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PROTEOMICS APPROACH TO IDENTIFY DIFFERENTIALLY EXPRESSED PROTEINS INDUCED BY IRON DEFICIENCY IN ROOTS OF *MALUS*

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Abstract

Malus xiaojinensis (*Malus xiaojinensis Cheng et. Jiang*) is a Fe-efficient apple. We report for the first time a systematic proteomic approach to iron stress-responsive proteins in roots of *Malus*. Plantlets of *Malus* were cultured in hydroponic system without Fe element for 3 d. After then, total proteins were extracted from roots of the treated plantlets and separated by two-dimensional gel electrophoresis (2-DE). More than 700 protein spots were reproducibly detected. The matrix-assisted laser desorption/ionisation-time of flight and peptide mass fingerprinting (PMF) analysis and database searching helped us identify 12 up-regulated protein spots representing 10 different proteins. Two spots were putative pyridoxine biosynthesis protein isoform B, 2 were identified as s-adenosylmethionine synthetase (SAMS) and other 8 as fructokinase (FK), isocitrate dehydrogenase (NADP), monodehydroascorbate reductase (MADR), AP003246 NID, actin, Hypothetical protein, probable catechol O-methyltransferase (COMT) and vacuolar H^+-ATPase (V-ATPase) catalytic subunit, respectively. These proteins were involved in regulation of carbohydrate and energy metabolism, stress-defense response, and membrane transport. Something relevant to Fe deficiency stress was discussed.

Introduction

Iron is one of the essential nutrients for plant growth and development. Although iron is present in large quantities in different types of soils, its low-soluble form as oxihydrates make it difficult for plants to uptake and use. Subsequently, plants show chlorosis due to Fe deficiency, which frequently results in poor productivity in agriculture.

Screening of iron-efficient crop plant appears to be the best and most economical means to combat Fe-deficient chlorosis (Han *et al*., 1994). *Malus* is an iron-efficient apple genotype selected by our laboratory. It responded to Fe deficiency by inducing a series of physiological and morphological changes in roots, such as the formation of root hairs and transformation cells, acidification of the rhizosphere and enhanced root ferric chelate reductase (FRO) activity. And then more research works have been done on some genes, as *FRO*, iron transport protein and *Myb et al.*, related to Fe-deficiency reactions and cloned from *Malus* by our group. But the specific mechanism by which *Malus* acquires resistance to Fe deficiency environment has as yet not been clear.

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In response to environmental stress, plants resort to various pro-survival strategies, most of which are preceded by specific changes in proteins whose biological functions are related to such environmental stress tolerance (Ndimba *et al*., 2005). Studies of protein expression involved in Fe deficiency may provide a detailed network of the Fe deficiency adaptation mechanism in *Malus*. Recent technical improvements in two-dimensional electrophoresis (2-DE) and Mass spectrometry (MS) make it possible to identify proteins involved in the network rapidly. Proteomics is believed to be useful for screening for proteins expressed under specific conditions such as following light, heat, cold, or hormone exposure (Marques *et al*., 2001).

In the present study, we deployed comparative proteome analysis using 2-DE to investigate the effects in the roots of *Malus* proteome of deprivation of the nutrition Fe. Results showed a comprehensive picture of the post-transcriptional response to Fe deficiency, which would be expected to serve as a basic platform for further characterization of gene function and regulation. Out of more than 700 spots visualized, we detected a number of iron deficiency responsive proteins. Up-regulated proteins identified and related with by Fe-deficiency in the present study was discussed. To our knowledge, this is the first report of a proteome analysis of *Malus*.

Materials and Methods

Plant materials and plant culture: Plantlets of *Malus*, grown *In vitro* with roots, were trans-cultured in 300 ml plastic bottles (4 plantlets per bottle) containing a complete nutrient solution (Han *et al*., 1994) and grown under light conditions as previously described by Han *et al*., (1994) for 4 weeks. Subsequently, the roots of the plantlets were washed with distilled water for 10 minutes and then the plants were grown either in a complete nutrient solution with 40 μ*M* FeNa-EDTA (control) or in nutrient solution without iron supply (stress). The pH of the nutrition solution was adjusted to 6.0 with KOH and renewed every day. The treatment of plant materials was repeated three times.

