ven by CaMV*35S* promoter during *Agrobacterium* co-cultivation, whereas for particle bombardment the gene was driven by the monocot promoter *Ubi1*. The difference in the ploidy level of bread and emmer wheat did not appear to interfere with the transformation efficiency as both varieties exhibited similar transformation efficiency with microprojectile or *Agrobacterium*-mediated transformation approaches. Differences in transformation efficiencies between the two different varieties of *T. aestivum* obtained by either of the strategies are not significant. There are few reports describing the transformation of Indian wheat cultivars, some of which have been demonstrated to be highly regeneration-dependent[22,23]. Thus, high transformation frequencies obtained in the present study may be attributed to a well-standardized regeneration system for these species and varieties. The choice of a vegetative tissue is also advantageous and intentional due to its year-round, non-seasonal availability. The present work thus paves the way for introduction of other agriculturally desirable traits in commercially popular Indian wheat types.

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Proteome maps of flood-tolerant FR 13A and flood-sensitive IR 54 rice types depicting proteins associated with O_2-deprivation stress and recovery regimes

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Rice crop, particularly in lowland ecosystem, is severely affected by flooding stress. To genetically engineer rice with improved flooding tolerance, it is important to gain an understanding of the proteins that constitute its flooding-stress response. A major component of the flooding stress is a decline in availability of O_2. Proteomics approach is a powerful tool for analysing global changes in protein profiles. We have constructed proteome maps of flood-sensitive IR 54 and flood-tolerant FR 13A rice types corresponding to several time points during their response to O_2 deprivation stress. Several up- and down-regulated proteins have been identified by 1D and 2D protein gel electrophoresis followed by silver staining. Based on peptide sequence analysis, we indicate that sucrose synthase, glyceraldehyde 3-phosphate dehydrogenase, UDP-glucose-6-dehydrogenase and asparagine synthetase are implicated in rice flooding-stress response.

RICE is the most important food crop in the world. Nearly 25% of the world rice (i.e. 38 mha) cultivated in the lowland ecosystem accounts for only 17% of the global rice supply[1]. Amongst the South Asian countries, India has the largest area (i.e. 17.2 mha) under rainfed lowland. The

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dearth of information on flooding tolerance-related genes is considered to be the limiting factor in raising of flooding-tolerant rice[2–7].

The recent upsurge in structural genomics is leading to accumulation of a huge wealth of literature on nucleotide sequences[8]. The science of proteomics is a possible approach to link the nucleotide sequence information to functional attributes of the cell[6]. Three major steps in proteomics are the separation of complex protein mixtures by 1D and 2D protein gel electrophoresis, characterization of the partial amino acid sequence of the separated proteins and unveiling identities of the proteins by database search[6]. Sachs *et al.*[9] analysed proteins induced in response to anaerobic stress in maize primary roots and reported that a small number of anaerobic polypeptides (ANPs) accounts for more than 70% of total protein synthesis after 5 h of anaerobic stress. Further work established that the enzymes of glycolysis and ethanolic fermentation pathways mainly constitute ANPs[4,10,11]. Moons *et al.*[12] reported that complete submergence of rice seedlings for 60 h increased accumulation of a 97 kDa protein in roots. The peptides generated by *in situ* tryptic digestion of this protein revealed significant homology with plant pyruvate orthophosphate dikinase (PPDK). Chang *et al.*[13] analysed the patterns of protein synthesis during hypoxic and anoxic conditions in maize by employing 2D protein gel electrophoresis. In this study, expression of as many as 262 individual proteins was altered with changes in O_2 tension regime. Further, 46 protein spots could be identified on the basis of MS analysis followed by database search in the latter study, and the identified proteins showed a wide base of functions.

