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Comparative proteomics analysis revealed increased expression of photosynthetic proteins in transgenic tobacco by overexpression of *AtCBF1* gene

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Abstract

CRT/DRE Binding Factor 1 (CBF1) overexpression enhanced freezing tolerance in *Nicotiana tabacum*. To unravel the proteomics changes caused by the ectopic overexpression of *AtCBF1* genes, we utilized 2-DE along with MALDI-TOF-MS and LC ESI-MS/MS to study the proteome profiles. We also tried to identify the proteins that significantly change in abundance in the *AtCBF1* transgenic tobacco compared to wild type plant. Totally, 68 proteins were up-regulated in *AtCBF1* transgenic tobacco, while 150 were down-regulated when the plants were cultured at room temperature. Sixteen up-regulated proteins were successfully identified and functionally annotated. These proteins mainly fell into 4 categories, including photosynthetic proteins, metabolic proteins, stress related proteins and chaperons. In all identified proteins, the photosynthetic proteins were the major category, suggesting *AtCBF1* overexpression has substantial positive effects on photosynthesis system. The chlorophyll *a* and *b* content were substantially increased by 20% and 40% in transgenic tobacco than in wild type plant, respectively. These data suggested that *AtCBF1* ectopic overexpression protected tobacco photosynthetic system under cold stress, which contributes the cold tolerance of this non-cold acclimation plant.

Keywords: CRT/DRE binding factor 1 (CBF1); cold stress; proteomics; photosynthesis; tobacco.
Abbreviation: *CBF1*-CRT/DRE Binding Factor 1; WT-wild type; CA-cold acclimated; NCA-non cold acclimated; COR-cold responsive; LEA-late-embryogenesis-abundant; GSH-Glutathione; SOD-superoxide dismutase; DREB-dehydration responsive element binding; MAPK-mitogen-activated protein kinase; CDPK-calcium-dependent protein kinase.

Introduction

Low temperature is one of the major adverse environmental factors that plants have to suffer in temperate regions. When the plants are exposed to cold stress, dramatic changes in gene expression, proteins composition, small molecules accumulation and the cell membrane compositions will occur (Hua, 2009; Thomashow, 2010), which help the plant to survive the cold injury. This process is called cold acclimation. Recent progresses in genomics revealed that quite amount of genes were related to cold acclimation (Guy et al., 1985; Stockinger et al., 1997; Gilmour et al., 1998; Hua, 2009; Su et al., 2010; Thomashow, 2010). These genes could be classified into two categories such as (1) encoding functional proteins protecting the cell from the damage of water stress (such as LEA protein, antifreeze proteins, ion channel proteins, chaperons and mRNA binding proteins etc.), synthases of osmotic pressure regulators (proline, betaine and soluble sugar etc.) and enzymes that help the cell maintaining the normal statues (GSH-S-transferase, soluble epoxide hydrolases, SOD, peroxidase and ascorbate peroxidase etc.); (2) encoding transcriptional factors that regulate gene expression (bZip, Myc, Myb and DREB etc.), kinases (MAPK, CDPK etc.) and proteases that transfer stress signals. In recent years, a CBF (CRT/DRE binding factor) pathway was identified in Arabidopsis, which is featured by transcriptional factor CBF and functional cold-regulated (COR) genes (Stockinger et al., 1997; Gilmour et al., 1998; Chinnusamy et al., 2003). CBF genes exist in both cold acclimate (CA) and non-cold acclimate (NCA) plants while COR family genes were only found in CA plants. Though these CBFs expression patterns were not identical (Zhang et al., 2004), their ectopic overexpression can increase freezing tolerance in plants without exception (Jaglo-Ottosen et al., 1998; Qin et al., 2004; Savitch et al., 2005; El Kayal et al., 2006; Xiong and Fei, 2006). Transgenic experiments proved that CBF overexpression enhanced freezing tolerance in both CA (Jaglo-Ottosen et al., 1998; Sharabi-Schwager et al., 2010) and NCA plants (Hsieh et al., 2002; Yang et al., 2010). It is still not clear how CBF overexpression enhances the freezing tolerance of NCA plants, in which *COR* gene is absent (Jaglo et al., 2001).

