Proteomic Analysis of Cold Stress Responses in Banana Leaves

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ABSTRACT. Cold stress is one of the most important environmental factors affecting crop growth and agricultural production. Induced changes of gene expression and metabolism are critical for plants responding and acclimating to cold stress. Banana (*Musa* sp.) is one of the most important food crops in the tropical and subtropical countries of the world. Banana, which originated from tropical regions, is sensitive to cold, which can result in serious losses in commercial banana production. To investigate the response of the banana to cold stress conditions, changes in protein expression were analyzed using a comparative proteomics approach. ‘Brazil’ banana (*Musa acuminata* AAA group) is a common banana cultivar in southern China. ‘Brazil’ banana plantlets were exposed to 5 °C for 24 hours and then total crude protein was extracted from treatment and control leaves by phenol extraction, separated with two-dimensional gel electrophoresis, and subsequently identified by mass spectrometry (MS). Out of the more than 400 protein spots reproducibly detected, only 41 protein spots exhibited a change in intensity by at least 2-fold, with 26 proteins increasing and 15 proteins decreasing expression. Of these, 28 differentially expressed proteins were identified by MS. The identified proteins, including well-known and novel cold-responsive proteins, are involved in several cellular processes, including antioxidation and antipathogen, photosynthesis, chaperones, protein synthesis, signal transduction, energy metabolism, and other cellular functions. Proteins related to antioxidation, pathogen resistance, molecular chaperones, and energy metabolism were up-regulated, and proteins related to ethylene synthesis, protein synthesis, and epigenetic modification were down-regulated in response to cold temperature treatment. The banana plantlets incubated at cold temperatures demonstrated major changes in increased reactive oxygen species (ROS) scavenging, defense against diseases, and energy supply. Increased antioxidation capability in banana was also discovered in plantain, which has greater cold tolerance than banana in response to cold stress conditions. Therefore, we hypothesized that an increased antioxidation ability could be a common characteristic of banana and plantain in response to cold stress conditions. These findings may provide a better understanding of the physiological processes of banana in response to cold stress conditions.

Cold stress is one of the major environmental factors that often affects crop growth, productivity, quality, and postharvest life (Sanghera et al., 2011). Cold stress can be classified as chilling (0 to 15 °C) or freezing (<0 °C) stress. To cope with such conditions, plants require specialized mechanisms to survive. Plants originating from temperate regions that are exposed to low, nonfreezing temperatures exhibit an increase in the extent to which they can tolerate freezing, which is a phenomenon known as “cold acclimation” (Thomashow, 1999). Significant progress has been made in understanding the molecular mechanisms that lead to cold acclimation in plants such as *Arabidopsis* [*Arabidopsis thaliana* (Miura and Furumoto,

2013)], winter wheat (*Triticum aestivum*), and rye (*Secale cereale*) (Chinnusamy et al., 2007). To adapt to cold stress, gene expression is reprogrammed and metabolism is modified through alterations in transcriptional and posttranscriptional regulation as well as translational and posttranslational regulation of cold stress signaling during cold acclimation (Barrero-Gil and Salinas, 2013; Miura and Furumoto, 2013). However, plants originating from tropical and subtropical origins are sensitive to chilling stress and lack this described cold acclimation mechanism. Proteomics is a powerful molecular tool for investigating the complete proteome in the organelle, cell, organ, or tissue at a given time point or under given physiological conditions and for comparing how the proteome is affected by different physiological conditions (Sobhanian et al., 2010). In addition, the combination of proteomics with measurements of changes in complementing transcriptome levels can identify translational and posttranslational regulations (Ghosh and Xu, 2014). A comparative proteomic approach has recently emerged as a powerful and promising tool for identifying genes and pathways that are crucial for stress responsiveness and tolerance of plants (Abdalla and Rafudeen, 2012; Gao et al., 2011; Zhang et al., 2010).

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Moreover, several proteomic studies have been successfully performed in banana (Carpentier et al., 2007; Esteve et al., 2013; Samyn et al., 2007; Toledo et al., 2012; Vanhove et al., 2012; Zhang et al., 2012).

Musa species, including bananas and plantains, are the fourth most important crop in developing countries (Lescot et al., 2008) and are giant perennial herbs from the monocot order Zingiberales, which originated in Southeast Asia and the western Pacific (Davey et al., 2013). Cultivated bananas and plantains are mainly triploids that derive from intraspecific hybridizations within *Musa acuminata* (A genome) and interspecific hybridizations with *Musa balbisiana* (B genome) (Davey et al., 2013). The current production mainly relies on somaclones derived from a single triploid genotype, 'Cavendish' (D'Hont et al., 2012). Banana, which originated from tropical regions, is cultivated in tropical and subtropical regions throughout the world (Heslop-Harrison and Schwarzhacher, 2007) and is sensitive to cold. Importantly, cold stress can result in serious loss in commercial banana production (Feng et al., 2010).

