IDENTIFICATION OF PROTEIN MARKERS LINKED TO DROUGHT TOLERANCE IN NEW IRANIAN RICE GERMPLASM

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> *Original Research Article*

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ABSTRACT

As with other crops, loss of enough water resources especially during low falling periods is one of the main problems in rice cultivation and production in most regions of Asia. Identification of protein markers linked to drought tolerance can facilitate rice breeding process for developing new elite drought tolerant varieties. In proteome analysis of studied rice germplasm, protein was extracted from leaves of the rice plants under natural growth condition by Damerval method. Concentration of the protein samples was measured by Bradford method. Then, Two-dimensional gel electrophoresis technique was used to identify protein markers linked to drought tolerance in studied rice germplasm. Protein spots available in gel profiles of studied samples were analyzed and compared together in Melanie software. Overall, molecular data analysis of protein spots available in 2D gel profiles indicated that 20 reproducible protein spots were linked to drought tolerance in the new Iranian drought tolerant genotype.

Keywords: Drought stress, new germplasm, protein spot, rice and two-dimensional gel electrophoresis.

INTRODUCTION

Rice (*Oryza sativa* L.) is the one of the oldest crop cereals in Asia that has been grown in nature so far [1]. Rice is the main staple food for more than half of world's population. Abiotic stresses are key limiting factors that restrict rice production and can directly or indirectly influence on the physiological situation of an organism by altering its metabolism, growth, and development [2]. Among them, drought is one of the major factors that induce several physiological, biochemical and molecular responses in plants [3-6]. Screening of rice varieties under drought stress is a prerequisite to produce stress tolerant rice cultivars [7]. Moreover, sustainable crop production is the major challenge in climate change scenarios in the world. Rice germplasm represents an interesting source of genes that control important agronomical

traits such as drought tolerance. This diversity is the basis for development of new cultivars with better adaptation to drought stress [8]. The analyses of differential protein expression of drought tolerant and susceptible genotypes can play a role in understanding of the genetic control of water use efficiency in rice [9]. In recent years, considerable information about molecular regulation has been obtained for understanding drought stress responses. The translational and post-translational machinery has clearly a main contribution in stress adaptation, especially in environmental stresses. Evaluation of protein expression patterns and post-translational protein modifications is necessary to reveal information about stress induced protein expression. This information cannot be revealed by genomic or transcriptomic analysis. Eventually, these processes will provide more direct insight into stress perception than genetic markers and possibly establish a complementary basis for marker-assisted selection of drought tolerance [10]. The technique of Two Dimensional Electrophoresis (2-DE) has the power to detect changes occurring in the protein complements of tissues and subcellular compartments [11]. So far, a lot of research have been conducted on identification of drought tolerance genes and characterization of drought tolerance mechanisms in different rice varieties by proteome analysis using 2-DE [1-6,12,13]. For example, to better understand traits controlling drought responsive mechanism in hybrid rice flag leaves during sensitive reproductive stage, Wang [14] conducted 2-DE to investigate proteomic profile of rice flag leaves at flowering and milk stages and identified 43 proteins at flowering stage and 54 proteins at milk stage by MS/MS that showed significant differences in silver stained gels. Rabello [9] analyzed the root proteome of upland and lowland varieties and revealed 408 reproducible spots in the 2D maps from treated and control plants. However, 44 spots were identified by mass spectrometry, including 15 differential proteins. Taken together, they found that possibly, the mechanisms of susceptibility to drought in tolerant upland rice variety "Três Meses Antigo" are similar to those in lowland varieties but the tolerant upland rice up-regulates proteins related to anti-oxidant and energy production in order to cope with water deficit. In this research, proteome analysis by Two-dimensional electrophoresis technique was done on studied rice germplasm for identification of new protein markers linked to drought tolerance in rice and marker assisted selection of drought tolerant genotypes.

MATERIALS AND METHODS

Phenotypic analysis of rice plants of Iranian landrace "Tarom Mahalli" was performed after two weeks of drought stress on standard evaluation system of International Rice Research Institute (IRRI) including two scales of leaf rolling and spike fertility [15]. Plant materials consisted of two early flowering and high yield drought tolerant mutant plants from a M1 generation plant, TM2-230-4(1) and TM2-230-4(2) that were selected from M2 generation population, a sensitive mutant plant (B-TM2) and a control plant. Sampling was done from these plants in three steps: the last day of drought stress, 24 h after rehydration and 7 days after rehydration. Moreover, sampling was done from a mutant plant prior to drought stress (TM2-230-4). Protein was extracted from leaf samples of above plant materials by Damerval et al. [16] method. Bradford [17] method was used to measure protein concentration of the samples. Standard protein concentration plot was created on different concentrations of standard protein (BSA) (0, 0.1, 0.3, 0.5, 0.7

and 1 mg/ml). If this plot is statistically significant that R^2 due to equation of the plot is more than 0.98. After creating standard protein concentration plot, protein concentrations in any sample were calculated in two replications and average protein concentrations were determined for any sample and then, needed amount of any protein sample for Two Dimensional Electrophoresis operation was calculated [18]. For rehydration of gels (IPG strips 17 cm), 320 µl rehydration solution (2% CHAPS, 8M urea, 0.018M DTT, 2% IPG buffer and 0.002% bromophenol blue) containing 120 µl protein was pipette into a channel of rehydration tray. Rehydration was done overnight which lasted for between 14 to 16 hours. PROTEAN IEF Cell (BIORAD Company) was used for performing first dimension (IEF). For running gel, power supply was turn on and running program was regulated on gradient. Running a gel 17 cm with pH=4-7, averagely needs to 42000Vh (42KVh). This Vh was supplied as a voltage gradient in continuous three steps. Cell PROTEAN II (BIORAD Company) was used for second dimensional electrophoresis. After preparation of second dimensional gel (12.5% acrylamide gel solution), the first dimension strip was soaked in equilibration buffer (Tris HCl 50Mm with pH=8.8, bromophenol blue 0.02%, DTT 1%, glycerol 30%, SDS 2% and urea 6M) for 15 minutes. After pouring enough electrophoresis buffer, power supply was turn on. Then, gel was run in two steps with 5 and 30 mAh for 1 and 5h, respectively. By finishing second dimensional electrophoresis, immediately, the gel was put in fixer solution. Finally, the gel was stained by Blum et al. [19] method. After staining, the gel was photographed using Gel Doc (BIORAD Company). Then, image of gel was imported into Melanie software version 6.2. All authorized protein spots available in studied gel profiles were scored. Using Melanie software, protein relative abundance (volume mean) of the spots in different replications and also, ratios of protein relative abundance of any spot in different times were calculated and compared together. Protein spots were selected that not only had considerable expression than the same spots in other samples or were expressed only in one or two specific times but also were significant statistically in student t-test in level of 1% or 5%.

RESULTS AND DISCUSSION

Four drought tolerant mutant plants (TM2-230-3, TM2-230-4, TM2-230-5 and TM2-B-14) were selected on two scales of leaf rolling and spikelet fertility in phenotypic analysis of mutant populations (Fig. 1).

