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Comparative proteome analysis in hot pepper (*Capsicum annuum* L.) after space flight

Análisis comparativo de proteomas en ají picante (*Capsicum annuum* L.) después de viajes en el espacio

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Abstract. Hot pepper (*Capsicum annuum* L.) is an important crop all over the world. To explore and identify differentially expressed proteins of hot pepper after space flight, three space-induced mutants (Y1, Y2 and Y3), which obtained new traits after space flight compared with their control lines (W1 and W2), were analyzed using comparative proteome analysis. In this study, leaf morphological characteristics of five kinds of hot pepper variations were evaluated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Results showed that obvious changes of cellular structure were seen in space induced mutants. Thirty nine out of the 255 differentially expressed proteins were successfully sequenced. Among these 39 proteins, 31 were homologous with known proteins, and the others were set as a hypothetical protein. All identified proteins were further classified into six groups including protein metabolism, energy metabolism and photosynthesis. In conclusion, these findings will benefit for the research of mutagenic mechanisms at the level of proteomics, and provide an effective way for new hot pepper breeding.

Keywords: *Capsicum annuum* L.; Proteomics; Space flight.

Resumen. El ají picante (*Capsicum annuum* L.) es un cultivo importante en todo el mundo. Tres mutantes inducidos por el espacio (Y1, Y2 e Y3), que obtuvieron nuevas características después de un vuelo espacial comparado con sus líneas control (W1 y W2), fueron analizados usando análisis de proteomas comparativo con el fin de explorar e identificar proteínas de ají picante expresadas diferencialmente después de un vuelo espacial. En este estudio, se evaluaron características morfológicas foliares de cinco clases de variaciones de ají picante utilizando microscopía electrónica de barrido (SEM) y microscopía electrónica de transmisión (TEM). Los resultados mostraron cambios obvios de estructura celular en los mutantes inducidos en el espacio. Treinta y nueve de 255 proteínas expresadas diferencialmente se secuenciaron exitosamente. Entre las 39 proteínas, 31 fueron homólogas a proteínas conocidas, y las otras se consideraron como una proteína hipotética. Todas las proteínas identificadas fueron además clasificadas en seis grupos incluyendo el metabolismo de proteínas y de la energía, y la fotosíntesis. En conclusión, estos hallazgos se beneficiarán por la investigación de mecanismos mutagénicos a nivel de proteomas, y proveerán una forma efectiva para nuevos cruzamientos en ají picante.

Palabras clave: *Capsicum annuum* L.; Proteomas; Vuelo espacial.

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INTRODUCTION

Hot pepper (*Capsicum annuum* L.) is an important crop on a global basis due to its sensory attributes including color, spiciness and flavor (Perucka & Oleszek, 2000). Generally, hot pepper fruit tastes spicy, because it contains capsaicin, which could increase appetite. In addition, hot pepper is mostly reported to have nutritional values based on the presence of individual phenolic acids, carotenoids and vitamin C, which are antioxidant constituents with the highest content among vegetables (Materska & Perucka, 2005).

To date, space mutation breeding is a good breeding technique and has shown a significant role in creating specific mutation genetic resources and cultivated varieties. Some researchers have obtained great progress in tomato, hot pepper and rice by space mutagenesis technology. Accordingly, those new varieties have been obtained and used for agricultural production (Liu et al., 2007; Yang & Guo, 2008). The influence of space environment on plant depends on the genetic plant background, flight time and altitude of space. Different studies have exhibited various test results. For example, Li et al. (2000) showed that the content of peroxidase in space-induced mutant leaf was higher than that it the control. However, Xu et al. (1997) found a decreased peroxidase activity in space-induced asparagus, suggesting that different biological responses might exist in different crops after space flight.

Gene expression results are embodied in the level of protein. Therefore, it is not fully revealed the exact gene function information just at the level of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA). Recently, the research on proteomics has grown by leaps and bounds (Jorr et al., 2015; Miernyk, 2014). Especially, the application of mass spectrometry technology provides a new method to better understand the regulatory mechanism of protein synthesis. It can analyze and identify gene function by comparing the differences between mutant or recombinant and wild types using comparative proteomics, providing the information of physiological and biochemical processes after inducement. For example, Zhang (2008) analyzed the gliadin and high molecular weight glutenin subunits (HMW-GS) content in space-induced wheat, and summarized subunit protein expression with varying degrees variation, finally affecting the quality of flour. Lv et al. (2009) and Zhang and Lv (2009) also reported similar information on agronomic traits and HMW-GS in wheat mutant.

In this work, we firstly analyzed the differentially expressed proteins in mutants and controls by protein two-dimensional electrophoresis (2-DE) technology, and detected variations at the level of proteomics to provide the theoretical basis for hot pepper space mutation breeding and mutagenic mechanism.

