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Patterns of protein expression in water-stressed wheat chloroplasts

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Abstract

The performance of control and water-stressed 10-d-old wheat seedlings was compared. During short-term water stress (irrigation was withheld for 9 d), rates of photosynthesis and transpiration, stomatal conductance, and relative water content decreased whereas the proline content increased. Chloroplast proteins were extracted from the leaves, separated by iso-electric focusing through two-dimensional electrophoresis, and stained with CBB R-250. Differentially expressed proteins were detected and analyzed with MALDI-TOF/TOF mass spectrometry. Under water stress, 9 proteins were up-regulated whereas 11 proteins were not affected. The ribulose-1,5-bisphospate carboxylase/oxygenase (Rubisco) small and large subunits, chloride carrier/channel family, and H^+-ATPase were up-regulated by water stress whereas membrane-bound ATP synthase subunit *b* and cytochrome *b6-f* complex were down-regulated.

Additional key words: chloride carrier/channel family, H^+-ATPase, MALDI-TOF/TOF-MS, *Triticum aestivum.*

Introduction

Plants are continuously subjected to periods of soil and atmospheric water deficits. Changes in the global climate further reduce the potential for maximizing crop yields (Chaves *et al.* 2002, 2003, Thapa *et al.* 2011). Breeding programs to introduce physiological traits that improve the yield and quality of wheat under water stress are very complicated and still ineffective. One reason for their lack of success has been the fact that traits for adaptations to water stress are often negatively correlated with yield and quality. Greater knowledge about the physiological and morphological plant characteristics that diminish yields under water stress now provides opportunities for wheat breeders (Richards *et al.* 2001). On exposure to water stress, plants show a wide range of responses at the cellular and molecular levels (Hasegawa *et al.* 2000) and plant metabolism (Rodrigues *et al.* 2011). Drought inhibits transpiration and photosynthesis within the first few days (Ribas-Carbo *et al.* 2005, Peng *et al.* 2009) due to decreased stomatal conductance (Quarrie and Jones 1979) and stimulates the accumulations of free proline (Johari-Pireivatlou *et al.* 2010). Leaf photosynthetic rate, which might be associated with yield, can be measured simply in the field suggesting a potential methodology for screening physiologically superior lines (Fischer *et al.* 1998).

Proteomics is a tool for understanding basic processes in plant growth and development as well as for examining changes in specific proteins in response to environmental fluctuations. Here, proteomics approach was used to investigate the effect of water stress in wheat chloroplasts. By analyzing differentially expressed proteins under drought we attempted to identify candidate for marker proteins (Peng *et al.* 2009). Sequencing of these stress-responsive proteins will then reveal that some of them have functions clearly consistent with the stress tolerance trait. As a final point, these proteins can be

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Abbreviations: 2-DE - two-dimensional electrophoresis, CLC - chloride carrier/channel, MALDI - matrix assisted laser desorption/ionization, Rubisco - ribulose-1,5-bisphospate carboxylase/oxygenase; TOF - time-of-flight.
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utilized in marker-assisted breeding or gene transformation programs (Salekdeh and Komatsu 2007). The main objectives of our experiments were *1*) to analyze changes in physiological parameters in response to drought stress, and *2*) to identify the set of proteins in wheat chloroplasts that are drought-responsive.

Materials and methods

Seeds of wheat (*Triticum aestivum* L.) cv. Keumganag were germinated and seedlings were grown in pots containing sandy loam soil in a growth chamber under 10-h photoperiod, irradiance of 200 µmol m^{-2} s^{-1}, temperature of 25 °C, and relative humidity of 70 %. At 10 d after sowing, irrigation was stopped for 3, 6, and 9 d (to induce water stress) while untreated seedlings were collected for control samples. All experiments were done in triplicate with one pot per treatment for biomass and biochemical analyses, plus three replications with well-developed leaves for evaluating protein expression.

To evaluate the water status during the stress period, relative water content (RWC) was used, determined according to Barrs and Weatherly (1962). Stomatal conductance and net photosynthetic rate were assessed on second leaves using a *LI-6400XT* portable photosynthesis system (*LI-COR Biosciences*, Lincoln, NE, USA). Test conditions included an irradiance of 1 000 µmol m^{-2} s^{-1}, temperature of 25 °C, relative humidity of 60 %, and data acquisition time at every 3 min with 5 replications. Leaf chlorophyll (Chl) was extracted with acetone, and Chl *a* and *b* content was determined spectrophotometrically (*UV-1700 PharmaSpec*, *Shimadzu*, Kyoto, Japan) at 663, 646, and 750 nm according Porra *et al.* (2002). Proline content was estimated by the ninhydrin reaction (Bates *et al.* 1973).