After protein extraction, a standard protein curve was plotted between concentration and absorbance of Bovine Serum Albumin (BSA). Equation derived from the plot was y = 0.0006x + 0.0259. The coefficient of determination (R^2) of the equation was approximately 0.993 and so, this concentration plot was correct and suitable for calculation of needed amount of any protein sample for Two Dimensional Electrophoresis operation (Fig. 2).

With including X amount in the equation, Y amount was calculated. Regarding that needed protein concentration of any sample for performing Two Dimensional Electrophoresis must be at least 120 µg/µl, Y amount (µg/µl) was divided to 120 (Table 1).

Fig. 1. Schematic pictures of tolerant and sensitive mutant plants in the landrace "Tarom Mahalli" (from left to right, respectively)

$$y = 0.0006x + 0.0259$$
$$R^2 = 0.9929$$
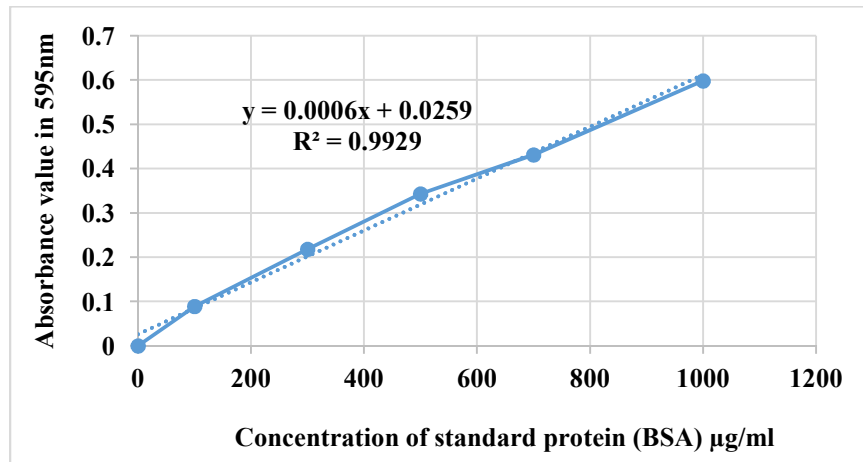
Fig. 2. Standard protein concentration plot

Required protein amount for two dimensional Electrophoresis operations in all studied samples was variable, in a range of 10-25 μl that is a suitable concentration for performing this operation.

Two Dimensional Electrophoresis of studied protein samples was performed in three replications (Figs. 3, 4, 5 and 6). Briefly, only 2D gel profiles of "1 day after rehydration" samples and "prior to drought stress" sample were represented for comparison.
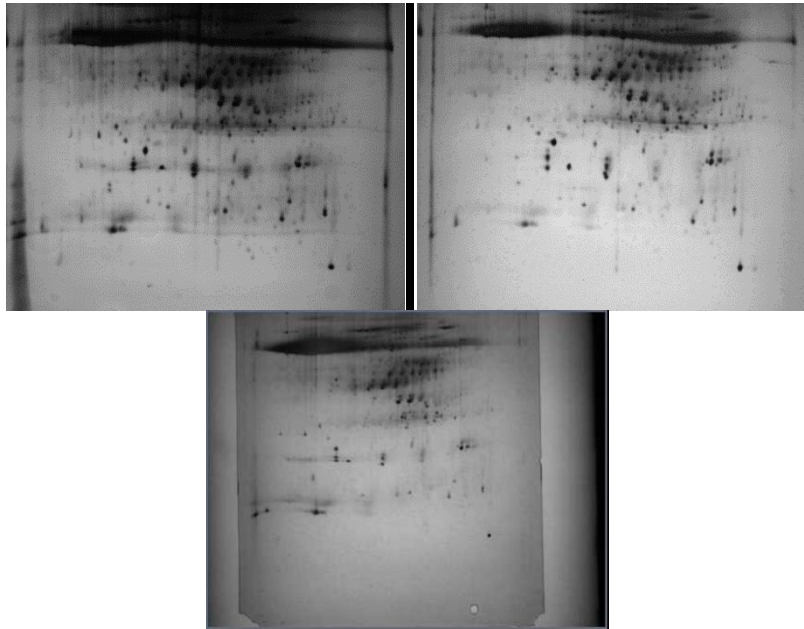
Fig. 3. Two dimensional electrophoresis profiles of sample "T3b- Tolerant, 1 day after rehydration"
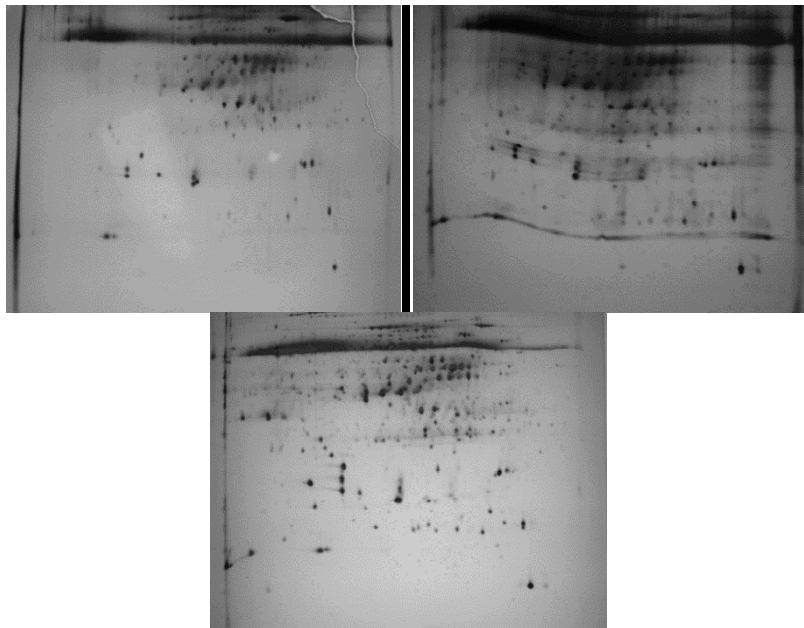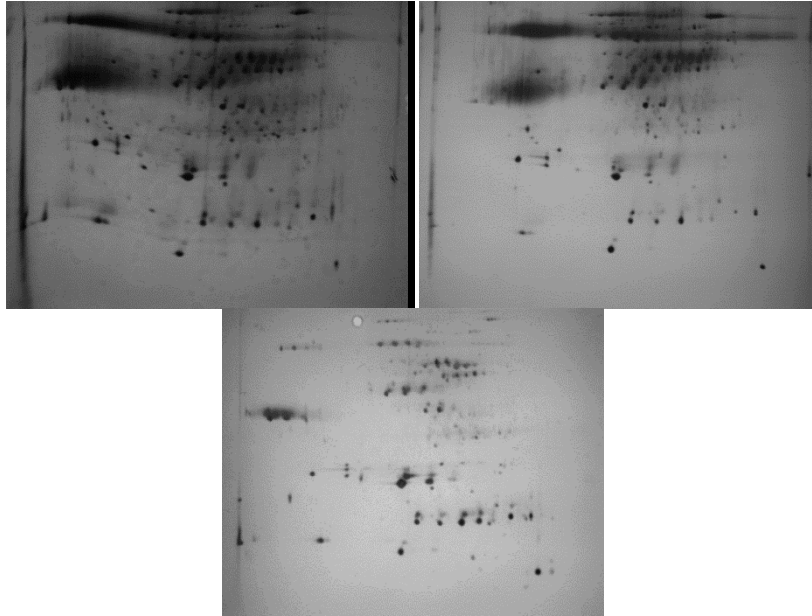