Proteomics profiling have been carried out to examine changes under cold stress in plants. A substantial amount of proteins are involved in photosynthesis, RNA metabolism, defense response, energy pathway, protein synthesis, folding and degradation, cell wall and cytoskeleton and signal transduction when plants are exposed to low temperature (Cui et al., 2005; Amme et al., 2006; Gao et al., 2009). Transcriptome and metabolome analyses were also used to explore the omic-es changes under cold stress (Cook et al., 2004; Hwang et al., 2005; Carvallo et al., 2011; Janmohammadi, 2012), which deepened our understanding of the physiological responses towards low temperature in NCA

plants. However, the proteomics data under *CBF* overexpression are missing, which would help to specify the *CBF* functions in NCA plants. A previous study demonstrated overexpression of Arabidopsis *AtCBF1* gene enhanced freezing tolerance in tobacco (Bian, 2003), but the mechanism is yet to be elucidated. In this work, we undertook a comparative proteomics analysis to identify the proteomic profile changes under AtCBF1 ectopic overexpression, and unravel the possible mechanism that *CBF* overexpression enhances freezing tolerance in NCA plants.

Results and discussion

Transgenic tobacco is more tolerant to freezing than wild type plant

The transgenic *AtCBF1* tobacco line used here was analyzed previously (Bian, 2003). The electrolyte leakage test results indicated that the transgenic plants exhibited a significant lower leakage percentage of 29.2%±5.8% at -6°C and 69.2%±5.9% at -6.5°C, respectively, compared to wild type tobacco which had 53.0%±5.7% at -6°C and 81.5%±4.2% at 6.5°C (Fig. 1). These results demonstrated that the *AtCBF1* transgenic plant were more freezing tolerant than wild type plant, which is consistent with the report that *CBF*s genes were functional in NCA plant (Hsieh et al., 2002).

2-DE profiling of the proteomes in wild type and transgenic tobacco

Fig. 2 shows the representative 2-DE results of total protein from 5 weeks old wild type and transgenic tobacco leaves. About 669 spots were detected from the wild type plant and 612 spots from *AtCBF1* transgenic plant, respectively. Table 1 summarizes the proteomes profile patterns.

Total detected protein spots were 679, in which 602 spots were consistently and reproducibly found in all gels. We regarded protein abundance of at least 2 folds increase or decrease as up-regulated or down-regulated, respectively. Among the 163 spots whose abundance was changed, 68 were up-regulated and 95 down-regulated in the *AtCBF1* transgenic plants. Supplementary Table 1 shows the spot abundance of the up- and down-regulated proteins.

Identification and functional categorization of proteins with altered expression

The 163 protein spots with 2-folds abundance were subjected to identification by MALDI-TOF MS and LC ESI-MS/MS. Totally, 26 proteins were successfully identified and function annotated, of which 16 were up-regulated and 10 were down-regulated. Table 2 categorizes the putative functional identities of the up-regulated proteins. Supplementary Table 2 lists the putative identities of the down-regulated proteins as well.

The identified up-regulated proteins were categorized as photosynthesis related (7 proteins), metabolism related (3 proteins), stress related (3 proteins), heat shock proteins (3 proteins) (Table 2). Fig. 3 shows spot abundance of photosynthetic proteins. The up-regulation of these proteins involved in Calvin cycle suggests *AtCBF1* overexpression has positive effects on photosynthetic system in *AtCBF1* transgenic plants. This result accorded with the previous microarray data which indicated genes involved in photosynthesis were up-regulated by overexpression of *CBF* homolog in *Brassica napus* (Savitch et al., 2005).

Chloroplast and photosynthesis is major site of cold injury (Browse, 2010). When the plants are placed to low temperature, photosynthesis is strongly inhibited in association with the reduced expression of photosynthetic genes (Strand et al., 1997). Photosynthesis provides the energy required for cold acclimation, which contributes to the attainment of freezing tolerance (Gray et al., 1997; Hüner et al., 2012). The up-regulation of the photosynthetic enzymes expression can potentially increase the photosynthesis capacity in *AtCBF1* transgenic tobacco, which contributes to cold tolerance by the accumulation of energy and carbohydrates.

