한작지(Korean J. Crop Sci.), 52(3): 281~288(2007)

Proteome Analysis of the Young Spikelets of Photoperiod-Sensitive Rice Mutant Treated in Different Photoperiods

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ABSTRACT Photoperiod sensitive genetic male sterile (PGMS) rice is sterile mutant controlled by photoperiod. A PGMS mutant 920S was sterile grown under long-day (LD) photoperiod (14 h light / 10 h dark) but fertile grown under short-day (SD) photoperiod (10 h light / 14 h dark). Proteome analysis revealed that 12 protein spots were differentially expressed in the spikelets of 920S plants either treated with LD or SD photoperiod. Among these proteins, three proteins including chlorophyll *a/b* binding protein, vacuolar ATPase β-subunit, α-tubulin and an unknown protein were more than three-fold abundant in the spikelet of the SD-treated plants than those of the LD-treated plants. On the other hand, eight proteins including acetyl transferase, 2, 3- biphosphoglycerate, aminopeptidase N, pyruvate decarboxylase, 60S acidic ribosomal protein and three unknown protein spots were more abundant in the spikelets of the LD-treated plants than those of the SD-treated plants. The results suggest that the observed proteins may be involved in sterile or fertile pollen development under LD or SD photoperiod respectively in the PGMS mutant rice.

Keywords : photoperiod, photoperiod sensitive genetic male sterile rice, PGMS, rice pollen sterility, rice proteomics

In the higher plants, the development of the male gametophyte is well organized into an elaborate process which plays a crucial role in plant reproduction (Bedinger, 1992). Pollen development consists of three major developmental stages which include sporogenesis, post meiotic development of microspores, and mitosis of microspores to form mature pollens (McCormick, 1993). Furthermore, cell wall biosynthesis, cytoskeletal development and the proper formation of tapetum are critical for the development and eventual release of pollen grains (Pacini *et al.*, 1985; Denis *et al.*, 1993; Honys and Twell, 2003). Any improper development of these male organs results in male sterilities.

Male sterility (MS) is caused by mutations in the male sex organs resulting from the lack of pollen grains in the anther or formation of nonfunctional pollen grains (Chaudhury, 1993). Currently, four types of male sterile in rice were reported. These include cytoplasmic male sterility (CMS), photoperiod sensitive genetic male sterility (PGMS), thermosensitive genetic male sterility (TGMS) and other male sterilities (Kurata *et al.*, 2005). PGMS rice mutant is sterile grown under long-day photoperiod condition (14 h light / 10 h dark) but is fertile grown under short-day photoperiod condition (10 h day / 14 h night) (Pandeya *et al.*, 2006). In PGMS rice, three different alleles for male sterility including *pms1, pms2* and *pms3* have been mapped on chromosome 7, 3 and 12, respectively (Zhang *et al.*, 1994; Mei *et al.*, 1999; Lu *et al.*, 2005). However, genes for thses alleles and the mechanisms for fertility alteration of the PGMS rice influenced by photoperiod are not revelaed yet.

Proteome analysis allows for qualitative and quantitative measurements of a large number of proteins which directly influences cellular biochemistry, and provides reliable analysis of the cellular state or system changes during growth, development and response to environmental factors (Chen and Harmon, 2006). Therefore, to identify proteins and genes involved in this PGMS phenomenon, here we analyzed the proteomic analysis of proteins isolated from

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spikelets of PGMS mutant rice 920S as response to LD or SD photoperiod treatments.

MATERIALS AND METHODS

Plant material

Rice (*Oryza sativa* L., cv Japonica) mutant 920S plants were grown in greenhouse conditions. The tillers from individual plants were divided and grown under two different photoperiod conditions (14 h light / 10 h dark - LD; 10 h day / 14 h night - SD). For the long-day treatment, plants were grown in natural long-day photoperiod conditions from June to mid of July in Gyeongsan, Korea. Simultaneously, for the short-day treatment, plants were exposed to 10 h day light and subsequently transferred to a dark room. The average temperatures were 30℃ during daytime and 22℃ at night. Young panicles of the 920S plants from both treatments were collected during the pollen development stages. Spikelets were detached from the panicle and immediately frozen in liquid nitrogen and stored in -80℃ until use.

Microscopy

Spikelets were collected from treated plants before noon. Three healthy flowering panicles from each plant were selected for pollen viability testing. Five spikelets from each panicle were collected and pollen grains from open anthers were placed on a glass slide, followed by staining with a 1% iodine potassiumiodode (I_2KI) solution. Pollen viability was studied using the Olympus BX51 light microscope (Olympus, Tokyo, Japan). Photographs were taken using the Olympus C-7070 digital camera.