It is possible that all flooding stress up- or down-regulated proteins may not necessarily be related to flooding tolerance. There is need to distinguish the proteins that play a direct role in flooding tolerance from those that are indirectly involved. One possible approach for meeting this objective is to employ contrasting germplasm for study. Use of contrasting types for the analysis of stress responses has earlier been documented for salt stress in barley[14] and rice[15–17]. IR series rice cultivars have a high yield and possess several desirable traits[18]. FR 13A and FR 43B are excellent flooding-tolerant rice types[1]. These rice types have been employed in several basic physiological studies[19–21], but little molecular work has been carried out employing the above types. The present study was aimed at identifying proteins that are specifically altered in flood-tolerant FR 13A (which is a selection from a local land race) and flood-sensitive IR 54 rice types (which has been generated through three-way cross method involving Nam Sagui/IR2071-88//IR2061-214-3-6-20, at the International Rice Research Institute, Philippines) during submergence stress and during recovery from stress.

Seedlings of IR 54 and FR 13A rice types (*Oryza sativa* L.) were raised, subjected to O_2-deprivation stress and transferred to aerobic condition for recovery, as described earlier[11]. For the O_2-deprivation treatment, 4-day-old germinated seedlings were transferred to an air-tight, water-filled container to which highly purified (approximately 99% pure) N_2 gas (obtained from SMS Multitech Ltd, New Delhi) was delivered through a tubing to simulate an O_2-deprivation atmosphere. The O_2-deprivation stress treatments were carried out at 26°C in dark to minimize any interference from photosynthetically-produced O_2. For 1D and 2D protein gel electrophoresis, samples were homogenized in liquid N_2 to a fine powder. Buffer-soluble proteins were extracted and analysed by 1.5 mm thick, vertical SDS-gels (Hoefer, USA), according to Pareek *et al.*[22] for 1D analysis. For 2D analysis, proteins were extracted essentially according to the protocol of Suzuki *et al.*[23]. Partially-purified protein samples were dissolved in the lysis buffer [9.5 M urea, 4% (v/v) NP-40, 5%(v/v) 2-mercaptoethanol and 2% (v/v) ampholines (pH 3.5–10 : 5–8 : 8–10.5 :: 2 : 8 : 1)]. Amount of protein in different extracts was estimated[22]. 2D analysis was performed according to Hames and Rickwood[24]. Silver staining of 2D gels was carried out as described by Pareek *et al.*[22]. Protein microsequencing analysis was carried out by the procedure described by Matsudaira[25]. The microsequencing analysis was carried out using an automated microsequencer (Perkin–Elmer Precise Protein Microsequencer). The sequence analysis and homology search were carried out using computer-aided Blast program[26,27]. The obtained sequences were compared with the sequences present in the SwissProt database.

FR 13A and IR 54 rice types showed comparable percentage germination under control conditions. When subjected to O_2-deprivation stress of 48 h (Figure 1 *d*), IR 54 roots showed stunted growth compared to FR 13A, which appeared to have a healthy growth with normal development of root hairs. When transferred to control conditions for 72 h (Figure 1 *e*), development of the first leaf in FR 13A was more pronounced compared to IR 54 seedlings. In FR 13A, there was more pronounced development of secondary roots and root hairs compared to IR 54 seedlings. O_2-deprivation treatment of 72 h (Figure 1 *f*) proved to be lethal for IR 54 rice seedlings. FR 13A seedlings on the other hand, showed relatively less chlorosis. When subjected to a recovery period of 72 h (Figure 1 *g*), fewer seedlings of IR 54 could recover compared to FR 13A in which case most of the seedlings appeared to have overcome the injury caused by the stress treatment.

Shoot and root samples of IR 54 and FR 13A seedlings harvested at variable intervals of O_2-deprivation stress were analysed for the protein profiles by 1D analysis. The major polypeptide alterations (both up- and down-regulated) noted in this analysis are shown in Figure 2. It is possible that differences in protein profiles between uninduced control and stressed samples could partly be due to growth of the seedlings (since this experiment continued from day-4 of the seedlings' age up to day-8). The