MATERIALS AND METHODS

Plant materials. Five hot pepper (*Capsicum annuum* L.) accessions, including three space-induced mutants (Yujiao1, Yujiao2 and Yujiao3) and two on-ground controls (Longjiao 1 and Longjiao 2), were collected from the Heilongjiang forest botanical garden, China (Fig.1). Y1 (Yujiao 1) and Y2 (Yujiao 2) were mutated from W1 (Longjiao 1); Y3 (Yujiao 3) was induced from W2 (Longjiao 5). More detailed agronomic characters of the five hot pepper varieties were shown in our previous study (Xie et al., 2014).

Observation of hot pepper variety leaves by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). To characterize the morphological changes in the leaves from species subjected to space induction, SEM and TEM were performed according to Meng et al. (2014). Fresh leaf sections (about 1 cm in length and 1 cm in width) of each accession were fixed in 3% glutaraldehyde (v/v) at 4 °C for 2 h, then washed twice in 0.1 M phosphate buffer (pH

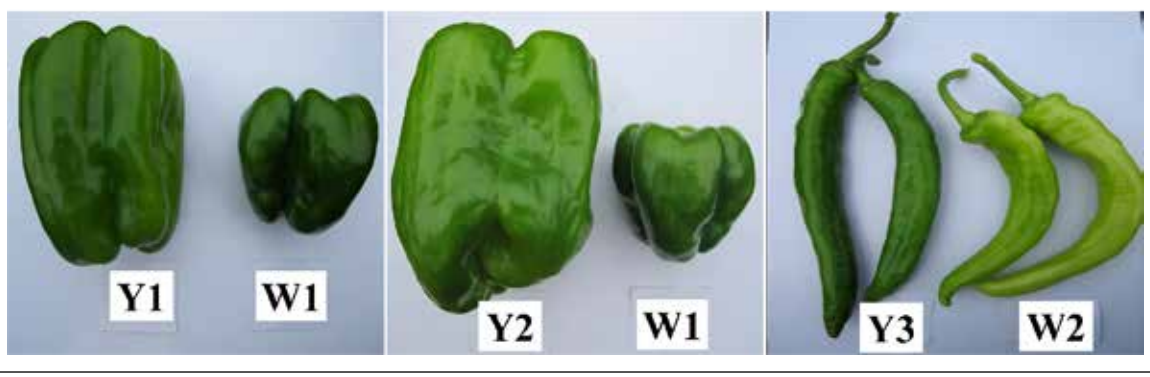

Fig. 1. The comparison of hot pepper mutants and their wild types. Y1, Yujiao 1; Y2, Yujiao 2; W1, Longjiao 2; Y3, Yujiao 3; W2, Longjiao 5. Ys are the mutants and Ws are the wild types.

Fig. 1. Comparación de los mutantes de ají picante y sus tipos silvestres. Y1, Yujiao 1; Y2, Yujiao 2; W1, Longjiao 2; Y3, Yujiao 3; W2, Longjiao 5. Ys son los mutantes y Ws los tipos silvestres.

6.8). After that, they were sequentially dehydrated in ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 10 min every time, and 100% ethanol was repeated twice (Meng et al., 2014). After dehydrating, they were further dried and pasted with copper, and then observed and photographed by scanning electron microscopy (JSM-5310LV, Japan).

Fresh leaf segments (1 cm × 1 cm) were fixed in 2.5% glutaraldehyde (v/v) at 4 °C for 2 h, then washed twice in 0.1 M PBS (sodium phosphate buffer, pH 6.8). Then, they were sequentially dehydrated in 50%, 70%, 90% and 100% acetone for 10 min every time, and embedded in Epon 812 for 2 h (Meng et al., 2014). Ultra-thin sections (70 nm thickness) were sliced, stained with uranyl acetate and lead citrate, and mounted on copper grids for TEM using transmission electron microscopy (H-7650, Hitachi, Tokyo, Japan).

Protein extraction and two-dimensional polyacrylamide gel electrophoresis. Fresh leaves (0.5 g) were collected for protein extraction. Total leaf protein was extracted using the trichloroacetic acid (TCA)/acetone precipitation method according to Wan and Liu (2008). Protein concentration was determined using Bradford reagents (Sigma, China) and bovine serum albumin as a standard.