Intact chloroplasts from fully developed leaves were isolated and purified on *Percoll* gradients following the procedure of Kamal *et al.* (2012a,b). For optimal yield, seedlings were first placed overnight in the dark at 4 °C to block starch accumulation. Briefly, 50 g of leaf tissue was washed with deionized water before being cut into 2 to 3 cm pieces. Afterward, 150 cm^3 of grinding buffer (330 mM sorbitol, 20 mM Tricine, 5 mM EGTA, 5 mM EDTA, 10 mM Na_2CO_3, 0.1 % (m/v) BSA, and 1.9 mM ascorbic acid) was added to the leaf segments, homogenised, filtrated through three layers of miracloth, and centrifuged at 3 000 *g* for 3 min (4 °C) (*Mega 17R* small high-speed refrigerated centrifuge, *Hanil Science Industrial*, Gangwondo, South Korea). After the white pellets were discarded, the supernatant was transferred to clean tubes and centrifuged at 9 000 *g* for 7 min (4 °C). The precipitate was again discarded and the supernatant was centrifugated at 4 000 *g* for 7 min (4 °C). The resultant green pellets were re-suspended in the grinding buffer and intact and broken chloroplasts were found. This chloroplast suspension was overlaid on a 40 % (v/v) *Percoll* solution and centrifuged at 8 000 *g* for 10 min (4 °C). Afterward, the upper phases containing the broken chloroplasts were carefully removed with a Pasteur pipette. The remaining intact chloroplasts were re-suspended in 0.5 cm^3 of storage solution (the grinding buffer but without 0.1 % BSA) and stored at -80 °C. For functional assays, intact chloroplasts were used as soon as possible to avoid losing protein and enzyme activities.

The purity of chloroplast proteins was determined by calculating the ratio of chloroplast proteins and impurities of other origin on the basis of 20 spots analyzed by MALDI-TOF/TOF-MS from 2-D gels. After MS analysis of these spots, 20 proteins were identified as chloroplast proteins which represent purity of 100 % using *Wolf PSORT* freeware software and *UniProt* database. Three replicates of chloroplast protein samples from 2-DE by tryptic digestion were analyzed by mass spectrometry and each sample was carried out with two methodological replications (Zorb *et al.* 2009, Kamal *et al.* 2012a,b).

From intact chloroplasts, protein was prepared for IEF by a modified version of the trichloroacetic acid (TCA)/acetone method (Damerval *et al.* 1986, Kim *et al.* 2010). Each 0.2 cm^3 sample was suspended in 4 cm^3 of TCA/acetone solution. After mixing, the solution was frozen at -20 °C for 1 h, then centrifuged at 14 000 *g* for 30 min. The precipitate was suspended in acetone solution. After standing at -20 °C for 12 h, the suspension was centrifuged at 14 000 *g* for 30 min. Afterward, the precipitate was dried *in vacuo*, and a part of the resultant powder was suspended in 0.5 cm^3 of the solubilization solution. After incubating at room temperature for 2 h with continuous shaking, the suspension was centrifuged at 14 000 *g* for 30 min and the resulting supernatant was subjected to IEF. Protein content in the sample was determined by the method of Bradford (1976) using a *Bio-Rad* protein assay kit (*Bio-Rad*, Hercules, CA, USA) and bovine serum albumin as the standard.

Soluble chloroplast proteins that are active under water stress were examined by 2-DE focusing according to the protocol of O'Farrell (1975) with some modifications. For the first dimension, sample solutions (400 µg) were loaded on the acidic side of the IEF tube gels, which was pre-run at 150 V for 1 h, 300 V for 1 h, and 500 V for 14 h. An IEF gel was utilized for a pH range of 3 to 10 (carrier ampholyte) in addition to those for acidic and basic pH values. Second dimension of the electrophoresis (*Nihon Eido*, Tokyo, Japan) was performed according to Kamal *et al.* (2012a,b). All gels were checked with an image scanner (*HP Scanjet G4010*, CA, USA; 300 dpi, 32 bits per pixel). Computer-assisted 2-DE image analysis was evaluated with *PDQuest* software according to Kim *et al.* (2011). Spots displaying reliable and significant differences ($P < 0.05$) were selected for MS analysis.