protein changes that are associated with the development of the seedlings in this time period were taken into consideration while documenting stress-induced protein alterations. In a separate experiment, IR 54 and FR 13A seedlings were initially subjected to O_2-deprivation stress for 24 and 48 h and then returned to control conditions for recovery (6 and 24 h). The major polypeptide alterations (both up- and down-regulated) noted in this analysis are shown in Figure 3. In order to analyse the steady-state polypeptide changes caused by O_2-deprivation stress in greater detail, 2D technique was employed. IR 54 and FR 13A rice shoot and root tissues were subjected to O_2-deprivation treatments for 6, 24, 48 and 72 h in this experiment. The 2D electrophorograms of the shoot samples are shown in Figure 4. The numerals marked on different protein spots in these electrophorograms represent proteins that showed marked alterations with respect to a sample of the contrasting rice type at the corresponding stage. In all, five polypeptides were selected for the amino acid sequence determination. Among these, three polypeptides were excised from the 2D gels representing 6 h O_2-deprivation stress-induced sample of FR 13A rice (Figure 5, spot numbers 1, 2 and 3; these proteins accumulated at higher levels in FR 13A than in IR 54). Two additional polypeptides were excised from 1D gels – one was down-accumulated (75 kDa polypeptide in Figure 2) and the other was up-regulated (92 kDa polypeptide in Figure 2) in response to O_2-deprivation stress. The possible homology of the amino acid sequences of these proteins is shown in Table 1.

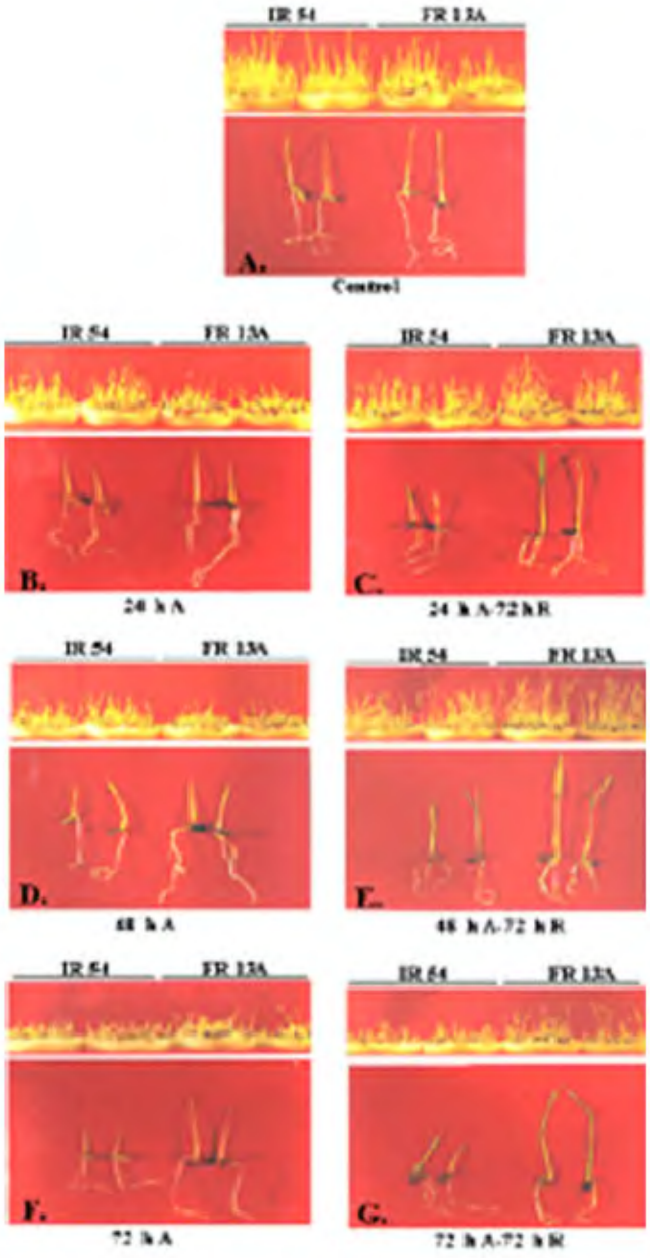

Figure 1. Growth pattern of IR 54 and FR 13A rice seedlings subjected to various durations of O_2-deprivation stress followed by recovery under control (non-anoxic) conditions. Petri plates reflecting the overall effect along with individual seedlings showing the effects of O_2-deprivation stress and recovery are shown in each panel. ***a***, Four-day-old seedlings grown under normal (control) conditions; ***b***, 24 h of O_2-deprivation stress; ***c***, 24 h of O_2-deprivation stress followed by 72 h recovery in control conditions; ***d***, 48 h of O_2-deprivation stress; ***e***, 48 h of O_2-deprivation stress followed by 72 h recovery in control conditions; ***f***, 72 h of O_2-deprivation stress; ***g***, 72 h of O_2-deprivation stress followed by 72 h of recovery in non-anoxic conditions. A, O_2-deprivation stress; R, Recovery in the control conditions following O_2-deprivation stress.