Our understanding to date of cold stress mechanisms in banana is limited. Therefore, in this study we used a comparative proteomic approach in an attempt to examine the effects of the earliest stages of cold stress on banana at the protein level. We show that 28 proteins altered in response to cold stress in banana, including well-known and novel cold-responsive proteins, are involved in several cellular processes, including antioxidation and antipathogen, photosynthesis, chaperones, protein synthesis, signal transduction, energy metabolism, and other cellular functions. The banana plantlets grown at cold temperatures demonstrated major changes including increased ROS scavenging, defense against diseases, and energy supply. Moreover, identification of cold stress response-related proteins could be useful biomarkers for facilitating conventional breeding approaches for cold-tolerant banana cultivars.

Materials and Methods

PLANT MATERIALS AND TREATMENT. 'Brazil' banana plantlets were cultivated in 15-cm plastic pots filled with a mixture of loam and coconut husk (60:40, v/v) under greenhouse conditions at the Institute of Tropical Bioscience and Biotechnology, Haikou, China. Greenhouse conditions were 12 h of light (300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ fluorescent lamps) at 28 °C and 12 h of darkness at 20 °C. The relative humidity was maintained at 80%. The plantlets were watered twice per week with tap water and once per week with a plant nutrient solution [0.7 g·L⁻¹ KNO₃, 0.7 g·L⁻¹ Ca(NO₃)₂, 0.8 g·L⁻¹ Ca(H₂PO₄)₂·H₂O, 0.28 g·L⁻¹ MgSO₄, 0.12 g·L⁻¹ Fe₂(SO₄)₃, 0.6 mg·L⁻¹ (NH₄)₆MO₇O₂₄·4H₂O, 0.6 mg·L⁻¹ H₃BO₃, 0.6 mg·L⁻¹ MnSO₄, 0.6 mg·L⁻¹ ZnSO₄, and 0.6 mg·L⁻¹ CuSO₄]. For the cold treatments, plantlets with four well-developed leaves were placed in climate cabinets (12 h light/12 h dark photoperiod at 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ fluorescent lamp) and left at 5 °C and 70% relative humidity for 24 h. Independent experiments were carried out three times, and five plantlets were used for each experiment. The mature flag-leaves from cold-treated plantlets and the control were cut by scalpel at 0800 HR, and harvested leaves were dipped in liquid nitrogen, and stored at -80 °C until needed.

PROTEIN EXTRACTION. Proteins from the leaves were extracted according to the phenol procedure previously described (Zhang et al., 2012). The resulting protein pellet was resuspended in an appropriate quantity of lysis buffer (15 L·kg⁻¹) containing

9.0 mol·L⁻¹ urea, 65 mmol·L⁻¹ dithiothreitol (DTT), 40 mmol·L⁻¹ Tris, and 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) supplemented with 0.8% immobilized pH gradients (IPG)-buffer and incubated at room temperature for 1.5 h. After centrifugation at 16,000g_n for 20 min at 4 °C, the supernatant was stored at -80 °C. The protein concentration was determined using the Bradford assay (Bradford, 1976). Protein concentration was calculated according to a standard protein curve using bovine serum albumin as a standard. The absorbance was measured using a spectrophotometer (UV2250; Shimadzu Corporation, Kyoto, Japan).

TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE). The 2-DE was carried out as previously described (Zhang et al., 2012). Protein concentration was normalized to 1 g·L⁻¹ in rehydration buffer [8 mol·L⁻¹ urea, 4% (w/v) CHAPS, 10% (v/v) glycerol, 0.002% (v/v) bromophenol blue, 0.5% (v/v) IPG-buffer, 1% (w/v) DTT] and 400 μg protein was loaded per gel. Gel strips (17 cm, pH 4-7; Bio-Rad, Hercules, CA) were hydrated for 14 h. Isoelectric focusing (IEF) was performed using the IEF system (Bio-Rad) at 17 °C with a current limit of 50 mA/strip: 0.5 h at 250 V, 1 h at 1000 V, 5 h at 10,000 V, and 60,000 V h at 10,000 V. Before separation along the second dimension, individual strips were equilibrated for 15 min in 5 mL equilibration solution [6 mol·L⁻¹ urea, 20% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), 0.002% (w/v) bromophenol blue, 1.5 mol·L⁻¹ Tris pH 8.8] containing 2% (w/v) DTT and subsequently for 15 min in 5 mL equilibration buffer containing 2.5% (w/v) iodoacetamide. Separation in the second dimension was performed on a vertical electrophoresis system (Bio-Rad) with 1 mm SDS polyacrylamide gels (12%). Electrophoresis was performed for 30 min at 120 V, followed by 200 V until the bromophenol blue line ran off. The protein spots were stained with colloidal Coomassie brilliant blue G-250 (cCBB). At least three replicates were performed for each sample.