Approximately 1000 μg of protein was loaded onto an immobilized pH gradient (IPG) strip holder (GE Healthcare, USA) with a 13-cm linear pH 4 to 7 gradient. The strip was actively hydrated at room temperature for 12 h. Then it was placed into an IPGphor apparatus for first dimension under the following conditions: 30 V for 2 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 0.5 h, and 8000 V for 6 h. The isoelectric focusing gel solution consisted of 8 M urea, 3.5% polyacrylamide, 2% Nonidet P-40, 2% Biolyte (pH 3.0-10.0 and pH 5.0-8.0), ammonium peroxodisulfate and tetramethylethylenediamine. After separation, SDS-PAGE in the second dimension was carried out using 15% polyacrylamide gel with 5% stacking gel. The electrophoresis was performed at 150 V for 30 min and then 220 V until the dye front reached the bottom of the gel using an Ettan DALT System (GE Healthcare, USA). The gels were stained with Coomassie brilliant blue (CBB), and image analysis was performed according to Wang et al. (2009). Each 2-DE separation was done three times for each accession. All reagents were purchased from Sigma, China.

Image acquisition and sequencing. A total of 15 CBB-stained 2-DE gels were analyzed using the ImageMaster 2-D Platinum Trial software (version 5.0, GE Healthcare) and the detection parameters were set according to the manufacturer's instructions (GE Healthcare). Protein spot detection, intensity measurement, background subtraction and spot matching were performed specifically after CBB staining of the gels using PDQuest software (version 7.1, Bio-Rad, USA). A criterion of $P<0.5$ or $P>2$ was used for defining significant differences. With this criterion, protein spots were selected and excised manually for protein identification. The isoelectric point (*pI*) and molecular weight (*Mr*) of each protein were determined using 2-DE SDS-PAGE standards. Following separation, proteins were collected in sterilized centrifuge tube and sequenced by Haihong Company (Beijing, China).

Data analysis. The obtained amino acid sequences were compared with those of known proteins in the Swiss-Prot, PIR, GenPept and PDB databases. To minimize false positives, a BLAST search at the NCBI was performed to confirm all matches. The identified proteins were then were divided into groups for functional classification using Gene Ontology (GO) terms (Conesa and Gotz, 2008).

RESULTS

Morphological changes of hot pepper leaves to space induction. In our study, space induction substantially changed the morphological patterns of hot pepper leaves (Fig. 2). Leaf morphological characteristics of five hot pepper species were evaluated, including stomatal dimensions and density. Stomatal dimensions (length and width) were measured in the upper and lower epidermis of mature leaves in all accessions, and three kinds of mutant lines showed significantly higher stomatal dimensions than those on-ground controls (Fig. 2). In addition, stomatal density in each leaf surface was higher in mutants than in the controls

The ultrastructural changes of hot pepper leaves were also observed using transmission electron microscopy (TEM) (Fig. 3). We observed that the shape changes occurred in the main cellular structures of the mitochondrion, chloroplast and vacuole of the leaves. At the cellular level, cells in control plants contained smaller vacuoles and less chloroplasts, while cells in mutants showed a loose arrangement with a thin cytoplasm, more vacuoles, smaller and more chloroplasts, and an irregular spongy tissue. Furthermore, at the organelles level, some evident changes were observed in mutants after space induction. They included swollen chloroplasts, accumulated plastoglobules and starch granules, and disordered thylakoids (Fig. 3). These results confirmed that changes on cellular structures were obvious in space induced mutants compared with their controls.

Protein expression profiling in hot pepper leaves. To identify the variation of space-induced hot pepper mutant at the protein level, we performed comparative proteomics in the mature leaves of mutants and on-ground controls. More than 1500 protein spots were detected with good reproducibility in the 2-DE gels; representative gels are shown in Figure 4. Protein spots with good reproducibility, and fold-changes in intensity < 0.5 or > 2 (in comparison with the corresponding control values) were further characterized. Compared with the corresponding control lines, protein spots 91, 74, and 90 were