The superior ability of FR 13A rice to survive and grow in flooded conditions has been noted under diverse experimental conditions[21,28–30]. The present work (Figure 1) is in concurrence with the above findings that FR 13A seedlings survive O_2-deprivation stress with better ability than IR 54 rice. The proteome analysis undertaken in this study was carried out with the overall objective of exploiting science of proteomics for gaining detailed understanding of the flooding response. The resolution of 2D gels was found to be extremely high and a large number of polypeptides (up to 1000 in the silver-stained gels and 600 in the Coomassie-stained gels) were observed with high resolution and almost negligible background. The analysis of the protein profiles was carried out by comparing photographic prints as well as by using the computer-aided image analysis with the help of a CCD camera[6]. A large number of protein alterations were noted in the course of this work (Figures 2–5). Several proteins (as represented by 112, 106 and 100 kDa in Figure 2) were below the level of detection in uninduced aerobic samples, but showed accumulation with the imposition of O_2-deprivation stress. These proteins showed a near-regular pattern of accumulation with the continuation of O_2-deprivation stress conditions. However, the accumulation profiles of a large number of proteins were dependent on the specific duration or level of the stress (98 and 81 kDa polypeptides, Figure 2 *a*; 92, 81, 65, 64, 53 and 50 kDa polypeptides, Figure 2 *b*). Most of the cellular proteins showed similar pattern of alterations in the shoot and root tissues of IR 54 and FR 13A seedlings (Figure 2). The 75 kDa

polypeptide showed pronounced decline in level with the onset of stress treatment. This protein was seen in uninduced control aerobic samples in both root and shoot tissues, but was not detected in all the stressed samples of IR 54 and FR 13A rice types. The 36, 35, 28 and 23 kDa proteins showed higher accumulation in IR 54 compared to FR 13A shoots (Figure 3). On the other hand, 39 kDa protein showed higher accumulation in the post-stress recovery period in FR 13A shoot samples. It is notable that transcripts of several respiratory pathway genes (such as those encoding phophofructokinase, glyceraldehydes 3-phosphate dehydrogenase, pyruvate kinase and pyruvate decarboxylase) show higher accumulation in IR 54 cultivar as against FR 13A rice during post-stress recov-