Protein preparation and 2-D electrophoresis: Primary white roots were removed from the controlled plants and treated plants 3 d after the treatments respectively. The samples were immediately frozen in liquid nitrogen and then stored at –80^{o}C until usage for protein extraction according to the method of Damerval *et al*., (1986). Briefly, samples were grounded in liquid nitrogen and suspended in 10% trichloroacetic acid (TCA) in acetone with 0.07% (w/v) β-mercaptoethanol at -20^{o}C for 1 h, followed by centrifugation for 20 minutes at 10,000 *g*. The pellets were resuspended in cold acetone with 0.07% (w/v) β-mercaptoethanol at -20^{o}C for 1 h and centrifuged at 4^{o}C for 20 minutes at 10,000 *g*. This step was repeated six times and then air-dried with vacuum pump. Proteins were then solubilized in lysis buffer containing 7 *M* urea, 2 *M* thiourea, 4% (w/v) CHAPS, 0.5% (v/v) CA (pH 3-10), 50 m*M* DTT and 1 m*M* PMSF. Protein quantity was analyzed according to Bradford's assay (Bradford, 1976). Three hundreds *ug* of total root proteins were loaded on a single IPG gel strip (170 mm, pH 5-8, Bio-Rad). IPG strips were actively re-hydrated at low voltage 50 V at 20^{o}C for 12 h. IEF was conducted by using Bio-Rad system. IEF conditions were as follows: 250 V for 15 minutes, linear ramping, followed by 10000 V for 5 h, linear ramping, 60000 VH, rapid ramping, 500 V for 2 h.

The second dimension was performed on a 12% polyacrylamide SDS gel using a PROTEAN plus Dodeca cell (Bio-Rad, Hercules, USA). High molecular mass standard marker (14-200 kDa) were used and appeared on the acid side of the special silver stained

gel. Silver stain was carried out according to a method of Shevchenko *et al*., (1996). Two-DE was repeated three times with independent protein samples and the representative gels were used.

Image and data analysis: The silver stained gels were scanned using a flatbed scanner. Image treatment, spot detection, and quantification were done using the PDQuest 6.2 software (Bio-Rad). The molecular masses of proteins on gels were determined by co-electrophoresis of standard protein marker. And pI of the proteins were determined by migration of the protein spots on 17 cm IPG strips. Spots were determined to be significantly up or down regulation when the relative abundance of spots increased or decreased at least twofold.

MALDI-TOF MS analysis: Protein spots of interest were manually excised from silver stained gels. The de-straining and trypsin in-gel digestion of the selected protein spots were carried out according to a method of Shevchenko *et al*., (1996). MS analysis was conducted by MALDI-TOF mass spectrometers (Autoflex Ⅱ, Brucker-Daltonics). Calibration was carried out using a standard peptide mixture. Spectra were obtained in a reflectron-delayed extraction mode over a mass range of 700-4000 Da.

Database query: The peptide mass fingerprint (PMF) obtained from each digested protein spot was analyzed using Mascot software (Matrix Science, London, UK) for protein identification. The PMF was matched against all green plant (*Viridiplantae*) entries in the MSDB and NCBInr database. The following parameters were set for searches: enzyme, trypsin; fixed modifications, carbamidomethyl cysteine; variable modifications, oxidised methionine and Pyro-glu (N-term Q); peptide mass tolerance, 0.01%; the parameters of missed cleavage, one missed cleavage site. Identifications were considered as the positive identifications when the following criteria were fulfilled: a significant Mascot score; at least five peptides matched and sequence coverage at least 20%.

Result and Discussion

The proteomic approach: To counteract iron deficiency, plants may change their gene expression and protein accumulation. Root is the first organ of plants to sense iron deficiency. Some iron deficiency-responsive genes were found to be induced mainly, or much stronger, in roots than in other organs. Therefore, in the present study, we also used roots of *Malus* to investigate iron deficiency-responsive proteins.

In order to better understand the processes involved in the adaptation to Fe-deficient stress and identify genes that are crucial to plant improvement for Fe-limited environments, the vast array of RNAs with altered expression levels provides numerous possibilities (Thimm *et al*., 2001). Without further work, it is difficult to identify promising candidate genes (Gygi *et al*., 1999). By the proteomic approach, focus could be centered on changes in actual protein levels, thus limiting the number of genes that initially must be considered as possible targets involved in Fe-induced changes (Hajheidari *et al*., 2005).