Protein extraction

For protein extraction, heading spikelets from the 920S rice plants treated either in LD photoperiod or SD photoperiod were used. Each of the proteins was extracted independently from more than 3 panicles collected from the each two different photoperiod treated plants. Spikelets were ground to fine powder with liquid nitrogen and the tissue powder was homogenized directly by mortar-driven homogenizer (PowerGen125, Fisher Scientific, Springfield, NJ, USA) in an extraction buffer containing 7 M urea / 2 M thiourea; 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate (CHAPS); 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte (pH 3.5-10.0, Amersham BioSciences, Piscataway, TX, USA) and 1 mM benzamidine. Proteins were extracted for 1h at 4℃ with vortexing. After centrifugation at 15,000 × g for 1 h at 15℃, insoluble material was discarded and soluble fraction was used for two-dimensional polyacrylamide gel electrophoresis (2-D-PAGE). The concentration of protein was determined by the Bradford method (1976) using a commercial dye reagent from Bio-Rad Laboratories (Hercules, CA, USA).

2-D electrophoresis and image analysis

The extracted proteins were analyzed by 2-D gel electrophoresis and this step was repeated twice independently. Immobilized pH gradient (IPG) dry strips (pH range of 4-9, 24 cm long, Genomine Inc., Korea) were equilibrated for 12-16 h with 7 M urea, 2 M thiourea containing 2% (w/v) CHAPS, 1% v/v DTT, 1% (v/v) pharmalyte (pH 3.5-10.0, Amersham BioSciences) and each loaded with 200 µg of sample. Isoelectric focusing (IEF) was performed at 20℃ using a Multiphor II electrophoresis unit and an EPS 3500 XL power supply (GE Healthcare, Germany) following manufacturer's instructions. For IEF, the voltage was linearly increased from 150 to 3,500 V within the 3 h run of the sample entry followed by a constant 3,500 V, with focusing completed after 96 kVh. Prior to the second dimension, strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl, pH 6.8, containing 6 M urea, 2% SDS, 1% DTT and 30% glycerol) and transferred to an alkylation buffer (2.5% iodoacetamide, 6 M urea, 2% SDS, 30% glycerol, 50 mM tris HCl, pH 6.8). Equilibrated strips were inserted onto SDS-PAGE gels (20-24 cm, 10-16% gradient SDS gel), and performed using Hoefer DALT 2D system (GE Healthcare) as per the manufacturer's instructions. The 2-D gels were run at 20℃ for 1.7 kVh and subsequently stained with silver nitrate (Sigma Chemical Co., St. Louis, MO.) as described by Oakley *et al.* (1980) with

the exclusion of fixing and sensitization steps with glutaraldehyde. Quantitative analysis of digitized images was carried out using the PDQuest software (version 7.0, Bio-Rad, Richmond, CA, USA) following the manufacturer's protocols. After spot detection, the quantity of each spot volume was normalized with total valid spot intensity. Protein spots, which showed more than two-fold difference in spot quantity between the 2-D gels of SD and LD spikelets, were selected for further analysis.

Enzymatic digestion of protein in gel

Protein spots with different expressions depending on photoperiod were excised from the stained gels and digested by modified porcine trypsin according to Shevchenko *et al.* (1996). Gel pieces were washed with 50% acetonitrile (Wako, Osaka, Japan) to remove SDS, salt and stain, vacuum-dried to remove solvent, rehydrated with trypsin (8-10 ng/μL, sequencing grade, Promega) and finally incubated for 8-10 h at 37℃. The proteolytic reaction was terminated by the addition of 5 μL 0.5% trifluoroacetic acid. Tryptic peptides were recovered by combining the aqueous phase from several extracts of gel pieces with 50% aqueous acetonitrile. After concentration, the peptide mixture was desalted using C_{18}ZipTips (Millipore Billerica, MA, USA), and were eluted in 1-5 μL of acetonitrile. Further, an aliquot of this solution was mixed with an equal volume of a saturated α-cyano-4-hydroxycinnamic acid solution in 50% aqueous acetonitrile and 1 μL of mixture was spotted onto a plate.