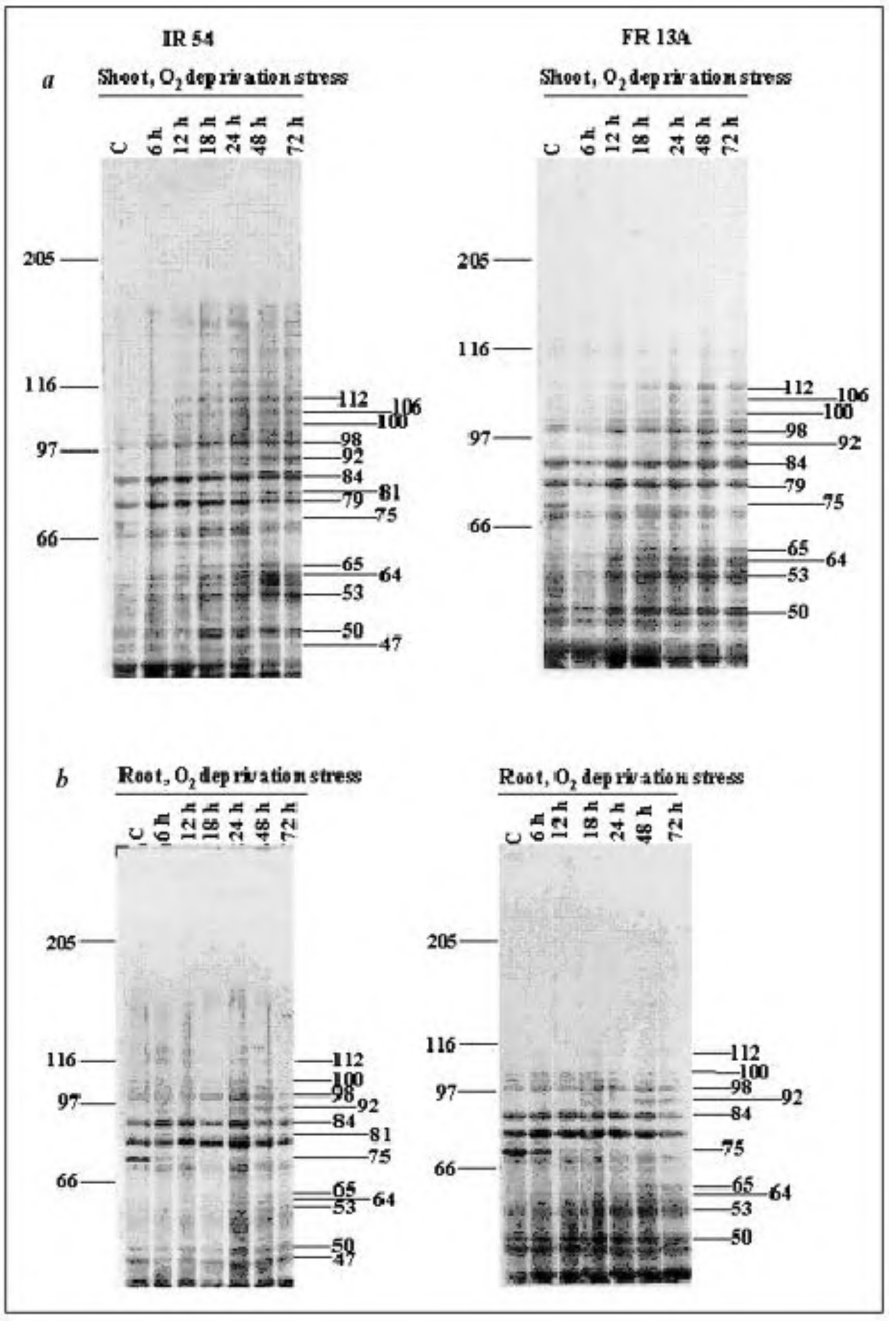

Figure 2. Electrophoretic profiles of proteins of shoot tissue of IR 54 and FR 13A rice seedlings as resolved on 7.5% uniform SDS-gel in response to 0–72 h O_2-deprivation stress. Equal amount of protein was loaded in each of the lanes. Gels were stained with silver nitrate. Numbers shown on the right side of each panel denote the molecular weights (in kDa) of the matching polypeptides. Duration of stress treatment is shown on the top of each lane. Positions of the standard molecular weight markers (kDa) are shown on the left side of each panel. C, Uninduced control.

ery period[11]. On the other hand, alcohol dehydrogenase encoding transcript shows higher accumulation in FR 13A rice compared to IR 54 under similar conditions of analysis[11]. Thus, it is possible that the differential protein alterations noted in this study reflect the polypeptides corresponding to these or other such transcripts. The analysis of cellular proteins by 2D protein gel electrophoresis method showed that the response of rice cells to O_2-deprivation stress is highly complex and the number of protein alterations scored by 1D analysis is a far underestimate. This analysis also revealed that a large number of proteins show growth stage-dependent, stress-induced alteration pattern in both IR 54 and FR 13A seedlings (Figure 4). From the number of polypeptides marked in 2D gels, it becomes amply clear that there is need to combine visual observations with computer-aided approach using requisite software to read 2D gels more effectively. Through the latter approach, it should be possible to (1) quickly read large number of gels, (2) identify minor variations in protein spots that are difficult to be apprehended by the naked eye, and (3) eliminate any probable bias due to human interventions. This needs more sophisticated laboratory set-up and is worth pursuing in future years.

