Amino Acids (2009) 36:115–123
DOI 10.1007/s00726-008-0039-4

ORIGINAL ARTICLE

Cold stress changes the concanavalin A-positive glycosylation pattern of proteins expressed in the basal parts of rice leaf sheaths

S. Komatsu · E. Yamada · K. Furukawa

Received: 14 October 2007 / Accepted: 25 January 2008 / Published online: 16 February 2008

Abstract Post-translational modifications such as glycosylation are important for changing the properties and functions of proteins. To analyze the importance of glycosylation during cold stress in rice, a proteomics approach was used. Proteins extracted from the basal part of rice leaf sheaths were separated by two-dimensional polyacrylamide gel electrophoresis, and subjected to lectin blot analysis using concanavalin A. From a total of 250 detected proteins, 22 reacted with the lectin, suggesting that they were *N*-glycosylated proteins. To determine how *N*-glycosylation of these proteins is affected by cold stress, rice seedlings were incubated at 5°C for 48 h, and proteins extracted from the basal parts of leaf sheaths were analyzed by the lectin blot assay. Cold stress changed the reactivity toward the lectin for 12 of the 22 glycoproteins. The identity of the 12 proteins was determined by protein sequencing and mass spectrometry with the majority of these glycoproteins being categorized as involved in energy production. Furthermore, calreticulin, one of the 12 glycoproteins, was also phosphorylated as a result of cold stress. These results indicate that cold stress of the basal parts of rice leaf sheaths changes the glycosylation and phosphorylation profiles of calreticulin, a key protein that regulates the quality control of other proteins.

S. Komatsu (✉) · E. Yamada
National Institute of Crop Science, Kannondai 2-1-18, Tsukuba 305-8518, Japan
e-mail: skomatsu@affrc.go.jp

E. Yamada · K. Furukawa
Nagaoka University of Technology, Nagaoka 940-2188, Japan

Keywords Rice · *Oryza sativa* · Basal parts of leaf sheath · Cold stress · Proteomics · Glycosylation · Calreticulin

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
IEF	Isoelectric focusing
PVDF	Polyvinylidene difluoride
CBB	Coomassie brilliant blue
PBS	Phosphate-buffered saline
MS	Mass spectrometry

Introduction

Low temperature is one of the most serious abiotic stresses of rice; suppression of rice plant growth and a rolled and withered appearance of the leaves are the prime indicators (Cui et al. 2005). Rice plants are most susceptible to low temperature at reproductive stages, resulting in pollen sterility (Imin et al. 2004; Hayashi et al. 2006). Rice plants in tropical and sub-tropical regions are seriously injured by temperatures below 12°C but above the freezing point (Lyons 1972; Raison and Lyons 1986). It is generally recognized that changes in protein synthesis patterns and mRNA transcript levels take place when rice plants are exposed to low temperature (Thomashow 1998; Koga-Ban et al. 1991). Although many cold-stress responsive genes and proteins have been analyzed in various plant organs, the mechanisms involved in plant cold stress responses have not been completely elucidated.

Rice plants have developed complicated mechanisms to perceive external signals and respond to cold stress with appropriate physiological changes. Cold stress especially limits rice production. Minimizing the loss caused by cold stress will not only help improve net production, but will also extend rice cultivation in marginal land previously not cultivated (Khush 1999; Tyagi and Mohanty 2000). Rice exposed to cold stress results in marked changes in gene expression, biomembrane lipid composition, and small molecule accumulation (Iba 2002). Most notably, rice plasma membrane protein levels change with cold stress (Yu et al. 2006; Komatsu et al. 2007), but not much is known about the regulatory mechanisms of this response. Therefore, identifying uncharacterized cold temperature responding genes and defining their functions will enrich the understanding of stress-signaling networks in rice and be important for improving rice tolerance to cold stress.

Furthermore, comprehensive analyses of transcript and protein levels have been developed for the functional characterization of genes and proteins responding to cold stress in rice as well as Arabidopsis. At the transcript level, various genes related to cold stress tolerance have been analyzed in detail with comprehensive analyses such as cDNA microarray (Kim et al. 2007; Rabbani et al. 2003; de los Reyes et al. 2003). At the protein level, a comparative proteomic approach was used to identify rice proteins responding to cold stress in leaves (Cui et al. 2005; Yan et al. 2006), leaf sheaths (Hashimoto and Komatsu 2007) and anthers (Imin et al. 2004; Hayashi et al. 2006). Although these results provided very useful information for identifying cold-stress responsive proteins, functional analysis of these proteins and a detailed description of the mechanisms of the cold-stress response are still needed.

In plants, the main target of stress is the endoplasmic reticulum, where the proteasome-degradation system and the detoxification system are involved in stress responses (Kariana et al. 2005; Martinez and Chrispeels 2003). Most proteins of the extracellular compartment and the endomembrane system are glycosylated by *N*-linked oligosaccharides in plants. The *N*-glycosylation of proteins has a great impact both on their physicochemical properties and on their biological functions. Glycosylation has been shown to act on unfolded proteins and is involved in the quality control of glycoprotein assembly in the endoplasmic reticulum (Rayon et al. 1998; Urada 2007). These prior reports suggest the importance of glycosylation for the optimal functioning of proteins involved in the plant stress response. In this study, lectin blot analysis was used to investigate the effect of cold stress on protein modification in the basal part of rice leaf sheaths. Among the rice glycoproteins whose abundance was changed by cold stress, calreticulin was analyzed in detail to reveal details about the importance of glycosylation for protein function.

Materials and methods

Plant materials and treatment

Rice (*Oryza sativa* L. cv. Nipponbare) was used in this study. Mature rice seeds were hushed, sterilized with 70% ethanol for 5 min and 1% NaClO for 30 min. The seeds were washed with sterile water and allowed to germinate on agar plate of MS medium (Abe et al. 1994) supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in an incubator at 25°C in darkness. After cells were proliferated for about 1 month, they were subcultured in N6 medium (Murashige and Skoog 1962) supplemented 1 mg/L 2,4-D for suspension culture. The cultured cells were subcultured once every 2 weeks.