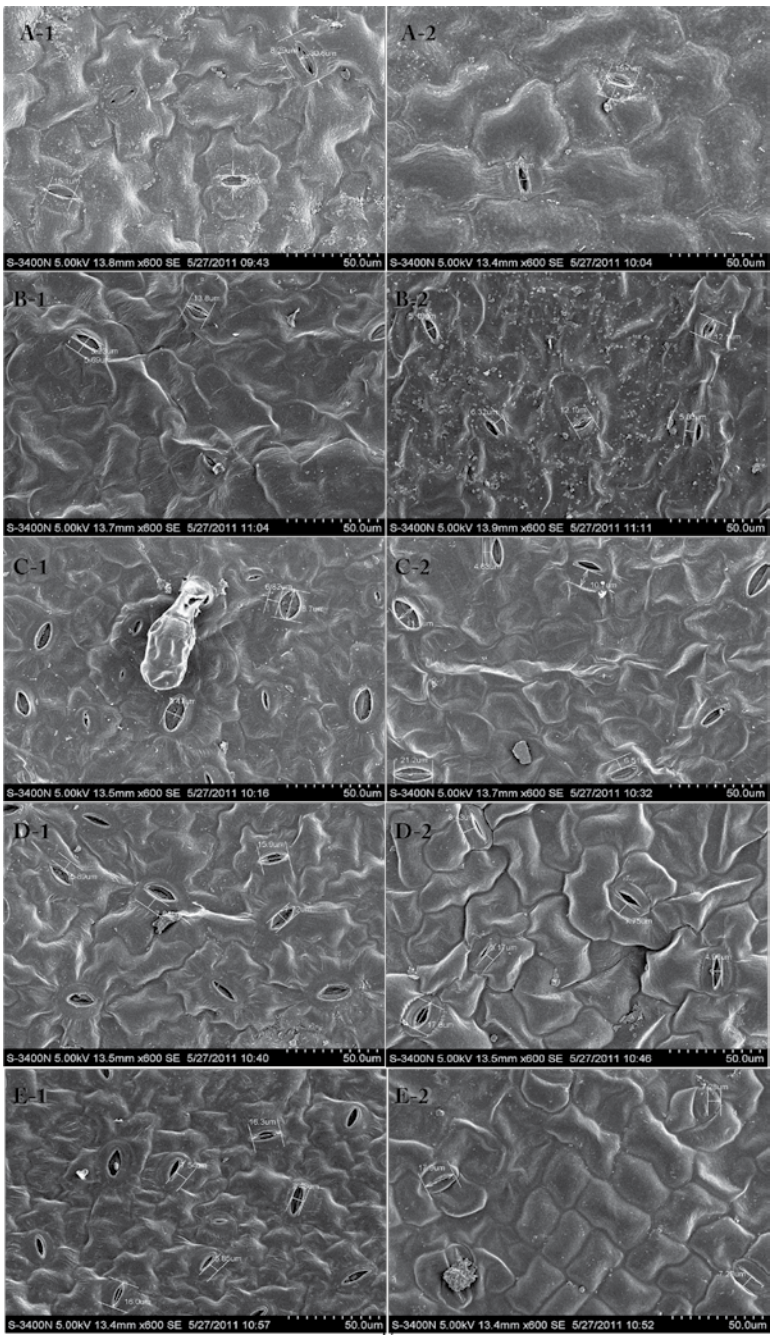

Fig. 2. Morphological changes on hot pepper leaves in five species by scanning electron microscopy. The detailed information of mutants or accessions was shown in Fig. 1. A-1, A-2: the upper and lower epidermis of mature leaves in the Y1 mutant, respectively; B-1, B-2: the upper and lower epidermis of mature leaves in the Y2 mutant, respectively; C-1, C-2: the upper and lower epidermis of mature leaves in the Y3 mutant, respectively; D-1, D-2: the upper and lower epidermis of mature leaves in the W1 accession, respectively; E-1, E-2: the upper and lower epidermis of mature leaves in the W2 accession, respectively.

Fig. 2. Cambios morfológicos en hojas de ají picante en cinco especies por microscopía electrónica de barrido. La información detallada de los mutantes o variedades se mostró en la Fig. 1. A-1, A-2: las epidermis superior e inferior de hojas maduras en el mutante Y1, respectivamente; B-1, B-2: las epidermis superior e inferior de hojas maduras en el mutante Y2, respectivamente; C-1, C-2: las epidermis superior e inferior de hojas maduras en el mutante Y3, respectivamente; D-1, D-2 las epidermis superior e inferior de hojas maduras en el cultivar W1, respectivamente; E-1, E-2: las epidermis superior e inferior de hojas maduras en el cultivar W2, respectivamente.