Chlorophyll contents increased in transgenic plants

Photochemical efficiency may result from the machinery coordination of photosynthetic pigments and the activity of Calvin cycle enzymes. Chlorophyll content has been shown to be linked to cold tolerance in rice and was successfully used to identify cold tolerant genotype (Glaszmann et al., 1990; Fracheboud et al., 1999). High chlorophyll content implies efficient energy capture ability and contributes to photosynthetic rate. Therefore, the chlorophyll *a* and *b* contents were measured and compared between wild type and transgenic tobacco (Fig. 4). In *AtCBF1* transgenic tobacco, the chlorophyll *a* and *b* content substantially increased by 20-% and 40%, respectively ($p<0.01$), indicating *AtCBF1* overexpression enhanced photosynthetic pigment accumulation.

In *Brassica napus*, overexpression of CBF like transcriptional factor partially mimicked cold-induced photosynthetic acclimation by increasing photosynthetic efficiency and capacity, pigment pool sizes and Calvin cycle enzymes capacities (Savitch et al., 2005). The high level of chlorophyll content and photosynthetic proteins found in *AtCBF1* transgenic tobacco leaves, suggesting higher level of photosynthesis capacity, which helps the transgenic plants to survive under cold stress, during which photosynthesis is suppressed.

Materials and methods

Plant materials and growth conditions

Wild type and *AtCBF1* transgenic tobacco (*N. tabacum* L.) seeds were obtained from Dr. Bian Hongwu (Zhejiang University). The plants were grown in the green house with a 16-h light (28 °C, 200 µmol m^{-2} s^{-1} light intensity) /8-h dark (25 °C). Humidity was maintained at 70%.

Chemicals

CHAPS, IPG DryStrip and buffer were from Bio-Rad (USA). Sequencing-grade modified trypsin was purchased from Promega (USA).

Proteomic extraction

To obtain reliable and reproducible 2-DE results, each leaves sample was collected from at least 10 individual seedlings and mixed together. At least three extractions were repeated from each sample.

For protein extraction, one gram leaves was ground to fine powder in a mortar under liquid nitrogen. 10 ml cold acetone containing 10% TCA, 0.07% 2-mercaptoethanol and 1 mM PMSF (Phenylmethanesulfonyl fluoride) was added and

Table 1. Protein expression profiles for *AtCBF1* transgenic tobacco compared with that of wild type plant, as resolved by 2-DE.

Protein pattern	Protein spots	No. spots identified
Constitutive expression	602	26
Abundance increased in transgenic plant	68	17
Abundance decreased in transgenic plant	95	9

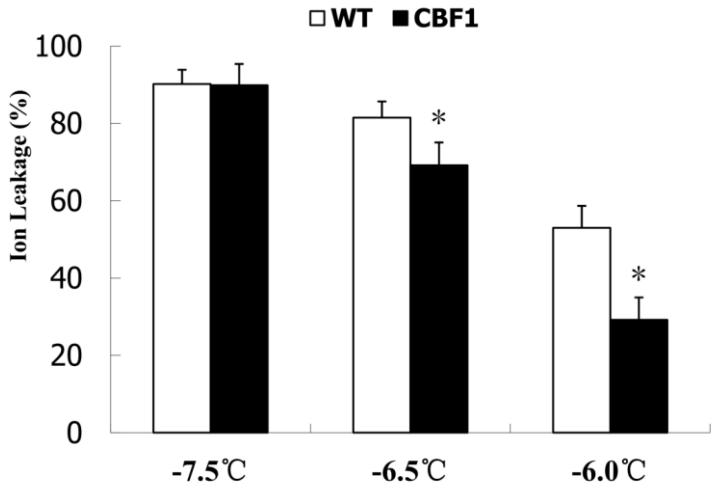

Fig 1. Electrolyte leakage rate of *AtCBF1* transgenic (CBF1) and wild type (WT) tobacco at different freezing temperatures. Electrolyte leakage was measured using leaf discs from six-week-old seedlings frozen to -6 °C, -6.5°C and -7 °C at a refrigerating rate of 1 °C per h from -1°C. Columns and bars give the mean ±SE of five experiment repeats. Data were analyzed with Student's T test and * shows significant differences between wild type and *AtCBF1* transgenic tobacco at indicated temperature.