Rice seedlings were grown in soil under fluorescent light (600 μmole/m^2/s, 16 h light/8 h dark) at 28°C and 70% relative humidity in a growth chamber. Two-week-old seedlings were transferred to 5°C cold chamber and left for 48 h under glimmer light. The basal parts of leaf sheath were collected after cold stress treatment or after a further incubated for 24 h at room temperature.

Preparation of crude protein extract

The following procedures were carried out at 4°C. For protein extraction, a portion (200 mg) of basal parts of leaf sheath or callus was homogenized in a mortar with a pestle in a 300 μL extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EGTA, 5 μM sodium vanadate and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000×*g* for 10 min, and the supernatant was used as the crude protein extract.

Gel electrophoresis

Proteins (200 μg, 100 μL) solubilized with lysis buffer containing 8 M urea, 2% Triton X-100, 2% ampholine (pH 3.5–10) and 10% polyvinylphyrrolidone-40 (O'Farrell 1975) were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in the first dimension by isoelectric focusing (IEF) and in the second dimension by SDS-PAGE (O'Farrell 1975). IEF tube gel solution consisted of 8 M urea, 3.5% acrylamide, 2% Nonidet P-40, 2% ampholines (pH 3.5–10.0 and pH 5.0–8.0), ammonium persulfate and TEMED. Electrophoresis was carried out at 200 V for 30 min, followed by 400 V for 16 h and 600 V for 1 h. After IEF, SDS-PAGE in the second dimension was performed using 15% polyacrylamide gel. pI and Mr of each protein were determined using 2D-PAGE markers (Bio-Rad, Hercules, CA, USA).

On the other hand, proteins (20 μg, 10 μL) solubilized with SDS-sample buffer containing 0.5 M Tris-HCl (pH 6.8), 10% glycerol, 2.5% SDS, and 5% 2-meraaptoethabol (Laemmli 1970) were separated by SDS-PAGE (Laemmli 1970) using 15% polyacrylamide gel.

Purification of proteins

Following protein separation by 2D-PAGE, gel pieces containing protein spots were excised and the proteins were electroeluted using an electrophoretic concentrator (Nippon-Eido, Tokyo, Japan) at 2 W constant power for 2 h. After electroelution, the protein solution was dialyzed against deionized water for 2 days and lyophillized. Proteins were redissolved in SDS-sample buffer, and separated by SDS-PAGE.

Western blot

The proteins separated 2D-PAGE or SDS-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY, USA) using a semidry transfer blotter (Nippon Eido). The PVDF membrane was incubated for 30 min in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Then it was allowed to react for 8 h at room temperature with a peroxidase-conjugated plant lectin, concanavalin A (Seikagaku Kogyo, Tokyo, Japan) that had been diluted 1:1,000 in PBS. Concanavalin A binds to high-mannose type and bi-antennary complex-type oligosaccharides. After the PVDF membrane had been rinsed in PBS containing 0.05% Tween 20, peroxidase activity was visualized by incubation with the horse radish peroxidase color development reagent (Bio-Rad). The image analysis was performed using Image Master 2D Elite software (GE Healthcare, Piscataway, NJ, USA).

On the other hand, proteins separated by 2D-PAGE or SDS-PAGE were electroblotted onto a PVDF membrane, the PVDF membrane was blocked with PBS containing 3% gelatin, cross-reacted with anti-calreticulin antibody (Komatsu et al. 1996), and treated with a secondly peroxidase-conjugated antibody. Peroxidase activity was visualized by incubation with the horseradish peroxidase color development reagent (Bio-Rad).

Amino acid sequence analysis

Following separation by 2D-PAGE, proteins were transferred onto PVDF membrane and stained by Coomassie brilliant blue (CBB), excised from the PVDF membrane and applied to a gas-phase protein sequencer Procise 494 (Applied Biosystems, Foster City, CA, USA). The amino acid sequences obtained were compared with those of known proteins in the Swiss-Prot, PIR, Genpept and PDB databases with Web-accessible search program FastA.

Analysis using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

CBB-stained proteins were excised from gels, washed with 25% methanol, 7% acetic acid for 12 h at room temperature, and destained with 50 mM NH_4HCO_3 in 50% methanol for 1 h at 40°C. After drying under vacuum, gel spots were incubated in 50 μl of a reduction solution containing 10 mM EDTA, 10 mM dethiothreitol and 100 mM NH_4HCO_3 at 60°C for 1 h. The gel spots were dried under vacuum and incubated in 50 μl of alkylation solution containing 10 mM EDTA, 10 mM iodoacetamide and 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. After washing with water, the gel spots were minced, dried under vacuum, and digested in 50 μl of 10 mM Tris–HCl (pH 8.0) containing 1 pM trypsin (Sigma, St Louis, MO, USA) at 37°C for 10 h. One hundred microliter of acetonitrile containing 0.1% trifluoroacetic acid was added to each gel piece and sonicated. Purification of the generated peptides was achieved using Nutip C-18 (Glygen, Columbia, MD, USA). The purified peptides were added to α-cyano-4-hydroxycinamic acid matrix and dried onto a plate for analysis using MALDI-TOF MS (Voyager-DE RP, Applied Biosystems). Calibration was external and data were collected in linear mode. Matching of empirical peptide mass values with theoretical digest and sequence information obtained from the database was performed using Mascot Version 2.0 software (Matrix Science Ltd, London, UK).

For MALDI-TOF MS analysis, 4 criteria were used to assign a positive match with a known protein: (1) the deviation between the experimental and theoretical peptide masses needed to be less than 50 ppm; (2) at least six different predicted peptide masses needed to match the observed masses for an identification to be considered valid; (3) the matching peptides needed to cover at least 30% of the known protein sequence; and (4) individual ions scores >51 identity or extensive homology ($P < 0.05$).