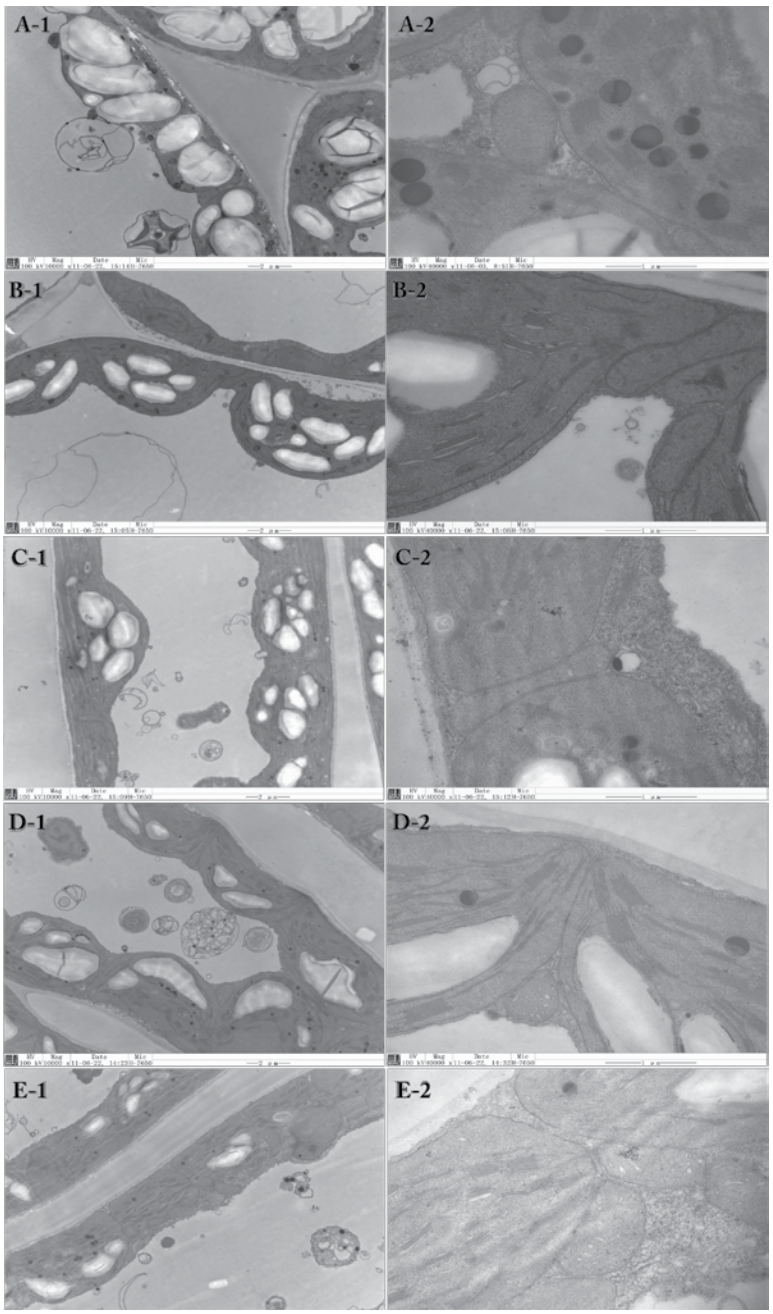

Fig. 3. Morphological changes on hot pepper leaves in five species by transmission electron microscopy. The detailed information of mutants or accessions was shown in Fig. 1. A-1, A-2: the upper and lower epidermis of mature leaves in the Y1 mutant, respectively; B-1, B-2: the upper and lower epidermis of mature leaves in the Y2 mutant, respectively; C-1, C-2: the upper and lower epidermis of mature leaves in the Y3 mutant, respectively; D-1, D-2: the upper and lower epidermis of mature leaves in the W1 accession, respectively; E-1, E-2: the upper and lower epidermis of mature leaves in the W2 accession, respectively.

Fig. 3. Cambios morfológicos en hojas de ají picante en cinco especies por microscopía electrónica de barrido. La información detallada de los mutantes o variedades se mostró en la Fig. 1. A-1, A-2: las epidermis superior e inferior de hojas maduras en el mutante Y1, respectivamente; B-1, B-2: las epidermis superior e inferior de hojas maduras en el mutante Y2, respectivamente; C-1, C-2: las epidermis superior e inferior de hojas maduras en el mutante Y3, respectivamente; D-1, D-2 las epidermis superior e inferior de hojas maduras en el cultivar W1, respectivamente; E-1, E-2: las epidermis superior e inferior de hojas maduras en el cultivar W2, respectivamente.

differentially expressed proteins on leaves of Y1, Y2 and Y3, respectively. Among them, 18, 11 and 30 proteins were visibly up-regulated, and 4, 1 and 6 proteins were down-regulated after space induction. Therefore, the changes of protein expression in mutants demonstrated that most of these proteins were significantly up-regulated.

Of the 255 differentially expressed proteins in the 2-DE gels, we selected 39 successfully sequenced proteins with significant difference. Among these proteins, 31 were able to assign a known function, and the others were set as hypothetical proteins (Table 1). The majority of the identified proteins (19; 50% or so) showed a strong sequence homology to the *Capsicum* proteins, followed by *Vitis vinifera* proteins, and the *Nicotiana tabacum* proteins. This indicated that hot pepper has a close relationship with *Vitis vinifera*, which is reflected in the sequence homologies of proteins in these two species.