MS ANALYSIS AND DATABASE SEARCH. 2-DE gels images were captured with a scanner (Powerlook-2100XL; UMAX Technologies, Dallas, TX) and scanned gel images (400 dpi) were imported into Image Master 2D Platinum (Amersham Biosciences, Geneva, Switzerland). The parameters were set as follows: Smooth 6, Mini Area 1, Saliency 8.00000. To verify the autodetected result, all spots were manually edited. Three well-separated gels of each sample were named as replicate groups. Statistical and quantitative analyses were used to compare the control group and the treated group. A paired *t* test and significance level of 95% were used for the analysis. In the quantitative analysis, the upper limit and the lower limit were set to 2.0 and 0.5, respectively. The spots were compared among three biological replicates. Only spots displaying reproducible change patterns were considered to be differentially expressed proteins and chosen for further analysis.

Each piece of excised gel was washed twice with 50 μL double-distilled water for 10 min and destained with 50 μL of NH₄HCO₃-CH₃CN (1:1, 50 mmol·L⁻¹) for 20 min at 37 °C. This step was repeated until the gel was completely destained. Subsequently, 50 μL of dehydration buffer containing CH₃CN was added and the pellet was vacuum filtered for 10 min. The protein in the gel was reduced with 20 μL of a solution containing 10 mmol·L⁻¹ DTT and 100 mmol·L⁻¹ NH₄HCO₃ for 1 h at 56 °C. After cooling to room temperature, 55 mmol·L⁻¹ iodoacetamide in 25 mmol·L⁻¹ NH₄HCO₃ (20 μL) was added quickly and samples were placed in a darkroom for 45 min. The peptides were subsequently extracted using 25 mmol·L⁻¹ NH₄HCO₃ for 10 min (twice), 25 mmol·L⁻¹ NH₄HCO₃ + 50%

CH₃CN for 10 min (twice), and CH₃CN for 10 min. They were then vacuum dried. The gel piece with the reduced protein was minced, lyophilized, and subsequently rehydrated in 25 mmol·L⁻¹ NH₄HCO₃ with 0.1 g·L⁻¹ trypsin at 37 °C overnight. After digestion, the minced gel was washed with 0.1% trifluoroacetic acid and centrifuged. The resulting peptide mixtures from each protein were analyzed by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS). The peptide mass fingerprinting obtained was analyzed using Mascot (Matrix Science, London, UK) for protein identification.

The MS protocols and database search parameters were as follows: database, NCBI nr; taxonomy, all entries/Viridiplantae (green plants); enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M) and Gln→pyro-Glu (N-term Q); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±100 ppm; peptide charge state, 1⁺; max missed cleavages: 1; report top, 20–50; and data file, peaklist. Identification was considered positive if the protein was obtained with at least two independent peptides with an expectation value of $P < 0.05$.

Results

2-DE. Triplicate gels were analyzed for each sample, which demonstrated a high level of reproducibility. Representative gels shown in Figure 1 indicate the differentially expressed protein spots in response to cold stress. More than 400 spots were reproducibly detected from digital image analysis on cCBB-stained gels over an isoelectric point range from 4 to 7 and molecular mass range from 10 to 100 kDa (Fig. 1). Quantitative image analysis showed that only 41 protein spots had a significant change in intensity of at least 2-fold relative to

the control. Of these, 26 protein spots were up-regulated and 15 protein spots were down-regulated after the cold treatment.

IDENTIFICATION OF DIFFERENTIALLY-EXPRESSED PROTEINS. Of the 30 protein spots resolved on the gel, 28 protein spots were successfully identified through a Mascot database search. Two of the spots did not match any protein in the database, which may represent novel cold-responsive proteins. Of the 28 proteins, 22 protein spots were up-regulated and six protein spots were down-regulated after cold stress treatment (Fig. 1; Table 1). All protein sequences detected and identified were also searched against a gene ontology tool (www.geneontology.org). These identified proteins were found to be involved in diverse biological processes, including antioxidation and antipathogen, photosynthesis, molecular chaperones, protein synthesis, signal transduction, energy metabolism, and others (Fig. 2). The largest functional category possessed proteins involved in the antioxidation and antipathogen (28%), including antioxidant responses, copper/zinc superoxide dismutase (SOD), ascorbate peroxidase (APX), ferredoxin-NADP reductase (FNR), and anthocyanidin synthase (ANS) as well as bio-stress responses, pathogenesis-related (PR) protein10.5, nucleotide-binding site leucine-rich repeat (NBS-LRR) protein, and 60 kDa jasmonate-induced protein (JIP60)-like isoform X2. The second largest category was photosynthesis (18%). Among the proteins involved in photosynthesis, four protein spots were linked to the ribulose biphosphate carboxylase/oxygenase (RuBisCo) and one protein spot was related to the chlorophyll binding protein. The next most prominent category was molecular chaperones (14%). Proteins of this group included heat shock protein (HSP), heat stress transcription factor A-2 (HSFA2)-like, and protein-disulfide isomerase (PDI). The next category was energy metabolism (11%), which included aconitate hydratase, phosphoglycerate mutase and short Ca²⁺-binding mitochondrial carrier (SCaMC). For the signal transduction (11%) and protein synthesis (11%) categories, proteins involved in ethylene signaling pathway and eukaryotic translation initiation and elongation factors were identified, respectively. The other functional categories (7%) contained S-adenosylmethionine (SAM)-dependent methyltransferases and putative myosin heavy chain (Table 1).