In vitro protein phosphorylation

Five micro liter crude protein extract was incubated in a 25 μL reaction mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.2 mM $CaCl_2$ and 39 μM [γ-^{32}P]ATP (Komatsu et al. 1996). The reaction mixture was incubated for 10 min at 30°C and terminated by the

addition of a lysis buffer (O'Farrell 1975). Denatured proteins were subjected to 2D-PAGE (O'Farrell 1975). The gels were stained with silver staining kit (Bio-Rad) or CBB, and exposed to X-ray film at −80°C for 5 days.

Results and discussion

Twelve out of 22 concanavalin A-positive glycoproteins are changed by cold stress

Post-translational modifications such as glycosylation are important for changing the properties and functions of proteins. To analyze the importance of glycosylation during cold stress in rice, a proteomics approach was used. In plants, *N*-linked glycans strongly influence glycoprotein conformation, stability and biological activity (Rayon et al. 1998). Proteins were extracted from callus and the basal parts of rice leaf sheaths, separated by 2D-PAGE, and subjected to lectin blot analysis with concanavalin A (Fig. 1). To determine if cold stress affects protein *N*-glycosylation, lectin blot analysis was conducted with concanavalin A.

Two-week-old rice seedlings were treated at 25°C (control) or 5°C (cold) for 48 h. After treatment, proteins were extracted from the basal parts of leaf sheaths, separated by 2D-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A (Fig. 1). Twenty-two out of 250 proteins (8.6%) resolved from the basal parts of leaf sheaths reacted with the lectin, a diagnostic feature of *N*-glycosylated proteins (Fig. 1). Cold stress altered the lectin reactivity of 12 proteins (Fig. 1). Among these, 7 proteins (spots 2, 5, 7, 8, 9, 10 and 11) increased and 5 proteins (spots 1, 3, 4, 6 and 12) decreased in reactivity in the lectin blot (Fig. 2b). From the comparison of lectin blot (Fig. 2b) and CBB staining (Fig. 2a) patterns, the increase in spot 5 and the decrease in spots 3, 4, 6 and 12 were alike with both detection protocols. This result suggests that for 5 out of 12 proteins increase or decrease of glycosylation are unrelated to cold treatment. After correction for protein abundance concanavalin A reactivity changed as a response to cold treatment in 7 out of 12 glycoproteins. Hashimoto and Komatsu (2007) reported that, in rice leaf sheaths, 9 proteins changed in abundance after cold stress, with 2 proteins increasing and 7 proteins decreasing in abundance in gels stained with CBB, and the percent change of these proteins, excepting RuBisCO LSU, was not large. Previous reports (Hashimoto and Komatsu 2007) and this study suggest that cold stress has a larger effect on protein glycosylation than on protein abundance.

At 5°C, all the biosynthetic processes are strongly inhibited and some relevant variation could be not observed. So, the basal parts of leaf sheath were collected after cold

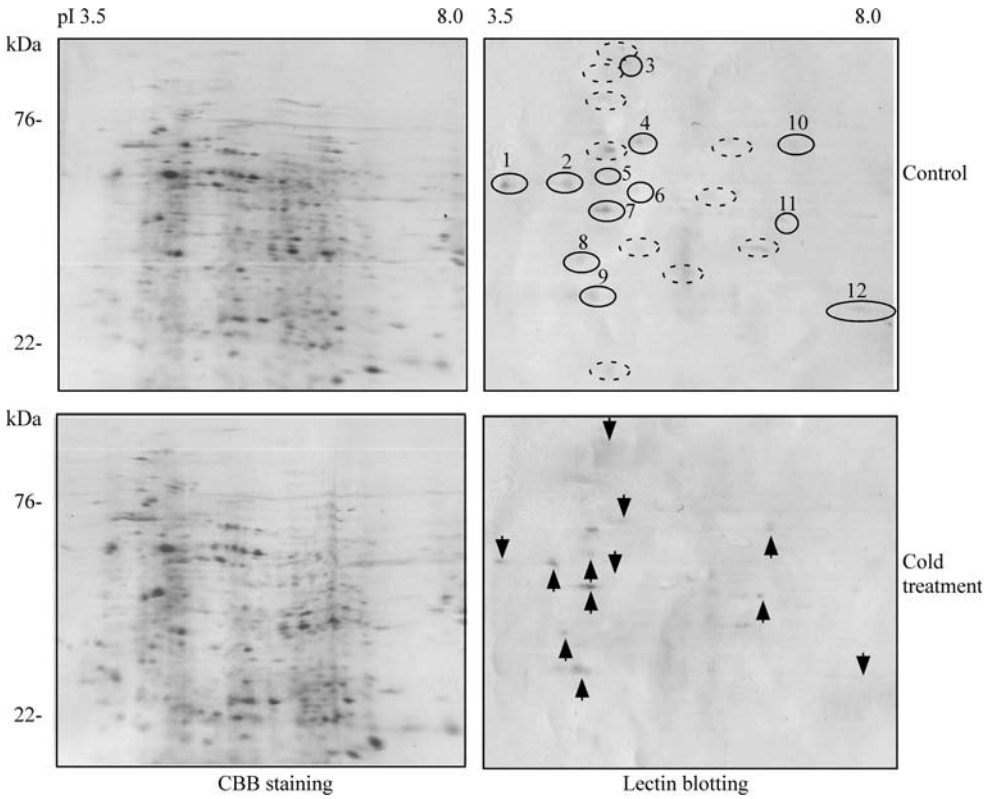

Fig. 1 Glycoprotein profiles in the basal parts of rice leaf sheaths after cold stress. Two-week-old rice seedlings were treated at 25°C (control, *upper*) or 5°C (cold, *lower*) for 48 h. After treatment, proteins were extracted from the basal parts of leaf sheaths, separated by 2D-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A (*right*). CBB-stained 2D-PAGE patterns were used as the loading control (*left*). *Circles* with a *solid line* and/or a *dotted line* represent the position of glycoproteins. *Arrows* indicate the glycoproteins changed by cold treatment and circles mark the positions of the same proteins from the control