MALDI-TOF-MS analysis and database search

Protein analysis was performed using Ettan MALDI-TOF (GE Healthcare). Peptides were evaporated with a N_2 laser at a wavelength of 337 nm using a delayed extraction approach, followed by acceleration with a 20 kV injection pulse for the time of flight analysis. Each spectrum was the cumulative average of 300 laser shots. Protein identification was performed by peptide mass fingerprinting using the Rockefeller University-developed search program ProFound (http://129.85.19.192/profound_bin/ WebProFound.exe). Spectra were calibrated with trypsin auto-digestion ion peak *m/z* (842.510, 2211.1046) as internal standards. Public databases including ExPASy (http://www.expasy. org/), PROSITE (http://www.expasy.org/prosite/), BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), BRENDA (http://www. brenda.uni-koeln.de/) and KEGG (http://www.genome.ad.jp/kegg/kegg2.html) were used for the database search.

RESULTS AND DISCUSSION

Fertility analysis of 920S rice in different photoperiods

Pollen viability was analyzed based on I_2KI stainablity and the spikelet fertility was analyzed based on seed setting rate of the individual PGMS 920S plants (Table 1). The wild type plants remained fertile regardless of photoperiod (Fig. 1A), however, PGMS mutant 920S plants remained fertile when exposed to the SD photoperiod (Fig.

Table 1. Pollen viability and spikelet fertility of PGMS 920S rice.

Plant	Photoperiods			
	Short day photoperiod condition (SD)		Long day photoperiod condition (LD)	
	Pollen viability (%)	Spikelet fertility (%)	Pollen viability (%)	Spikelet fertility (%)
920S-1	73.13	29.41	58.71	6.5
920S-2	23.28	25.0	23.12	1.59
920S-3	86.54	18.75	63.45	2.62
920S-4	82.15	34.88	13.2	2.45
920S-5	70.83	47.45	33.83	3.31
920S-6	73.32	55.88	5.52	2.47
920S-7	80.57	37.88	9.36	4.10
Average	69.97	35.6	29.59	3.29
Jinmibyeo	93.0	89.60	94.50	93.5

Note: Jinmibyeo is a wild type cultivar.

1B), and became sterile when exposed to the LD photoperiod (Fig. 1C). According to Datta *et al.* (2002), insufficient starch accumulation results in sterile pollen, thus, pollen staining with iodine potassium iodide (I_2KI) solution was used to detect starch accumulation in the pollen grains in the present study. The results of stainability test illustrated that pollen grains from plants grown under the SD photoperiod were round and darkly stained with I_2KI solution (Fig. 1E) which is similar to the pollen grains of the wild type rice (Fig. 1D). However, pollen grains of the 920S plants grown under the LD photoperiod were not stained with I_2KI (Fig. 1F) and hence, most of the spikelets were empty and sterile (Fig. 1C).

Spikelet fertilities for the wild type rice in both SD and LD photoperiod treatments were at 89.6% and 93%, respectively (Table 1). However, 920S plants subjected to the SD photoperiod had an average pollen viability of 69.9% and spikelet fertility of 35.6% (Table 1). On the other hand, 920S plants subjected to a LD photoperiod have a low average pollen viability of 29.59% and spikelet fertility of only 3.29% (Table 1). Since the average spikelet fertility showed 3.29% under LD photoperiod and 35.6% under SD photoperiod, the present study revealed that 920S mutant rice is a tyical PGMS mutant. Therefore, our observations in this study clearly indicate that less than 5% fertility was obtained from more than 14 h in day length suggesting that 14 h under light in LD photoperiod is a critical requirement of sterile PGMS plants.

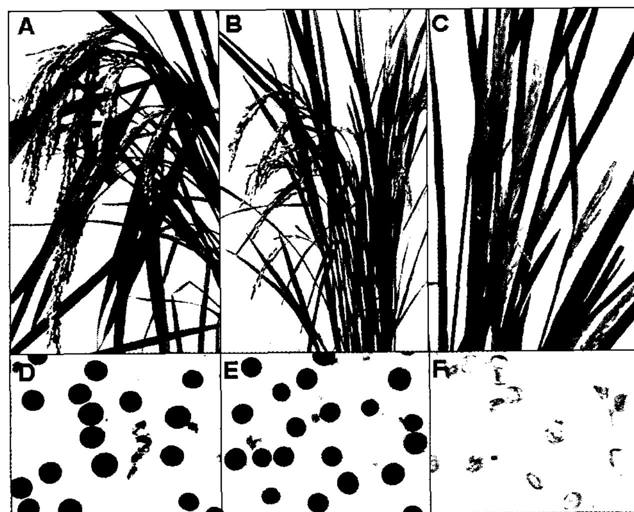

Fig. 1. Phenotypes of wild type (Jinmibyoe) and 920S rice. Ripening statges of wild type panicles (A), panicles of 920S rice grown under short-day (SD) photoperiod condition (B) and long-day (LD) photoperiod condition (C). The pollen grains of wild type (D), SD photoperiod condition (E) and LD photoperiod condition (F) were stained with iodine potassiumiodide (I_2KI) solution.