Fig. 4. Two dimensional electrophoresis profiles of sample "S4- Sensitive, 1 day after rehydration"

Table 1. Calculation of needed protein amount of any sample for Two Dimensional Electrophoresis on standard protein concentration plot

Sample No.	Symbol	Absorbance 1	Absorbance 2	X (Average absorbance) in 595 nm	Y (Protein concentration) (µg/ml)	Y *12 (Elution factor) (µg/ml)	Y /1000 (µg/µl)	Needed protein amount for Two Dimensional Electrophoresis (120/Y) µl
TM*2-230-4-1 Tolerant (14 days after drought stress)	T3a	0.471	0.444	0.4575	734.125	8809.5	8.8095	13.62
TM2-230-4-1 Tolerant (1 day after rehydration)	T3b	0.355	0.327	0.341	536.774	6441.288	6.441288	18.63
B-TM2 Sensitive (7 days after rehydration)	S2	0.602	0.583	0.5925	962.815	11553.78	11.55378	10.39
TM2-230-4 (Prior to drought stress)	4R	0.272	0.262	0.267	411.418	4937.016	4.937016	24.31
B-TM2 Sensitive (14 days after drought stress)	S1	0.434	0.405	0.4195	669.753	8037.036	8.037036	14.93
TM2-230-4-1 Tolerant (7 d2ays after rehydration)	T1	0.447	0.461	0.454	754.95	9059.352	9.059352	13.25
B-TM2 Sensitive (1 day after rehydration)	S4	0.361	0.391	0.376	596.064	7152.768	7.152768	16.78
TM2-230-4-2 Tolerant (1 day after rehydration)	4S	0.324	0.338	0.331	519.834	6238.008	6.238008	19.24
TM2-230-4-2 (14 days after drought stress)	4P	0.493	0.514	0.5035	812.049	9744.588	9.744588	12.31
Control (14 days after drought stress)	B0	0.341	0.314	0.3275	513.905	6166.86	6.16686	19.46
Control (1 day after rehydration)	B1	0.42	0.479	0.4495	720.573	8646.876	8.646876	13.88
Control (7 days after rehydration)	B7	0.476	0.457	0.4665	749.371	8992.452	8.992452	13.34

*TM is abbreviation to Tarom Mutant. Number 230 indicate 230 Gy mutant rice population

Fig. 5. Two Dimensional Electrophoresis Profiles of sample "B1- Control, 1 day after rehydration"
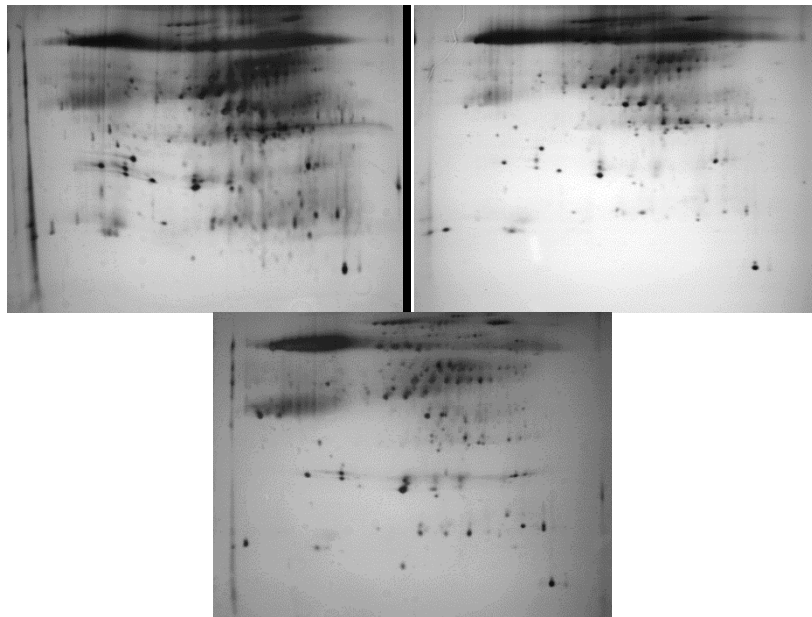
Fig. 6. Two Dimensional Electrophoresis Profiles of sample "4R- Prior to drought stress"

- Results of inter and intra class analysis of studied protein samples in different times

Totally, 47 comparisons were made between studied samples in different times. Among them, 11 comparisons were due to intra class analysis whereas 36 comparisons were related to inter class analysis. For example, in inter class analysis of tolerant and control samples, 1 day after rehydration (T3b and B1), protein relative abundance of 95 spots was significant statistically in student t-test in levels of 5% and 1%. Protein relative abundance of these spots in one of these samples was considerably higher than this of the same spots in other sample or possibly haven't been expressed in one of the samples. From 95 spots, protein relative abundance of 61 spots in sample T3b increased by approximately two-fold in comparison to sample B1. Expression of 12 spots in sample B1 was about two-fold higher than expression of these in sample T3b. As high molecular weight protein spots in second dimensional gel weren't related to drought tolerance [20, 21] and most of these spots possibly have been sequenced, 8 spots were omitted. Also, protein relative abundance of 22 spots wasn't about two-fold higher than this of the same spots in other sample. In total, 65 different protein spots likely related to drought tolerance were characterized in comparisons of two Dimensional Electrophoresis Profiles of these two samples. In following, the important results of inter class analyses have been discussed.

Inter class analyses of "1 day after rehydration" samples and "prior to drought stress" sample represented that protein relative abundance of 61 and 29 spots in tolerant sample (T3b) increased considerably in comparison to control and sensitive samples, B1 and S4. However, 41 protein spots were up-regulated 1 day after rehydration in tolerant sample (T3b) compared to "prior to drought stress" sample (4R). Whereas, only 7 protein spots were down-regulated in tolerant sample (T3b) compared to "prior to drought stress" sample (4R). Moreover, protein relative abundance of 12 and 5 spots in tolerant sample, T3b decreased greatly in control and sensitive samples, B1 and S4.

In addition to, inter class analyses of "14 days after drought stress" samples and "prior to drought stress" sample showed that expression of 7 and 17 protein spots in tolerant sample (T3a) increased considerably in comparison to control and sensitive samples, B0 and S1. Although 22 protein spots were up-regulated 14 days after drought stress in tolerant sample, T3a compared to "prior to drought stress" sample. Whereas, 12 protein spots were down-regulated in tolerant sample (T3a) compared to "prior to drought stress" sample (4R). However, expression of 8 and 10 protein spots in tolerant sample (T3a) decreased greatly in control and sensitive samples, B0 and S1.

