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Proteome Analysis of Mutant Drought Tolerant Iranian Rice Using 2D Gel Electrophoresis

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Authors' contributions

This research was accomplished with collaboration of all authors. Authors MTH, HM and MRN managed the study. Author MTH assessed the research results and wrote the article. Authors SSJ, MTH and MRN performed proteome analysis of mutant drought tolerant Iranian rice using 2D gel electrophoresis. Authors MTH and SSJ performed molecular data analysis from protein spots using Melanie software. Author MA helped writing the article. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Major regions of rice cultivation in Iran and Asia are affected by drought stress, especially at the end of the flowering stage. Therefore, it is inevitable to develop efficient crops by water utilization. The technique of proteome analysis by 2D-gel electrophoresis is useful to monitor comprehensive changes that occur in the protein relative abundance of tissues and organisms under stresses. Phenotypic analysis of mutant rice plants under drought stress was performed on standard evaluation systems of International Rice Research Institute. Protein extraction was done from studied samples in different times (control, fourteen days of stress, one day after rehydration, seven days after rehydration). Then, proteome analysis of the samples was performed by 2D electrophoresis. Molecular

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analysis of protein spots available in gel profiles of selective plants was done using Melanie software version 6.2. Inter class analysis of the samples indicated that protein relative abundance of 83, 88, 92, 118 and 180 spots in tolerant sample T1, seven days after rehydration was significantly increased than other samples. Moreover, inter class analysis represented that relative abundance of 7 and 8 spots in tolerant sample T3a, fourteen days after drought stress was more than 2-fold to other tolerant samples T1 and T3b, respectively. However, inter class analysis of tolerant sample T1, seven days after rehydration with tolerant samples T3a, fourteen days after drought stress and T3b, one day after rehydration and 4R, control condition sample, wild type sample B7, seven days after rehydration and sensitive sample S2, seven days after rehydration indicated that protein relative abundance of 88, 92, 118, 180 and 83 spots was significant statistically at 1% and 5% levels in student t-test, respectively. In conclusion, after proteome analysis of studied plants, twenty protein spots with No. 29, 30, 72, 91, 97, 107, 108, 112, 114, 132, 137, 143, 305, 311, 313, 345, 352, 366, 401, 405 and 466 were detected as candidate spots linked to drought tolerance.

Keywords: Proteome; 2D electrophoresis; Oryza sativa; drought stress and protein spot.

1. INTRODUCTION

Rice (*Oryza sativa*) is the member of cereal (*Poaceae*) family and an important cereal grown in about one-third of the world's total cereal crop area. Drought stress is a major problem in crop cultivation and production in the world. Such as other crops, loss of enough water resources especially during low falling periods is one of problems in rice cultivation and production. Sometimes, this damage reaches to 50-70% of reduction in yield in some regions of the world. Intermittent drought stress in rice has a significant effect on various cell cycle processes such as cell division, cell elongation, and maturation, which may be associated with peduncle growth arrest during water deficit [1]. Tahmasebi Sarvestani et al. [2] conducted a field experiment during 2001 - 2003 to evaluate the effect of water stress on yield and yield components using four rice cultivars, Tarom, Kazar, Fajr and Nemat that are commonly cultivated in Mazandaran province, Iran. Genetic improvement of adaptation to drought through the conventional approach is slow in attaining to progress, because of the low heritability of yield under stress, the inherent variation in the field and agronomical limitations. In plants, mutations can be artificially induced by mutagenic agents and utilized for production of new superior varieties of species from traditional variety. IAEA/FAO reported that 443 rice varieties have been developed by induced mutations during a period of 40 years, 1966 - 2004 [3]. The analysis of stress-responsive proteins in plants is an important route to the discovery of genes conferring stress tolerance and their use in breeding programs. Proteomics is fast emerging as an alternative field for studying the differential response of crops undergoing different stress [4]. Proteomics of rice embryo and endosperm [5]; green and etiolated shoot [6]; root [7]; cultured suspension cells [8]; leaf sheath [9]; anther [10], and different organelles such as mitochondria [11] has been performed by different researchers. Mikami et al. [12] has opened a route to critically understand the functions of rice proteins. The proteomics analysis of drought-conditioned leaves of three week-old plants in the drought-tolerant rice cultivars CT9993 and IR62266 has been reported by Salekdeh et al. [13]. Karim Khan and Komatsu [14] indicated that proteome analysis provides information on functional genomics, including identification of open reading frames (ORFs) from genome sequences, determining the proteins for sub cellular compartment localization, and identifying novel components involved in the components 'biogenesis'. Previous studies consistently identify regions on chromosomes 1, 4, 8 and 9 that influence a

range of drought-resistance traits, yield and yield components under stress [15]. So, evaluation of drought tolerance may be more effective using molecular methods to select for specific regions rather than individual traits. Ali and Komatsu [16] analyzed proteome of rice leaf sheath of two weeks- old rice seedlings during drought stress and found that actin depolymerizing factor is one of the target proteins induced by drought stress. Mirzaei et al. analyzed long-distance drought signaling within root systems in rice (*Oryza sativa* L. cv. IR64) [17]. Quantitative label-free shotgun proteomic analysis of four different root tissues resulted in identification of 1487 nonredundant proteins, with nearly 900 proteins present in triplicate in each treatment. Jagadish et al. [18] studied the morphology of the reproductive organs and pollen number, and changes in anther protein expression, in response to high temperature at anthesis of three rice (*Oryza sativa* L.) genotypes and showed 46 protein spots changing in abundance in a 2D-gel electrophoresis, of which 13 differentially expressed protein spots were analyzed by MS/MALDI-TOF. Goal of present study was analysis of responsive proteins to drought stress using two-dimensional electrophoresis system and determination of protein spots linked to drought tolerance in new tolerant and high yield rice plants [18].

2. MATERIALS AND METHODS

2.1 Determination of Irradiation Optimum Dose, Establishment of Different Mutant Generations and Drought Stress Application on Mutant rice Plants

As plant genotypes and cultivars indicate different responses to gamma irradiation, optimum dose is different in various genotypes and cultivars. In order to obtain to high frequency of favorable mutations and maximum of favorable effects and so, to obtain to optimum dose, seeds of Iranian rice landrace "TaromMahalli", that is a high quality genotype and sensitive to drought stress [2], were irradiated with different doses (0, 150, 180, 200, 220, 250 and 300 Gy) of gamma ray from Co₆₀ source in gamma cell [19]. Three weeks after sowing of seeds (two weeks after seed germination), LD₅₀ for survival of seedlings was estimated in different doses. Prior to impose drought stress, field soil was drained up to field capacity. Field capacity was determined by gravimetric method and tensiometer. After soil moisture approached field capacity, water stress was imposed about 10 days before flowering up to four days after flowering [20]. It is necessary to mention that the last stage of flowering is coincident with a very sensitive stage to drought stress.

2.2 Phenotypic Analysis of Mutant rice Plants under Drought Stress

Phenotypic analysis of rice plants under drought stress was performed on standard evaluation systems of IRRI [21]. The first phenotypic evaluation system of plants was on leaf rolling scale 0-9. 0, leaves healthy; 1, leaves start to fold (shallow); 3, leaves folding (deep V-shape); 5, leaves fully cupped (U-shape); 7, leaf margins touching (O-shape); 9, leaves are tightly rolled (V-shape). The second phenotypic evaluation system of plants was on spikelet fertility. 1, more than 80%; 3, 61 - 80%; 5, 41 - 60%; 7, 11 - 40%; 9, less than 11%.

2.3 Proteome Analysis of Candidate Plants

Protein extraction was performed from leaves a tolerant mutant plant to drought stress selected from M2 generation population on both leaf rolling and spike fertility scales (TM2-230-4), a sensitive mutant plant (B-TM2) and a wild type plant of genotype TaromMahalli. Sampling was done from these plants in three steps: last day of drought stress, 24 h after

rehydration (one day after stress finishing) and seven days after rehydration. Meanwhile, sampling was done randomly from a mutant plant prior to drought stress (control condition) (230-4-TM2) Table1.

Table 1. Characteristics of plant samples selected for 2D electrophoresis

Sample no.	Tolerance level	Prior to stress (control condition)	14 days after stress	1 day after rehydration	7 days after rehydration
Wild type	Sensitive (9)	-	x	x	x
TM*2-230-4	Tolerant (0)	-	x	x	x
B-TM2	Sensitive (9)	-	x	x	x
230-4-TM2	-	x	-	-	-

* TM2 represents M2 generation of genotype TaromMahalli. 230 indicate dose 230 Gy population. B is equal to Sensitive Bulk

2.4 Protein Extraction

Protein was extracted from leaf samples of above selective mutant plants by Damerval et al., method [22]. Plant materials were collected and frozen rapidly in liquid nitrogen. 1g of plant material was ground in a mortar containing a minimum quantity of liquid nitrogen until a fine powder is obtained. The powder was transferred to cold 14 ml acetone – proof Falcon centrifuge tube. 10 ml of TCA Extraction Solution (10 g TCA, 70 mg DTT, Acetone to 100 ml) (pre-cooled at -20°C) was added. Tubes were centrifuged in pre-cooled rotor (run at 4°C) at 35000g for 15 minutes. Tubes were transferred to dry ice and then supernatants were discarded carefully. The tubes containing the pellets were kept on dry ice. To each tube, 10 ml of Sample Washing Solution (70 mg DTT, Acetone to 100 ml) (pre-cooled at -20°C) was added and broke up the pellet. The tubes were returned to -20°C for 1 hour. Tubes were centrifuged at 12000g at 4°C for 15 minutes. Steps 6 to 9 were repeated once or twice depending on the nature of the plant material. After the final supernatant has been removed with care, the mouth of each tube was covered with parafilm. A needle was used to make several small holes in the parafilm. The pellet was lyophilized for one hour. The lyophilized samples can be stored at -80°C. During the lyophilization, 10 ml urea sample (lysis) buffer (5.4g (9M) urea, 0.4g CHAPS, 0.1g DTT, 250µl (40% stock) pH 3-10 ampholytes, 0.0424g (35mM) Tris base, Double distilled water to 10 ml) was prepared and filtered. 20mg of lyophilized power was suspended in 300µl of the Lysis Buffer. The solution was incubated at 37°C for 2h with continuous stirring. The solution was centrifuged at 12000g (= 10000 rpm on bench top centrifuge) at room temperature for 30 minutes. A small aliquot was used to determine protein content (Bradford assay). Supernatants were transferred to clean, labeled Eppendorf tubes and Stored at -80°C.