Fig. 1. Representative two-dimensional electrophoresis (2-DE) gels of proteins extracted from banana leaves. (A) A 2-DE gel of the control sample treated at 5 °C for 0 h. (B) A 2-DE gel of the sample treated at 5 °C for 24 h. Total protein was extracted and separated by 2-DE. In isoelectric focusing, 400 µg of protein was loaded onto immobilized pH gradient strips (17 cm, pH 4–7). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 12% gels. The protein spots were stained with colloidal Coomassie brilliant blue G-250. Statistical and quantitative analyses were performed to compare the control and treated groups. For the statistic sets, the paired t test was used at a significance level of 95%. In the quantitative analysis, the upper and lower limits were set to 2.0 and 0.5, respectively. The spots were compared among three biological replicates. Quantitative analysis revealed a total of 41 spots that had significant changes in intensity by at least 2-fold relative to the control. The 30 proteins identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), of these, 28 proteins indicated with spot numbers (1–28) were known proteins and two proteins were novel proteins. Mr = molecular weight; pI = isoelectric point. The circles and arrows indicate the positions of MALDI-TOF MS-identified proteins whose expression levels were altered in cold-treatment and control gels.

Discussion

Cold stress has been regarded as a major stress for crops, especially tropic and subtropic crops, such as rubber (*Hevea brasiliensis*), cassava (*Manihot esculenta*), papaya (*Carica papaya*), and banana, and its negative effects have been studied extensively in many crops. At present, comparative proteomics analysis is often used to assess the complex cellular changes and characterization in plant responses to cold stress. In this study, proteome

Table 1. Cold-responsive proteins identified in banana leaves by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).^z

Spot ^y	Accession ^x	Protein name	Organism	EMr ^w	EpI	TMr	TpI	Score	M	C	Exp
Antioxidation and anti-pathogen											
Antioxidation											
7	AFV46365	Copper/zinc superoxide dismutase CSD2A-1 (SOD)	<i>Musa acuminata</i>	23.29	6.12	22.86	5.40	94	7	43	Up
17	AFV46369	Copper/zinc superoxide dismutase (SOD)	<i>M. acuminata</i>	22.05	7.19	23.11	6.11	57	5	21	Down
27	AAP42501	Ascorbate peroxidase (APX)	<i>Ipomoea batatas</i>	27.75	5.32	24.53	4.89	55	10	25	Up
11	CAL58586	Ferredoxin-NADP reductase (FNR)	<i>Ostreococcus tauri</i>	40.82	7.56	41.90	5.66	70	12	32	Up
28	AAB82287	Anthocyanidin synthase (ANS)	<i>Matthiola incana</i>	40.79	5.54	30.50	5.04	57	7	27	Up
Anti-pathogen											
23	EOY06623	Pathogenesis-related protein 10.5 (PR)	<i>Theobroma cacao</i>	17.55	5.14	38.36	5.10	73	9	54	Up
25	AFC90534	Nucleotide-binding site leucine-rich repeat protein partial (NBS-LRR)	<i>Rhododendron formosanum</i>	28.65	5.94	37.56	6.05	58	8	33	Up
26	XP_004982822	60 kDa jasmonate-induced protein-like isoform X2 (JIP60)	<i>Setaria italica</i>	18.80	6.89	34.53	6.23	53	9	24	Up
Photosynthesis											
3	ABG22613	Ribulose biphosphate carboxylase/oxygenase (RuBisCo) activase	<i>Oryza sativa</i>	38.71	5.36	43.18	5.14	92	15	38	Up
6	AGB56405	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, chloroplast (RuBisCo)	<i>Renealmia cernua</i>	19.71	6.11	26.57	5.47	102	7	33	Up
8	Q7X999	Ribulose biphosphate carboxylase/oxygenase (RuBisCo) activase 2, chloroplastic	<i>Larrea tridentata</i>	48.26	6.78	27.10	5.03	67	11	22	Up
13	AAM98163	Magnesium chelatase subunit of protochlorophyllide reductase (MCPR)	<i>Arabidopsis thaliana</i>	46.50	5.36	47.57	5.90	65	15	38	Up
19	XP_002277357	RuBisCo large subunit-binding protein subunit alpha, chloroplastic-like (Rlsbp)	<i>Vitis vinifera</i>	62.06	5.20	104.93	4.92	121	14	25	Down
Molecular chaperones											
4	CAL56865	Chaperone HSP104 and related dependent Clp proteases (HSP)	<i>O. tauri</i>	86.44	5.18	27.54	5.56	58	6	10	Up
16	NP_001042949	Protein-disulfide isomerase (PDI)	<i>O. sativa</i>	41.12	6.43	34.58	5.99	90	10	28	Up
18	AFB35144	Small heat shock protein (HSP)	<i>M. acuminata</i>	17.75	6.15	21.13	4.55	83	8	25	Up
20	XP_003549228	Heat stress transcription factor A-2-like (HSFA2)	<i>Glycine max</i>	46.58	6.26	45.90	5.68	63	13	36	Up
Protein synthesis											
5	AAQ08197	Eukaryotic translation initiation factor 4A1 isoform, partial (eIF4A)	<i>T. cacao</i>	37.78	5.55	24.29	5.94	68	7	26	Up
9	ACJ84967	Eukaryotic translation initiation factor 3 subunit E-like (eIF3e)	<i>Medicago truncatula</i>	24.57	4.81	22.29	5.75	76	8	33	Down
21	CAL53714	Protein translation elongation factor Ts (EF-Ts)	<i>O. tauri</i>	36.45	5.55	50.27	5.99	84	14	52	Down
Signal transduction											
10	XP_004250139	14-3-3 protein 1-like	<i>Solanum lycopersicum</i>	28.36	4.76	23.56	4.47	56	7	32	Up
22	ADM64331	S-adenosyl-L-methionine (SAM) synthase	<i>M. acuminata</i>	43.69	5.72	55.81	6.14	113	13	38	Up
24	NP_001149427	1-aminocyclopropane-1-carboxylate oxidase 1 (ACO)	<i>Zea mays</i>	34.76	4.99	34.50	4.74	52	6	18	Down