Protein spots were manually excised from the 2-DE gel and digested by trypsin (Kamal *et al.* 2012a,b). Peptides were dissolved in 0.5 % (v/v) trifluoroacetic acid

(TFA) and desalted with a *ZipTip* C_{18} (*Millipore*, Bedford, MA, USA). Those purified peptides were then eluted directly onto a MALDI plate by using an α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution [10 mg per cm^3 of CHCA in 0.5 % (v/v) TFA + 50 % (v/v) acetonitrile; 1:1]. All mass spectra were acquired in the reflection mode with 0 - 4000 *m/z* by a *4700* proteomics analyzer (*Applied Bio-systems*, Framingham, MA, USA). External calibration was performed using a standard peptide mixture of des-Arg bradykinin, angiotensin, Glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) clip 1-17, ACTH clip 18-39, and ACTH clip 7-38.

Acquired MS/MS spectra were evaluated, and auto submitted to *Flex* analysis *v. 3.3* (for the analysis of the spectrum and the assignment of the peaks) using *Mascot Generic File* (MGF) with an in-house licensed *MASCOT* search engine (*Mascot v. 2.3.01, Matrix Science*, London, UK) against the *viridiplantae* within the *UniProt_Sprot* and *NCBInr* database. The carbamidomethylation of cysteines was set as a fixed modification whereas the oxidation of methionines was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Mass tolerance of the precursor ion was ± 25 - 50 μg g^{-1}; that of the fragment ions 0.5 Da, threshold level 0.05, and missed cleavages 1. The instrument setting was specified as MALDI-TOF/TOF. Protein hits were validated if the identification involved at least 10 top-ranking peptides with $P < 0.05$ and peptide scores > 25, and also selected false positive rate < 0.05. When those peptides matched multiple members of a protein family, the presented protein was selected based on the highest score and the greatest number of matching peptides. The positive identification had to meet the following criteria: a significant *MASCOT* score and at least two matched peptides in MS/MS analysis. For functional categorization based on gene ontology, the protein information resources (PIR) (http://pir.georgetown.edu) were utilized according to Huang *et al.* (2003) and Kamal *et al.* (2012a,b).

Data for physiological and biochemical parameters were statistically analyzed by analysis of variance (*ANOVA*) and Duncan's multiple range test (DMRT) by using the statistical package *PSAW*, *v. 17* (*SPSS Inc.*, Chicago, IL, USA).

Results and discussion

Ten-day-old wheat seedlings were transferred into soil with a water content of about 50 - 60 % for the control plant. Under water stress, leaf relative water content (RWC) gradually decreased to 96, 95, and 92 % after 3, 6, and 9 d, respectively (Table 1). These rather high values are in consequence of lower water absorption from the soil but the regulation of water loss by stomata. Similar reactions have been reported in rice (Farooq *et al.* 2010), bean (Rosales-Serna *et al.* 2004), soybean (Shen *et al.* 2010), wheat (Grigorova *et al.* 2011), and maize (Efeoğlu *et al.* 2009).

Water stress significantly reduced stomatal conductance and rates of transpiration and photosynthesis. Stomatal conductance and transpiration rate in treated seedlings were reduced to 59 and 66 % of the control at 3 d, 28 and 30 % at 6 d, and 17 and 24 % at 9 d, respectively (Table 1). Stomata generally are closed due to diminished leaf pressure potential or in consequence of chemical signals, especially abscisic acid, generated by the roots under drought (Flexas *et al.* 2007). The changes in mesophyll conductance may be linked to physical alterations in the structure of the intercellular spaces (Lawlor and Cornic 2002) or because of biochemical alterations in membrane permeability. Net photosynthetic rate declined to 90 % of the control at 3 d, 65 % at 6 d, and 60 % at 9 d (Table 1). Photosynthesis, in conjunction with cell growth, is one of the primary processes affected by drought (Chaves 1991) because either the availability of CO_2 is decreased by diffusion restrictions through the stomata or mesophyll (Nilsen and Orcutt 1996, Flexas *et al.* 2007) or photosynthetic metabolism is altered (Lawlor and Cornic 2002). A secondary effect may arise due to the generation of reactive oxygen species which also interrupt leaf physiological processes (Ort 2001, Chaves and Oliveira 2004).