2.5 Measurement of Protein Concentration Available in Any Sample

It was used from Bradford method to measure concentration of protein samples [23]. Absorbance amount of samples were measured in Wavelength 595 nm. Standard plot was drawn on different concentrations of standard protein (BSA) (0, 0.1, 0.3, 0.5, 0.7 and 1 mg/ml). If concentration plot of standard protein sample is statistically significant that R^2 due to equation of the plot is more than 0.98. After drawing standard plot, concentration of protein samples was calculated in two replications and their averages were determined and then,

needed amount of protein sample was calculated for two-dimensional electrophoresis operation.

2.6 Two-Dimensional (2D) Electrophoresis

At first, gel strips must be rehydrated. For rehydration of gels, 320 μ l rehydration solution (2% CHAPS, 8M urea, 0.018M DTT, 2% IPG buffer and 0.002% bromophenol blue) that contains 120 μ l protein was pipetted into channel available in rehydration tray. The gel was put on channel slowly, whereas side of gel was in top direction. Rehydration took along at night averagely 14-16 hours. In this research, it was used from the set (made in Biorad Company) for performing first dimension (IEF). For running gels, power supply was turn on and its program was regulated on gradient. To run a gel 17 cm with pH=4-7, averagely needs to 42000Vh (42KVh). This Vh amount can be supplied gradient in continuous three steps. We supplied this Vh in three steps 250V, 250 to 10000V and 45000Vh in 10000V. Of course, this is better to begin electrophoresis with lower voltage, so that salts and other pollutants are exited from gel prior to move proteins. In all of these steps, stream intensity on 1mA and power on 5W was constant. Averagely, first dimension took along 15 to 16h for a gel 17cm. After completion of first dimension, gels can be kept in freezer -20°C or prepared for second dimension directly. Vertical electrophoresis set "Cell Protein II (Biorad Company) was used to accomplish second dimension electrophoresis of sample. After preparation of second dimension gel (12.5%), first dimension strip was soaked in equilibration buffer (TrisHCl 50 mM with pH=8.8, Bromophenol blue 0.02%, DTT 1%, Glycerol 30%, SDS 2% and urea 6M) for 15 minutes. After putting strip gel on level of second dimension gel, the top of strip gel was overlaid with melted agarose 0.5% (70°C). After throwing enough electrophoresis buffers, power was turn on and gel was run with mA in several steps (first step, 5mA for 1h and second step, 30mA for 5h). By finishing second dimensional electrophoresis, gel was exited from gel cassette and cut to some extent acidity side of gels for more convenient diagnosis and labeled. Immediately, gel was put in fixer solution. Then, staining steps were done continued on Blum method [24].

2.7 Photography of Gel

After staining, the gels were photographed with Gel Doc set (Biorad Company) and images were saved to raw format of two dimensional gels.

2.8 Molecular Data Analysis from Protein Spots Available in Gel Profiles of Selective Plants using Melanie Software

Images with quantity one format were converted to TIFF format. Then, gels were imported to Melanie version 6.2 software with reduction factor: 3. Gels were detected with Saliency: 5, Smooth: 2 and Min area: 5. All of authorized spots available in studied gels were scored. Using Melanie software, volume of protein spots available in different replications of any sample in different times was calculated. Then, volume mean of protein spots in different replications was estimated for any sample and also, ratios for any sample in different times were calculated. Protein spots were selected on the criterion that their relative abundance was more than 2 fold change to the same spots in other samples. Also, protein spots were selected that had expressed only in one or two specific times. As number of time treatments was 4, if any comparison in different times was significant statistically at 5% and 1% levels, that numerical amount of student t-test became more than 2.353 and 4.541, respectively. Protein spots were selected that not only their relative abundance than to other spots were

more than 2 fold change but also significant statistically at 5% and 1% levels. In proteome analysis, protein spots with higher molecular weight that are not likely linked to tolerance to drought stress and their correlation with tolerance in different samples and times have not been completely confirmed were not selected [25,26]. Protein spots that had low or medium molecular weight and their relative abundance in different comparisons between samples had been proved more than 2 fold change and preferably were significant at 1% level, were introduced as protein markers linked to drought tolerance.

3. RESULTS AND DISCUSSION

3.1 Data Analysis Outputs from Phenotypic Evaluations of Mutant Plant Populations under Drought Stress

After two weeks drought stress, sixty-four tolerant plants selected on leaf rolling scale and eighty one early flowering mutant plants on flowering date were selected among M₂ generation population. Furthermore, four mutant plants were tolerant and early flowering (TM₂-230-3, TM₂-230-4 and TM₂-230-6). Sixty-four tolerant selective plants on leaf rolling scale were analyzed on spikelet fertility scale and identified only three tolerant mutant plants on two scales (TM₂-230-3, TM₂-230-4 and TM₂-230-5). In the second year of evaluation, thirty-three mutant plants of M₃ generation expressed tolerance to drought stress on leaf rolling scale from sixty-four tolerant selective plants of M₂ generation. Among them, fifteen plants were identified tolerant to drought stress on spikelet fertility and had high yield. Of course, only tolerant plant TM₂-230-4 was used in protein extraction and 2D electrophoresis.

3.2 Results Due to Extraction and Quantification of Protein from Leaf Samples of Selective Plants

Standard plot was drawn on different concentrations of standard protein (BSA) (0, 0.1, 0.3, 0.5, 0.7 and 1 mg/ml). Concentration plot of standard protein samples (BSA) was drawn and equation ($y=1694x-40.88$) was obtained Fig. 1. R² of resulted equation was 0.992 and so, drawn concentration plot was correct and suitable for calculation of needed protein amount of samples for two-dimensional electrophoresis operation.

With including X amount (absorbance value in wavelength 595 nm) in the equation, Y amount (concentration of standard protein) was calculated and as concentration of needed protein sample for doing two-dimensional electrophoresis operation must be at least 120 µg/µl, Y amount (µg/µl) was divided to 120 Table 2.

3.3 Two-Dimensional Electrophoresis Results of Studied Samples in this Research

Two-dimensional electrophoresis operations of selective samples were done in three replications Figs. 2, 3, 4 and 5.

3.4 Results of Molecular Data Analysis from Protein Spots Available in Gel Profiles of Selective Plants by Melanie Software

Totally, forty seven comparisons were done between studied samples in different times that eleven comparisons were due to intra class analysis and thirty six comparisons were related

to inter class analysis. From thirty six inter class analysis, only twenty one analyses due to comparisons of tolerant samples with other samples, have been discussed in this research.

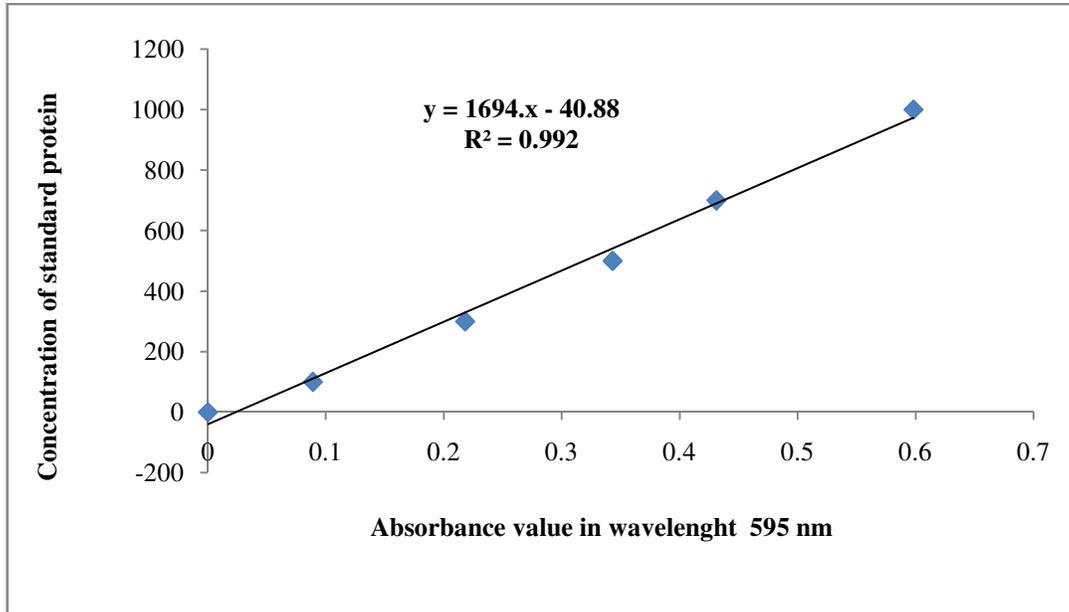


Fig. 1. Concentration plot of standard protein sample



Fig. 2. Two-dimensional electrophoresis profile of sample "4R - control condition"

