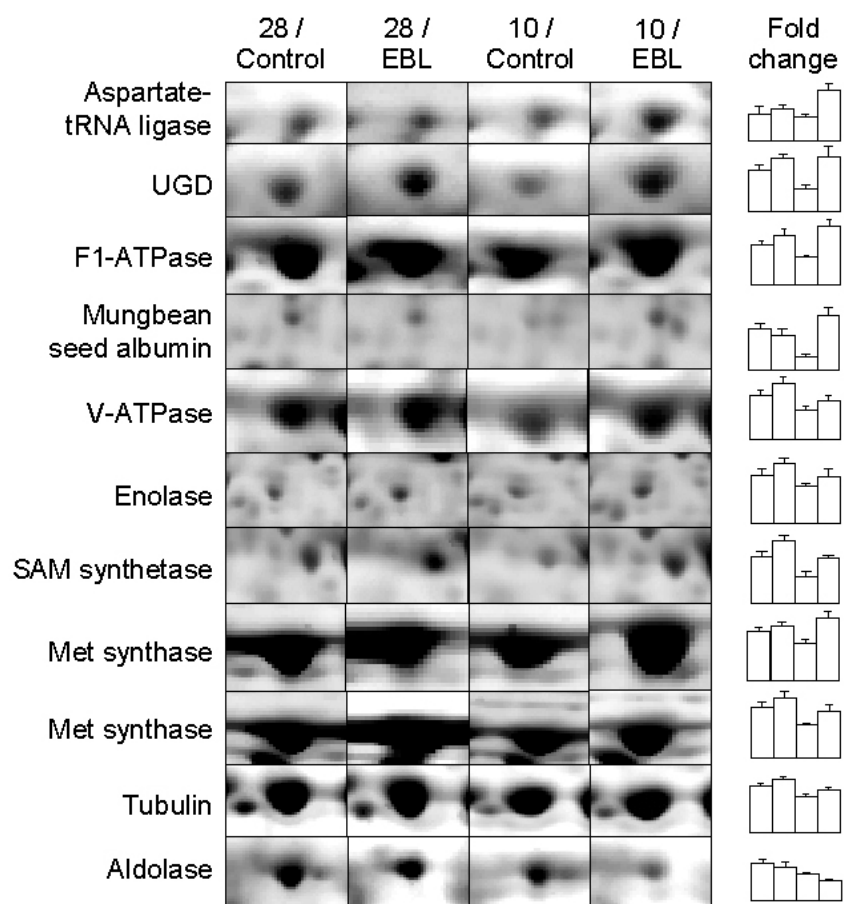
Fig. 5. The comparisons of MS/MS identified proteins. Eleven MALDI-TOF MS/MS identified proteins were compared, and the fold change was statistically calculated from three repeats.

monitor the intermediate metabolites of glycolysis, such as glyceraldehyde-3-phosphate and fructose-1,6-biphosphate.

Seed albumins are a major group of storage proteins present as plant food allergens. They have been observed to be highly compact, which possibly confers stability against thermal denaturation and digestion by proteolytic enzymes, and enables plants to resist biotic and abiotic stresses [27]. In this study, mung bean seed albumin (MBSA) was re-up-regulated by BR specifically under chilling conditions but not under normal conditions (Fig. 5). Regardless of other seed albumin functions, here we conclude that they might be involved in the chilling response.

Exogenous treatment of methionine-promoted epicotyl elongation under normal and chilling conditions

Among the proteinogenic amino acids, methionine (Met) and *S*-adenosylmethionine (SAM) display many direct or indirect essential functions in cellular metabolism. In growing *Arabidopsis* seedlings, a synchronized increase in the levels of methionine synthase and SAM synthetase was found [28]. The accumulation of SAM synthetase genes at a high concentration of NaCl shows a possible role of SAM synthetase in the adaptation to salt stress [29]. Here, we showed that two methionine synthases and one SAM synthetase were up-regulated by EBL under both normal and chilling conditions. Coincidentally, epicotyl elongation was observed to be promoted by treatment with Met under normal and chilling conditions (Fig. 6) just as observed upon BR treatment (Fig. 2). The importance of methionine in cell growth has been reported for both plant and animal cells, particularly for the human cancer cell [30, 31]; however, the crosstalk between BR's action and methionine's is minimal. Therefore, the possible involvement of methionine assimilation downstream of BR's action may be considered a novel project for future research.
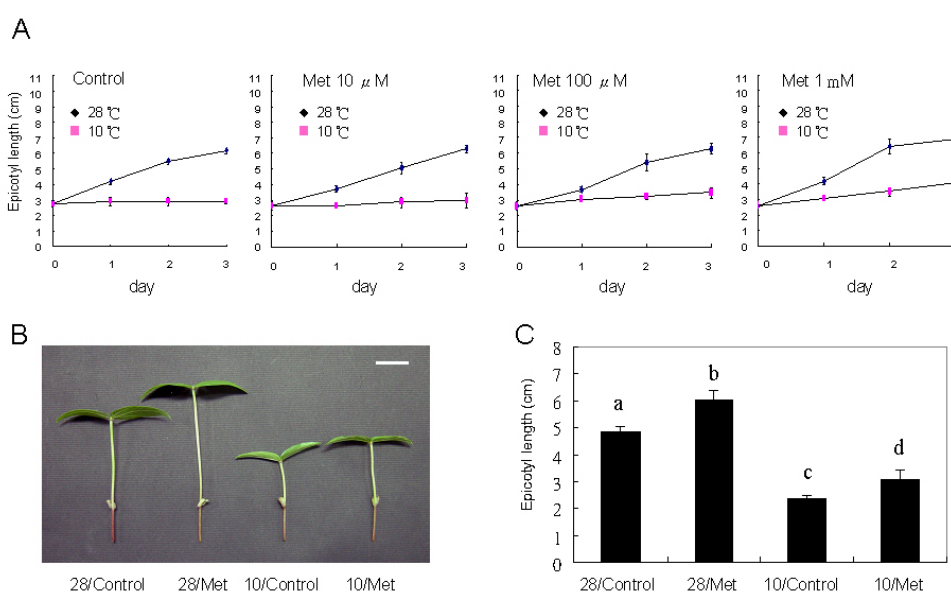

Fig. 6. Methionine-promoted seedling growth. After spraying with 2 ml of the serial dilution of methionine or the control treatment, the 5-day old seedlings were transferred to a 28ºC or 10ºC growth chamber for three days (A). The closer comparison (B) and the statistical data (C) for epicotyl length promoted by pre-treatment with 1 mM methionine were shown. Statistic bars marked with a different letter were significantly different using Fisher's LSD ($p < 0.05$).

CONCLUSIONS

Using proteomics, we identified ten down-regulated proteins that were re-up-regulated after BR treatment under chilling conditions. Most of these proteins were predicted to be involved in cell growth, wall formation, ATP production, the stress response, and methionine assimilation. In addition to its role in plant growth and development, the molecular function of BR in the response to chilling stress was revealed. Moreover, the involvement of methionine in plant growth under chilling conditions and its correlation to BR's action were also considered. In this study, the proteomes of chilling treatment, BR response and the BR-antistress reaction were established for the mung bean; their physiological functions require further investigation.

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