Continued next page

expression of banana leaves was investigated in response to cold stress. Although we identified differentially expressed proteins that are involved in a number of cellular functions, including antioxidation and antipathogen, photosynthesis, chaperone, signal transduction, protein synthesis, energy

metabolism, and other cellular functions, the largest changes occurred in proteins involved in antioxidation, pathogen defense, and energy supply in response to cold stress. Together, these data suggest that plant cells are able to monitor cold stress by modulating the expression of specific proteins.

Table 1. Continued.

Spot ^y	Accession ^x	Protein name	Organism	EMr ^w	EpI	TMr	TpI	Score	M	C	Exp
Energy metabolism											
1	BAJ92965	Putative aconitate hydratase, cytoplasmic	<i>Hordeum vulgare</i>	107.89	6.83	106.82	6.58	78	19	23	Up
2	XP_002323696	Predicted phosphoglycerate mutase	<i>Populus trichocarpa</i>	61.38	5.40	82.75	5.68	63	13	21	Up
14	XP_002277297	Calcium-binding mitochondrial carrier protein (SCaMC)	<i>V. vinifera</i>	54.95	5.95	27.20	4.21	57	5	11	Up
Other cellular functions											
12	XP_002440149	Putative myosin heavy chain	<i>Sorghum bicolor</i>	161.65	6.07	42.11	6.20	78	18	14	Up
15	ACN40367	S-adenosylmethionine (SAM)-dependent methyltransferases	<i>Picea sitchensis</i>	39.74	5.80	34.31	5.87	60	6	24	Down

^zBanana plantlets were grown under growth chamber conditions (5 °C, relative humidity 70%, 12 h photoperiod and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR, the control sample treated for 0 h and the sample treated for 24 h). Total protein was extracted and separated by two-dimensional gel electrophoresis. In isoelectric focusing, 400 μg of protein was loaded onto immobilized pH gradient strips (17 cm, pH 4–7). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 12% gels. The protein spots were stained with colloidal Coomassie brilliant blue G-250. Statistical and quantitative analyses were performed to compare the control and treated groups. For the statistic sets, the paired *t* test was used at a significance level of 95%. In the quantitative analysis, the upper and lower limits were set to 2.0 and 0.5, respectively. The spots were compared among three biological replicates. Quantitative analysis revealed a total of 41 spots that had significant changes in intensity by at least 2-fold relative to the control. Of the 41 proteins, 30 proteins were digested with trypsin, and analyzed with MALDI-TOF MS. The peptide tags were submitted to sequence query with Mascot (Matrix Science, London, UK) for database search in NCBI nr. 28 of the 30 proteins were known proteins and two proteins were novel proteins.

^yProtein spot number corresponding to the 2-DE gels (Fig. 1).

