Proteome Analysis of Vernalization-Treated *Arabidopsis thaliana* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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In order to gain insight into the molecular changes at the protein level in plants exposed to low temperature for a long period of time (vernalization), proteome analyses of vernalization-treated *Arabidopsis thaliana* have been carried out by two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. Fourteen proteins including ATP binding/GTP binding/translation elongation factor and glycine-rich RNA-binding protein 7 (GRP7) showed differential expression in vernalization-treated *Arabidopsis thaliana*. GRP7 showed the most dramatic increase in expression suggesting its involvement in response to vernalization treatment.

Key Words : *Arabidopsis thaliana*, Vernalization, MALDI-TOF MS, GRP7

Introduction

Flowering time of plants is regulated by both endogenous chemical signals and environmental cues. The two major environmental factors influencing flowering time are light and temperature.[1] Many plants recognize a seasonal change for flowering through photoperiod, a length of day. Recent analysis showed that circadian rhythm of a gene called CONSTANS (CO) and coincidental light exposure at the peak time of CO is the molecular basis of photoperiodic flowering response in both monocots and dicots.[2]

Temperature is another important factor in determining the flowering time. It has been reported that flowering is accelerated in winter annuals of many plant species (such as wheat, barley, cabbage and Arabidopsis) if exposed to low temperature for a long time. It is called vernalization.[3] Vernalization is an evolutionarily advantageous mechanism preventing premature flowering during late fall and allowing flowering in the following spring in response to favorable environments.[3,4] The effect of vernalization treatment depends on the growth status and genotype of the plant as well as the length of the treatment.[3,4] The recent molecular genetic analyses of Arabidopsis winter annuals revealed the epigenetic regulatory mechanism of vernalization such that vernalization changes the chromatin structure of a strong floral repressor, *FLOWERING LOCUS C (FLC)* from active to silenced state.[5] However, what causes such chromatin modification is not understood. Therefore, it is of critical importance to see proteomic changes induced by vernalization. It is hoped that the identification of proteins differentially expressed in vernalization-treated *A. thaliana* could provide a clue to understanding, at the molecular level, key steps in controlling the flowering time of plants in general. In this paper, we report identification, by 2-D electrophoresis and MALDI-TOF mass spectrometry (MS), of several proteins that seem to be associated with vernalization.

Experimental Section

Chemicals. Immobilized pH gradient strips (pH 5-8, ReadyStrip, $0.5 \times 3.3 \times 170$ mm), acrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate, coomassie Brilliant Blue (CBB) R-250, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), protein assay solution, and ampholyte (Bio-Lyte, 3/10) were from Bio-Rad (Hercules, CA, USA). Urea, thiourea, N,N'-methylenebisacrylamide, Triton X-100, dithiothreitol (DTT), trypsin, and iodoacetamide (IAA) were from Sigma (St. Louis, MO, USA). Tris-(hydroxymethyl)-aminomethane α-cyano-4-hydroxy cinnamic acid (CHCA) were from Aldrich (Milwaukee, WI, USA).

Vernalization treatment. *A. thaliana* was grown under the long-day condition (16 h light period, 8 h dark period). After stratification for 3 days at 4 °C, the control plant was grown for 11 days at 23 °C. Vernalization involved normal growth at 23 °C for 9 days followed by 30 days at 4 °C.

Protein extraction and 2-D electrophoresis. The plant tissue was pulverized in liquid nitrogen using a mortar and pestle. Lysis buffer (6 M urea, 2 M thiourea, 50 mM DTT, 2% CHAPS, 0.5% Triton X-100, 40 mM Tris, 0.2% Bio-Lyte) was added, and the mixture was vortex mixed for 30 min and centrifuged at 14,000 rpm for 30 min at 4 °C. Protein was precipitated with 80% acetone for 1 h at −20 °C. The protein pellet was washed with 80% acetone, dried, and dissolved in a minimum volume of the lysis buffer. The amount of protein in the supernatant was determined by the Bradford method.

Isoelectric focusing (IEF) followed by polyacrylamide gel electrophoresis was performed in a standard manner. Image Master™ (Swiss Institute of Bioinformatics, Switzerland) was used to calculate the volume percent of the protein spots on the gel.