Also, inter class analyses of "7 days after rehydration" samples and "prior to drought stress" sample indicated that protein relative abundance of 107 and 54 spots in tolerant sample (T1) increased considerably in comparison to control and sensitive samples, B7 and S2. However, 94 protein spots were up-regulated 7 days after rehydration in tolerant sample, T1 compared to "prior to drought stress" sample. Whereas, only 10 protein spots were down-regulated in tolerant sample (T1) compared to "prior to drought stress" sample (4R). Moreover, protein relative abundance of 48 and 4 spots in tolerant sample (T1) decreased greatly in control and sensitive samples, B7 and S2.

Finally, inter class analyses of tolerant samples in different times (14 days after drought stress, 1 day after rehydration and 7 days after rehydration) indicated that protein relative abundance of 81, 74, 117 and 112 spots, in tolerant sample, 7 days after rehydration (T1) increased considerably in comparison to tolerant samples, 14 days after drought stress and 1 day after rehydration (T3a, T3b, 4P and 4S). Whereas expression of 7, 13, 10 and 7 protein spots decreased greatly in tolerant sample (T1) compared to other tolerant samples. Moreover, protein relative abundance of 48 spots in tolerant (1) sample, 1 day after rehydration (T3b) increased considerably compared to tolerant (1) sample, 14 days after drought stress (T3a). However, expression of 8 protein spots decreased

greatly in tolerant (1) sample (T3b) compared to tolerant (1) sample (T3a). In addition to, expression of 16 protein spots in tolerant (2) sample, 14 days after drought stress (4P) decreased considerably compared to tolerant (2) sample, 1 day after rehydration (4S). Whereas protein relative abundance of 28 spots in tolerant (2) sample increased greatly compared to tolerant (2) sample (4P).

Results of inter and intra class analysis of the gel profiles studied in this research indicated that there were significant considerable differences in number and expression of protein spots detected in 2D gel profiles of tolerant, sensitive and control samples in three time steps compared to "Prior to drought stress" sample.
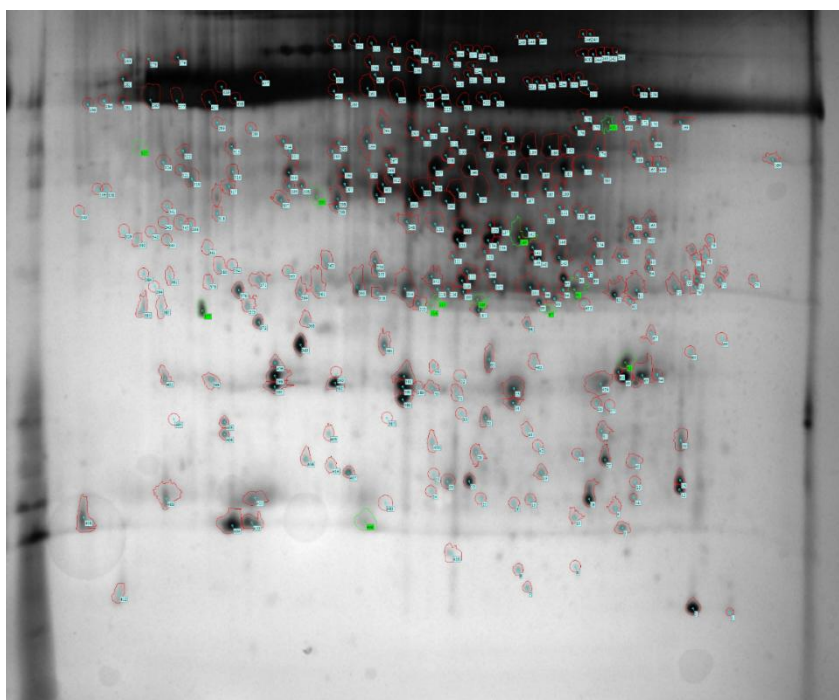
Fig. 7. Protein spots possibly linked to drought stress tolerance in profile of tolerant sample, 1 day rehydration (T3b) (Rep. 1)
*All spots have been shown with number. Green spots are likely related to drought stress tolerance.*
Rep.1 is abbreviation of Repeat 1

**- Identification of protein spots linked to drought stress tolerance**

1. Comparisons between two dimensional electrophoresis profiles of tolerant, sensitive and control samples after 1 day rehydration (T3b, B1 and S4)

Twelve protein spots No. 62, 91, 97, 108, 114, 115, 143, 305, 325, 451 and 466 were selected in tolerant sample (T3b) as candidate spots linked to drought tolerance in profiles of 1 day after rehydration. These spots were expressed in tolerant sample (T3b) about two-fold more than S4 and B1 samples and or expressed only in T3b sample. However, difference in expression levels of these proteins between tolerant sample and other samples was significant in student t-test (Figs. 7 and 8). From 12 different spots available in profile of tolerant sample (T3b) (Rep.1), there were 2 protein spots in profile of control sample (B1) and only 1 protein spot in profile of sensitive sample (S4).