From the range of polypeptides that were found to be altered in response to O_2-deprivation stress by 1D and 2D

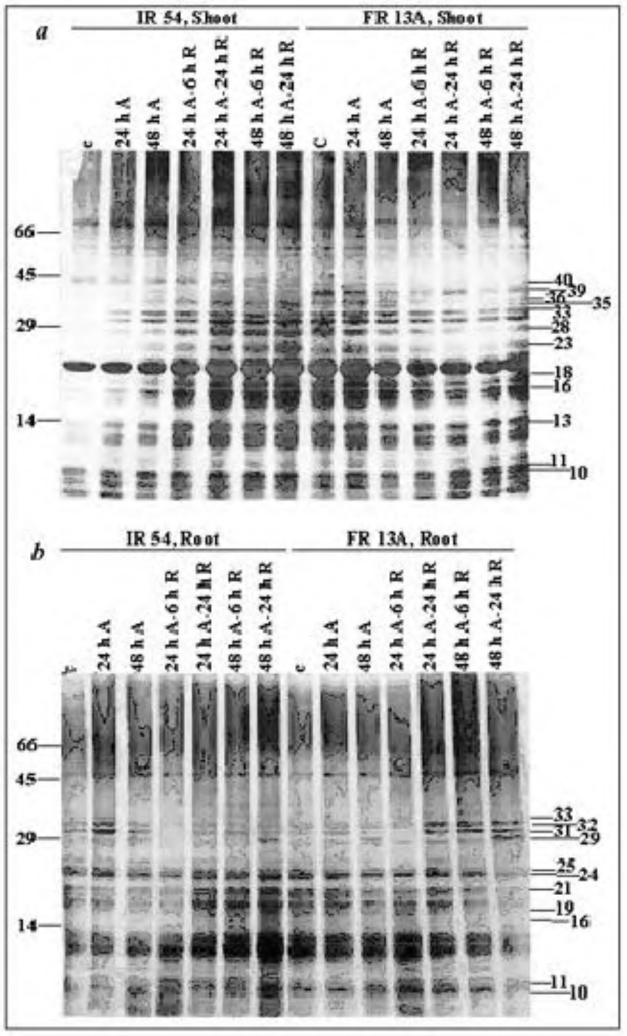

Figure 3. Electrophoretic profiles of proteins of shoot tissues of IR 54 and FR 13A rice seedlings as resolved on the 5–20% linear acrylamide gradient SDS-gel in response to 24 and 48 h O_2-deprivation stress (A) followed by recovery (R) of 6 and 24 h. Equal amount of protein was loaded in each lane. Gels were stained with silver nitrate. Numbers shown on the right side of each panel denote the molecular weights (kDa) of the matching polypeptides. Duration of stress and recovery treatment is shown on the top of each lane. Positions of the standard molecular weight markers (kDa) are shown on the left side of each panel. C, Uninduced control.