MALDI-TOF MS of tryptic peptides. The protein spot from the gel was sliced and digested overnight at 37 °C with

10 μL of a trypsin solution (2 μg/mL in ammonium bicarbonate). For MS analysis, 1.5 μL of the matrix solution (2 mg CHCA in 60% ACN with 0.1% TFA) was mixed with an equal volume of the tryptic digest, and a 0.5 μL aliquot was loaded on AnchorChip^{TM} target plate. Auto Flex II MALDI-TOF MS/MS (Bruker Daltonics) equipped with a 337 nm nitrogen laser, delayed extraction, and reflectron was used in the positive ion mode. MASCOT peptide mass fingerprint search program (http://www.matrixscience.com/cgi/search_form.pl?SEARCH=PMF) was used for protein identification. The search options were: NCBI database, *Arabidopsis thaliana*, maximum of 2 miss cleavages, 100 ppm mass error.

Transcriptional analysis. To characterize the transcriptional level of *AtGRP7*, total RNA was extracted from 11 days old seedlings using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Through reverse transcription-polymerase chain reaction (RT-PCR) amplification, the expression level of each gene was analyzed. The sequences of primers used were AtGRP7F (TGCTTCGTTGGAGGTCTAGC)/AtGRP7R (GTAACCTCCTCCTTCACCAC) for *AtGRP7*, and TUB-F (CTCAAGAGGTTCTCAGCAGTA)/TUB-R (TCACCTTCTTCATCCGCAGTT) for *TUBULIN*.

Results and Discussion

Results from 2-D electrophoretic separation of proteins extracted from control and vernalization-treated *A. thaliana* are shown in Figure 1. Some distinct changes were evident by visual inspection. A total of 41 proteins were identified. Among them, 27 spots (indicated with arrows) were observed in both samples without major changes (Table 1). They include Rubisco activase, glutathione transferase, nucleoside diphosphate kinase, and ribulose-bisphosphate carboxylase. In some cases the changes were certain but minor. In several cases, two spots separated horizontally were identified as the same protein (1/2, 3/4, 9/10/11/15, 13/14, 23/24, 25/26) and probably represent modification of the acidic or basic group resulting in the shift in isoelectric pH (pI) with a small change in the molecular weight. Most of the proteins not affected by vernalization correspond to the functional group of metabolism and indicate that vernalized plants carry out basic metabolic activity at the normal rate.

A total of 14 protein spots (12 different proteins) showing increase or decrease in intensity by a factor of about two or more are circled. Again, proteins related by modification were observed (3/4, 5/6). These differentially expressed proteins are summarized in Table 2. Proteins showing decreased and increased expression are listed separately in the increasing order of differential expression. The percent volume of the protein spots in Table 2 are average of results from 16 separate gels (multiple harvests and extractions from control and vernalization-treated *A. thaliana*).

Eight proteins showing significant decrease in the expression level upon vernalization-treatment include glutathione transferase, translation elongation factor, transcription factor, putative major latex protein, glyceraldehyde 3-phosphate dehydrogenase, and carbonic anhydrase. Since the vernalization-treatment was essentially a cold-treatment, decrease in certain metabolic and regenerative proteins was expected. However, why these particular proteins are significantly lowered in cellular concentration in a reproducible manner is not understood and poses an interesting question in terms of their structure and function. The reproducibility in the decreased expression of putative major latex protein (spot 14) is shown in Figure 2 as an example. The observation was reproducible in duplicate extractions from two separate harvests of the plant.

We were more interested in finding proteins that are overexpressed in vernalization-treated *A. thaliana*. Three pro-