^xNCBI accession number.

^wEMr = experimental molecular weight; EpI = experimental isoelectric point; TMr = theoretical molecular weight; TpI = theoretical isoelectric point; Score = Mascot score for the most significant hits; M = number of mass values matched; C = percent sequence coverage; Exp = expression.

Proteins involved in antioxidation and antipathogen

INCREASED ANTIOXIDATION. Cold stress has previously been shown to induce ROS accumulation in plant cells (Suzuki and Mittler, 2006). ROS are produced as byproducts of aerobic metabolism in plants (Apel and Hirt, 2004), and have a dual function as both damaging and signaling compounds (Mittler et al., 2004; Møller and Sweetlove, 2010). On one hand, stress-induced ROS are hazardous to oxidizing lipids, proteins, and nucleic acids, but on the other hand, they can also act as signals that activate defense pathways in response to biotic attack (Keunen et al., 2013), including the hypersensitive response to pathogens (Rhoads et al., 2006). Surprisingly, we found that cold stress induced an increase in the expression of SOD and APX in banana leaves, while the expression of another iso-enzyme of SOD slightly decreased (Table 1). The simultaneous up- and down-regulation in different isoenzymes has been detected under cold stress conditions, similar to what has been described in zoysiagrass (*Zoysia japonica*) stolons exposed to cold stress, and Xuan et al. (2013) confirmed that functionally different APX proteins differentially respond to cold stress. Scavenging for increased ROS at low temperatures can be achieved by shifting protein expression to produce isoforms that have improved performance at low temperature. In addition, specific FNR and ANS genes were up-regulated in response to cold stress in banana leaves (Table 1). FNR is an important protein involved in plant photosynthesis and plays a role in quenching ROS (Lintala et al., 2012; Mulo, 2011). Moreover, the expression level of FNR genes has been shown to increase upon drought stress (Lehtimäki et al., 2010). The anthocyanins, which are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH, belonging to the flavonoid family, are found in plant tissues as diverse as leaves, flowers, fruit, and seeds (Lim et al., 2013), and have

been reported to possess a variety of biomedical properties, including antioxidative, antimutagenic, antimicrobial, antiinflammatory, and antihypertensive activity (Cheng et al., 2007). ANS, which is an enzyme involved in anthocyanin biosynthesis, catalyzes the reaction from the colorless leucoanthocyanidins to the colored anthocyanidins (Nakajima et al., 2001). The abundant activities of functional proteins that work cooperatively can contribute to establishing a new cellular redox homeostasis under cold stress conditions in banana leaves.

INCREASED PATHOGEN RESISTANCE. Plants that survive through winter are simultaneously exposed to abio- and bio-stress. Therefore, it is not surprising that cold stress induced an increase in the abundance of defense-related proteins. To have protection from microbial pathogen infection, plants have evolved a large number of immune receptors, such as PR proteins, resistance proteins, and disease resistance proteins, which sense pathogen-derived molecules and trigger a defense response (Jones and Dangl, 2006). Treatment with cold significantly increased the abundance of three defense-related proteins (a PR protein 10.5, a NBS-LRR protein, and a JIP60-like isoform X2) in this study (Table 1). PR proteins have been defined as defense-related proteins in plants that are encoded by the host plant but induced by pathogens and stress (Agarwal and Agarwal, 2014). The abundance of PR proteins in moss was found to increase in response to cold stress (Wang et al., 2009). NBS-LRR proteins make up the majority of disease resistance proteins in plants (Yue et al., 2012). In addition, ribosome-inactivating proteins (RIPs) belong to a large family of ubiquitous proteins with antiviral and antifungal properties (Van Damme et al., 2001). JIP60 is a RIP (Chaudhry et al., 1994) involved in the plant stress response (Reinbothe et al., 1994). Therefore, cold stress can lead to an increase in the expression of disease resistance proteins in plants (Dumont et al., 2011).

Fig. 2. Distribution of the functional categories of the proteins identified as having differential expression patterns after cold exposure compared with control banana leaves. Total protein was extracted and separated by two-dimensional gel electrophoresis. In isoelectric focusing, 400 μ g of protein was loaded onto immobilized pH gradient strips (17 cm, pH 4–7). Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed with 12% gels. The protein spots were stained with colloidal Coomassie brilliant blue G-250. Statistical and quantitative analyses were performed to compare the control and treated groups. For the statistic sets, the paired *t* test was used at a significance level of 95%. In the quantitative analysis, the upper and lower limits were set to 2.0 and 0.5, respectively. The spots were compared among three biological replicates. Quantitative analysis revealed a total of 41 spots that had significant changes in intensity by at least 2-fold relative to the control. Of the 41 proteins, 30 proteins were digested with trypsin, and analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The peptide tags were submitted to sequence query with Mascot for database search in NCBI nr. 28 of the 30 proteins were known proteins and two proteins were novel proteins. The known proteins that showed a statistically significant change were functionally sorted into seven functional categories according to the Gene Ontology annotation.