L-proline content was measured for determining how

Table 1. Water stress-induced changes in selected parameters in 10-d-old wheat seedlings. Means ± SE, $n = 3$. Means in each row followed by the same letters are not significantly different according to DMRT at $\alpha = 0.05$.

Parameters	Control	3 d	6 d	9 d
Photosynthetic rate [μmol(CO_2) m^{-2} s^{-1}]	14.45 ± 0.25a	13.10 ± 0.30b	9.45 ± 0.11c	8.67 ± 0.17c
Stomatal conductance [mol m^{-2} s^{-1}]	0.54 ± 0.02a	0.32 ± 0.00b	0.15 ± 0.04c	0.09 ± 0.00c
Transpiration rate [mol m^{-2} s^{-1}]	7.19 ± 0.29a	4.74 ± 1.16ab	2.18 ± 0.55bc	1.76 ± 0.04d
Proline [μmol g^{-1}(f.m.)]	0.73 ± 0.01c	0.89 ± 0.02b	0.92 ± 0.02b	1.05 ± 0.01a
Chlorophyll *a* content [μg g^{-1}(f.m.)]	11.20 ± 0.01a	11.04 ± 0.13a	10.84 ± 0.16a	10.00 ± 0.23a
Chlorophyll *b* content [μg g^{-1}(f.m.)]	9.76 ± 0.25a	9.12 ± 0.16a	8.48 ± 0.05a	7.92 ± 0.12a
Relative water content [%]	96.49 ± 1.33a	95.79 ± 0.12a	94.86 ± 0.36a	92.03 ± 0.67b

drought influences the production of osmoprotective substances in wheat plants. Similar to results reported by Vendruscolo *et al.* (2007), water stress resulted in a increased accumulation of proline in the leaves in a time-dependent manner (Table 1). Proline has numerous functions under stress conditions including osmotic adjustment (Voetberg and Sharp 1991) and restoring carbon and nitrogen reserves upon stress recovery (Díaz *et al.* 1999). Although researchers agreed that proline has an important role in the adaptation of cells to osmotic stress, doubts persist about whether the accumulation of this amino acid has an adaptation benefit or if it is only result of changed metabolism due to various stresses (Serraj and Sinclair 2002).

Image analysis with *PDQuest* software enabled to identify 135 highly reproducible chloroplast protein spots on each gel (Fig. 1). From those spots, 20 spots were revealed as differentially expressed proteins (DEPs) based on three replications. These spots were broadly distributed having pI values that ranged from 3 to 10 and with relative molecular massees of 10 to 250 kDa. After 3, 6, and 9 d of stress, 19, 11, and 14 DEPs were up-regulated while 1, 9, and 6 DEPs were down-regulated, respectively. Nine DEPs (No. 1, 6, 9, 10, 11, 14, 16, 19, and 20) were up-regulated under water stress. Another eight DEPs (No. 3, 4, 5, 7, 13, 17, 18, and 19) were not expressed in untreated sample because those proteins are activated upon water stress (in all the three sets of experiments) (Fig. 1). Interestingly, some DEPs displayed fluctuating patterns of expression. Similar results have been reported in leaves of wheat (Peng *et al.* 2009), rice (Salekdeh *et al.* 2002), sugar beet (Hajheidari *et al.* 2005), and soybean (Alam *et al.* 2010).

Several proteins were differentially expressed in untreated and treated seedlings. Using 2-DE coupled with high through-put MALDI-TOF/TOF-MS verified that nearly all were chloroplastic proteins except for a putative protein in spot #1 and a hypothetical protein in spot #13. Seventeen proteins were confirmed by *WolF PSORT* software and 5 proteins by *UniProt* database (Nakai and Kanesia 1991, Horton *et al.* 2007). Unfortunately, some identified proteins contained low sequence percentages (Table 2). The possible reason might be that concentration of peptide loaded in a mixture with other peptides is suppressed in comparison with that when it is loaded as single peptide. Other reason for the relatively low sequence coverage is the fact that long sequence stretches without lysine and arginine provides signals appearing outside the recorded mass-to-charge interval (m/z > 4000). Additionally, some sequence