From 12 different spots in "1 day after rehydration" samples, 10 protein spots No. 325, 305, 143, 108, 91, 114, 115, 97, 451 and 466 weren't expressed in sensitive and control samples (S4). In spite of these spots, protein spot No. 62 wasn't expressed in sensitive sample (S4). In following, expression and protein relative abundance of these spots were analyzed in "prior to drought stress" sample (4R) using Melanie software and. Then, four protein spots that weren't related to drought tolerance, were omitted. Totally, statistical analysis of proteome data from "1 day after rehydration" samples and "prior to drought stress" sample indicated that possibly 8 reproducible protein spots No. 143, 91, 72, 108, 97, 305, 466 and 114 are possibly linked to drought tolerance in tolerant sample T3b (Tables 2 and 3 and Fig. 9). There were significant statistical differences in protein relative abundance of selective spots linked to drought tolerance in these samples.
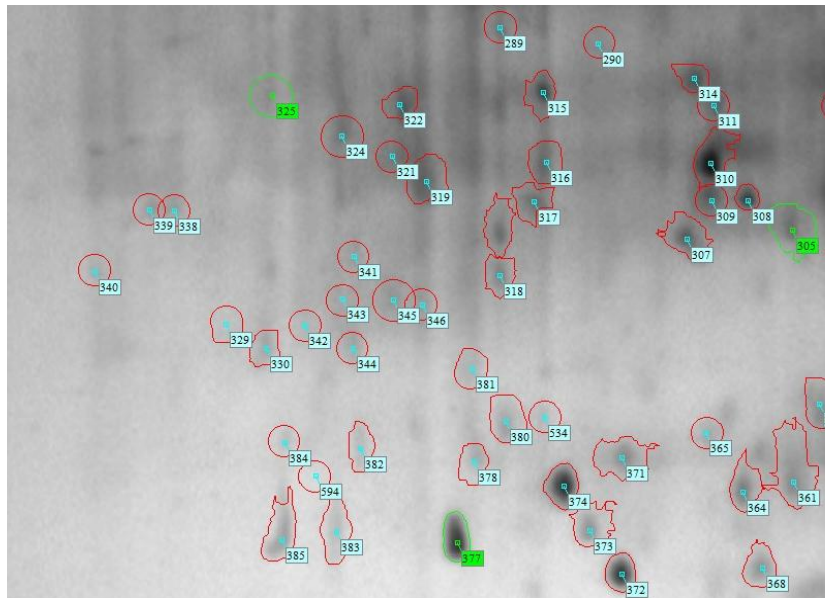
(a)
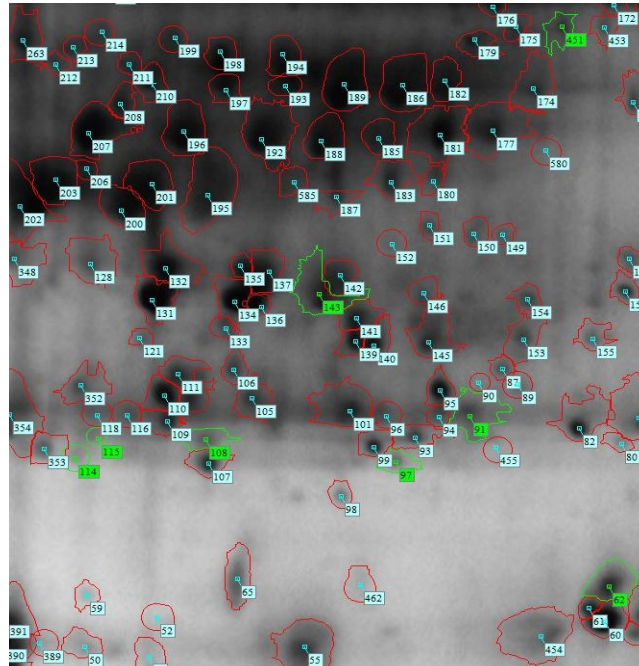
(b)
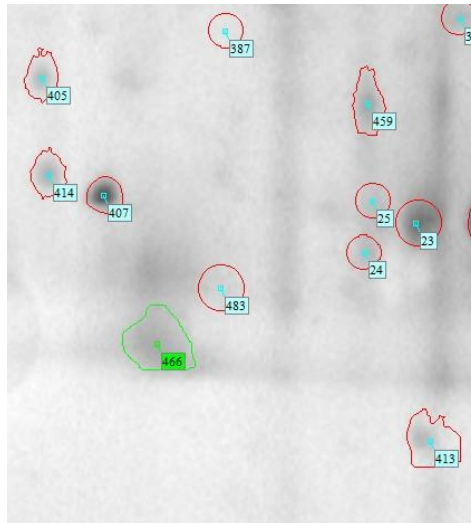


(c)

**Fig. 8. Magnified picture of protein spots possibly linked to drought stress tolerance
in profile of tolerant sample, 1 day after rehydration (T3b) (Rep.1) with their
expression level**

*Green spots are likely related to drought stress tolerance. (a) Protein spots No. 325, 305 and 377. (b) Protein spots
No. 143, 114, 115, 108, 91, 97, 451, and 62. (c) Protein spot No. 466.*

(301)

Table 2. Determination of protein relative abundance of selective spots possibly linked to drought stress in "1 day after rehydration" samples using Melanie software

Match ID	Spot No.	Mean (100%)	Variance	M.S.D	Mean of protein relative abundance in sample T3b[*]	Mean of protein relative abundance in sample S4	Mean of protein relative abundance in sample B1	Mean of protein relative abundance in sample 4R
1857	143	0.32	0.29	0.92	0.61	0.28	0	0.95
1882	91	0.19	0.19	1.00	0.44	0	0	0.31
1887	72	0.08	0.10	1.20	0.22	0.14	0	0
1895	108	0.06	0.10	1.73	0.24	0	0	0.23
1903	97	0.05	0.08	1.55	0.17	0	0	0.24
2135	305	0.13	0.18	1.36	0.39	0.15	0	0.34
2246	466	0.4	0.50	1.26	0.53	0	0	0
2258	114	0.046	0.11	2.26	0.23	0	0	0

T3b, S4, B1 and 4R are abbreviation of tolerant, sensitive and control samples of 1 day after rehydration and "prior to drought stress" sample, respectively
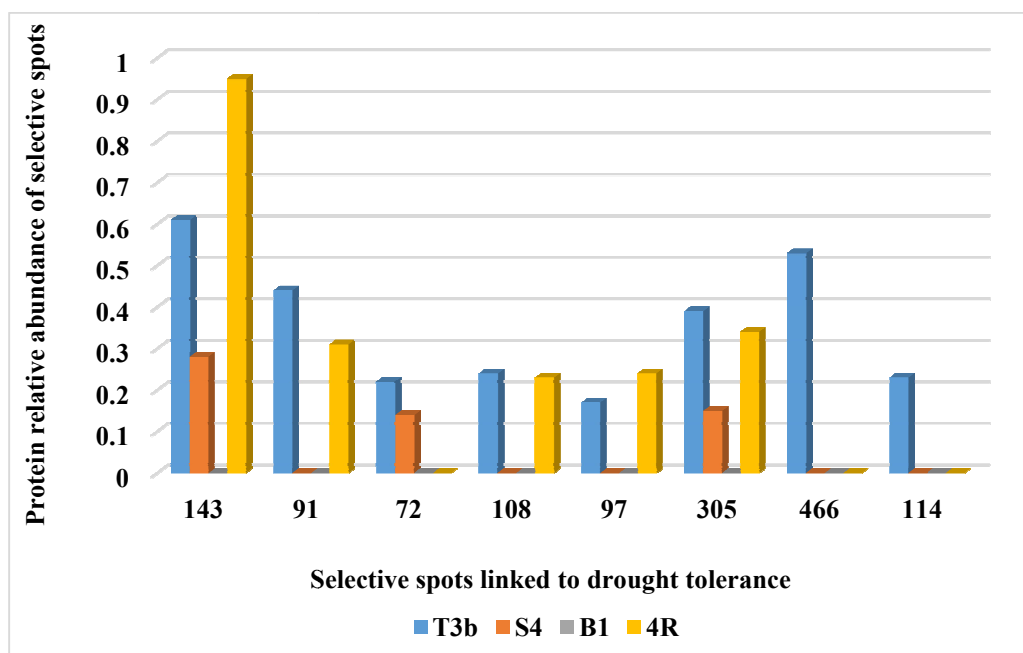
Fig. 9. Differential expression of selective spots possibly linked to drought tolerance in "1 day after rehydration" samples

T3b, S4, B1 and 4R are abbreviation of tolerant, sensitive and control samples of 1 day after rehydration and "prior to drought stress" sample, respectively

Table 3. Comparison and statistical analysis of protein spot possibly linked to drought tolerance in "1 day after rehydration" samples using Melanie software