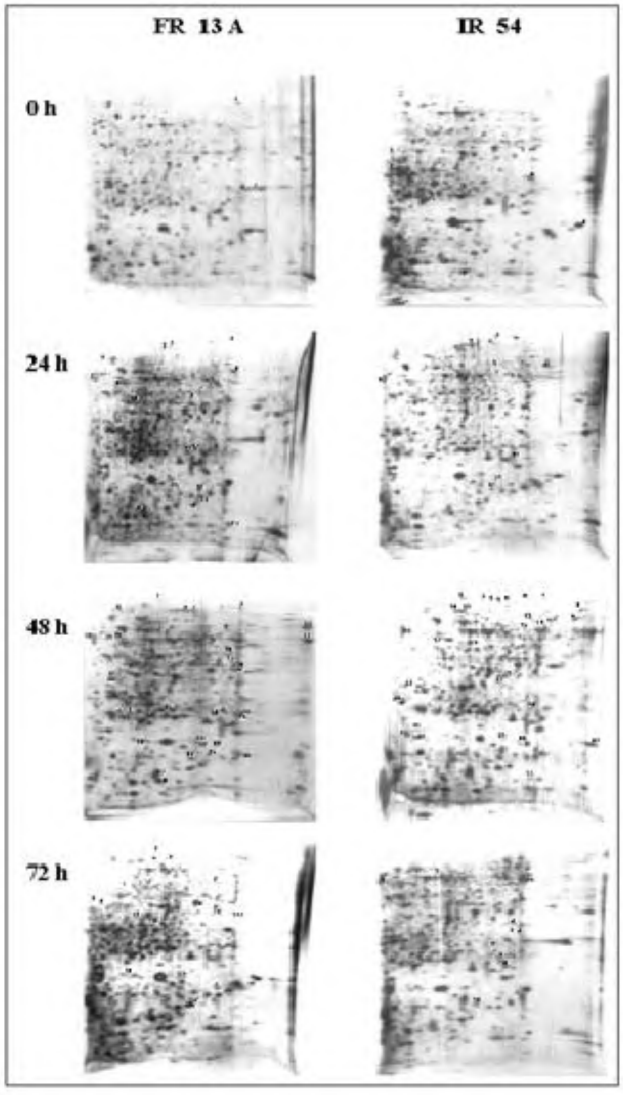

Figure 4. 2D analysis proteins from shoot tissues of IR 54 and FR 13A rice types in response to O_2-deprivation stress. Soluble proteins were first resolved by isoelectric focusing (1–5 mm) rod gels followed by separation on 12.5% (1.5 mm) uniform concentration SDS-gel. Next,100 µg of total soluble protein fraction was loaded for analysis. Gels were stained using silver nitrate. Panels show computer-aided CCD-image analysis. Prominent variations are scored in the numerals. Duration of stress treatment (h) is indicated.

methods, five polypeptides were analysed for the amino acid sequence. Among these, three were excised from 2D gels representing O_2-deprivation stressed shoot samples of FR 13A rice (Figure 5). These three polypeptides accumulated at higher levels in FR 13A than IR 54 rice. Further, two polypeptides were excised from ID gels. One of these

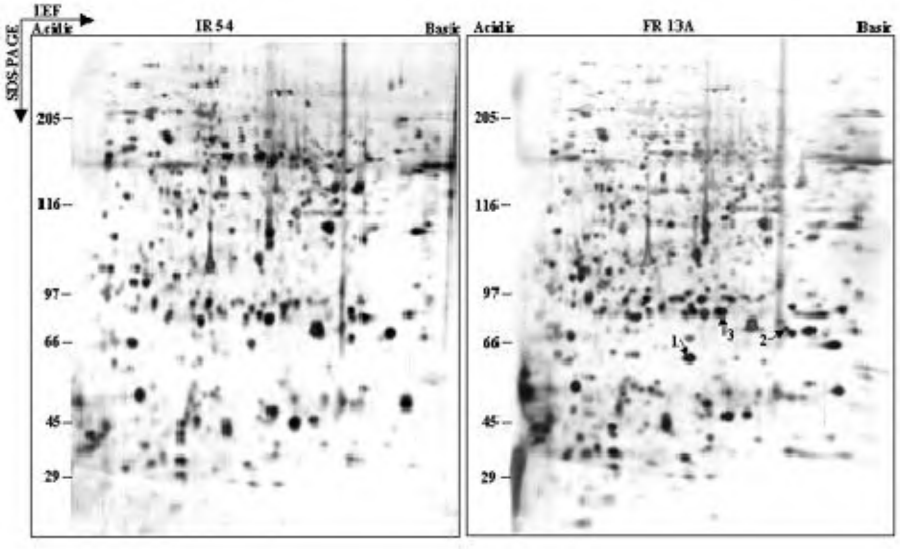

Figure 5. 2D analysis of proteins from shoot tissues of IR 54 and FR 13A rice types in response to O_2-deprivation stress. Samples corresponding to 6 h treatment are shown. Other details are the same as in Figure 4. Prominent polypeptide variations in FR 13A scored by numerals 1, 2 and 3 with arrows indicate the polypeptides excised for amino acid microsequencing analysis.