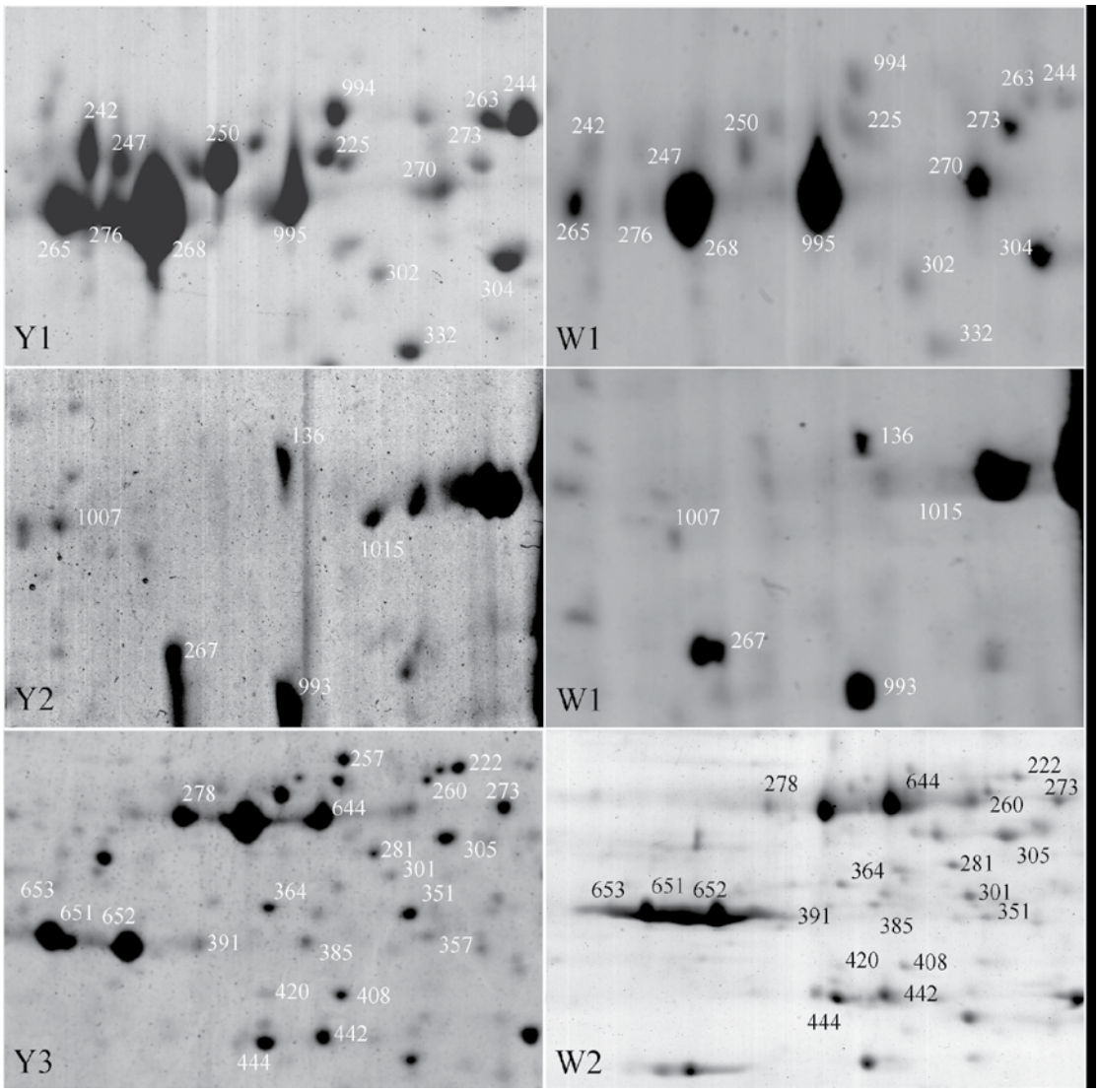

Fig. 4. Representative 2-DE image of hot pepper leaf proteome. Total proteins were separated by 2-DE and stained with CBB. The differentially expressed proteins were marked with numbers on the 2-DE gels. Y1, Y2 and Y3 represent hot pepper mutants after space flight. W1 and W2 represent control samples. More detailed information about mutants and controls could be found in materials and Fig. 1. Thirty-nine identified protein spot numbers were listed in Table 1.

Fig. 4. Imagen 2-DE representativa del proteoma foliar de ají picante. Las proteínas totales fueron separadas por 2-DE y coloreadas con CBB. Las proteínas expresadas diferencialmente fueron marcadas con números en los geles 2-DE. Y1, Y2 y Y3 representan mutantes de ají picante después del vuelo espacial. W1 y W2 representan las muestras control. Información más detallada acerca de los mutantes y controles se puede encontrar en materiales y en la Fig. 1. Treinta y nueve números de agrupaciones proteicas identificadas se mencionaron en la Tabla 1.

Table 1. List of differentially expressed protein spots in hot pepper leaves after space induction.
Tabla 1. Lista de agrupaciones proteicas expresadas diferencialmente en hojas de ají picante después de la inducción espacial.