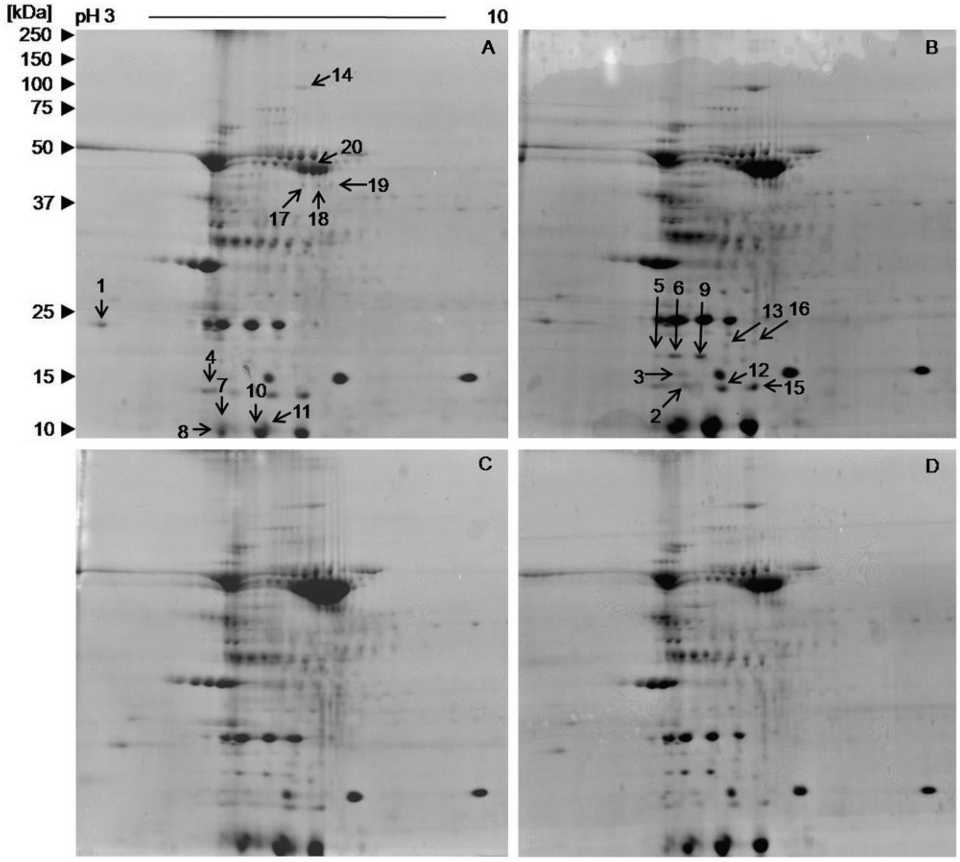

Fig.1. Effects of water stress on protein expression in wheat untreated control seedlings (*A*; standard irrigation) and plants that received no water for 3 (*B*), 6 (*C*), or 9 d (*D*). Proteins were extracted from leaf chloroplasts, separated by 2-DE, and visualized by CBB R-250 staining. The M_r of each protein was determined by 2-DE markers. *Arrows* indicate protein spots whose expression was changed by drought treatment as measured with *PDQuest* image analyzer.

Table 2. General features of wheat chloroplast proteins in response to water stress, as identified *via* MALDI-TOF/TOF-MS. Some proteins (spot No. 3, 4, 5, 7, 13, 17, 18, and 19) were not detected in control sample. ctr - control, gi - *NCBI* acc. No., Q or P - *SWISSPORT* acc. No., EMM/pI - experimentally determined molecular mass/pI value, TMM/pI - theoretical molecular mass/theoretical pI value, PM - peptide matches, PC - protein coverage, Loc - sub-cellular localization using *WolF PSORT* identity [%], U - identified from *UniProt* databases.