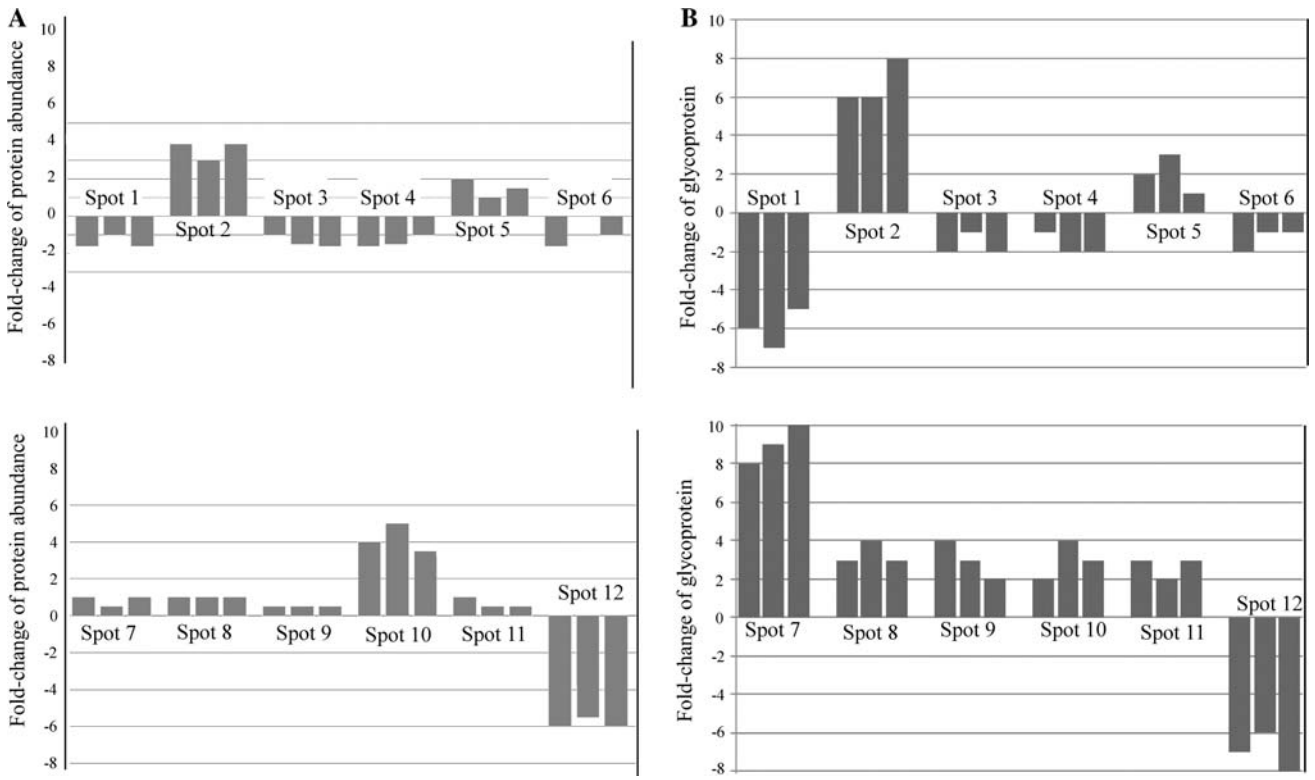

Fig. 2 Relative expression of changed glycoproteins in the basal parts of rice leaf sheaths after cold stress. Two-week-old rice seedlings were treated at 25°C (control) or 5°C (cold) for 48 h. The changes in protein abundance (**a**) and glycoprotein (**b**) were calculated with Image-Master 2D Elite software and plotted in reference to the relative intensities of 12 spots indicated in Fig. 1 *left* and *right*, respectively. The *histogram* shows the fold-changes following cold treatment as calculated from the pixel density for each of the identified protein spots in three independent experiments. Each *bar* represents an independent experiment

stress treatment and further incubated for 24 h at room temperature (Fig. 3). Spots 1, 2, 7 and 9 were clearly affected, but other glycoproteins did not change during recovery at room temperature after cold stress.

The abundance of UDP-glucose pyrophosphorylase and RuBisCO subunit binding-protein alpha subunit are changed by cold stress

Ohtsu et al. (2005) developed new procedures to identify proteins after they were detected by Western blotting or other interactions such as lectin blotting on membranes. Using this method, a glycoprotein detected by lectin blotting with concanavalin A was identified (Ohtsu et al. 2005). In our experience, however, glycoproteins on PVDF membranes after lectin blotting were unsuitable for identification. Therefore, the glycoproteins purified from 2D-PAGE gels were identified by protein sequencing and mass spectrometry (MS). Homology searches were carried out using the FASTA or Mascot search tools (Table 1). N-terminal sequences were successfully obtained for one (spot 1) of the 12 proteins, identified as calreticulin (spot 1). The remaining 11 proteins (spots 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12) were N-terminally blocked, and MS was used to identify homologous proteins (Table 1). Out of 11 proteins, two proteins (spots 7 and 8) did not receive significant matches, indicating they might be novel proteins (Table 1). Previously, Hashimoto and Komatsu (2007) reported that the abundance of UDP-glucose pyrophosphorylase and RuBisCO subunit binding-protein alpha subunit was changed by cold stress.