Spot no.	NCBI no.	Function category protein name	Plant species	pI	Mr	Protein score	Sequence coverage	Up/ down
188	82623425	enolase-like	*Solanum tuberosum*	5.96	48.52	516	15%	↑
222	1346523	S-adenosylmethionine synthase 1	*Capsicum annuum*	5.47	43.19	616	23%	↓
244	193290730	S-adenosylmethionine synthetase	*Capsicum annuum*	5.66	43.06	723	31%	↑
250	1169931	Glutamine synthetase	*Capsicum annuum*	5.24	39.62	108	15%	↑
251	193290696	glutamine synthetase 2	*Capsicum annuum*	6.48	47.93	726	23%	↑
257	218312	chloroplast elongation factor TuB (EF-TuB)	*Nicotiana sylvris*	5.70	46.79	415	13%	↑
264	225449432	hypothetical protein	*Vitis vinifera*	5.50	48.21	232	8%	↑
265	226503019	malate dehydrogenase, cytoplasmic	*Zea mays*	5.76	35.85	70	4%	↑
270	12230332	Nucleoside diphosphate kinase	*Capsicum annuum*	6.31	16.37	488	41%	↑
273	147782894	hypothetical protein	*Vitis vinifera*	5.67	39.35	322	16%	↑
279	350439988	chloroplast-specific ribosomal protein	*Solanum lycopersicum*	5.93	34.89	131	10%	↑
281	302797092	hypothetical protein	*Selaginella moellendorffii*	6.85	78.03	44	11%	↓
297	25555593	phosphoribulose kinase	*Ricinus communis*	5.83	45.22	125	5%	↑
301	804973	L-ascorbate peroxidase	*Capsicum annuum*	5.32	27.66	267	24%	↑
327	56786934	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*Cremastosperma magdalenae*	5.96	51.40	502	23%	↑
351	357495999	Serine-type peptidase	*Medicago truncatula*	6.79	45.95	128	7%	↓
364	125563861	hypothetical protein	*Orza sative Indica Group*	5.97	25.49	208	13%	↓
377	147823108	hypothetical protein	*Vitis vinifera*	6.45	27.63	147	16%	↓
385	154259484	ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Olea europaea*	4.92	28.98	427	20%	↑
420	147791852	hypothetical protein	*Vitis vinifera*	5.87	33.44	476	19%	↑
442	47169163	chloroplast ferredoxin-NADP+ oxidoreductase precursor	*Capsicum annuum*	5.08	33.65	839	41%	↑
493	11465963	ATP synthase epsilon subunit	*Nicotiana tabacum*	5.18	14.60	88	26%	↑
644	58531054	pathogenesis related protein	*Capsicum chinense*	5.22	17.37	95	10%	↑
651	15218090	mitochondrial-processing peptidase subunit alpha-1	*Arabidopsis thaliana*	5.94	54.54	100	8%	↑
664	117607073	mRNA-binding protein	*Capsicum annuum*	6.96	18.89	274	34%	↑
665	225449432	hypothetical protein	*Vitis vinifera*	5.50	48.21	232	8%	↑
669	68299213	malate dehydrogenase	*Capsicum chinense*	5.36	20.21	257	37%	↑
675	154259484	ribulose-1,5-bisphosphate carboxylase/oxygenase activase	*Olea europaea*	4.92	28.98	427	20%	↓
676	19896	photosystem II 23kDa polypeptide	*Nicotiana tabacum*	5.26	24.59	186	9%	↓
963	19875	glutamate-1-semialdehyde 2,1-aminomutase	*Nicotiana tabacum*	7.05	51.26	547	16%	↑
965	117607073	mRNA-binding protein	*Capsicum annuum*	6.96	18.89	274	22%	↑
985	22633	fructose-bisphosphate aldolase	*Spinacia oleracea*	6.57	42.74	180	6%	↑
991	222641066	hypothetical protein OsJ_28391	*Oryza sativa Japonica Group*	5.06	12.78	25	8%	↑
993	154259484	chloroplast rubisco activase	*Capsicum annuum*	4.92	28.98	349	17%	↓
994	114421	ATP synthase subunit beta, mitochondrial	*Capsicum annuum*	5.95	59.93	863	23%	↑
999	47169163	chloroplast ferredoxin-NADP+ oxidoreductase precursor	*Capsicum annuum*	6.08	33.65	821	40%	↓
1015	71842522	alanine aminotransferase	*Vitis vinifera*	6.13	53.08	289	12%	↑
1025	146464486	23kDa polypeptide of the oxygen evolving complex of photosystem II	*Sonnertia alba*	5.98	25.25	222	10%	↑
1030	12230332	Nucleoside diphosphate kinase	*Capsicum annuum*	6.31	16.37	488	25%	↑

The 39 identified proteins were further classified into six groups based on their main functions as defined by the GO Functional Catalogue (Fig. 5). Among these six groups, the most important pathway belonged to protein metabolism (e.g., S-adenosylmethionine synthase, Nucleoside diphosphate kinase, carboxylase/oxygenase activase, mRNA binding protein). This demonstrated that space induction mostly affected the metabolic processes. Other important identified pathways were involved with energy metabolism and photosynthesis.