In the present study, a combination of 2-DE, trypsin in gel digestion, peptide mass fingerprint and database search techniques, proved to be a useful strategy in studies of Fe-efficient associated proteins in *Malus*. We were able to identify 10 different proteins using the cross-species database on the base of homology or conservation.

2-D gel and analysis of proteins altered under stress: Previous efforts to optimize the protocols showed that 2-D gels performed in a narrow linear range of pI 5-8 allowed a higher resolution of the total proteins of *Malus* species, containing more than 80% of the proteins visualized under a pI range 3-10 (data not shown).

More than 700 protein spots were reproducibly detected on silver-stained gels (Fig. 1). Of these, 36 protein spots showed significant up-regulated changes in the iron deficiency stressed roots. Out of them, 17 spots revealed peptide fingerprint peaks. There were still a few down-regulated proteins spots but producing no good spectra. Changes in proteins under the stress conditions were shown in Fig. 2. Spots that showed consistent positions on different gels were considered to be the same proteins. Standard marker proteins also were found at the same positions on different gels.

Mass spectrometry analysis and database searching helped us identify 12 spots representing 10 different proteins. Among the 12 identified proteins, 6 were characterized as enzymes and 6 as of stress and defense responsive proteins or functional unknown (Table 1). Of these, SAMS, NADP, FK and H^+-ATPase catalytic subunit were previously reported (Li *et al.*, 2008). And putative pyridoxine biosynthesis protein isoform B, probable COMT, MADR, AP003246 NID and actin were revealed to be new iron deficiency responsive proteins.

Proteins involved in primary metabolism: Under iron deficiency the activities of enzymes in roots were affected and the basic metabolisms were disturbed. Our proteomic analysis indicated that response of roots of *Malus* to iron deficiency involved the expression of various enzymes which were involved in the energy metabolism. Spot 3, 4, 5and 7 were identified as the NAD-dependent isocitrate dehydrogenase, FK and SAMS. All of the three proteins showed increased expression under iron-deficiency stress (Fig. 2, Table 1).

In order to maintain homeostasis under stress conditions, plants require an extra energy supply to fortify the resistance mechanisms. An expression analysis of genes in glycolysis, the tricarboxylic acid cycle revealed an induction of several enzymes, indicating an increase in respiration in response to Fe deficiency (Thimm *et al*., 2001). NAD-dependent isocitrate dehydrogenase (NAD-IDH, EC 1.1.1.42) was a critical enzyme of the tricarboxylic acid cycle that converts malate and NAD into oxaloacetate and NADH (Chen & Gddal, 1999).

FK is an enzyme involved in sucrose to hexose-phosphate conversion. The increasing activity of FK has been identified in juvenile cluster roots (Massonneau *et al*., 2001). Suppression of *Frk2*, one of the cDNA clones encoding FK, inhibited root growth (Odanaka *et al*., 2002). FK may play a role in regulation of energy metabolism, possibly by providing fructose-6-phosphate for glycolysis or through conversion to UDP-glucose (UDPG) to support biosynthesis of cell wall material (Karni & Aarni, 2002). The increased abundance of FK suggested an absolute necessity for the formation of root hairs and transformation cells induced by Fe-deficient stress.

SAMS catalyzed the conversion of ATP and L-methionine into SAM. Recent results confirmed that SAMS was involved in tolerance to abiotic stresses such as wounding (Kim *et al*., 1994), salinity stress (Sanchez-Aguayo *et al*., 2004), water stress (Chang *et al*., 1996), drought stress (Mayne *et al*., 1996) and heat shock (Sule *et al*., 2004). Loss of resistance of the Jubilant cultivar to abiotic stress factors could be partially due to the absence of this protein (Sule *et al*., 2004). Under Fe deficiency stress the up-regulated protein spots (spot 5 and 7) identified as SAMS have also been detected in our study. SAM was a substrate of Nicotianamine synthase for nicotianamine(NA) biosynthesis. SAMS was the important enzyme to biosynthesize NA with SAM. NA played a key role in Fe metabolism and homeostasis in all higher plants (Higuchi *et al*., 2001).

Table 1. Putative identities of proteins by peptide mass fingerprint query.