Match ID	Spot No.	T3b*/S4 ratio	Student t test (T3b/S4)	T3b/B1 ratio	Student t test (T3b/B1)	T3b/4R ratio	Student t test (T3b/4R)
1857	143	2.14	**5.33**	#DIV/0!	**32.49**	0.64	0.61
1882	91	#DIV/0!	**13.35**	#DIV/0!	**13.35**	1.42	**13.35**
1887	72	1.53	**1.82**	#DIV/0!	**4.56**	#DIV/0!	**4.56**
1895	108	#DIV/0!	**482.57**	#DIV/0!	**482.57**	1.01	0.07
1903	97	2.73	**5.31**	#DIV/0!	**5.31**	0.72	1.11
2135	305	#DIV/0!	**2.56**	2.607757	**3.17**	1.15	1.66
2246	466	#DIV/0!	**50.31**	#DIV/0!	**50.31**	#DIV/0!	**50.31**
2258	114	#DIV/0!	**2.98**	#DIV/0!	**2.98**	#DIV/0!	**2.98**

*T3b, S4, B1 and 4R are abbreviation of tolerant, sensitive and control samples of 1 day after rehydration and "prior to drought stress" sample, respectively. Bold numbers represent significant differences (p < 0.05, p < 0.0) in expression of the spots between the samples. #DIV/0! Represents that protein relative abundance in one of samples is 0. Number of time treatments in this study, was 4. So, if numerical values of student t test due to the comparison in levels of 5% and 1%, is 2.353 and 4.541, respectively, comparison will be significant in level of 1% or 5%

2. Comparisons between two dimensional electrophoresis profiles of tolerant, sensitive and control samples after 7 days rehydration (T1, S2 and B7)

22 protein spots No. 29, 30, 107, 124, 132, 173, 175, 264, 288, 289, 292, 295, 311, 313, 319, 321, 323, 325, 326, 328, 345 and 352 were selected in tolerant sample (T1) as candidate spots linked to drought tolerance in profiles of 7 days after rehydration. These spots were expressed about two-fold more than sensitive (S2) and control (B7) samples and "prior to drought stress" sample (4R) or expressed only in tolerant sample (T1). Also, difference in expression levels of these proteins between tolerant sample and other samples was significant in student t-test. From 22 spots available in profile of tolerant sample (T1) (Rep.2), there were 14, 15 and 4 protein spots in profiles of sensitive sample (S2), control sample (B7) and "prior to drought stress" sample (4R), respectively. In other words, 8 protein spots No. 328, 326, 323, 321, 288, 352, 124 and 29 weren't expressed in sensitive sample (S2). Also, 7 protein spots No. 352, 288, 313, 107, 124, 30 and 29 weren't expressed in control sample (B7). Protein relative abundance of 16 spots No. 345, 328, 326, 325, 313, 311, 295, 264, 289, 288, 352, 173, 124, 30 and

29 in tolerant sample (T1) was about two-fold more than "prior to drought stress" sample (4R) or these spots were present only in tolerant sample (T1). Moreover, protein relative abundance of spots No. 292, 132 and 107 was changed in 4R sample than tolerant sample (T1). Totally, statistical analysis of proteomic data from of "7 days after rehydration" samples and "prior to drought stress" sample indicated that 10 reproducible protein spots No. 311, 345, 132, 401, 29, 30, 313, 112, 137 and 352 are possibly related to drought tolerance (Tables 4 and 5 and Fig. 10). There were significant statistical differences in protein relative abundance of selective spots linked to drought tolerance in these samples.

3. Comparisons between two dimensional electrophoresis profiles of tolerant, sensitive and control samples after 14 days drought stress (T3a, B0 and S1)

Only one protein spot No. 466 was selected in tolerant sample (T3a) as candidate spots linked to drought tolerance in profiles of 14 days after drought stress. This spot was expressed only in sample T3a. However, expression level of this protein was significant in student t-test (Fig. 11). Totally,

statistical analysis of proteome data from "14 days after drought stress" samples and "prior to drought stress" sample indicated that there is only a reproducible protein spot No. 466 possibly related to drought tolerance in tolerant sample T3a (Tables 6 and 7 and Fig. 11). There was significant statistical difference in protein relative abundance of selective spot linked to drought tolerance in these samples.
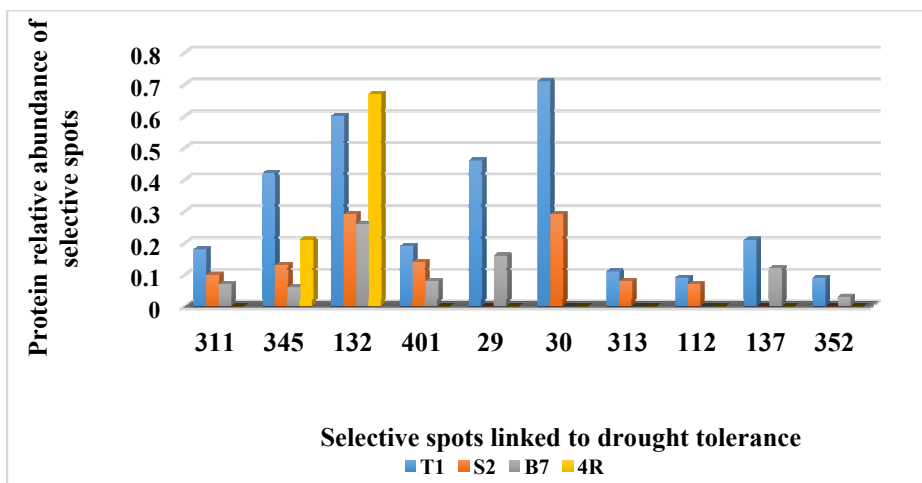
Fig. 10. Differential expression of selective spots possibly linked to drought tolerance in "7 days after rehydration" samples

T1, S2, B7 and 4R are abbreviation of tolerant, sensitive and control samples of 7 days after rehydration and "prior to drought stress" sample, respectively
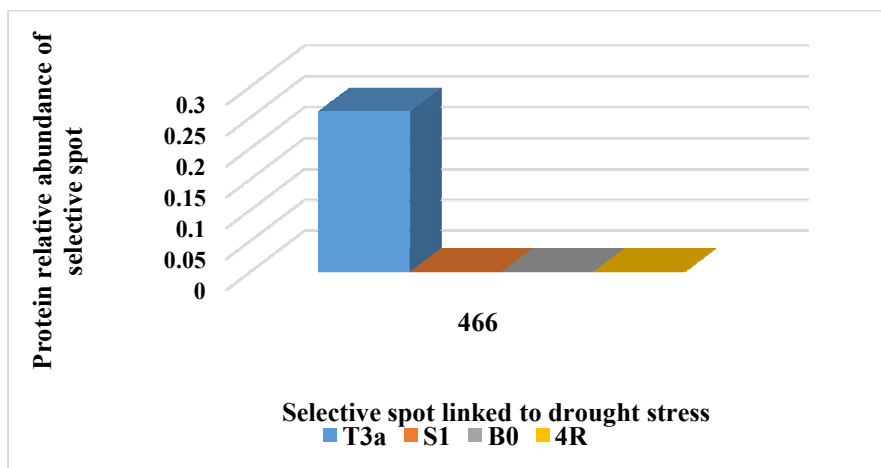
Fig. 11. Differential expression of selective spot linked to drought tolerance in "14 days after drought stress" samples

T3a, S1, B0 and 4R are abbreviation of tolerant, sensitive and control samples of 14 days after drought stress and "prior to drought stress" sample, respectively