Proteomic analysis of 920S spikelets

The protein expression patterns were compared next to the total protein extracted from the young rice spikelets of different photoperiod treated 920S rice plants. On the silver-stained representative 2-DE gel, approximately 1,600 protein spots were reproducibly detected in gels with silver staining across the two biological replicates (Fig. 2). Among them, 26 protein spots showed a greater than three fold discrepancy between spikelet proteins of SD- and LD-

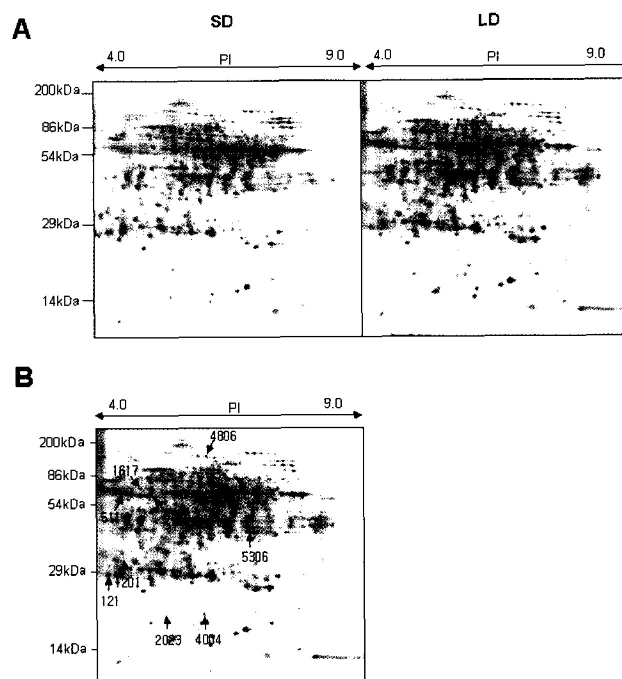

Fig. 2. Proteomic data of the spikelets of 920S rice grown under short-day (SD) photoperiod condition and long-day (LD) photoperiod condition. (A) The 2-D electrophoresis gel pictures of proteins isolated from young spikelets of 920S rice proteins from SD and LD treatments. (B) Differentially expressed proteins between SD and LD. Analyzed 12 protein spots are indicated with arrow heads. Isoelectric focusing was performed with IPG Dry Strips (pH 4-9, 24 cm long). The gels were stained with silver nitrate.

treated plants as detected. Of these 26 protein spots, 12 spots were excised for further MALDI-TOF-MS analysis (Table 2) and quantitative expression level comparison (Fig. 3). The amino acid sequences obtained from this analysis were compared with those of known proteins in the BLAST, Swiss-Port and NCBI protein databases. According to the published database, the identified proteins were related to energy, cell growth, metabolism and protein synthesis (Table 2). Of the proteins which expressed a difference in abundance as a function of photoperiod, chlorophyll *a/b*-binding protein (Spot ID 0121), vacuolar ATPase β-subunit (Spot ID 1617), α-tubulin (Spot ID 0511) and an unknown protein (Spot ID 2023) were highly abundant in the spikelet subjected to a SD photoperiod treatment. However, protein spots including acetyl transferase (Spot ID 2503), 2,3- biphosphoglycerate (Spot ID 5704), aminopeptidase N (Spot ID 4806), pyruvate decarboxylase (Spot ID 5721), 60S acidic ribosomal protein (Spot ID 5306) and three unknown protein spots (Spot ID's 3512, 1201, 4004) were abundant in the spikelets of plants subjected to a LD photoperiod treatment. Identification of these proteins provides useful clues on how the 920S plants become sterile when subjected to a LD photoperiod. Therefore, we attempt to describe their possible roles in pollen development.