Spot no.	Accession no.	Matched proteins	% cov	Matched peptides	Mascot score	Experim-ental Mr/pI	Theoreti-cal Mr/pI	Matched species
	Primary metabolism							
3	S28423	isocitrate dehydrogenase	13	5	72	42/7.1	46567/ 6.19	alfalfa
4	Q42896_LYCES	Fructokinase	25	11	72	35/5.9	34969/ 5.76	*Lycopersicon esculentum*
5	gi|10443981	S-adenosylmethion-ine synthetase	40	13	155	42/6	43309 /5.37	*Brassica juncea*
7	AAB40094	SLU60481 NID	41	8	82	47/6.2	37265/ 5.28	*Lycopersicon esculentum*
	Stress response							
8	T06189	probable catechol O-methyltransferase	27	6	62	36/7	41343/ 5.66	barley
9	Q6QND3_TOBAC	Putative pyridoxine biosynthesis protein isoform B	25	10	105	33/6.8	33068/ 5.93	*Nicotiana tabacum*
10	Q6QND3_TOBAC	Putative pyridoxine biosynthesis protein isoform B	30	10	117	33/6.3	33353/ 5.93	*Nicotiana tabacum*
11	BAB93255	AP003246 NID	34	5	60	32/6.2	28817/ 5.31	*Oryza sativa*
12	JU0182	Monodehydroascor-bate reductase	20	8	62	30/6	47501/5.29	*Cucumis sativus*
	Membrane transport							
1	gi|60592632	vacuolar H^+-ATPase catalytic subunit	37	19	168	70/6.2	68 999 / 5.43	*Pyrus communis*
	Cytoskeleton-related protein							
2	Q7XZI9_GOSHI	Actin	45	14	117	48/5.5	41936/ 5.37	*Gossypium hirsutum*
	Unknown protein							
6	Q8LCS4_ARATH	Hypothetical protein	20	5	69	43/6.3	38635/ 9.4	*Arabidopsis thaliana*

Column 1: spot numbers from the analyses shown in Figure 2; column 2: database accession number for the homologue; column 3: putative identity of the protein based on the peptide mass fingerprint query; column 4: sequence coverage percentage; column 5: Number of the matched peptides; column 6: Mascot similarity score; column 7: *Mr* and p*I* of the polypeptide estimated from the gel analysis; column 8: *Mr* and theoretical p*I* of the identified protein; column 9: plant species of the matched protein.