3.5 Results of Inter Class Analysis of Studied Samples in Different Times

Inter class analysis of tolerant sample T1, seven days after rehydration with tolerant samples T3a, fourteen days after drought stress and T3b, one day after rehydration and 4R, control condition sample, wild type sample B7, seven days after rehydration and sensitive sample S2, seven days after rehydration indicated that protein relative abundance of 88, 92, 118, 180 and 83 spots was significant statistically at 1% and 5% levels in student t-test, respectively. Also, protein relative abundance of 57, 74, 81, 94 and 107 spots in sample T1 was more than 2-fold to samples T3a and T3b and 4R, B7 and S2, respectively. As mentioned, high molecular weight protein spots in second dimensional gel, which are not

related to tolerance to drought stress and or possibly have been sequenced and also, protein relative abundance of some spots in sample T1 that were not more than 2-fold to the same spots in above samples, were omitted. So, 61, 83, 85, 95 and 144 different protein spots likely related to drought tolerance were selected.

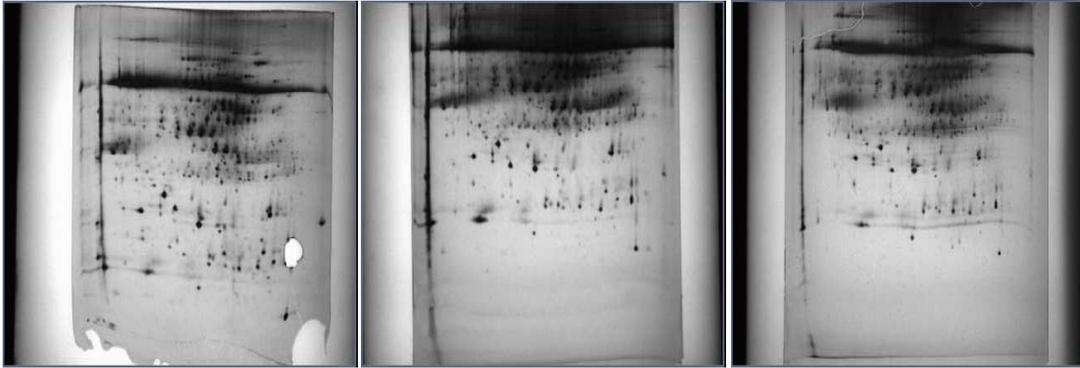


Fig. 3. Two-dimensional electrophoresis profiles of sample “T1- Tolerant seven days after rehydration”

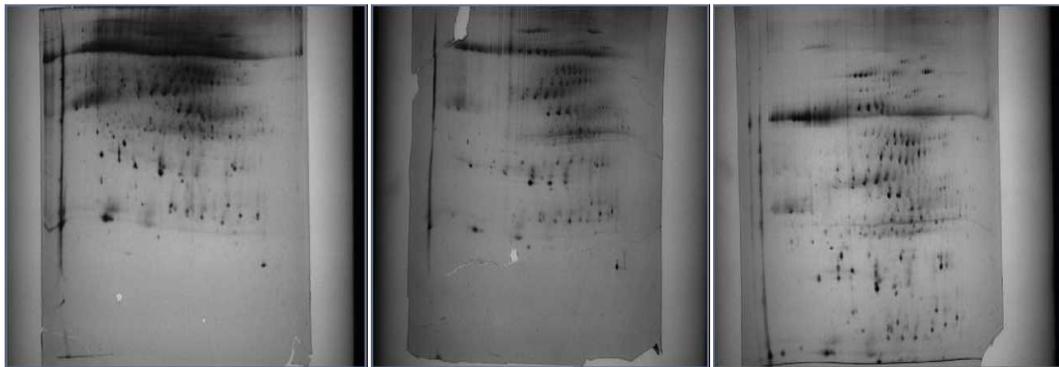


Fig. 4. Two-dimensional electrophoresis profile of sample “S2- Sensitive seven days after rehydration”



Fig. 5. Two-dimensional electrophoresis profile of sample “B7- wild type seven days after rehydration”

Inter class analysis of tolerant sample T3b, one day after rehydration with tolerant sample T3a, fourteen days after drought stress, control condition sample 4R, wild type sample B1, one day after rehydration and sensitive sample S4, one day after rehydration represented that protein relative abundance of 48, 58, 61 and 95 spots was significant statistically than other samples at 1% and 5% levels, respectively. Also, protein relative abundance of 29, 38, 41 and 61 spots in sample T3b was more than 2-fold to samples T3a, 4R, B1 and S4, respectively. Totally, 29, 42, 43 and 65 different protein spots likely related to drought tolerance were identified. It is necessary to mention that inter class analysis of tolerant sample T3b with tolerant sample T1, seven days after rehydration indicated that protein relative abundance of thirteen spots in T3b sample was more than 2-fold to T1 sample. In inter class analysis of tolerant sample T3a, fourteen days after drought stress with control condition sample 4R, sample B0 (wild type), fourteen days after drought stress and sensitive sample S1, fourteen days after drought stress, protein relative abundance of 26, 37 and 44 spots in tolerant sample T3a was significant than other samples statistically at 1% and 5% levels. Also, protein relative abundance of 38, 26 and 38 spots in sample T3a was more than 2-fold to samples 4R, B0 and S1, respectively. Totally, 28, 15 and 26 different protein spots likely related to drought tolerance were selected. It is necessary to mention that interclass analysis of tolerant sample T3a with tolerant samples T1, seven days after rehydration and T3b, one day after rehydration indicated that protein relative abundance of seven and eight spots in T3a sample was more than 2-fold to T1 and T3b samples, respectively.

Overall, fourteen days after drought stress, protein relative abundance of thirty eight spots in tolerant sample was more than 2-fold than 4R, control condition sample, whereas, one and seven days after rehydration of the plants, protein relative abundance of forty one and ninety four spots in tolerant sample was more than 2-fold than 4R. Inter class analysis of seven days after rehydration samples using Melanie software represented that relative abundance of 164 proteins in tolerant sample has been increased or reduced than sensitive and wild type samples. Also, molecular analysis of fourteen days after drought stress and one day after rehydration samples indicated that relative abundance of 63 and 143 proteins in tolerant sample have been changed than sensitive and wild type samples, fourteen days after drought stress and one day after rehydration, respectively.

In total, inter class analysis of the protein spots available in the profiles using Melanie software represented that relative abundance of 254 proteins in tolerant sample T1, seven days after rehydration have been increased or reduced than other tolerant, sensitive and wild type samples in this research. Also, relative abundance of 211 proteins in tolerant sample T1 have been increased or reduced than sensitive and wild type samples (S2 and B7), seven days after rehydration. Molecular analysis of 254 proteins indicated that relative abundance of 119 proteins in tolerant sample T1, have been changed than 4R in control condition. This issue indicated that most of responsible genes involved in relative abundance of these proteins have been inactive or down regulated in drought stress period and, seven days after rehydration have been activated by plant and recovered again. Protein relative abundance of ninety four spots was up-regulated seven days after rehydration in tolerant sample (T1) than control condition sample (4R sample).

3.6 Determination of Possible Protein Spots Linked to Tolerance to Drought Stress

Seven days after rehydration, relative abundance of twenty two protein spots (No. 289, 288, 264, 326, 321, 345, 311, 323, 319, 132, 175, 328, 107, 313, 124, 30, 292, 173, 352, 29, 295 and 325) available in tolerant sample T1 was increased more than 2-fold to sensitive and

wild type samples (S2 and B7) or these spots were expressed only in sample T1 Tables 3, 4 and 5. From twenty two spots available in tolerant sample T1 (Rep.2) profile, there were fourteen spots in sensitive sample S2 (Rep.1) profile, fifteen spots in wild type sample B7 (Rep.1) profile and four spots in control condition sample 4R (Rep.1) profile Figs. 6, 7, 8 and 9.

From twenty two spots available in tolerant sample T1 (Rep.2) profile, there were fourteen spots in sensitive sample S2 (Rep.1) profile, fifteen spots in wild type sample B7 (Rep.1) profile and four spots in control condition sample 4R (Rep.1) profile Figs. 6, 7, 8 and 9.