No.	Fold change			Acc. No.	Protein description	EMM/p*I*	TMM/p*I*	PM	PC	Loc
	3 d/ctr	6 d/ctr	9 d/ctr							
1	4.05↑	2.75↑	4.82↑	gi|14140139	putative protein	23/3.6	24.8/4.3	2	18.4	17
				gi|115448701	H+-transporting two-sector ATPase	23/3.6	26.2/4.9	4	27.4	77
2	1.19↑	1.66↓	1.69↓	Q7X9A6	cytochrome *b6-f* complex iron-sulfur subunit, chloroplastic	12.5/5.2	24.1/8.4	2	39	70
				Q08479	adenylate kinase a	12.5/5.2	26.6/8.4	5	27.9	16
6	3.90↑	2.70↑	6.28↑	gi|94549022	ribulose-1,5-bisphosphate carboxylase/oxygenase activase	18/5.8	34.8/5.5	2	22.5	14
8	3.08↓	2.42↑	3.11↑	P26667	ribulose bisphosphate carboxylase small chain pw9, chloroplastic	7.91/5.35	19.7/8.5	3	21.7	100
9	5.17↑	2.01↑	2.18↑	gi|24210613	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	16.21/5.68	43.3/6.4	2	19.2	U
10	2.28↑	2.15↑	2.09↑	gi|207080698	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	7.61/5.84	7.8/9.4	2	18.2	17
11	3.63↑	3.01↑	2.10↑	Q40004	ribulose bisphosphate carboxylase small chain, chloroplastic	8.32/5.88	19.6/8.9	3	21.8	100
12	1.06↑	1.69↓	1.63↓	Q40004	ribulose bisphosphate carboxylase small chain, chloroplastic	11.63/5.94	19.6/8.9	3	21.8	100
14	4.10↑	1.39 ↑	6.40↑	gi|226462786	chloride carrier/channel family	100/6.17	69.8/5.8	3	41.2	14
15	1.00↑	2.27↓	2.75↓	Q7X9A6	cytochrome *b6-f* complex iron-sulfur subunit, chloroplastic	11.56/6.32	24.1/8.4	2	30.8	70
16	1.15↑	4.38↑	1.25↑	P05698	ribulose bisphosphate carboxylase large chain	18.12/6.33	53.6/6.2	2	20.6	U
20	3.39↑	4.13↑	2.24↑	gi|62861063	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	44/6.4	52/6.3	2	26.3	U
3	↑	1.13↓	1.55↑	gi|147945622	chloroplast oxygen-evolving enhancer protein 1	15/4.9	34.7/6	8	46.4	77
4	↑	1.19↓	5.27↑	gi|212896622	membrane-bound atp synthase subunit *b*	15/5.18	50.6/5.2	2	38.9	12
5	↑	1.41↓	2.61↑	gi|94549022	ribulose-1,5-bisphosphate carboxylase/oxygenase activase	18/5.44	34.8/5.5	2	18.5	14
7	↑	2.92↓	3.11↑	gi|207080698	chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit	8.49/5.3	7.8/9.4	2	18.2	17
13	↑	1.81↓	1.74↓	gi|147799937	hypothetical protein	22/5.84	95.3/8.9	2	31.2	14
17	↑	3.72↑	1.14↓	A1EA16	ribulose bisphosphate carboxylase large chain	45/6.2	53.4/6.2	2	23.3	U
18	↑	1.42↓	2.06↓	gi|37702769	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	45/6.3	48.1/6.3	2	31.8	13
19	↑	7.57↑	2.17↑	gi|215272094	ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	43.5/6.2	47.6/6	2	24.7	U

stretch might contain too many lysines and arginine and cause signals having m/z ratios lower than 500. Conversely, this might be observed as part of incomplete cleavage of peptides (Hjernø and Roepstorff 2005).