UDP-glucose pyrophosphorylase is involved in the production/metabolism of UDP-glucose, a key metabolite for sucrose and cell wall biosynthesis. Meng et al. (2007) indicated that UDP-glucose pyrophosphorylases were differentially expressed at the tissue level and in response to metabolic feedback and cold stress, and pointed to a tight post-transcriptional/translational control and distinct roles for these genes in *Populus*. In rice, cold stress had a negative effect on the level of glycosylation of UDP-glucose pyrophosphorylase (Fig. 2). Furthermore, the role of UDP-glucose pyrophosphorylase is to provide UDP-glucose for the synthesis of the carbohydrate moieties of glycoproteins and for glycosylation reactions (Kleczkowski et al. 2004). In this study, UDP-glucose

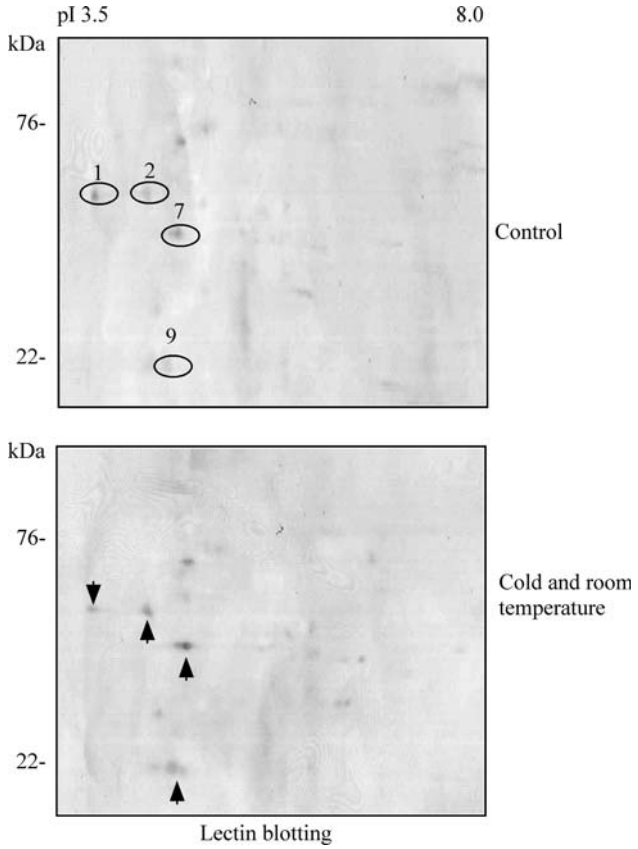

Fig. 3 Glycoprotein profiles in the basal parts of rice leaf sheaths after cold stress and a further incubation at room temperature. Two-week-old rice seedlings were treated at 5°C (*upper*) for 48 h, and then kept at room temperature for 24 h (*lower*). After treatment, proteins were extracted from the basal parts of leaf sheaths, separated by 2D-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A. *Arrows* indicate the changed glycoproteins and *circles* mark the positions of the same proteins from the control

pyrophosphorylase was changed by cold stress leading to a changed capacity for glycosylation. This result suggests that glycosylation has an important role in the response mechanism against cold stress.

RuBisCO subunit binding-protein alpha subunit participates in the formation of the RuBisCO complex. This chaperone protein functions to ensure the correct assembly of the complex (Demirevska-Kepova et al. 1999), by assembling the mature large and small RuBisCO subunits into competent units. Large RuBisCO subunit fragments after cold stress (Hashimoto and Komatsu 2007), suggesting that the observed change of RuBisCO subunit binding-protein alpha subunit with cold stress is linked with RuBisCO complex formation. Previous reports and this result suggest that glycosylation levels of RuBisCO subunit binding-protein monitored by lectin blotting correspond to changes in protein abundance.

Glycoproteins changed by cold stress are mainly categorized into energy production

Assessment of each protein was carried out using the classification system described by Bevan et al. (1998). Five proteins (mitochondrial F1-ATPase, 6-phosphogluconate dehydrogenase, NADP-dependent malic enzyme, enolase and UDP-glucose pyrophosphorylase) were classified as proteins associated with energy production, two proteins (elongation factor 1 beta′ and RuBisCO subunit binding-protein alpha subunit) were placed in the protein synthesis category, one protein (heat shock protein 90) was associated with protein destination, and the remaining protein (calreticulin) was categorized as a signal transduction

Table 1 Changed glycoproteins in basal parts of leaf sheath of rice by cold stress

Spot no.	Homologous protein (Homology percentage or score*)	Accession no.	N-terminal
01	Calreticulin (100)	Q9SP22	N-EVFFQEKFDA
02	Calreticulin (127*)	AK065341	N-blocked (MS)
03	Heat shock protein 90 (59*)	AB111810	N-blocked (MS)
04	Mitochondrial F1-ATPase (65*)	D10491	N-blocked (MS)
05	6-Phosphogluconate dehydrogenase (57*)	AF486280	N-blocked (MS)
06	UDP-glucose pyrophosphorylase (82*)	AB062606	N-blocked (MS)
07	Not significantly hit		N-blocked (MS)
08	Not significantly hit		N-blocked (MS)
09	Elongation factor 1 beta′ (90*)	D12821	N-blocked (MS)
10	NADP dependent malic enzyme (91*)	AB053295	N-blocked (MS)
11	Enolase (110*)	AY335488	N-blocked (MS)
12	RuBisCO subunit binding-protein alpha subunit (82*)	AK100602	N-blocked (MS)

Spot no.: spot numbers are given in Fig. 2, N-: N-terminal amino acid sequences as determined by Edman degradation, N-blocked (MS): N-terminal was blocked and homologous protein was determined by mass spectrometry. Accession no.: accession number in NCBI database

* Shows the score of protein determined by mass spectrometry

protein. Overall, glycoproteins changed by cold stress are mainly placed into the category of energy production.