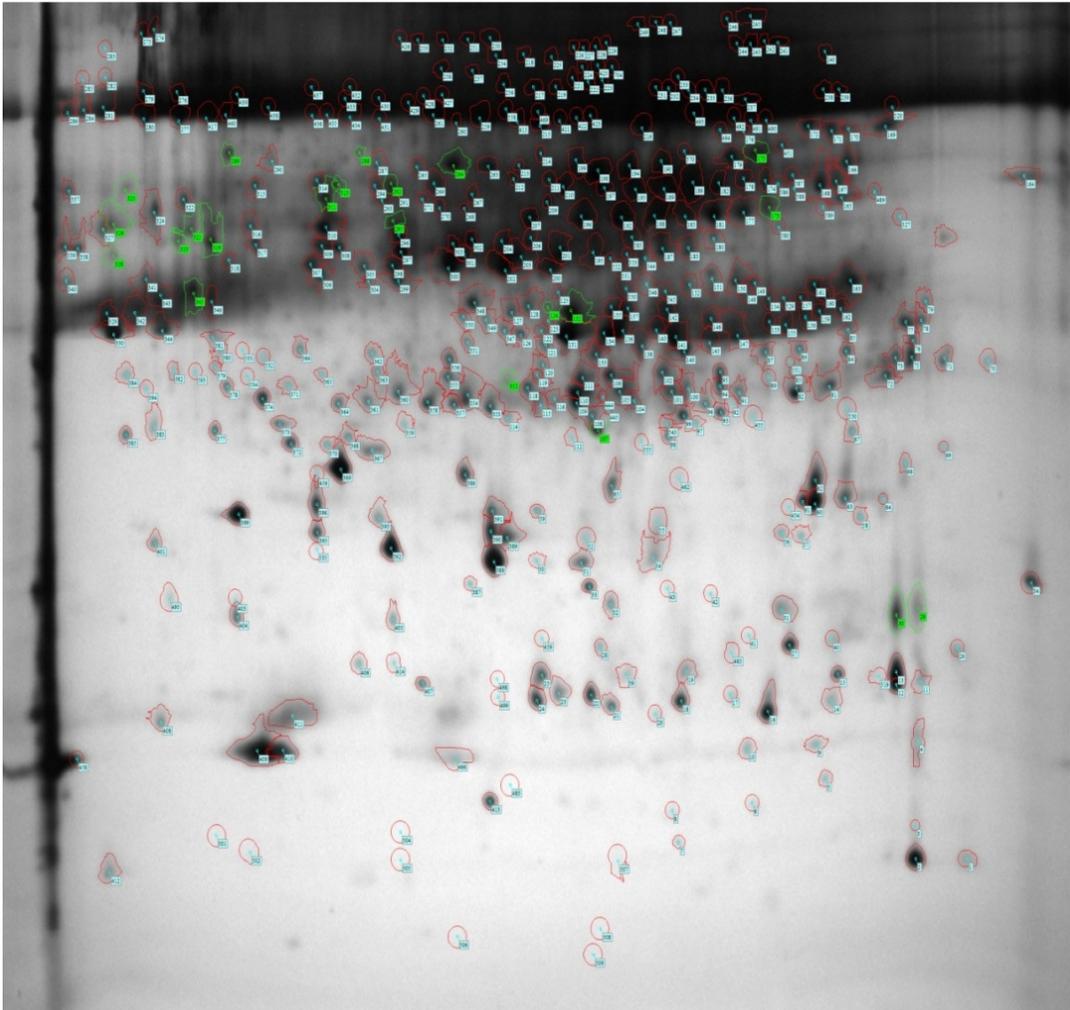
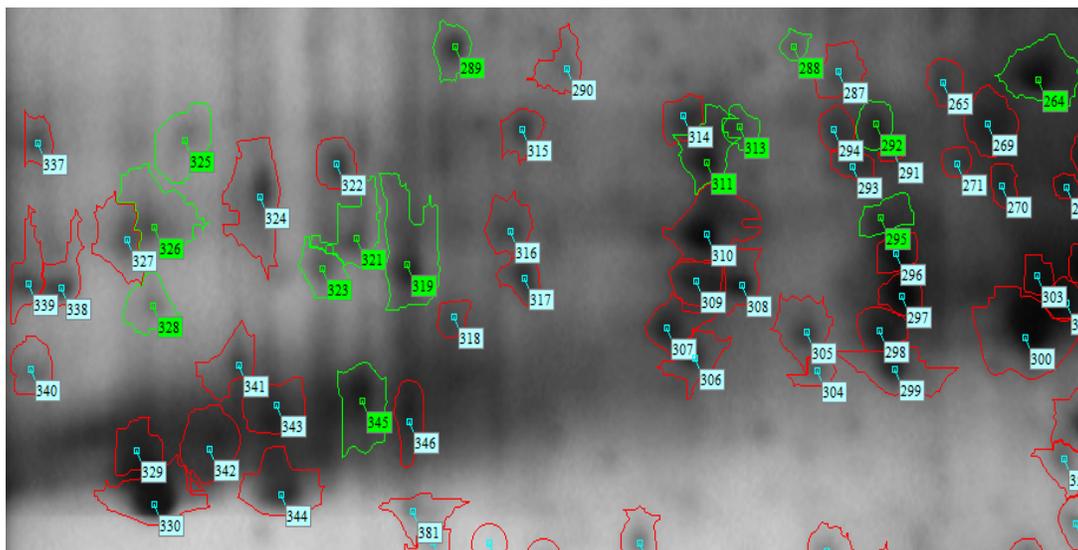
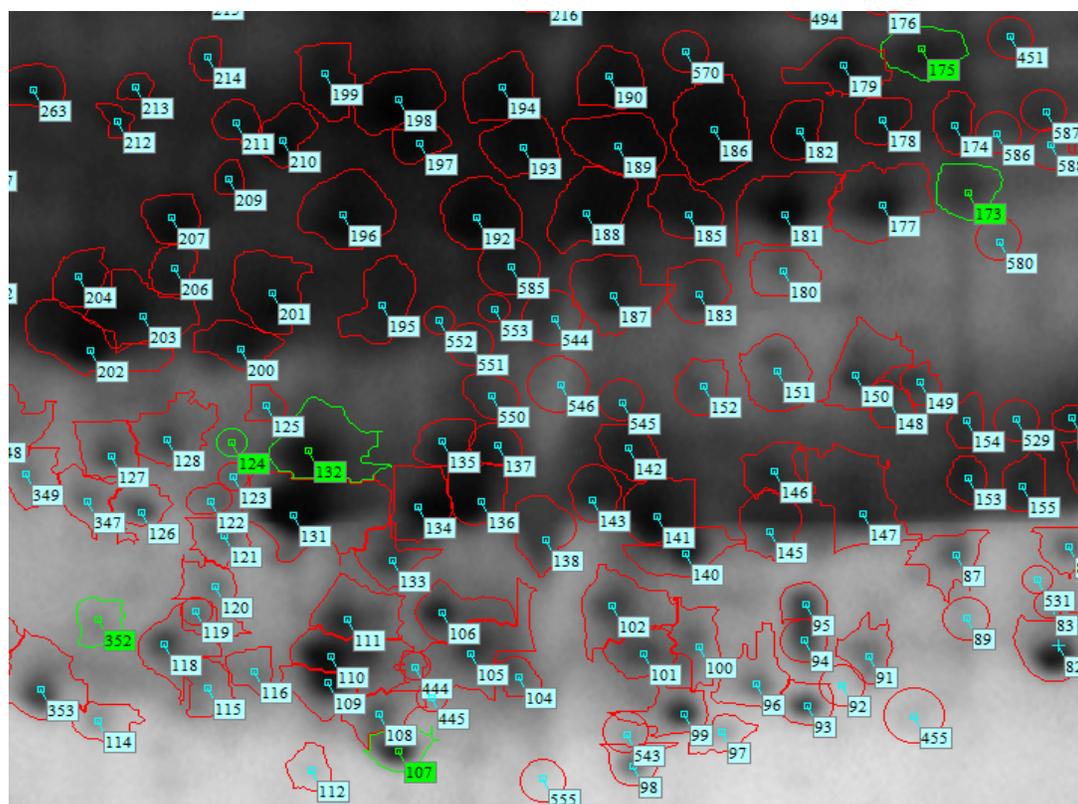


Fig. 6. Possible protein spots linked to drought tolerance in tolerant sample seven days after rehydration (T1) (Rep.2) profile