Involvement in photosynthesis

Plant photosynthesis is inhibited at low temperature, because the capacity of many photosynthetic enzymes, such as RuBisCo, is affected by cold stress (Yamori et al., 2014). RuBisCo is the key regulatory enzyme responsible for CO₂ fixation during photosynthesis. It is localized in the chloroplast stroma and is composed of eight small subunits encoded by a small nuclear multigene family as well as eight large subunits encoded by a single gene in the multicopy chloroplast genome (Basha et al., 2009). RuBisCo activase maintains maximal photosynthetic rates by using the energy from adenosine triphosphate (ATP) hydrolysis to release tight binding inhibitors from the active site of RuBisCo (Keown et al., 2013). It also has another role as a chaperone during stress (Sobhanian et al., 2010). RuBisCo large subunit-binding protein (Rlsbp) subunit alpha is implicated in the assembly of the RuBisCo oligomer (Roy and Cannon, 1988). Magnesium chelatase subunit of protochlorophyllide reductase (MCPR) catalyzes light-dependent reduction of the divinyl- and monovinyl-derivatives of the non-esterified chlorophyll precursor protochlorophyllide in the biosynthesis of chlorophylls (Frick et al., 2003). RuBisCo activase and MCPR were found to be up-regulated in banana leaves under cold stress in this study, but the alpha subunit of Rlsbp was decreased (Table 1). Cold treatment can increase the abundance of RuBisCo and RuBisCo activase (Dumont et al., 2011; Wang et al., 2009; Xuan et al., 2013). When photosynthesis is greatly inhibited by low temperature in various crops (Allen and Ort, 2001; Jin et al., 2011), they can increase the levels of RuBisCo, RuBisCo activase, and MCPR, which are needed to compensate for decreased activities of the enzymes at low temperatures (Yamori et al., 2014). Although the alpha subunit of Rlsbp may inhibit RuBisCo activities under cold stress conditions, the reason for the decreased levels after exposure to cold stress will require further clarification.

Involvement with molecular chaperones

Molecular chaperones are involved in the assistance of protein folding together with the prevention and recovery of polypeptide aggregation, which may occur during protein

biosynthesis and during stress situations (Hinault and Goloubinoff, 2007). As molecular chaperones, Hsps are required to protect plant cells from cold damage (Wang et al., 2009) and can help other proteins maintain or regain their native conformation by stabilizing partially unfolded states (Boston et al., 1996). In response to heat stress, HSFA2 can act as a transcription activator of genes involved in metabolism and redox homeostasis (Liu and Charnig, 2013). In addition, HSFA2 also regulates a large number of chaperones and co-chaperones, as well as genes involved in multiple phytohormone signaling pathways (Liu and Charnig, 2013). PDIs also play a role as chaperones in the quality control system for correct protein folding (Onda et al., 2011). In this study, two Hsps and one HSFA2 were up-regulated by cold stress, and therefore may play important roles similar to that under heat stress conditions. In addition, one PDI gene was induced by cold stress in this study (Table 1). The up-regulation of molecular chaperones is important for the folding, assembly, and stabilization of membranes and proteins. This mechanism also guarantees the quality of proteins that are essential for plant tolerance to cold stress conditions.

Involvement in protein synthesis

Eukaryotic translation initiation factor 4A (eIF4A), ATP-dependent ribonucleic acid (RNA) helicase, and the binding of eIF4A together with eIF4B promotes unwinding of mRNA secondary structure (Aitken and Lorsch, 2012). It has been shown that eIF4A silencing inhibits plant growth, but tobacco mosaic virus (TMV) accumulation did not decrease in these plants (Hwang et al., 2013). Eukaryotic translation initiation factor 3E (eIF3e) has been implicated in the regulation of translation (Paz-Avram et al., 2008). Translation elongation factors Ts (EF-Ts) is an important component of the protein translation system, whereby it has inhibitory effects on the guanosine triphosphatase activity of elongation factor Tu and enhances guanosine diphosphate release (Palmer et al., 2013). In this study, we found that the expression of proteins involved in protein synthesis, such as eIF3e and EF-Ts, was repressed under cold stress conditions, but eIF4A expression was induced (Table 1). One hypothesis is that eIF3e and EF-Ts preset in the host may be commandeered to synthesize pathogenic proteins, but eIF4A is not used by pathogens under cold stress conditions, so the host only represses the expression of eIF3e and EF-Ts in response to cold stress. The increased accumulation of eIF4A suggests that under cold stress conditions, plants may synthesize important proteins involved in cold resistance or acclimation.