incubated at –20 °C for at least 45 min. After two washes with acetone containing 0.07% 2-mercaptoethanol and 1 mM PMSF, the precipitate was dried in a Speedvac. Proteins were dissolved from dried precipitate using 20 µl/mg of lysis buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, 20 mM DTT, 1 mM PMSF). After vortex vigorously for 5 min and sonication in an ultrasonic bath (15 °C) for 5 min, the insoluble material was eliminated by centrifugation for 30 min at 32 000 g. The supernatant was additionally clarified by once further centrifugation (10 min at 32 000 g). The protein samples were quantified according to Bradford (Bradford 1976) using BSA as a standard.

Two-dimensional gel electrophoresis

For 2-DE, 1000 µg total protein was loaded on a 17 cm non-linear IPG strip with pH gradients of 3-10. Second dimension SDS-PAGE was run at 200 V for 6 hours using a PROTEAN™ Plus Dodeca system (Bio-Rad, CA, USA). The gel was stained with the colloidal CBB G250 and scanned with a GS-800 calibrated densitometer (Bio-Rad, CA, USA). Image analysis was carried out using PDQuest software version 7.4.0 (Bio-Rad, CA, USA) according to the manufacturer's instructions. All the spots detected automatically were edited manually for verification. After spot detection and background subtraction, the gels were aligned, matched and normalized, and the protein spot abundance was estimated by detecting stained spot volumes.

In-gel digestion, protein identification and database search

Protein spots volume showing at least 2-fold changes between gels were selected and excised using a ProteomeWorks™ Plus Spot Cutter (Bio-Rad, CA, USA). All the spots excised were inspected manually for the accuracy. The gel pieces were washed briefly with pure water and destained, and then dried in a Speedvac. Proteins were digested in 10µl trypsin solution (10 ng/µl in 20 mM ammonium bicarbonate/5% ACN) for 12 hours at 37 °C. The reaction was stopped by adding 2 µl 5% TFA.

The digested peptides mixtures were extracted and mixed with α-cyano-4-hydroxy-trans-cinnamic acid saturated solution in 0.1% TFA, 50% ACN, and then dispensed onto a 96-well plate for MALDI-TOF MS analysis (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA). MS data acquisition was first calibrated with lock mass adrenocorticotropic hormone fragment (amino position, 18–39, m/z 2,465.199 Da, ACTH) and then with a self-digested peak of porcine trypsin (m/z 2,211.105 Da). Protein identification was performed by using Mascot (Matrix Science; London, UK) searching the NCBInr database. The parameters were as follow: enzyme, trypsin; monoisotopic mass accuracy, 100 ppm; missed cleavages, 1; allowed variable modifications, oxidation (Met) and propionamide (Cys). Identifications were considered with certainty when met the following criteria: a score more than 67, at least 5 peptides matched with a mass tolerance of 100 ppm and one missed cleavage site.

For ESI-MS/MS, samples were run on a Q-TOF2 hybrid quadrupole/TOF mass spectrometer (Micromass, UK) with a nanoflow Z-spray source. Peptides separation was achieved on a reverse phase C18 column (PepMap C18, 0.075 × 150mm) using a 35-min linear gradient of 10-60% ACN. The mass spectrometer was operated in the positive ion mode

Table 2. Putative identities classification of proteins up-regulated in *CBF1* transgenic tobacco.