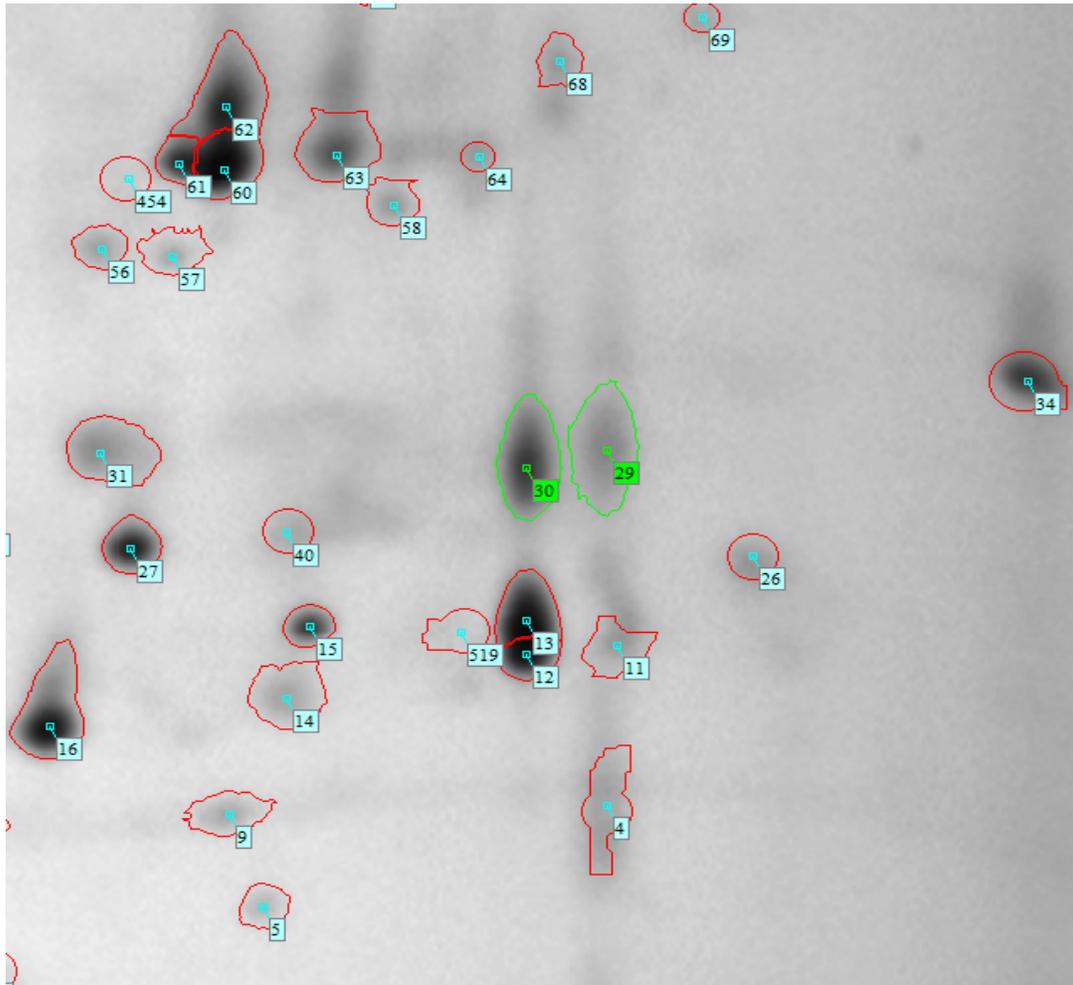
All spots have been shown with number. Green spots are likely related to tolerance to drought stress. Rep.2 is abbreviation of Repeat 2



(a)



(b)



(c)

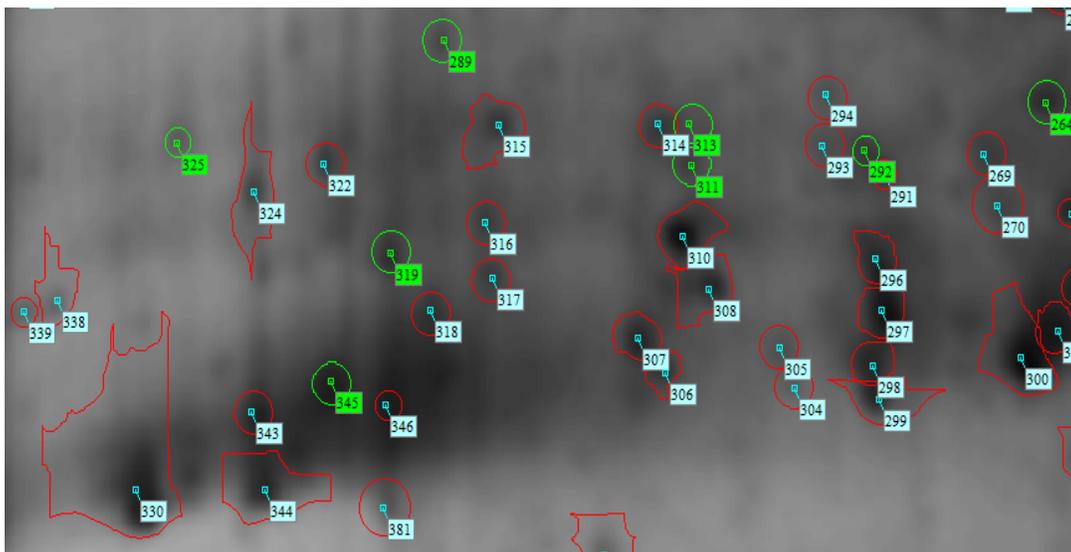
Fig. 7. Magnified picture of possible protein spots linked to drought tolerance in tolerant sample seven days after rehydration (T1) (Rep.2) profile with their protein relative abundance

All spots have been shown with number. Green spots are likely related to tolerance to drought stress.

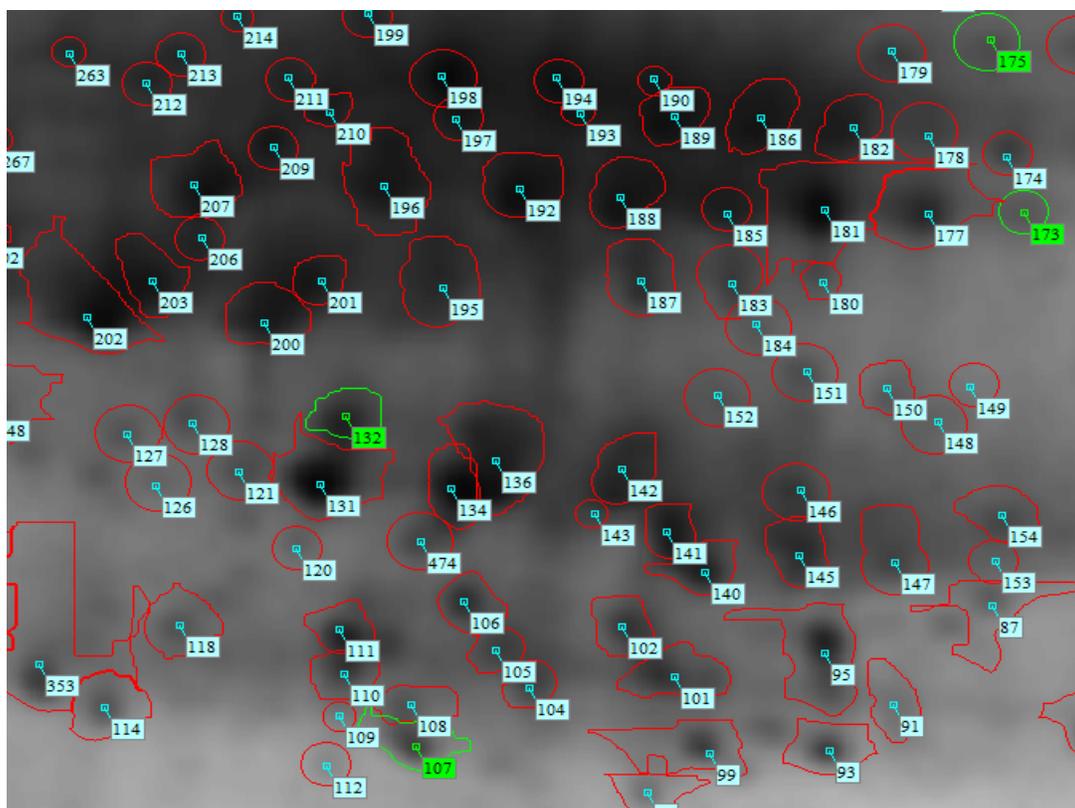
(a) Protein spots No. 328, 326, 325, 323, 321, 319, 345, 311, 313, 289, 288, 295, 292 and 264. (b)

Protein spots No. 175, 173, 132, 124, 352 and 107. (c) Protein spots No. 30 and 29

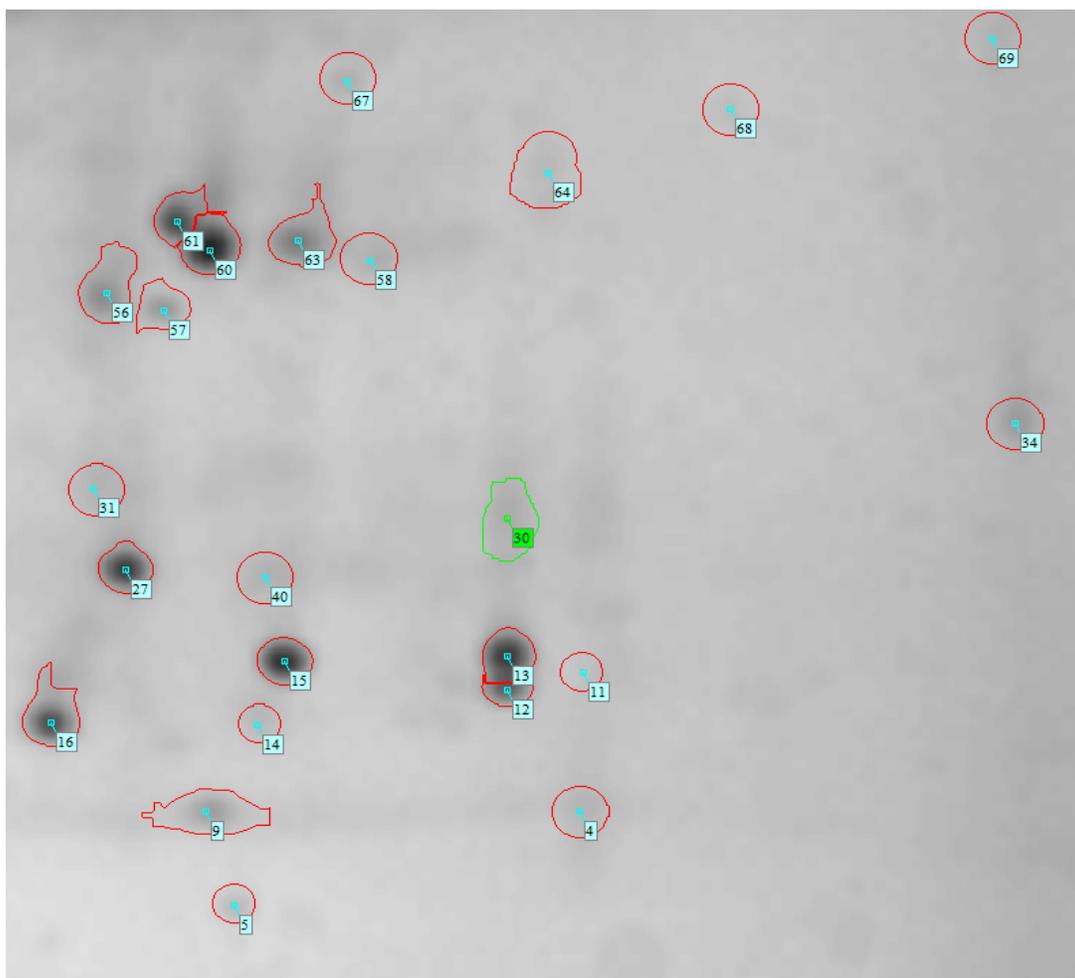
From twenty two protein spots in tolerant sample T1, eight spots No. 328, 326, 323, 321, 288, 352, 124 and 29 were not expressed in sensitive sample S2 (Rep.1) Fig. 9.