Involvement in signal transduction

Ethylene is a gaseous plant hormone that regulates essentially all plant growth and developmental processes, including germination, leaf and flower senescence and abscission, nodulation, fruit ripening, and the response to a wide variety of stresses (Yoon and Kieber, 2013). SAM is a precursor for the biosynthesis of ethylene and polyamines (PAs) and is synthesized by SAM synthetase (Cui et al., 2005). The 1-aminocyclopropane-1-carboxylic acid (ACC) is converted into ethylene by ACC oxidase (ACO), which is the final step of ethylene biosynthesis (Yang and Hoffman, 1984). In this study, SAM was up-regulated, while ACO was down-regulated by cold stress (Table 1), which suggests that biosynthesis of ethylene may be inhibited leading to the accumulation of PAs in response to cold stress. PAs can control the levels of abscisic acid (ABA) in response to cold by modulating ABA biosynthesis at the transcriptional level (Cuevas

et al., 2009). In addition, PAs play important roles in the tolerance of plants against cold stress, which contributes to the enhancement of ROS scavenging systems (Abavisani et al., 2013; Çakmak and Atici, 2009; Zhang et al., 2009). The 14-3-3 proteins are phosphoserine-binding proteins that modulate the functions of a diverse range of proteins via direct protein–protein interactions, such as with kinases, transcription factors, structural proteins, ion channels, and pathogen defense-related proteins (Abreu et al., 2013; Denison et al., 2011). In addition, 14-3-3 proteins appear to be involved in the brassinosteroids, ABA, and gibberellic acid signaling pathways during stress response (Denison et al., 2011). One 14-3-3 protein was induced by cold stress in our study (Table 1), and numerous proteomic studies have shown a differential accumulation of 14-3-3 proteins in response to cold stress in plants (Chattopadhyay et al., 2011; Hashimoto and Komatsu, 2007; Wang et al., 2012; Xu et al., 2012).

Involvement in energy metabolism

Alteration of energy metabolism under cold stress conditions can be affected by enzymes involved in glycolysis (Yan et al., 2006) and the tricarboxylic acid (TCA) cycle (Li et al., 2011). SCaMC mediates ATP-Mg/Pi exchange and plays important roles in energy metabolism (Yang et al., 2014). Aconitate hydratase is involved in the TCA cycle and was found to be up-regulated under cold stress conditions, and a similar trend was observed for phosphoglycerate mutase, which is involved in glycolysis, and SCaMC, which is involved in energy metabolism (Table 1). Thus, enhanced levels of these primary metabolism-related enzymes indicate that adequate energy is a prerequisite for how plants deal with cold stress conditions.

Involvement in other cellular functions

In this study, SAM-dependent methyltransferases were down-regulated and putative myosin heavy chain was up-regulated by cold stress conditions (Table 1). Methylation of proteins, nucleotides, and sugars is crucial for metabolism, cell signaling, and epigenetic programming, and a methyl group is most often added by SAM-dependent methyltransferases (Grove et al., 2011). The plant myosins use the energy from ATP hydrolysis to drive transportation of various intracellular cargos, including membranous organelles, protein complexes, mRNAs, along tracks of cytoskeletal polymers (Wang et al., 2014).

Yang et al. (2012) hypothesized that greater cold resistance found in plantain may be associated with increased ROS scavenging capability, reduced ROS production, and decreased lipid peroxidation. Surprisingly, our results showed that increased antioxidation capability to scavenge ROS and reduce ROS production also exists in response to cold stress conditions in banana. Therefore, we postulate that increased antioxidation ability could be a common characteristic of banana and plantain in response to cold stress conditions. In addition, an increased defense capability and energy supply were also found to occur in response to cold stress conditions in banana.

Conclusions

In conclusion, 28 differentially-expressed proteins were identified in response to cold stress conditions in banana that are involved in several cellular processes, including antioxidation and antipathogen, photosynthesis, chaperones, protein synthesis, signal transduction, energy metabolism, and other

cellular functions. Proteins related to antioxidation, pathogen resistance, molecular chaperones, and energy metabolism were increased, while proteins related to ethylene synthesis, protein synthesis, and epigenetic modification were decreased in response to cold temperature treatment. The banana plantlets incubated at cold temperatures demonstrated major changes in increased ROS scavenging, defense against diseases, and energy supply. Based on these findings, ROS, pathogens, and energy deficiency would be predominantly deleterious to banana plants under cold stress conditions. Increased antioxidation capability in banana was also discovered in plantain, which has greater cold tolerance than banana in response to cold stress conditions. Therefore, we hypothesized that an increased antioxidation ability could be a common characteristic of banana and plantain in response to cold stress conditions. Changes in the expression levels of these proteins contribute to our understanding of the physiological processes of banana in response to cold stress conditions. Importantly, some of the identified cold stress response-related proteins may be useful biomarkers to facilitate conventional breeding approaches for cold-tolerant banana cultivars.

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