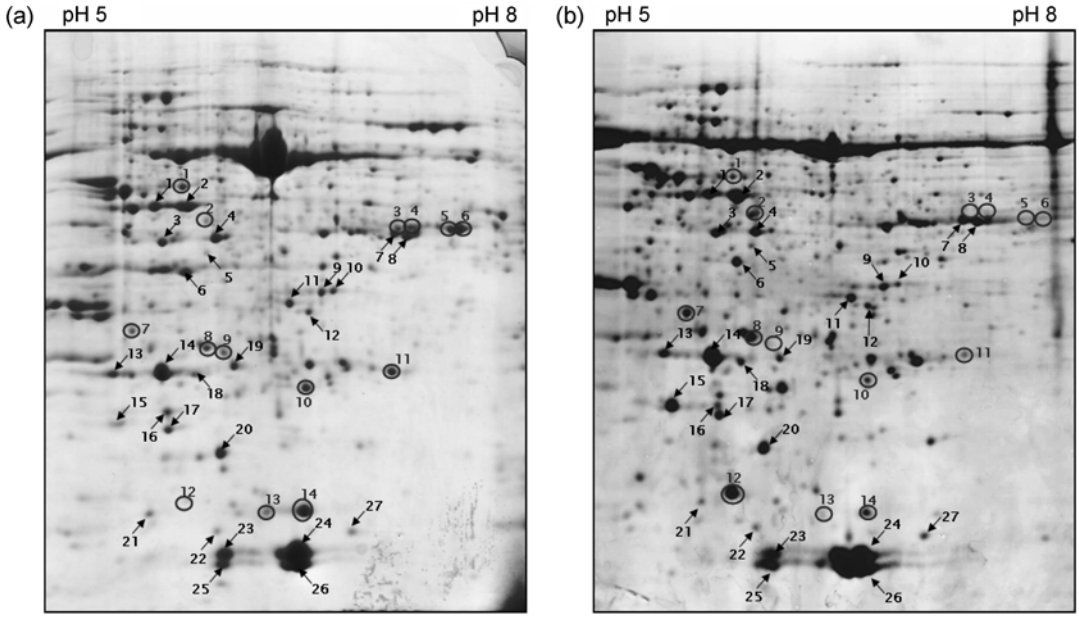

Figure 1. 2-DE map of control (a) and vernalization-treated *A. thaliana* (b).

Table 1. Identified proteins with no major differences in both samples

Spot No.	MW/pI	Accession No.	Name
1	49069/7.55	gi|30687995	RCA(Rubisco avtivase)
2	"	"	"
3	41781/5.94	gi|79322651	fructrose-biphosphate aldolase
4	41318/9.07	gi|42573227	"
5	41630/8.13	gi|15224351	OASB; cysteine synthase
6	32096/5.46	gi|21594017	putative lectin
7	36966/6.62	gi|21593240	glyceraldehyde-3-phosphate dehydrogenase C subunit
8	36890/6.67	gi|15222166	NAD binding/glyceraldehydes-3-phosphate dehydrogenase
9	47464/6.12	gi|27752811	ribulose-1,5-bisphosphate carboxylase
10	"	"	"
11	"	"	"
12	26054/6.04	gi|1143394	V-type proton-ATPase
13	28078/6.90	gi|15222166	PSBP(oxygen-evolving enhancer protein 2); calcium ion binding
14	"	"	"
15	47464/6.12	gi|27752811	ribulose-1,5-bisphosphate carboxylase
16	25979/6.21	gi|15233115	LHCA1; chlorophyll binding
17	25653/9.04	gi|15233985	hydrogen-transporting ATP synthase
18	23626/5.56	gi|15223576	glutathione dehydrogenase(ascorbate)
19	25634/5.80	gi|18411929	glutathione transferase 8
20	28190/8.83	gi|15228674	ROC4; peptidyl-prolyl cis-trans isomerase
21	16569/5.58	gi|15235002	ATGRP8(glycine-rich protein 8); RNA binding
22	14490/5.84	gi|5881700	ATPase epsilon subunit
23	20271/8.82	gi|15240901	ribulose-biphosphate carboxylase
24	"	"	"
25	14689/5.69	gi|13926229	F1019.10/F1019.10
26	"	"	"
27	16229/7.03	gi|16396	nucleoside diphosphate kinase

Table 2. Proteins differentially expressed in control and vernalization-treated AT

Spot No.	MW/pI	Accession No.	Name	% Volume (std dev)	
				Control	Treated
showing decrease					
11	24131/6.17	gi|15224581	ATGSTF9; glutathione transferase	0.23 (0.06)	0.10 (0.02)
1	51598/5.84	gi|15237059	ATP binding/GTP binding/translation elongation factor	0.21 (0.02)	0.09 (0.01)
10	33871/8.73	gi|18410804	transcription factor	0.28 (0 .07)	0.09 (0.02)
14	17507/5.91	gi|7269215	putative major latex protein	0.77 (0.10)	0.22 (0.03)
5	37652/7.00	gi|166702	glyceraldehyde-3-phosphate dehydrogenase A subunit	0.23 (0.17)	0.04 (0.03)
6	"	"	"	0.29 (0.20)	0.03 (0.02)
13	17507/5.91	gi|15236566	unknown protein	0.16 (0.03)	n.d.
3	42820/8.16	gi15222111	NAD binding/glyceraldehyde 3-phosphate dehydrogenase	0.09 (0.06)	n.d.
4	"	"	"	0.16 (0.12)	n.d.
9	28166/5.29	gi|62320917	carbonic anhydrase, chloroplast precursor	0.10 (0.04)	n.d.
showing increase					
7	34858/8.37	gi18404496	unknown protein	0.10 (0.04)	0.18 (0.04)
2	38270/5.65	gi|15236768	fructose-bisphosphate aldolase	0.08 (0.01)	0.18 (0.05)
8	27138/5.24	gi|742408	triose phosphate isomerase	0.18 (0.03)	0.41 (0.15)
12	16880/5.85	gi|15226605	AtGRP7; RNA binding	0.06 (0.02)	0.73 (0.19)