(a)



(b)

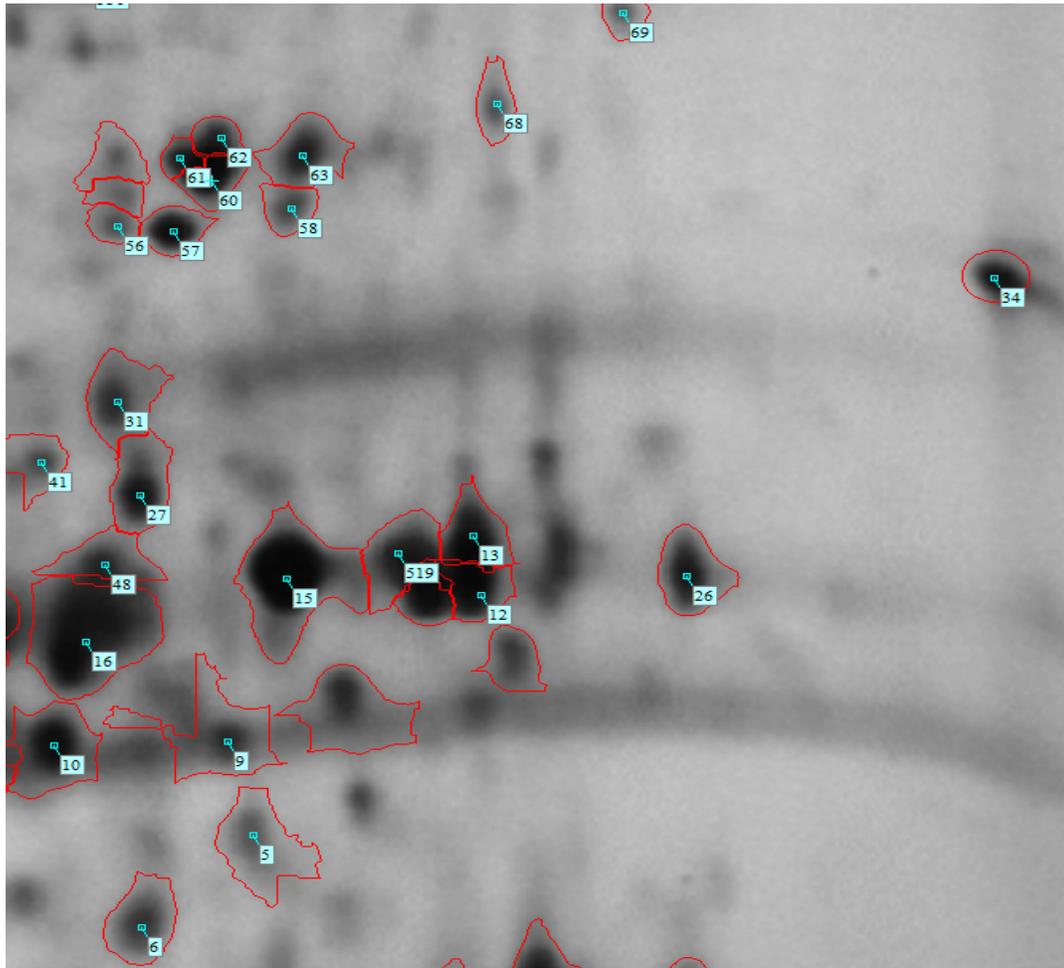


(c)

Fig. 8. Magnified picture of possible protein spots linked to drought tolerance in sensitive sample seven days after rehydration (S2) (Rep.1) profile with their protein relative abundance.

All spots have been shown with number. Green spots are likely related to drought tolerance. (a) Protein spots No. 325, 319, 345, 311, 313, 295, 292 and 264. (b) Protein spots No. 175, 173, 132, 124 and 107. (c) Protein spot No. 30.

Also, from twenty two protein spots in tolerant sample T1, seven spots No. 326, 288, 313, 124, 30 and 29 were not expressed in wild type sample B7 (Rep.1) Fig. 9



(c)

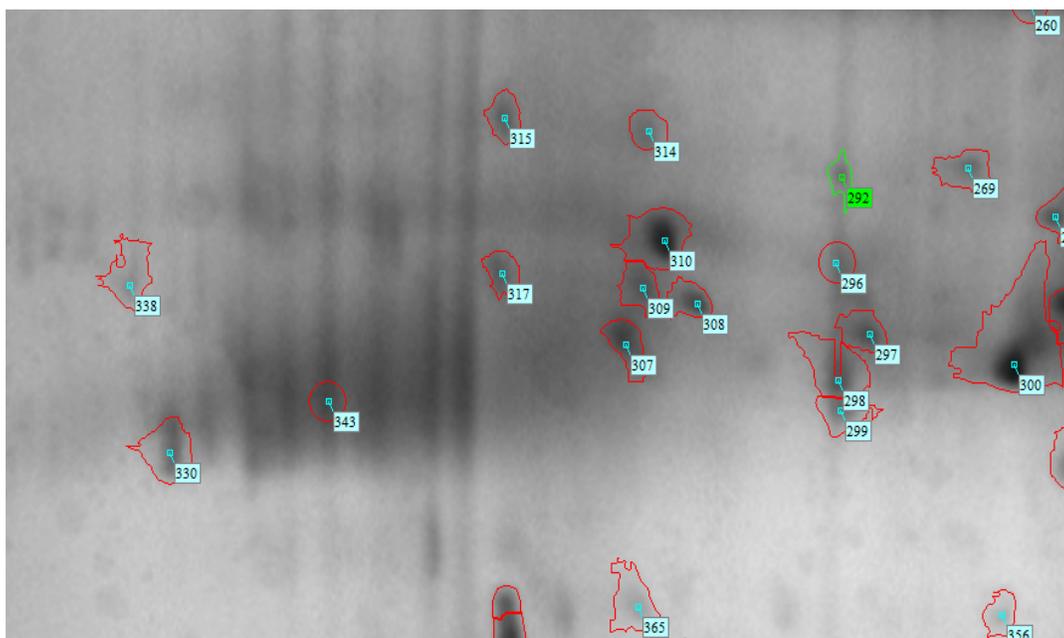
Fig. 9. Magnified picture of possible protein spots linked to drought tolerance in wild type sample seven days after rehydration (B7) (Rep.1) with their protein relative abundance

All spots have been shown with number. Green spots are likely related to tolerance to drought stress.