Table 4. Determination of protein relative abundance of selective spots possibly linked to drought stress in "7 days after rehydration" samples using Melanie software

Match ID	Spot No.	Mean (100%)	Variance	M.S.D	Mean of protein relative abundance in sample T1[*]	Mean of protein relative abundance in sample S2	Mean of protein relative abundance in sample B7	Mean of protein relative abundance in sample 4R
735	311	0.08	0.09	1.14	0.18	0.10	0.07	0
767	345	0.16	0.20	1.25	0.42	0.13	0.06	0.21
771	132	0.38	0.19	0.49	0.60	0.29	0.26	0.67
867	401	0.09	0.08	0.94	0.19	0.14	0.08	0
878	29	0.17	0.27	1.60	0.46	0	0.16	0
880	30	0.27	0.33	1.24	0.71	0.29	0	0
1130	313	0.04	0.05	1.19	0.11	0.08	0	0
1175	112	0.03	0.04	1.29	0.09	0.07	0	0
1176	137	0.08	0.10	1.19	0.21	0	0.12	0
1257	352	0.03	0.04	1.29	0.09	0	0.03	0

T1, S2, B7 and 4R are abbreviation of tolerant, sensitive and control samples of 7 days after rehydration and "prior to drought stress" sample, respectively

Table 5. Comparison and statistical analysis of protein spots possibly linked to drought tolerance in "7 days after rehydration" samples using Melanie software

Match ID	Spot No.	T1[*]/S2 ratio	Student t test (T1/S2)	T1/B7 ratio	Student t test (T1/B7)	T1/4R ratio	Student t test (T1/4R)
735	311	1.91	**2.53**	2.79	**2.93**	#DIV/0!	**3.62**
767	345	3.26	**3.77**	6.67	**4.30**	1.98	**3.05**
771	132	2.10	**2.59**	2.32	**4.11**	0.34	**0.89**
867	401	1.36	1.79	2.52	**4.43**	#DIV/0!	**6.93**
878	29	#DIV/0!	**2.12**	2.81	**1.82**	#DIV/0!	**2.12**
880	30	2.49	**4.49**	#DIV/0!	**7.2**	#DIV/0!	**7.23**
1130	313	1.42	**2.55**	#DIV/0!	**5.40**	#DIV/0!	**5.40**
1175	112	1.31	1.35	#DIV/0!	**8.13**	#DIV/0!	**8.13**
1176	137	#DIV/0!	**3.62**	1.80	1.60	#DIV/0!	**3.62**
1257	352	#DIV/0!	**5.04**	2.65	**3.72**	#DIV/0!	**5.04**

T1, S2, B7 and 4R are abbreviation of tolerant, sensitive and control of samples of 7 days after rehydration and "prior to drought stress" sample, respectively. Bold numbers represent significant differences ($p < 0.05$, $p < 0.0$) in expression of the spots between the samples. #DIV/0! Represents that protein relative abundance in one of samples is 0. Number of time treatments in this study, was 4. So, if numerical values of student t test due to the comparison in levels of 5% and 1%, is 2.353 and 4.541, respectively, comparison will be significant in level of 1% or 5%

Table 6. Determination of protein relative abundance of selective spot possibly linked to drought stress in "14 days after drought stress" samples using Melanie software

Match ID	Spot No.	Mean (100%)	Variance	M.S.D	Mean of protein relative abundance in sample T3a[*]	Mean of protein relative abundance in sample S1	Mean of protein relative abundance in sample B0	Mean of protein relative abundance in sample 4R
5689	466	0.05	0.11	2.18	0.26	0	0	0

[*]T3a, S1, B0 and 4R are abbreviation of tolerant, sensitive and control samples of 14 days after drought stress and "prior to drought stress" sample, respectively

Table 7. Comparison and statistical analysis of protein spot possibly linked to drought tolerance in "14 days after drought stress" samples using Melanie software

Match ID	Spot No.	T3a[*]/S1 ratio	Student t test (T3a/S1)	T3a/B0 ratio	Student t test (T3a/B0)	T3a/4R ratio	Student t test (T3a/4R)
5689	466	#DIV/0!	**6.02**	#DIV/0!	**5.04**	#DIV/0!	**5.04**

[*]T3a, S1, B0 and 4R are abbreviation of tolerant, sensitive and control samples of 14 days after drought stress and "prior to drought stress" sample, respectively. Bold number represents significant difference (p < 0.05, p < 0.0) in expression of the spots between the samples. #DIV/0! Represents that protein relative abundance in one of samples is 0. Number of time treatments in this study, was 4. So, if numerical values of student t test due to the comparison in levels of 5% and 1%, is 2.353 and 4.541, respectively, comparison will be significant in level of 1% or 5%

Table 8. Determination of protein relative abundance of selective spots possibly linked to drought stress in tolerant samples in different times using Melanie software

Match ID	Spot No.	Mean (100%)	Variance	M.S.D	Mean of protein relative abundance in sample T1[*]	Mean of protein relative abundance in sample T3a	Mean of protein relative abundance in sample T3b	Mean of protein relative abundance in sample 4P
881	405	0.1	0.09	0.84	0.2	0.07	0.12	0.3
1176	137	0.26	0.22	0.85	0.21	0.12	0.51	0.11
1240	295	0.08	0.08	1.1	0.19	0.06	0.005	0.05

[*]T1, T3a, T3b and 4P are abbreviation of samples of tolerant 7 days after rehydration, tolerant (1) 14 days after drought stress, tolerant (1) 1 day after rehydration and tolerant (2) 14 days after rehydration, respectively

4. Comparisons between two dimensional electrophoresis profiles of tolerant sample (T1) and other tolerant samples

16 protein spots No. 97, 112, 137, 271, 275, 288, 291, 293, 295, 321, 322, 323, 327, 328, 366 and 405 were selected in tolerant sample of 7 days after rehydration (T1) as candidate spots linked to drought tolerance in profiles of tolerant samples. These spots were expressed about two-fold more than other tolerant samples or were expressed only in T1 sample. However, difference in expression levels of these proteins between tolerant samples was significant in student t-test. From 16 different spots available in profile of "7 days after rehydration" sample (T1), there were 6, 6 and 4 protein spots in profiles of "14 days after drought stress" sample (T3a), "1 day after rehydration" sample (T3b) and "14 days after drought stress" sample (4P), respectively. However, any protein spot in profile of "1 day after rehydration" sample (4S) wasn't observed.

Totally, statistical analysis of proteome data from tolerant samples in different times indicated that possibly three protein spots No. 405, 137 and 295 are linked to drought tolerance in studied drought tolerant mutant samples (Tables 8 and 9 and Fig. 12). However, there were significant statistical differences in protein relative abundance of selective spots linked to drought tolerance in tolerant mutant samples of this research. Generally, the spots that were expressed approximately in all tolerant samples, were selected as protein markers linked to drought tolerance. Regarding that none of the above three protein spots weren't expressed in tolerant (2) sample (4S), this sample hasn't been listed in Tables 8 and 9.