Spot No.	Gi	Cal./Obs. Mass (kDa)	Cal./Obs. *p*I	S/SC/PM	Protein name	Source
Photosynthetic protein						
33*	12644171	34.93/32.59	5.91/5.11		Oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	Tomato
35	61697115	33.01/35.12	5.15/5.61	92 / 47 / 12	Oxygen evolving complex 33 kDa photo system II protein	Tobacco
53	19992	45.4/25.91	5.24/5.01	119 / 48 / 10	RuBisCO activase	Tobacco
61*	19898	21.93/27.87	5.33/7.03		Photo system II 23 kDa polypeptide	Tobacco
79*	12643758	48.31/45.13	8.14/5.34		RuBisCO activase 2	Tobacco
98*	1345550	20.91/28.36	5.42/7.63		23-kDa polypeptide of photo system II oxygen-evolving complex	Tobacco
291*	19896	24.48/21.12	5.26/5.29		Photo system II 23 kDa polypeptide	Tobacco
Metabolic protein						
171*	119354	47.77/56.29	5.68/6.26		Enolase 2	Tomato
275	50725061	15.96/30.61	5.13/9.59	98 / 28 / 9	Beta-Glucosidase homolog 1	Rice
297*	3334149	46.60/45.33	6.64/5.15		Magnesium-chelatase subunit chlI	Tobacco
Stress related protein						
9	2632088	34.95/29.39	5.01/4.83	78 / 18 / 6	Plastid-lipid-Associated Protein	Tobacco
31	19773	26.64/27.62	5.11/5.08	84 / 24 / 8	Acidic chitinase PR-Q	Tobacco
293*	3901016	6.89/20.91	9.3/5.42		ABA-inducible protein	Beechnut
Chaperon						
100*	37704437	14.00/15.45	5.80/5.42		Cytosolic class I small heat shock protein 3B	Tobacco
277	37704403	15.63/15.63	5.39/5.39	79 / 43 / 7	Cytosolic class I small heat shock protein 1B	Tobacco
296*	3256372	26.58/22.20	6.63/5.28		Heat shock protein 26 (Type I)	Tobacco

*Refers to the proteins identified by LC ESI-MS/MS.

S: Score; SC: Sequence Coverage; PM: Peptides Matched

Protein identification was performed by searching the NCBInr database with Mascot (Matrix science; London, UK). The parameters were: monoisotopic mass accuracy of 100 ppm; one missed cleavages; allowed variable modifications, oxidation (Met) and propionamide (Cys). Identifications were considered with certainty when meeting the following criteria: a score more than 67, at least 5 peptides matched with a mass tolerance of 100 ppm and one missed cleavage site. The MS/MS data acquired were searched with the parameters: monoisotopic peptide masses, ±0.2 Da peptide mass tolerance, one missed cleavage, modifications allowed for oxidation of methionine and carboxyamidomethylation of Cys.

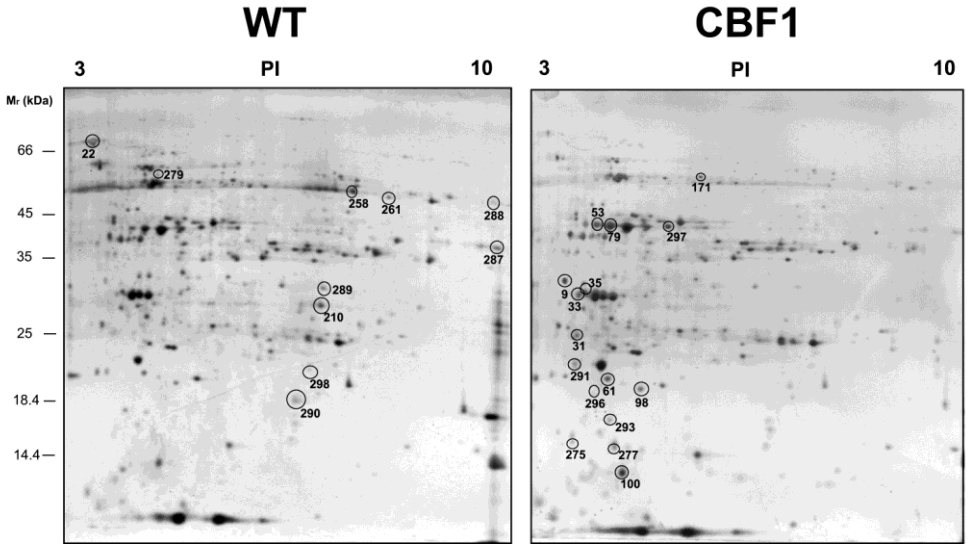

Fig 2. Proteomics profiles of tobacco seedlings subjected to cold stress. Annotated 2-D gels correspond to total proteins extracted from wild type (WT) and *AtCBF1* transgenic tobacco leaves (CBF1). 2-DE and gel staining were performed as described in "Materials and methods" section. The experiment was repeated three times, and protein spots abundance were calculated and compared between gels. Numbers correspond to the identified spots listed in Table 2 and supplementary Table 2. The up- and down-regulated protein spots in *AtCBF1* transgenic tobacco compared with those in wild type were marked on CBF1 and WT gels, respectively.