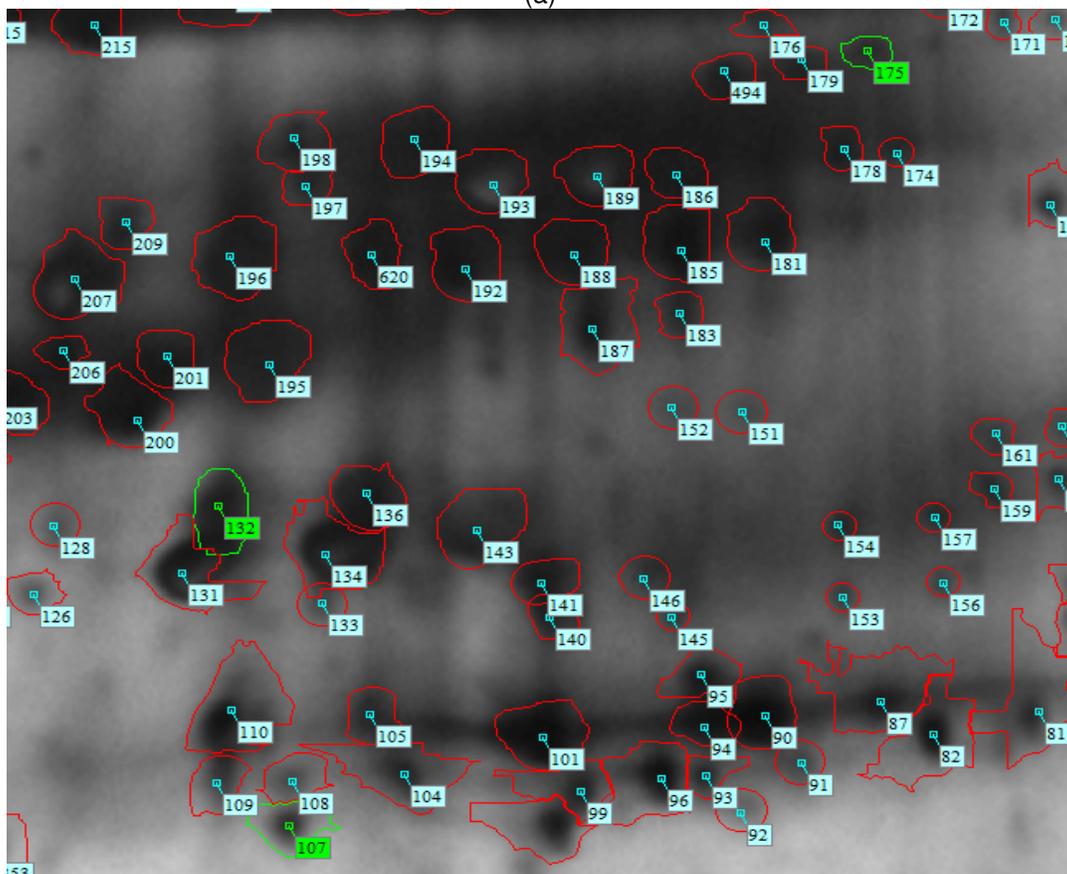
(a) Protein spots No. 328, 326, 325, 323, 321, 319, 345, 311, 295, 292 and 264. (b) Protein spots No.

175, 173, 132 and 107. (c) There are not protein spots No. 30 and 29

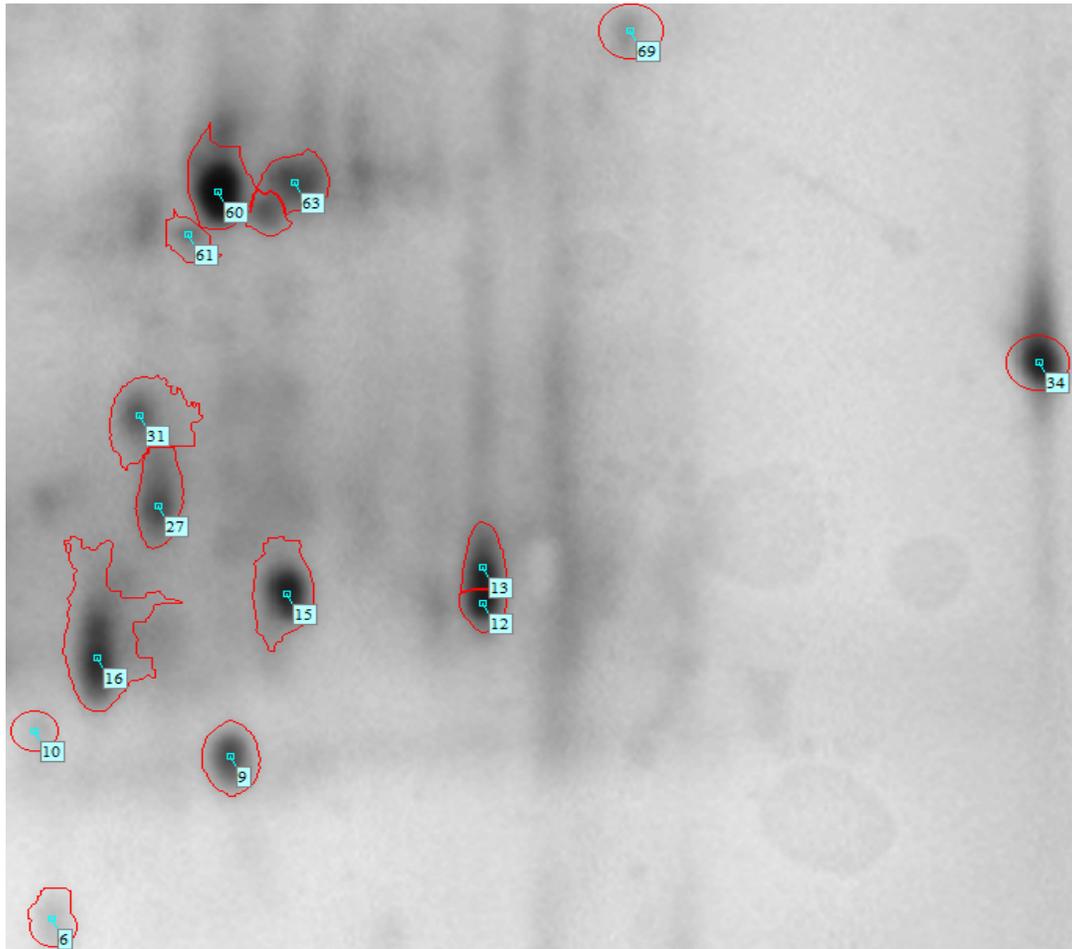
From twenty two protein spots (No. 289, 288, 264, 326, 345, 311, 175, 29, 328, 313, 124, 30, 173, 352, 295 and 325), protein relative abundance of sixteen spots in tolerant sample T1 was increased more than 2-fold to 4R sample or these spots were present only in tolerant sample T1 profile Fig. 10. Fifteen spots No. 345, 328, 326, 325, 313, 311, 295, 264, 289, 288, 352, 173, 124, 30 and 29 were not expressed in control condition sample (4R) (Rep.1) than tolerant sample T1 Fig. 10 whereas protein relative abundance of spots No. 292, 132 and 107 was not changed in 4R sample. After analysis and comparison of protein spots available in seven days after rehydration and control condition profiles, it was characterized that 16 genes were up-regulated in tolerant sample (T1) seven days after rehydration.



(a)



(b)



(c)

Fig. 10. Magnified picture of possible protein spots linked to drought tolerance in control condition sample 4R (Rep.1) profile with their protein relative abundance

(a) Protein spot No. 292 has not been changed in 4R sample than tolerant sample (T1). (b) Relative abundance of protein spot No. 175 has been decreased and relative abundance of protein spots No. 132 and 107 have not been changed in 4R sample than tolerant sample (T1). (c) There are not protein spots No. 30 and 29

One day after rehydration, relative abundance of twelve protein spots in tolerant sample (T3b) was increased more than 2-fold to sensitive and wild type samples (B1 and S4) or these spots were expressed only in T3b sample and significant in student t-test. From twelve spots (No. 108, 305, 97, 62, 325, 143, 91, 466, 377, 115, 451 and 114) available in tolerant sample T3b (Rep.1) profile, there were two spots in wild type sample B1 (Rep.1) profile, and only one spot in sensitive sample S4 (Rep.1) profile. Relative abundance of sixteen protein spots (No. 288, 322, 327, 321, 323, 328, 366, 97, 405, 291, 293, 137, 271, 295, 275 and 112) seven days after rehydration in tolerant sample was increased more than 2-fold, in comparison with other times or these were present only seven days after rehydration. From sixteen spots available in tolerant sample seven days after rehydration (T1) (Rep.1) profile, there were only six protein spots in tolerant samples fourteen days after drought stress (T3a)

(Rep.1) and one day after rehydration (T3b) (Rep.1) profiles. There were many differences between profiles of tolerant samples (one day after rehydration and fourteen days after drought stress) and control condition sample. For example, one day after rehydration, protein spots No. 401 and 462 were present in only tolerant sample whereas these spots had not been expressed in control condition. Relative abundance of protein spot No. 72 in fourteen days after drought stress and one day after rehydration samples was 2 fold in comparison with control condition. Also, fourteen days after drought stress, relative abundance of protein spots No. 186 and 147 were present in tolerant sample was increased more than 2-fold to control condition. Protein spots No. 186 and 147 were present in tolerant sample. Only spot No. 186 was present in control condition sample (4R) but its relative abundance had been decreased.

Inter class analysis of tolerant sample T3a with tolerant samples T1, seven days after rehydration and T3b, one day after rehydration indicated that relative abundance of seven and eight spots in T3a sample was more than 2-fold to T1 and T3b samples, respectively. It is necessary to mention that inter class analysis of tolerant sample T3b with tolerant sample T1, seven days after rehydration indicated that protein relative abundance of thirteen spots in T3b sample was more than 2-fold to T1 sample.