teins (fructose-bisphosphate aldolase, triose phosphate isomerase, and an unknown protein) showed about 2-fold increase. Most remarkable was AtGRP7; RNA binding protein, which showed more than 10-fold increase in a reproducible manner as shown in Figure 3. The MALDI-TOF mass spectrum of AtGRP7 tryptic peptides is shown in Figure 4.

To address if the increase of AtGRP7 protein is due to the

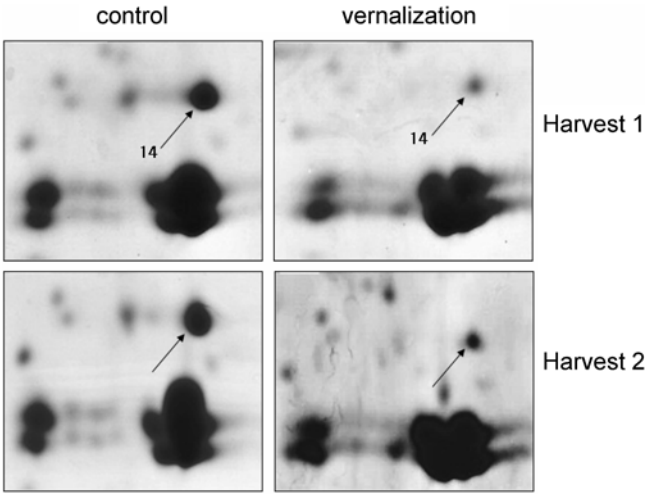

Figure 2. Reproducibility in the decreased expression of putative major latex protein.

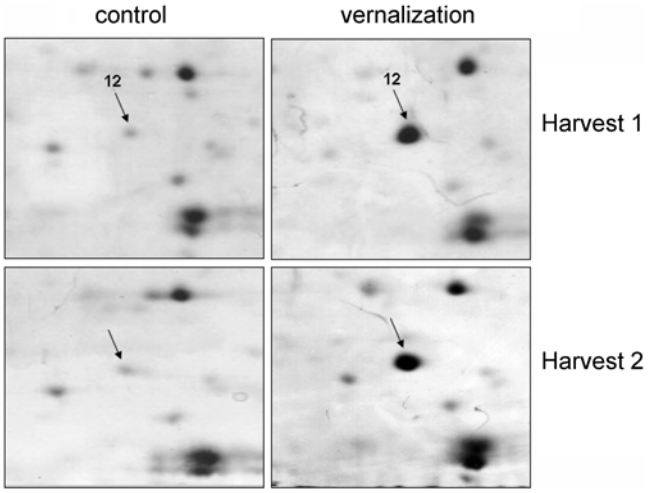

Figure 3. Reproducibility in the increased expression of AtGRP7; RNA binding protein.

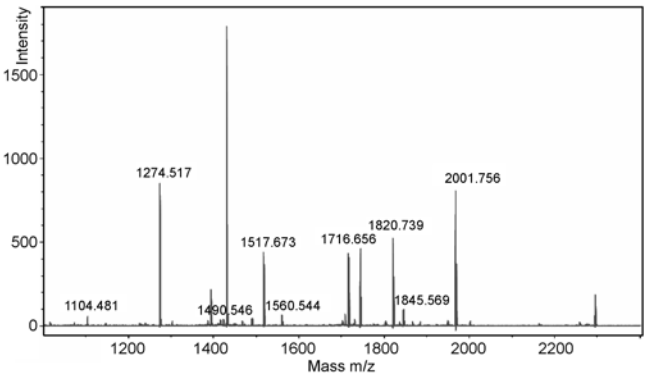

Figure 4. Mass spectrum of AtGRP7; RNA-binding protein.