Chlorophyll *a/b* binding protein (CAB protein)

The primary functions of the CAB protein in higher plants are the absorption of light through chlorophyll excitation and transfer of the absorbed energy to the photochemical reaction centers (Green and Durnford, 1996). Gene expression of some families of chlorophyll binding proteins are solely triggered by light stress, and their transcription and protein accumulation are light intensity dependent (Anderson *et al.*, 2003). In this experiment, CAB protein (Spot ID 0121) was abundant in the spikelets of the SD-treated plants whereas only trace amounts were present in LD-treated plants (Table 2, Fig. 3). This suggests that CAB protein gene expression may have been suppressed under LD photoperiods. The low CAB protein level in the spikelets may have caused the failure of functional pollen grain development resulting to sterility in PGMS 920S rice.

Alpha-tubulin (TubA)

Microtubules of the eukaryotic cytoskeletons are composed of a heterodimer of α-tubulin (TubA) and β-tubulin (TubB) which is involved in the maintenance of cell shape and mediation of chromosome migration in mitosis (Ducher, 2001). In plants, microtubules are organized in different arrays associated with the different phases of cell cycle

Table 2. Proteome data of PGMS 920S spikelets treated with short and long photoperiod conditions.

Protein type	Spot ID	Profound search detail protein	Source	Accession	MM	pI	Quantity		No. of peptide	Coverage %	Ec No.
							SD	LD			
Energy	0121	Chlorophyll *a/b*-bindingprotein	*O. sativa*	gi|115484898	30.27 kDa	4.30	3096.8	14.81	8	28	NA
Unknown	2023	Unknown	*O. sativa*	gi|18644694	12.69 kDa	5.00	2529.26	0.15	5	49	NA
Cell growth	0511	Alpha tubulin (TubA)	*O. sativa*	gi|50428726	50.54 kDa	4.48	493.83	115.05	18	24	EC 2.3.1.108
Metabolism	1617	Vacuolar ATPase B subunit	*O. sativa*	gi|14150751	54.15 kDa	4.72	569.75	155.94	17	32	EC 3.6.3.14
Metabolism	2503	Putative acetyl transferase	*O. sativa*	gi|34912654	46.14 kDa	4.86	761.31	1620.29	31	53	EC 2.3.1.48
Metabolism	4806	Aminopeptidase N	*O. sativa*	gi|50948547	98.64 kDa	5.31	268.15	583.11	16	14	EC 3.4.11.1
Unknown	3512	ESTsAU033004	*O. sativa*	gi|34897738	55.27 kDa	5.18	0	50.2	6	9	NA
Unknown	1201	OJ000315_02.8	*O. sativa*	gi|38569173	33.96 kDa	4.53	0	174.232	5	20	NA
Metabolism	5704	2,3- biphosphoglycerate	*O. sativa*	gi|15128394	61.21 kDa	5.43	0	591.35	8	16	EC 2.3.1.48
Metabolism	5721	Pyruvate decarboxylase (PDC1)	*O. sativa*	gi|7436707	65.37 kDa	5.57	0	595.18	9	11	EC 4.1.1.1
Protein synthesis	5306	60S acidic ribosomal protein	*O. sativa*	gi|50940807	34.47 kDa	5.48	0	913.92	10	18	NA
Unknown	4004	Unknown	*A. thaliana*	gi|9758488	17.59 kDa	5.29	0	1920.02	9	45	NA

Note: Spot ID refers to the spot number as given in the figure 3 and 4. Quantity refers to the average amount of proteins intensity of two independent analyses, MM; experimental molecular masses, pI; experimental isoelectric points, SD; Short-day photoperiod condition, LD; Long-day photoperiod condition, EC No; SwissPort/UniPort identity number.