Table 1. Salient findings on the amino acid microsequencing analysis of protein spot number. In column alignment with the database sequence, identical amino acid is shown by dot (.) and non-identical amino acid is shown by respective symbol

Comment on the spot	Amino acid sequence of the polypeptide	Sequence producing significant alignment	Identity (%)	Alignment with database sequence
1. 2D spot, FR 13A, 6 h shoot, O_2-deprivation stress	AVMWLCKCGW	Membrane protein, nosy precursor	60	AVMWLCKCGW . . L . . . LLA .
		Bovine amiloride-sensitive sodium channel alpha-subunit	66	AVMWLCKCG . . L . . . TF .
		Human amiloride-sensitive sodium channel alpha-subunit	66	AVMWLCKCG . . L . . . TF .
2. 2D spot, FR 13A, 6 h shoot, O_2-deprivation stress	WCHHYHFACV	Sucrose synthase 3, *Oryza* sp.	83	HYHFAC . . . . S .
		Sucrose synthase 2, *Oryza* sp.	83	HYHFAC . . . . S .
		Sucrose synthase 2, *Hordeum vulgare*	83	HYHFAC . . . . S .
3. 2D spot, FR 13A, 6 h shoot, O_2-deprivation stress	SDNYPWVKDG	Salty transcriptional regulator	66	DNYPWVKDG N . T . . . A . .
		Glyceraldehyde 3-phosphate dehydrogenase (larval)	75	NYPWVKDG . I . . D . . .
		Cellulase (endo-1, 4-beta-glucanase) precursor	50	SDNYPWVKDG NS . . . APP .
4. 75 kDa protein, FR 13A, 6 h shoot,O_2-deprivation stress	WPTPGKK	Ribulose bisphosphate carboxylase small chain precursor(small subunit)	85	WPTPGKK . . . T . . .
		UDP-glucose 6-dehydrogenase	71	WPTPGKK . . . . . SR
5. 92 kDa protein, FR 13A, 6 h shoot, O_2-deprivation stress	AKGFGPAI	*Oryza* sp., asparagine synthetase	83	GFGPAI L
		Oryza sp., lipoxygenase, chloroplast precursor	100	GFGP . . . .
		Oryza sp., ATP synthase, β-chain	50	AKGFGPAI S . . . Y . AV

(75 kDa protein in Figure 2) was down-regulated by the stress treatment in shoot and root tissues of both IR 54 and FR 13A rice types. The other protein (92 kDa protein in Figure 2) was up-regulated in response to the application of stress. The peptide microsequencing data showed that none of these proteins has a perfect match with any known protein in the literature (Table 1). BLAST search results showed proteins that have partial homology to the amino acid sequences of the test proteins. The amino acid sequence of spot number 2 showed homology to sucrose synthase protein. O_2-deprivation stress induced increase in sucrose synthase enzyme activity and transcript levels have already been shown[31]. It may thus be inferred that the identified protein represents a novel sucrose synthase form. Likewise, spot number 3 showed homology to a specific form of glyceraldehyde 3-phosphate dehydrogenase. The down-regulated 75 kDa polypeptide showed homology to UDP-glucose-6-dehydrogenase, while the up-regulated 92 kDa protein showed homology to rice asparagine synthetase. Based on the results obtained, it is suggested that the above enzymes are implicated in flooding response of rice. However, it is important that as far as possible, extended amino acid sequences must be read to reduce errors accruing through matching of shorter pockets of sequences in the database search. Additionally, it is important that all candidate proteins identified through this approach must further be tested through requisite experiments (such as through mutant- or transgenic-based methods).

The protein alterations scored in the present work can be looked at with a viewpoint that both the induced and repressed proteins may be important in cellular adaptation to O_2-deprivation stress. The induced proteins may be the result of increased transcription, while those repressed may be due to decreased transcription or activated degradation[6]. The identities of the altered stress proteins in terms of (1) what role they play in metabolism, and (2) whether they represent altogether novel proteins, can possibly be worked out by obtaining information on the amino acid sequences of the short peptides of these proteins. It is amply clear that the understanding of flooding tolerance response in rice is far from being complete.

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