The most important purpose of this research was identification of new protein spots linked to drought tolerance in the novel rice germplasm. Western and northern blotting experiments of Sato and Yokoya [20] indicated that overexpression of a small heat-shock protein (sHSP17.7) with low molecular weight in high temperature condition in transgenic mutant rice plants enhances tolerance to drought stress. According to Sato and Yokoya [20] and Convener [21], one of the criteria for choosing protein spots related to drought tolerance in this research was low molecular weight of the spots. So, low or medium molecular weight reproducible protein spots were introduced as protein markers linked to tolerance to drought stress. However, protein relative abundance of these spots in tolerant samples was about 2-fold higher than other samples and expression of them was significant in student t-test in level of 1% or 5%. Totally, analysis of protein profiles of tolerant and sensitive mutant samples and control sample in three sampling times (14 days after drought stress, 1 day and 7 days after rehydration) and "prior to drought stress" sample indicated that 20 reproducible protein spots No. 29, 30, 72, 91, 97, 108, 112, 114, 132, 137, 143, 295, 305, 311, 313, 345, 352, 401, 405 and 466 are possibly related to drought tolerance. Also, Muthurajan et al. [22] discovered 31 protein spots representing significant differential relative abundance under drought stress condition. Among them, 10 protein spots were newly induced by drought stress. Moreover, five spots were up-regulated whereas 16 spots were significantly down-regulated. In research of Hosseini Salekdeh et al. [13], among 2000 protein spots detected in leaf extracts using proteome analysis, 42 spots showed significant changes in abundance under stress. Finally, they identified 27 spots with different responses in two studied varieties (CT9993 and IR62266). However, Rabello et al. [8] identified 22 protein spots possibly

related to drought stress tolerance in plants of traditional upland rice (*Oryza sativa* L. var. Japonica) varieties. Ali and Komatsu [3] indicated that considerable increasing in concentration of actin depolymerizing factor in drought tolerant plants. Moreover, in this research, abundance of 10 proteins increased in leaf sheath of two weeks' rice seedlings exposed to drought stress and was found that these proteins are involved in defense, signaling, cell structure and energy metabolism. Jagadish et al. [23] identified 46 protein spots with variable abundances in response to high temperature in anthesis stage in three rice genotypes (*Oryza sativa* L.) after performing two dimensional gel electrophoresis. Finally, they analyzed 13 protein spots with differential expression by MS/MALDI-TOF. On the other hand, Wang et al. [14] identified 43 proteins at flowering stage and 54 proteins at milk stage representing significant changes in silver stained 2D gel profiles of rice flag leaves. In research of Maksup et al. [24], 53 proteins

showed significant differences in tolerant cultivars exposed to drought stress especially in NSG19. These tolerant cultivars responded to increasing of cell and DNA repair proteins and fast closing of stomata in order to keep photosynthesis activities. Also, proteome analysis of drought susceptible rice cultivar "Zhenshan97B" and tolerant rice cultivar "IRAT109" by Jangpromma et al. [25] indicated that expression of LEA genes in transgenic rice and wheat, confers salt and drought tolerance and or increase efficiency in water using and biomass production. However, some conservative mechanisms might be induced by drought stress when expression of ATP synthase, Peptidyl prolyl ci-trans isomerase and Rubisco activase enzymes increased under drought stress [4]. Research results of some scientists such as Muthurajan et al. [22], Wang et al. [14], Jagadish et al. [23], Hosseini Salekdeh et al. [13] and Rabello et al. [8,9] confirm the results of this research.
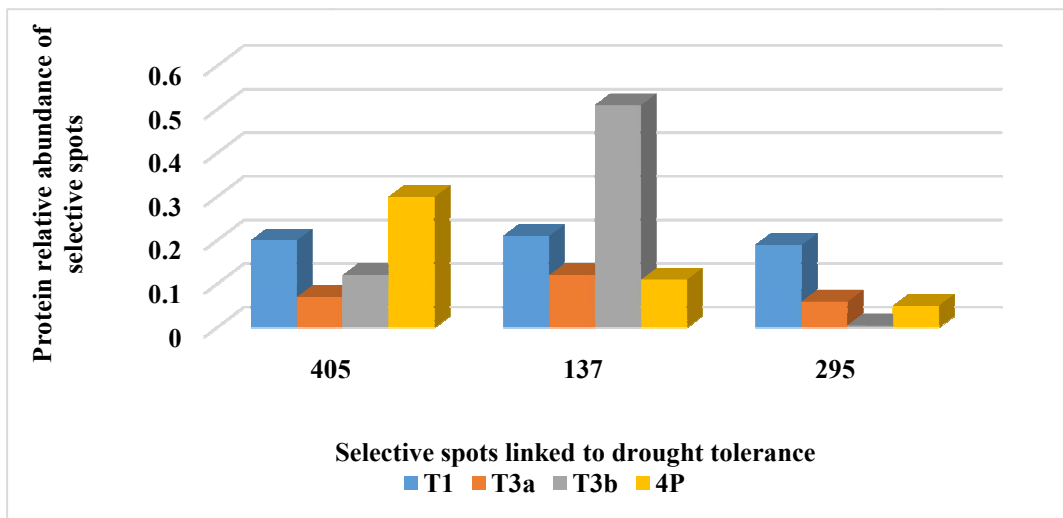
Fig. 12. Differential expression of selective spots possibly linked to drought tolerance in tolerant samples in different times

T1, T3a, T3b and 4P are abbreviation of tolerant sample after 7 days rehydration, tolerant (1) sample after 14 days drought stress, tolerant (1) sample after 1 day rehydration and tolerant (2) sample after 14 days drought stress, respectively

Table 9. Comparison and statistical analysis of protein spot possibly linked to drought tolerance in tolerant samples in different times using Melanie software

Match ID	Spot No.	T1*/T3a ratio	Student t test (T1/T3a)	T1/T3b ratio	Student t test (T1/T3b)	T1/4P ratio	Student t test (T1/4P)
881	405	2.96	**6.28**	1.69	2.68	0.67	**3.27**
1176	137	1.8	2.45	0.41	**2.93**	1.9	2.62
1240	295	3.14	**5.65**	1130	**13.14**	3.8	**8.24**

T1, T3a, T3b and 4P are abbreviation of samples of tolerant 7 days after rehydration, tolerant (1) 14 days after drought stress, tolerant (1) 1 day after rehydration and tolerant (2) 14 days after rehydration, respectively. Bold numbers represent significant differences (p < 0.05, p < 0.01) in expression of the spots between the samples. #DIV/0! Represents that protein relative abundance in one of samples is 0. Number of time treatments in comparison of tolerant samples, was 3. So, if numerical values of student t test due to the comparison in levels of 5% and 1%, is 2.92 and 5.841, respectively, comparison will be significant in level of 1% or 5%

CONCLUSION

Proteome analysis of new Iranian rice germplasm by 2D gel electrophoresis indicated that 20 protein spots are linked to drought tolerance. These protein markers can be used in breeding programs and production and characterization of new drought tolerant rice varieties. However, more comprehensive studies will be needed to characterize the genes and mechanisms responsible for drought tolerance in the new Iranian germplasm.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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