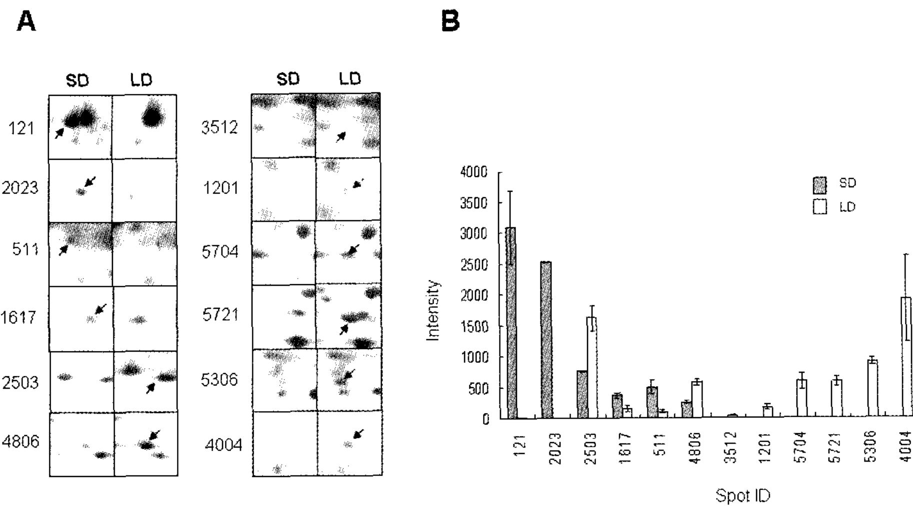

Fig. 3. Comparision of differentially expressed proteins in the young spikelets of 920S rice grown under short-day (SD) photoperiod and long-day (LD) photoperiod conditions. (A) Enlarged sections of differentially expressed protein spots from SD and LD. (B) Comparison of differentially expressed proteins intensity of 920S pollens from SD and LD photoperiod conditions. Values are the average ± SE of protein intensities from two independent experiments. Gray bars indicate SD photoperiod condition and white bars indicate LD photoperiod condition.

(Goddard *et al.*, 1994). Microtubules are basic components of plant's cytoskeleton which are involved in many fundamental cellular processes, such as cell division, cell elongation and cell wall synthesis (Quin *et al.*, 1997). Elevated expression of α-tubulin was reported in the pollen of sunflower (Evrard *et al.*, 2002) and in the rapidly growing tissues such as root tips and young flowers of rice (Jeon *et al.*, 2000). In the present study, α-tubulin (Spot ID 0511) was less abundant in the spikelets of LD-treated plants than the SD-treated plants (Table 2, Fig. 3). This indicates that insufficient amounts of microtubules in the LD treated plants may have delayed microspore division leading to the incomplete formation of viable pollen grains, thereby resulting in sterility of the PGMS 920S rice when subjected to long-day photoperiod conditions.

Vacuolar ATPase B subunit (V-ATPase)

The vacuolar ATPase is a multi-subunit complex consisting of two distinct domains including a transmembrane proton channel (V_0) and an attached catalytic complex (V_1) (Nishi and Forgac, 2002). The plasma membrane V-ATPases is also functional in a variety of normal and diseased processes including sperm maturation (Brown *et al.*, 1997). In Arabidopsis, V-ATPase function is essential for Golgi organization, development of male gametophytes and pollen maturation (Dettmer, 2005). As V-ATPase is involved in sperm maturation in animals and male gametophyte development in plants, it might be involved in the development of fertile pollen grains. Proteome analysis revealed a two-fold abundance of V-ATPase (Spot ID 1617) in the spikelets of SD-treated plants as compared to LD-treated plants (Table 2, Fig. 3). The V-ATPAse deficiency in spikelets of LD-treated PGMS 920S rice may have impaired functional pollen grain development. Therefore, this suggests that V-ATPase may play a role in functional pollen development.

Specific proteins influenced by long-day photoperiod

Five protein spots including 2, 3 biphosphoglycerate (Spot ID 5704), pyruvate decarboxylate (Spot ID 5721), 60S ribosomal protein (Spot ID 5306) and two unknown proteins (Spot ID 4004 and Spot ID 1201) were highly abundant in the spikelets of LD-treated plants, but not detected in the SD-treated plants (Table 2, Fig. 3). The amino acid of protein spot ID 1201 was highly homologus to a protein encoded in unknown cDNA (OJ000315) of rice. Furthermore, the two proteins, acetyltransferase (Spot ID 2503)

and the aminopeptidase N (Spot ID 4806) were present in more than twice the amount in the spikelets of LD-treated plants than SD-treated plants. Although a significant differential pattern of these protein expressions have been identified in the spikelets of the LD- and SD-treated plants, interpretation of their functions was not easy in relation to the sterility observed from the LD-treated plants. However, since these proteins are known to be involved in protein biosynthesis, they are considered to play a vital role during active vegetative growth of sterile spikelets during LD photoperiod conditions. In this study, differentially expressed proteins have been successfully identified from the spikelets of 920S rice plants grown under either LD or SD photoperiod conditions.

ACKNOWLEDGEMENTS

We thank Md. Nurul Matin of Yeungnam University for their technical assistance. A student fellowship for Devendra Pandeya was supported by the Korean Research Foundation Grant funded by the Korean Government (KRF-2006-211-F00002). This research was supported through a grant (CG1213) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

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