Mitochondrial F1F0-ATPase is a multimeric enzyme, in which F1 is a hydrophilic sector carrying the catalytic sites for ATP synthesis/hydrolysis and F0 includes hydrophobic subunits embedded in the membrane so as to constitute a proton channel (Pedersen et al. 2000). Zang et al. (2006) reported that mitochondrial ATP synthase small subunit is a nuclear-encoded gene (*RMtATP6)* that functions as a component of the F0-part of F1F0-ATPase. Expression of *RMtATP6* is induced by salt and other osmotic stress treatments.

One of the key enzymes of the pentose phosphate pathway, an important plant metabolic pathway, is 6-phosphogluconate dehydrogenase. Huang et al. (2003) reported that 6-phosphogluconate dehydrogenase in plants plays a role in cell division and salt response. These previous reports and our results indicate that 6-phosphogluconate dehydrogenase and mitochondrial F1-ATPase respond similarly to salt and cold stresses. Furthermore, hyperhydricity is considered a physiological disorder that can be induced by different stressing conditions. Saher et al. (2005) reported metabolic and energetic states of hyperhydric carnation shoots resulting from altered metabolism of pyruvate in hyperhydric tissues by the induction of pyruvate synthesis via NADP-dependent malic enzyme. These results also suggest that hyperhydric plant tissues have adapted to hypoxia stress conditions by the induction of the oxidative pentose phosphate and fermentative pathways.

Yan et al. (2006) has also reported that enolase, elongation factor 1 beta′ and heat shock protein respond to cold stress in rice. Primary metabolism, such as metabolism of carbon, nitrogen, sulfur, and energy, need to be modulated to establish a new homeostasis under cold stress (Thomashow 1998). Cold stress up-regulates expression of enolase, an enzyme involved in glycolysis. This up-regulation might help to produce more energy needed in various defense processes.

Calreticulin among 12 glycoproteins is also phosphorylated by cold stress

Furthermore, to analyze the importance of post-translational modifications during cold stress, in vitro protein phosphorylation was monitored. Two-week-old rice seedlings were treated at 5°C for 48 h. After treatment, the proteins were extracted from the basal parts of leaf sheaths, separated by 2D-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A (Fig. 4 left). In parallel, protein extracts were incubated in a reaction mixture containing [γ-^{32}P]ATP. After in vitro protein phosphorylation, proteins were separated by 2D-PAGE, and the 2D-PAGE gels were autoradiographed (Fig. 4 right). Nine proteins were phosphorylated or de-phosphorylated in the basal parts of leaf sheaths by cold stress. Two calreticulins were phosphorylated (spot 2) and de-phosphorylated (spot 1) as well as glycosylated by the cold stress (Fig. 4).

In rice, calreticulin has been identified as a calcium-binding phosphorylated protein that appears to be associated with the regeneration of cultured rice cells (Komatsu et al. 1996). Calreticulin was also present in rice suspension culture cells, and was developmentally regulated during regeneration (Li and Komatsu 2000). The role of calreticulin as a stress-induced molecular chaperone protein of the endoplasmic reticulum is becoming more apparent (Chapman et al. 1998). Rice calreticulin has been shown to be involved and phosphorylated in the signaling

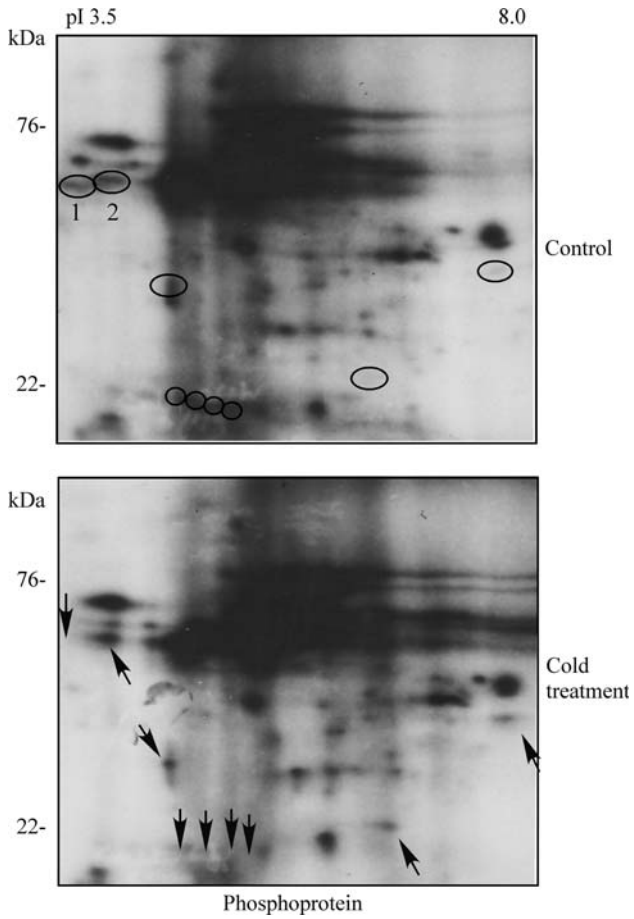

Fig. 4 Phosphoprotein profile from the basal parts of rice leaf sheaths after cold stress. Two-week-old rice seedlings were treated at 25°C (control, *upper*) or 5°C (cold, *lower*) for 48 h. Protein extracts were incubated in a reaction mixture containing [γ-^{32}P]ATP. After in vitro protein phosphorylation, proteins were separated by 2D-PAGE, and exposed to X-ray film. *Arrows* indicate the phosphorylated proteins whose abundance changed by cold treatment and *circles* mark the positions of the same proteins from the control. Protein spots 1 and 2 were the positions of two calreticulins