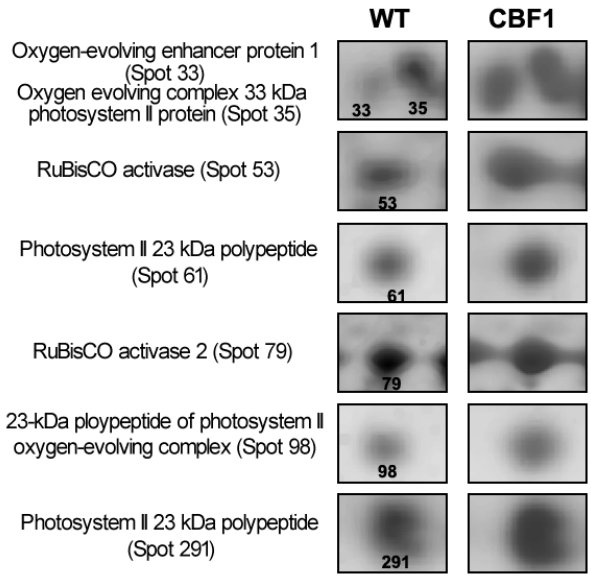

Fig 3. Quantitative changes of the 7 identified photosynthetic proteins up-regulated by AtCBF1 overexpression in transgenic tobacco leaves. Total protein extracted from wild type (WT) and *AtCBF1* transgenic tobacco leaves (CBF1). 2-DE and gel staining were performed as described in "Materials and methods" section. Quantitative changes of the 7 identified photosynthetic proteins which were up-regulated in *AtCBF1* transgenic plant were analyzed.

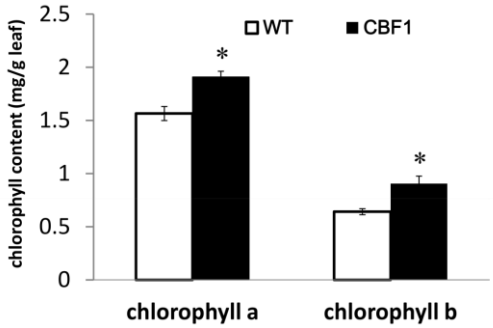

Fig 4. Chlorophyll *a* and *b* content of *AtCBF1* transgenic (CBF1) and wild type (WT) tobacco plants. Chlorophyll *a* & *b* content were measured using leaves from six-week-old seedlings. Columns and bars give the mean ±SE of five experiment repeats. Data were analyzed with Student's T test and * shows significant differences between wild type and *AtCBF1* transgenic tobacco for each chlorophyll contents.

with a source temperature of 80 °C. A voltage of 2.35 kV was applied to the nanospray probe tip. The MS/MS data acquired were searched with the NCBInr database using MASCOT MS/MS ion search engine. The search parameters were: monoisotopic peptide masses, ±0.2 Da peptide mass tolerance, one missed cleavage, modifications allowed for oxidation of methionine and carboxyamidomethylation of Cys.

Electrolyte leakage test and chlorophyll measurement

Tobacco freezing tolerance was tested by measuring the percentage of electrolyte leakage as previous described (Xing et al., 2011). Two hundred milligrams tobacco leaves were collected (five repeats for each sample) and analyzed.

For chlorophyll measurement, leaves were extracted with 80% acetone overnight, the *A645* and *A663* were determined, and chlorophyll *a* and *b* contents were calculated. The electrolyte leakage and chlorophyll content data were analyzed with Student's T test to determine statistically significant differences.

Conclusion

In conclusion, a substantial number of proteins expression was altered in *AtCBF1* transgenic tobacco compared with those in wild type plants. Functional classification revealed photosynthetic proteins, metabolic proteins, stress related proteins and chaperons constituted the major part of the proteins that up-regulated in transgenic plant. Both photosynthetic proteins and chlorophyll content were increased in transgenic tobacco, suggesting that *AtCBF1* ectopic overexpression protected tobacco photosynthetic system when subjected to cold stress and contributed the freezing tolerance of this non-cold acclimation plant.

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