In total, after proteome analysis of studied plants, twenty protein spots with spot numbers 29, 30, 72, 91, 97, 107, 108, 112, 114, 132, 137, 143, 305, 311, 313, 345, 352, 366, 401, 405 and 466 were detected as candidate spots linked to drought tolerance. These spots had low or medium molecular weight and their protein relative abundance in different comparisons between samples had been proved more than 2 fold change and preferably was significant at 1% level. Sato and Yokoya [25] indicated that over relative abundance of a small heat shock protein, sHSP17.7 enhances tolerance to drought stress in transgenic rice plants. Salekdeh et al. [13] represented that from more than 2000 protein spots detected in leaf extracts by proteomics analysis, forty two proteins showed a significant change in abundance under stress, with twenty seven of them exhibiting a different response pattern in both cultivars (CT9993 and IR62266). Also, Rabello et al. [27] identified twenty two proteins putatively associated with drought tolerance. Ali and Komatsu [16] represented that a marked increase in the concentration of actin depolymerizing factor in drought-tolerant plants, suggesting its importance in drought stress. In two-week-old rice seedlings subjected to drought stress, abundance of ten proteins were increased in the leaf sheath and was found to be involved in defense, signal transduction, cell structure and energy metabolism. Muthurajan et al. [1] detected thirty one protein spots show significant differential protein relative abundance under drought. Of these, ten proteins were found to be newly induced by drought; five up-regulated and sixteen proteins identified to be significantly down-regulated. In leaf tissues of three contrasting cultivars, IR20, Nam Sa-Gui 19 and KDML105, subjected to drought stress, fifty three proteins showed significant difference. Proteins for cell and DNA repair and stomatal activity, were over expressed in NSG19, whereas proteins related to phytochrome A signaling, DNA repair, and superoxide dismutase (SOD) transcript were readily observed in KDML105 [28]. Ribulose-1, 5-Bisphosphate Carboxylase (RuBisCo) content is down-regulated in rice as a result of drought stress [16]. In addition to, proteomics analysis represented that Late Embryogenesis Abundant proteins (LEA proteins) genes in transgenic rice and wheat have been shown to confer tolerance to salt and drought stress or to increase efficiency in water usage and biomass productivity [29]. However, some protective mechanisms might be induced by drought stress, since the abundances of ATP synthase, Rubisco activase and peptidyl-prolylcis-trans isomerase were enhanced under the drought stress [30]. Studies of Ali and Komatsu [16], Mirzaei et al. [17] and Jagadish et al. [18] support results of this research.

Table 2. Calculation of needed protein amount of any sample for two-dimensional electrophoresis

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 (fourteen days after drought stress)	T3a	0.47	0.44	0.46	734.13	8809.50	8.81	13.62
TM2-230-4 (one day after rehydration)	T3b	0.36	0.33	0.34	536.77	6441.29	6.44	18.63
TM2-230-4 (seven days after rehydration)	T1	0.45	0.46	0.45	754.95	9059.35	9.06	13.25
B-TM2 (fourteen days after drought stress)	S1	0.43	0.41	0.42	669.75	8037.04	8.04	14.93
B-TM2 (one day after rehydration)	S4	0.36	0.39	0.38	596.06	7152.77	7.15	16.78
B-TM2 (seven days after rehydration)	S2	0.60	0.58	0.59	962.82	11553.78	11.55	10.39
Wild type (fourteen days after drought stress)	B0	0.34	0.31	0.33	513.91	6166.86	6.17	19.46
Wild type (one day after rehydration)	B1	0.42	0.48	0.45	720.57	8646.88	8.65	13.88
Wild type (seven days after rehydration)	B7	0.48	0.46	0.47	749.37	8992.45	8.99	13.34
230-4 (Prior to drought stress: control condition)	4R	0.27	0.26	0.27	411.42	4937.02	4.94	24.31

TM is abbreviation to Tarom Mutant. Number230 indicates 230 Gy mutant rice population

Table 3. Comparison and statistical analysis of selective protein spots linked to drought tolerance in seven days after rehydration and prior to drought stress samples using Melanie software

Match ID	Spot No.	Mean (100%)	Variation	M.S.D	Mean of protein Relative Abundance in T1 * sample	Mean of protein Relative Abundance in S2 sample	Mean of protein Relative Abundance in B7 sample	Mean of protein Relative Abundance in 4R sample	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	0.06	0.09	1.44	0.18	0.10	0.07	0	1.91	2.53	2.79	2.93	#DIV/0!	3.62
758	305	0.06	0.10	1.56	0.09	0.06	0	0.34	1.63	1.56	#DIV/0!	3.13	0.27	0.17
767	345	0.14	0.19	1.34	0.42	0.13	0.06	0.21	3.26	3.77	6.67	4.30	1.98	3.05
771	132	0.45	0.25	0.55	0.60	0.29	0.26	0.67	2.10	2.59	2.32	4.11	0.89	0.34
790	366	0.06	0.06	1.10	0.11	0.14	0.06	0	0.79	0.32	1.83	3.66	#DIV/0!	7.67
806	72	0.08	0.14	1.61	0.26	0.22	0	0	1.20	1.62	#DIV/0!	2.90	#DIV/0!	2.90
817	91	0.15	0.13	0.85	0.18	0	0.13	0.31	#DIV/0!	3.28	1.43	0.65	0.59	1.29
831	108	0.18	0.10	0.58	0.14	0.23	0.28	0.23	0.64	0.07	0.51	0.38	0.62	1.57
832	114	0.13	0.17	1.32	0.13	0.38	0	0	0.35	2.52	#DIV/0!	5.18	#DIV/0!	5.18
836	97	0.08	0.08	1.11	0.11	0.18	0.15	0.24	0.60	0.79	0.72	1.12	0.46	0.36
838	107	0.32	0.21	0.66	0.33	0.52	0	0.41	0.64	0	#DIV/0!	6.25	0.81	14.23
867	401	0.09	0.08	0.94	0.19	0.14	0.08	0	1.36	0	2.52	0	#DIV/0!	6.93
878	29	0.13	0.25	1.94	0.46	0	0.16	0	#DIV/0!	3.24	2.81	4.43	#DIV/0!	5.12
880	30	0.20	0.31	1.55	0.71	0.29	0	0	2.49	4.49	#DIV/0!	7.23	#DIV/0!	7.23
881	405	0.15	0.11	0.71	0.20	0.26	0.15	0.19	0.79	0.30	1.31	0.99	1.07	2.13
1130	313	0.03	0.05	1.50	0.11	0.08	0	0	1.42	2.55	#DIV/0!	5.40	#DIV/0!	5.40
1175	112	0.03	0.04	1.29	0.09	0.07	0	0	1.31	1.35	#DIV/0!	8.13	#DIV/0!	8.13
1176	137	0.08	0.10	1.19	0.21	0	0.12	0	#DIV/0!	3.62	1.80	1.60	#DIV/0!	3.62
1178	143	0.35	0.53	1.54	0.22	0.13	0.19	0.95	1.68	1.50	1.17	0.84	0.23	1.30
1257	352	0.03	0.04	1.60	0.09	0	0.03	0	#DIV/0!	5.04	2.65	3.72	#DIV/0!	5.04
1331	466	0.51	0.69	1.36	0.46	1.75	0.70	0	0.26	1.66	0.66	1.34	#DIV/0!	1

*T1, S2, B7 and 4R are equal to tolerant, sensitive, wild type (seven days after rehydration) and control (prior to drought stress) samples, respectively. Bold numbers indicate significance of differences ($p < 0.05$ or $p < 0.01$) between the spots. #DIV/0! represents protein relative abundance in one of the samples is 0

Table 4. Comparison and statistical analysis of selective protein spots linked to drought tolerance in *one day after rehydration samples using Melanie software*

Match ID	Spot No.	Mean (100%)	Variation	M.S.D	Mean of protein Relative Abundance in T3b* sample	Mean of protein Relative Abundance in 4S sample	Mean of protein Relative Abundance in B1 sample	T3b/S4 Ratio	Student t-test (T3b/S4)	T3b/B1 Ratio	Student t-test (T3b/B1)
1857	143	0.32	0.29	0.92	0.61	0.57	0	2.14	---	#DIV/0!	32.49
1895	108	0.06	0.10	1.73	0.24	0	0	#DIV/0!	482.57	#DIV/0!	482.57
1903	97	0.05	0.08	1.55	0.17	0	0	2.73	5.31	#DIV/0!	5.31
2246	466	0.4	0.50	1.26	0.53	1.06	0	#DIV/0!	50.31	#DIV/0!	50.31

*T3b, B1 and S4 are equal to tolerant, Wild type and sensitive (one day after rehydration) samples, respectively. Bold numbers indicate significance of differences ($p < 0.05$ or $p < 0.01$) between the spots. #DIV/0! represents protein relative abundance in one of the samples is 0

Table 5. Comparison and statistical analysis of selective protein spot linked to drought tolerance (No.405) in *tolerant samples in different times using Melanie software*

Match ID	Spot No.	Mean (100%)	Variation	M.S.D	Mean of protein Relative Abundance in T1* sample	Mean of protein Relative Abundance in T3a sample	Mean of protein Relative Abundance in T3b sample	T1/ T3a Ratio	Student t-test (T1/T3a)	T1/ T3b Ratio	Student t-test (T1/T3b)
881	405	0.1	0.09	0.84	0.2	0.07	0.12	2.96	6.29	1.69	2.68

*T1, T3a and T3b are equal to tolerant samples in seven days after rehydration, fourteen days after drought stress and one day after rehydration, respectively. Bold numbers indicate significance of differences ($p < 0.05$ or $p < 0.01$) between the spots

4. CONCLUSION

Fifteen drought tolerant mutants were identified on leaf rolling and spikelet fertility scales from M3 generation population of genotype "TaronMahalli" after two continuous years of drought stress. Proteome analysis of tolerant and sensitive mutant plants and wild type in three sampling stages (after drought stress, one day and seven days after rehydration) and control condition plant indicated that twenty protein spots with spot numbers 29, 30, 72, 91, 97, 107, 108, 112, 114, 132, 137, 143, 305, 311, 313, 345, 352, 366, 401, 405 and 466 are possibly candidate spots linked to drought tolerance. It is necessary to accomplish more investigations on these protein spots in order to determine responsible genes and mechanisms for drought tolerance in rice.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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