Among the identified proteins, several Rubisco proteins are represented by spots located on different gel areas. These multiple spots could designate different splice variants or post-translationally modified isoforms and cleaved isoforms of the same protein (Weiss and Gorg 2007). Rubisco is a bi-functional enzyme which catalyzes two contending reactions, photosynthetic CO_2 assimilation and photorespiratory carbon oxidation in the stroma of a chloroplast and it records for 40 to 55 % of soluble protein. Those that were up-regulated by stress included isoforms of Rubisco, a finding that agrees with that by Salekdeh *et al.* (2002). Rubisco amount increases during leaf expansion and it reached the highest content after full expansion of leaf (Ishida *et al.* 1997). Some Rubisco proteins were down-regulated under water stress (Table 2). Rubisco degrade very fast in barley and wheat leaves during senescence (Wardley *et al.* 1984) whereas both reversible and irreversible inactivation of Rubisco occur during drought stress. However, a complementary enzyme Rubisco activase release of tight binding inhibitors from Rubisco which is itself regulated by redox control (Foyer and Parry 2001). The removal of inhibitors by Rubisco activase may be impaired because ATP content is decreased (Tezara *et al.* 1999). It is also susceptible to high temperatures that may be associated with drought (Crafts-Brandner and Salvucci 2000). Moreover, the Rubisco content can be increased by drought as reported in birch (Paakkonen *et al.* 1998) and wheat (Demirevska *et al.* 2009). In this study, number of Rubisco subunits was unevenly expressed in chloroplasts of water-stressed seedlings (Table 2). Isoforms of Rubisco activase likely play an important role in stabilizing and controlling proteolysis (Schwartz *et al.* 1995) and in maintaining chloroplast functioning during drought stress (Huo *et al.* 2004). Regulation of Rubisco activity has yet unclear under water stress. The regulation of photosynthesis during the senescence of leaves in soybean that display markedly different photosynthetic rates is mediated primarily by Rubisco activity and decreases in the accumulation of the holoenzyme in leaves is accompanied by coordinate decrease in the content of the rbcL and rbcS mRNA (Jiang *et al.* 1993) which is showed similarly in protein level in this report. Here, the co-variation of stomatal conductance, transpiration rate, and RWC, often observed during water stress, was impaired to evaluate the same effects of these factors on Rubisco activity (Flexas *et al.* 2006).

Cytochrome *b6-f* complex is located as a single-pass transmembrane domain in the thylakoid membrane. It mediates electron transfer between photosystems I and II as well as cyclic electron flow around PSI and state transitions (Hurt and Hauska 1981). The cytochrome *b6-f* complex was up-regulated by 3 d of stress, a result similar to that in drought-stressed rice (Ali and Komatsu 2006). However, it was down-regulated at 6 d and 9 d.

Expression of chloroplast oxygen-evolving enhancer protein 1 (OEE1) was up-regulated after 9 d of stress. Murota *et al.* (1994) have reviewed the functioning of this protein in salt adaptation using photo-autotrophically cultured tobacco cells. OEE1 was also reported in salt-stressed mangrove and rice (Abbasi and Komatsu 2004, Sugihara *et al.* 2000).

Adenylate kinase works in the metabolism of purine nucleotides. This small ubiquitous enzyme is essential for cell maintenance and growth. Here, it was up-regulated after 3 d, but down-regulated after both 6 and 9 d due to possibly cell death (Table 2). Adenylate kinase catalyzes a reversible transphosphorylation reaction which converts ADP to ATP and AMP (Pradet and Raymond 1983).

Plasma membrane proteins such as H^+-ATPase and membrane-bound ATP synthase subunit *b* were also identified. These proteins were down-regulated at 6 d but up-regulated at 3 d and 9 d. A multi-subunit non-phosphorylated ATPase is involved in ion transport. F-type enzymes in the inner mitochondrial and thylakoid membranes act as ATP synthases. This movement is driven by the H^+ electrochemical potential gradient. V-type and A-type enzymes have a similar structure and functions under stress conditions resulting they pump H^+ rather than synthesize ATP (Stefanovic *et al.* 2009). Integral membrane proteins, the chloride carrier/channel, were identified. They were up-regulated by water stress. All functionally characterized members of the chloride carrier (ClC) family are involved in a voltage-regulated process. The channels serve a variety of physiological roles, *e.g.*, cell volume regulation, stabilization of membrane potential, and signal transduction (Worden *et al.* 2009) which is actively involved with stress. Nitrate is required for plant growth and it's stored in the central vacuole. Some members of the ClC family are anion channels and may be a involved in nitrate homeostasis. According to *WolF POSRT*, CLC proteins displayed at 14 % similarity (Table 1) with phytoene synthase proteins which are located in chloroplasts (Roemer *et al.* 1993).

In conclusion, this study introduces new insights into how water stress affects protein expression in wheat chloroplasts. This proteomics approach enables researchers to make improvements in plants at the molecular level by discovery of marker proteins, marker-assisted breeding and gene transformation programs. Significant number of proteins related to photosynthesis and carbon metabolism which was up-regulated under mild to moderate water stress was revealed. Identifying these proteins provides information about chloroplast metabolism under a depleted water supply.

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