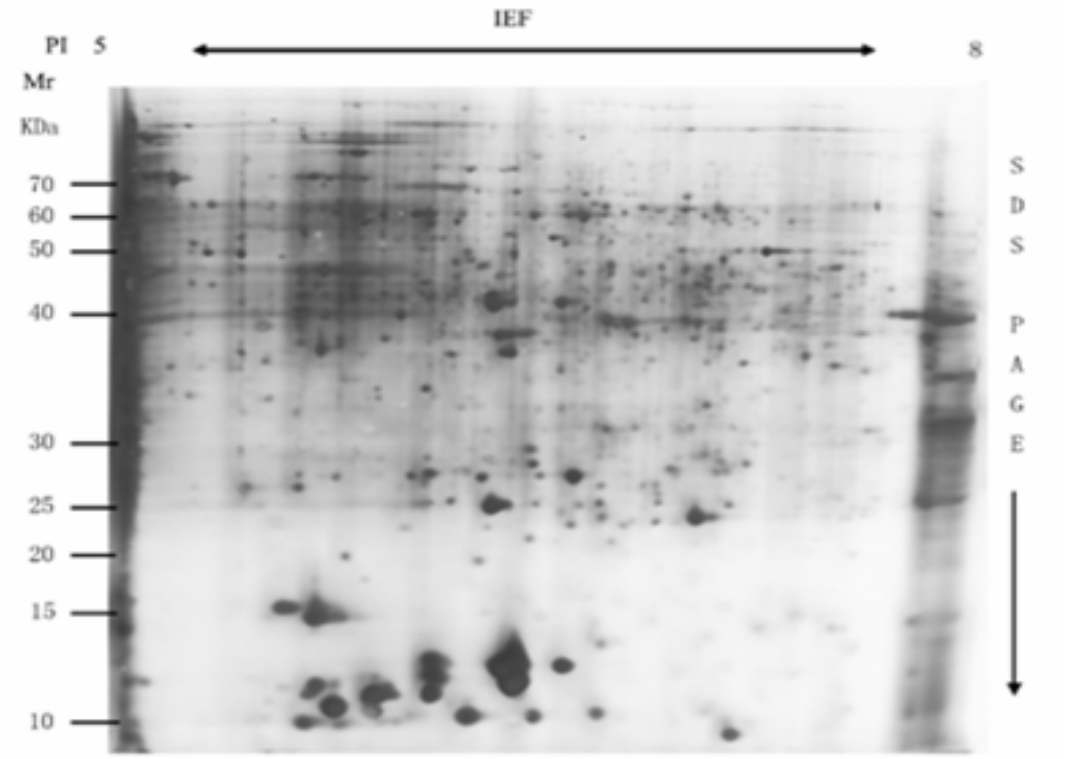

Fig. 1. 2-D gel analysis of proteins extracted from roots of *Malus* harvested under iron deficient condition for 3 d. In the first dimension, 300 *ug* of protein was loaded on a 17 cm IPG strip with a nonlinear gradient of pH 5-8. In the second dimension, 12% SDS-PAGE gels were used. Proteins were visualized by silver staining.

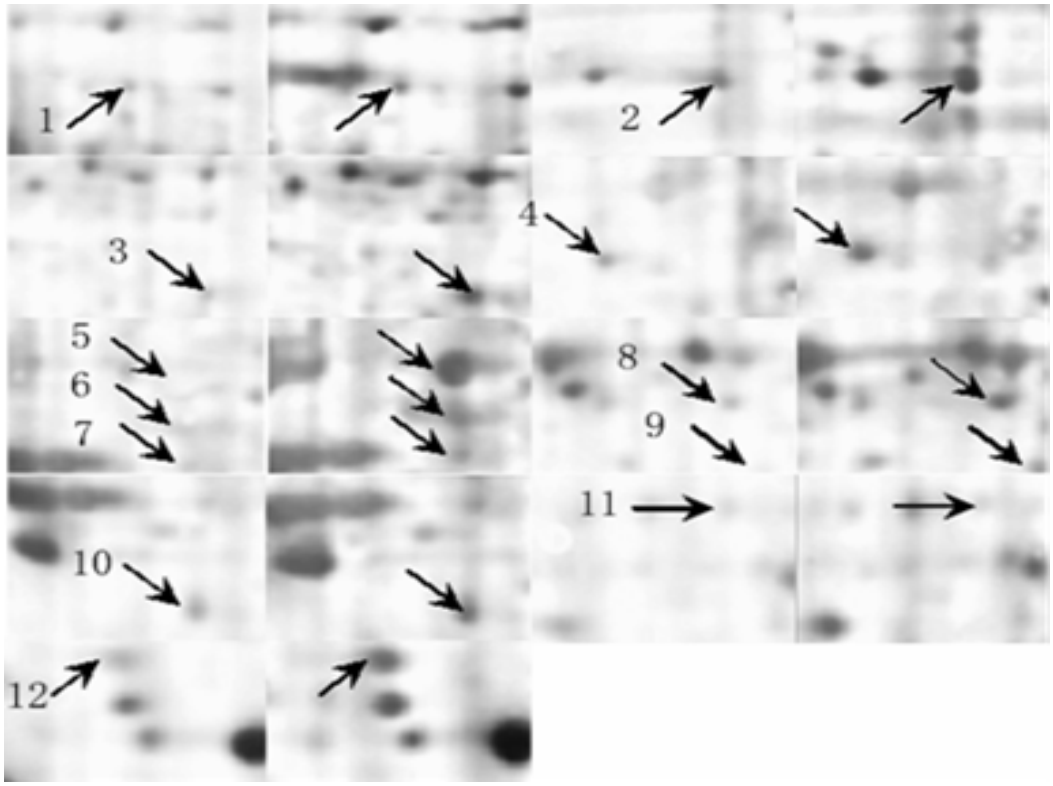

Fig. 2. Examples of up-regulated changes in protein abundance in roots of *Malus* under iron deficiency conditions. Arrows: polypeptides significantly increased in the gels obtained from treated plants compared to those from obtained from control plants.

Proteins involved in stress and defense response: Plants responded to stress conditions by expression of specific proteins. In our work, many of the spots showing up-regulated profile were matched to proteins related to defense-related reactions. Thus, proteomic analysis allowed us to identify 3 enzymes related to stress response from roots of *Malus* (Table 1). This is the first report of these enzymes involved in the defensive reactions to iron deficiency stress.