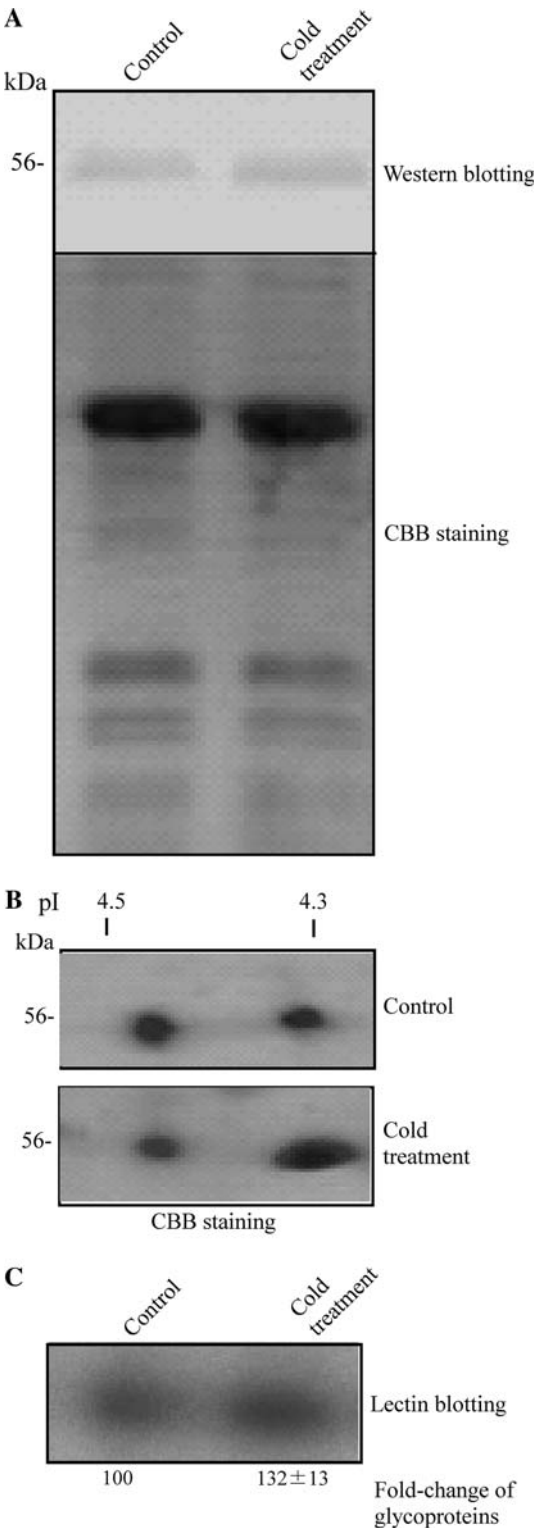

Fig. 5 Expression analysis of calreticulin in the basal parts of rice leaf sheaths after cold stress. Two-week-old rice seedlings were treated at 25°C (*control*) or 5°C (*cold*) for 48 h. After treatment, the proteins were extracted from the basal parts of leaf sheaths, separated by SDS-PAGE, transferred to a PVDF membrane, and cross-reacted with anti-calreticulin antibody (**a** *upper*). CBB-stained SDS-PAGE patterns were used as the loading control (**a** *lower*). In parallel, after extraction of proteins from the basal parts of leaf sheaths after cold treatment, proteins were separated by 2D-PAGE and stained by CBB (**b**). Calreticulin purified from 2D-PAGE gels was separated by SDS-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A (**c**). The detected bands represent glycosylated calreticulin

pathway leading to the cold stress response (Li et al. 2003; Khan et al. 2005). Previous reports and this study indicate that calreticulin is regulated not only by phosphorylation but also by glycosylation.

Glycosylation and phosphorylation of calreticulin but not expression level are affected by cold stress

Two-week-old rice seedlings were treated at 5°C for 48 h. After treatment, the proteins were extracted from the basal parts of rice leaf sheaths, separated by SDS-PAGE, transferred to a PVDF membrane, and cross-reacted with anti-calreticulin antibody (Fig. 5a). At the same time, after extraction of proteins from the basal parts of rice leaf sheaths after cold treatment, proteins were separated by 2D-PAGE and stained by CBB (Fig. 5b). Furthermore, calreticulin purified from 2D-PAGE gels was separated by SDS-PAGE, transferred to a PVDF membrane, and cross-reacted with peroxidase-conjugated concanavalin A (Fig. 5c). The expression level of calreticulin in cold treatment was the same as that in control (Fig. 5a), but the lectin blot with purified calreticulin showed that glycosylation activity increased with cold stress (Fig. 5c). By 2D-PAGE analysis, calreticulin spot with lower pI was found shifted by cold treatment to higher pI. These results indicate that cold stress of the basal parts of rice leaf sheaths changes the glycosylation and phosphorylation profiles of calreticulin, a key protein that regulates the quality control of other proteins.

Concluding remarks

Rayone et al. (1998) indicated that *N*-glycosylation of concanavalin A is important for its transport to the vacuole and the regulation of its lectin activity. The regulation of the lectin activity is crucial since a lectin with high affinity for high-mannose-type *N*-glycans, such as mature concanavalin A, could bind to newly synthesized *N*-linked glycoproteins in the endoplasmic reticulum preventing their transport down-stream in the secretory pathway (Rayone et al. 1998). Calreticulin is also a protein localized in the endoplasmic reticulum, suggesting that the pathway through the endoplasmic reticulum is changed as a result of cold stress. These results indicate that cold stress to the basal parts of rice leaf sheaths affects particularly post-translational modifications

such as those demonstrated by calreticulin. As a result, the quality control of many other proteins passing through the secretory pathway can be influenced.