increased transcript level, we performed reverse transcription-polymerase chain reaction (RT-PCR). As shown in Figure 5, *AtGRP7* transcript was increased after 1 week cold treatment which does not affect the flowering time.[6] In addition, such an increase was maintained for 4 weeks of vernalization treatment which strongly accelerates flowering

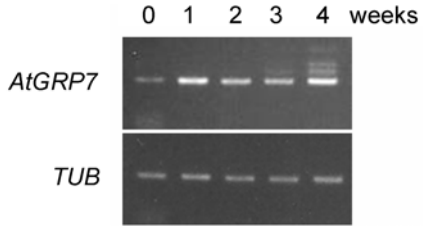

Figure 5. Expression level of AtGRP7 during vernalization treatment characterized using RT-PCR method.

time. It has been reported that *AtGRP7* transcript is increased by cold stress.[7] The AtGRP7 gene has also been known to be involved in the circadian rhythm,[8] thus suggesting the possible link between circadian rhythm and cold response. However, it has not been elucidated in the past if AtGRP7 is involved in the vernalization process or flowering time regulation.

In contrast to AtGRP7, another member of the same gene family, AtGRP8, showed no significant change at the protein level upon vernalization, which was confirmed by more than 3 independent biological replicates (Table 1). It is an interesting result because *AtGRP8* also has been reported to be induced by cold stress.[7] When the plants were continuously exposed to cold, the transcript level of *AtGRP8* was increased during the first day but was gradually decreased afterwards. Thus, it is likely that AtGRP8 level is increased by short-term cold stress but gradually decreased during acclimation to a long period of cold, *i.e.*, vernalization. In contrast, AtGRP7 is not only induced by cold stress but also maintained during vernalization. Therefore, it is likely that AtGRP7 has a divergent function compared to AtGRP8.

A variety of RNA binding proteins involved in post-transcriptional control are known.[8-10] The RNA recognition motif (RRM) toward the N-terminal side of the RNA binding proteins consists of 80-90 amino acids. The RNP1 (RGFGFVTF) and RNP2 (CFVGGL) sequences in the RRM are directly involved in target RNA recognition and binding. Typical cellular RNA binding proteins have one or two RRM toward the N-terminal and various auxiliary motifs toward the C-terminal end. Auxiliary motifs known include glycine-rich region, zinc finger motif, and acidic domain.[11,12] The AtGRP7 protein that showed over 10-fold increase upon vernalization-treatment has a RNP1 (RGFGFVTF) sequence at residue 49-56 and a glycine-rich region toward the C-terminus as shown below.

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1   MASGDVEYRC FVGGLAWATD DRALETAFAQ YGDVIDSKII NDRETGRSRG
51  FGFVTFKDEK AMKDAIEGMN GQDLDGRSIT VNEAQSRGSG GGGGHRGGGG
101 GGYRSGGGGG YSGGGGSYGG GGGRREGGGG YSGGGGGYSS RGGGGGSYGG
151 GRREGGGGYG GGEGGGYGGS GGGGGW
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Although vernalization is the same as cold treatment except for the length of the low temperature treatment, it is

physiologically different in that the cold treatment usually does not affect flowering time significantly whereas vernalization affects flowering time dramatically.[3] The molecular basis of vernalization has been elucidated recently.[5] The final outcome of vernalization in Arabidopsis is the suppression of *FLC*, a strong repressor of flowering. Interestingly, many RNA binding proteins function as repressors of *FLC*.[13] For example, *FCA* gene encodes the protein with the RRM motif,[14] *FY* gene encodes polyadenylation factor,[15] and *FPA* and *FLK* encodes proteins with plant-specific KH type RNA binding domain.[16,17] The mutation of any of these genes causes a strong activation of *FLC*, thus resulting in late flowering.[13] Our results showing increase in AtGRP7, a RNA binding protein, upon vernalization which causes the suppression of *FLC* are consistent with the earlier results that many of the RNA binding proteins are involved in the repression of *FLC*. It has also been known that the cellular level of transcripts for RNA binding proteins is significantly increased under stress such as cold, drought, or viral infection.[10,11] Because AtGRP7 gene is also increased by cold stress, it is possible that AtGRP7 is involved in the response to cold stress. This question can be addressed by generating a loss-of-function mutant and over-expression of transgenic line of AtGRP7.

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