MS analysis identified spot 8 as COMT, which plays an important role in the synthesis of lignin (Morrow *et al*., 1997). COMT utilizes SAM to transmethylate caffeic acid, which results in the production of ferulic acid. Coniferyl alcohol (as well as sinapyl alcohol) is ultimately generated from the ferulic acid. This combination of coniferyl, ferulic and sinapyl alcohols in the presence of free radicals results in the production of lignin (Morrow *et al*., 1997). The average number of lignified cells in vascular bundles was significantly great in plants under salt stress (Sanchez-Aguayo *et al*., 2004). And the methyl-lignin accumulation was also observed in some epidermal cells of the submergence-tolerant rice (Kang *et al*., 2004). The relation of COMT to Fe deficiency stress has not been reported before.

One protein (spot 9 and 10), putative pyridoxine biosynthesis protein isoform B, that was up-regulated in the iron deficiency-stressed root, was known to participate in the pyridoxine biosynthesis. Pyridoxine (vitamin B6) was an important cofactor in amino acid biosynthesis and involved in many protein biological reactions. The work of Bilski *et al*., (2000) and Ehrenshaft *et al*., (1999) showed a clear role for pyridoxine as a quencher of singlet oxygen and the superoxide radical in fungi. Pyridoxine has recently been added to a list of potent antioxidants found in plants (Wang *et al*., 2004). Shi *et al*. (2002) have shown a link between the production of pyridoxal-5-phosphate and salt tolerance. But there have been no reports of increased pyridoxine as a result of stress conditions. The increased abundance of the pyridoxine biosynthesis protein may show an increased pyridoxine in the iron deficiency stressed root. There were results which suggested that the major action of pyridoxine in plants may be through its role as a cofactor for numerous enzymes rather than through its direct effect on active oxygen species (AOS) (Graham *et al*., 2004).

Spot 12 was identified as MDAR, up-regulated in the iron deficiency stressed root. It could reduce monodehydroascorbate to ascorbic acid. And ascorbic acid could be oxidated to monodehydroascorbate by ascorbate peroxidase. Because monodehydroascorbate was unstable, it could be reduced to ascorbic acid again by MDAR. Thus MDAR was critical for regeneration of ascorbic acid (Smirnoff, 1996, Smirnoff & Wheeler, 2000). A high abundance of the MDAR may be a result of increasing need for ascorbic acid by roots under the iron deficiency. Ascorbic acid, well known for its function as an antioxidant, probably provided protection to the developing roots against oxidative stress that occurred during the period of iron deficiency. Efficient regeneration of ascorbic acid may be helpful to eliminate the AOS induced by the iron deficiency stress.

Spot 11 was identified as AP003246 NID (Pir7b protein) from rice. The Pir7b is one of the accumulation transcripts whose increase is an observable aspect of defense response (Waspi *et al*., 1998).

Proteins involved in membrane transport: Spot 1 matched to known protein involved in membrane transport was highly expressed in roots of *Malus* under Fe-deficiency stress. V-ATPases played a critical role in maintaining the pH of endomembrane compartments

in eukaryotic cells (Strompen *et al*., 2005). V-ATPases, which generated a H^+ electro-chemical gradient across the tonoplast membrane (Bageshwar *et al*., 2005), were also involved in plants' defense against environmental stress (Magnotta & Gogarten, 2002). The proton-translocating plant V-ATPase was of prime importance for acidification of intracellular compartments, which was known to energize ion and metabolite transport (Padmanaban *et al*., 2004) and essential for processes such as secondary activated transport, maintenance of ion homeostasis, and adaptation to environmental stress (Seidel *et al*., 2004).

The response in intact Fe-deficient roots was localized to root hairs, which developed on secondary roots during the period of Fe deficiency (Buckhout *et al*., 1989). New root developments depended on the cellur acidification. The increased expression of V-ATPase may help acidify the celluar environment which was necessary for the cell elongation and new roots developing.

Conclusions

Results from the present study indicated that specific proteins enhanced in certain region of *Malus xiaojinensis* 2-DE image showed a coordinated response to Fe stress. By using a proteomic approach, a series of proteins, showing differential changes in profiles during the course of iron stress, were identified. Some of them were related to carbon metabolism and photosynthesis, thus indicating adaptive changes in physiology. We suggest that genes functioning in many physiological events coordinate the response to iron deficiency. The effects of prolonged Fe deficiency stress on the proteome of roots of *Malus xiaojinensis* is under investigation.

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