References

Abe T, Oka Y, Sasahara T (1994) Varietal variations in biochemical changes during growth and redifferentiation of rice callus cultures. Jpn J Genet 69:385–396

Bevan M, Bancroft I, Bent E, Love K et al (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. Nature 391:485–488

Chapman R, Sidrauski C, Walter P (1998) Intracellular signaling from the endoplasmic reticulum to the nucleus. Annu Rev Cell Dev Biol 14:459–485

Cui S, Huang F, Wang J, Ma X, Cheng Y, Liu J (2005) A proteomic analysis of cold stress responses in rice seedlings. Proteomics 5:3162–3172

Demirevska-Kepova K, Simova-Stoilova L, Kjurkchiev S (1999) Barley leaf rubisco binding protein and rubisco activase and their protein/protein interactions. Bulg J Plant Physiol 2:31–44

Hashimoto M, Komatsu S (2007) Proteomic analysis of rice seedlings during cold stress. Proteomics 7:1293–1302

Hayashi T, Yamada T, Nakayama K, Komatsu S, Koike S (2006) Susceptibity to coolness at the young microspore stage under high nitrogen supply in rice (*Oryza sativa* L.). Plant Prod Sci 9:212–218

Huang J, Zhang H, Wang J, Yang J (2003) Molecular cloning and characterization of rice 6-phosphogluconate dehydrogenase gene that is up-regulated by salt stress. Mol Biol Rep 30:223–227

Iba K (2002) Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance. Annu Rev Plant Biol 53:225–245

Imin N, Kerim T, Rolfe BG, Weinman JJ (2004) Effect of early cold stress on the maturation of rice anthers. Proteomics 4:1873–1882

Kariana ME, Meyer DJ, Gibbon BC, Jung R, Boston RS (2005) Identification and characterization of endoplasmic reticulum-associated degradation proteins differentially affected by endoplasmic reticulum stress. Plant Physiol 138:218–231

Khan M, Takasaki H, Komatsu S (2005) Comprehensive phosphoproteome analysis in rice and identification of phosphoproteins responsive to different hormones/stresses. J Proteome Res 4:1592–1599

Khush GS (1999) Green revolution: preparing for the 21st century. Genome 42:646–655

Kim SH, Kim JY, Kim SJ, An KS, An G, Kim SR (2007) Isolation of cold stress-responsive genes in the reproductive organs, and characterization of the OsLti6b gene from rice (*Oryza sativa* L.). Plant Cell Rep (on line)

Kleczkowski LA, Geisler M, Ciereszko I, Johansson H (2004) UDP-glucose pytophosphorylase. An old protein with new tricks. Plant Physiol 134:912–918

Koga-Ban Y, Abe M, Kitagawa Y (1991) Alteration in gene expression during cold treatment of rice plant. Plant Cell Physiol 31:901–905

Komatsu S, Masuda T, Abe K, (1996) Phosphorylation of a protein (pp56) is related to the regeneration of rice cultured suspension cells. Plant Cell Physiol 37:748–753

Komatsu S, Konishi H, Hashimoto M (2007) The proteomics of plant cell membranes. J Exp Bot 58:103–112

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Li Z, Komatsu S (2000) Molecular cloning and characterization of calreticulin, a calcium-binding protein involved in the regeneration of rice cultured suspension cells. Eur J Biochem 267:737–745

Li Z, Onodera H, Ugaki M, Tanaka H, Komatsu S (2003) Characterization of calreticulin as a phosphoprotein interacting with cold-induced protein kinase in rice. Biol Pharm Bull 26:256–261

Lyons JM (1972) Phase transitions and control of cellular metabolism at low temperatures. Cryobiology 9:341–350

Martinez I, Chrispeels MJ (2003) Genomic analysis of the unfolded protein response in *Arabidopsis* shows its connection to important cellular processes. Plant Cell 15:561–576

Meng M, Geisler M, Johansson H, Mellerowicz EJ, Karpinski S, Kleczkowski LA (2007) Differential tissue/organ-dependent expression of two sucrose- and cold-responsive genes for UDP-glucose pyrophosphorylase in Populus. Gene 389:186–195

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of protein. J Biol Chem 250:4007–4021

Ohtsu I, Nakanishi T, Furuta M, Ando E, Nishimura O (2005) Direct matrix-assisted laser desorption/ionization time-of-flight mass spectrometric identification of proteins on membrane detected by Western blotting and lectin blotting. J Proteome Res 4:1391–1396

Pedersen PL, Ko YH, Hong S (2000) ATP synthase in the year 2000: evolving views about the structures of these remarkable enzyme complexes. J Bioenerg Biomembr 32:325–332

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol 133:1755–1767

Raison JK, Lyons JM (1986) Chilling injury: a plea for uniform terminology. Plant Cell Environ 9:685–686

Rayon C, Lerouge P, Faye L (1998) The protein *N*-glycosylation in plants. J Exp Bot 49:1463–1472

de los Reyes BG, Morsy M, Gibbons J, Varma TS, Antoine W, McGrath JM, Halgren R, Redus M (2003) A snapshot of the low temperature stress transcriptome of developing rice seedlings (*Oryza sativa* L.) via ESTs from subtracted cDNA library. Theor Appl Genet 107:1071–1082

Saher S, Fernandez-Garcia N, Piqueras A, Hellin E, Olmos E (2005) Reducing properties, energy efficiency and carbohydrate metabolism in hyperhydric and normal carnation shoots cultured in vitro: a hypoxia stress? Plant Physiol Biochem 43:573–582

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol 118:1–8

Tyagi AK, Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Sci 158:1–18

Urada R (2007) Cellular response to unfolded proteins in the endoplasmic reticulum of plants. FEBS J 274:1152–1171

Yan SP, Zhang QY, Tang ZC, Su WA, Sun WN (2006) Comparative proteomic analysis provides new insights into chilling stress responses in rice. Mol Cell Proteomics 5:484–496

Yu X, Peng YH, Zhang MH, Shao YJ, Su WA, Tang ZC (2006) Water relations and an expression analysis of plasma membrane intrinsic proteins in sensitive and tolerant rice during chilling and recovery. Cell Res 16:599–608

Zhang X, Takano T, Liu S (2006) Identification of a mitochondrial ATP synthase small subunit gene (*RMtATP6*) expressed in response to salts and osmotic stresses in rice (*Oryza sativa* L.). J Exp Bot 57:193–200