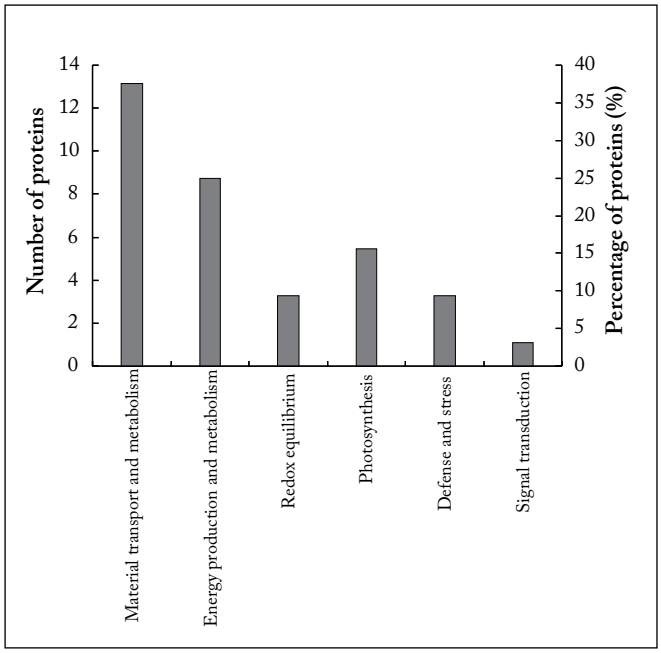

Fig. 5. Functional classification of differentially expressed proteins in five species.

Fig. 5. Clasificación funcional de proteínas expresadas diferencialmente en cinco especies.

DISCUSSION

Although the direction and variability of space mutagenesis is unknown and incontrollable, this technique is still effective to induce and produce mutants. It is an effective method to increase crop yield and improve some resistances (Lv Haixia, 2013). Our previous reports indicated that genetic variation in hot pepper mutant after space flight was obvious compared with that at their corresponding controls at a genetic level (Xie et al., 2014). Here, we firstly detected the changes of hot pepper after space flight using comparative proteome analysis. The results further indicated that hot pepper can obtain genetic variation by space flight at the proteomic level.

Leaf morphological characteristics are directly related to plant photosynthesis, including leaf ratios, stomata size and density, and ultrastructure of chloroplast in leaves (Meng et al., 2014). Therefore, the size and density of stomata play a vital role to regulate photosynthetic rate. Our SEM and TEM reports also suggested that space flight disturbed the formation and development of hot pepper leaves. Similar findings were reported by Wan and Liu (2008) using TEM, which confirmed that the shapes of many organelles, and the generation and accumulation of starch granules in mesophyll cells were highlighted after external environmental changes. In our study, both SEM and TEM indicated that leaves of hot pepper mutants showed a high range of stomata size and density, and accumulated more chloroplasts and starch grains, which might contribute to provide a great supply of energy for hot pepper. Accordingly, these phenomena resulted in higher yields in hot pepper mutants compared with their controls. This result is in agreement with the study of Zhang et al. (2004), who found that there were thinner cytoplasms, thicker cytoderms, bigger vacuoles, irregular and smaller chloroplasts, smaller grana lamella and accumulated starch granules in leaves of sainfoin after space.

In our work, large numbers of differentially expressed proteins were observed and sequenced in different hot pepper varieties (Table 1). Most proteins were mainly involved in protein metabolism, and energy metabolism processes (Fig. 5). Out of 39 differential proteins, most of them showed identity with available data, suggesting that some of them in the present study represented unknown proteins, which need to be further studied (Table 1). For example, some protein spots (spot 327, 993, 385 and 675) have homology with the Ribulose-1,5-bisphosphate carboxylase oxygenase and the Rubisco activase, which catalyze the first major carbon reaction in the calvin cycle of photosynthesis, and convert atmospheric free carbon dioxide to energy storage elements (Feller et al., 1998; Hasse et al., 2015). Compared with the controls, these proteins in mutants showed up-regulation, suggesting that induced hot pepper mutants had the ability to enhance carbon fixation in the organelles, which resulted in a higher energy production and raising of photosynthetic rates. Additionally, protein spots 250 and 251 were homologous with glutamine synthetase (GS), which is an enzyme in the nitrogen metabolism, and important in the nitrogen assimilation process that converts inorganic into organic nitrogen (glutamic acid, glutamine, etc.) after absorption and utilization by plants (Li et al., 2001). Thus, GS has a very positive meaning in the amino acid anabolism and catabolism processes (Masalkar and Roberts, 2015). In this study, the expression level of two expressed proteins was up-regulated, indicating that this protein may promote nitrogen assimilation.

Malate dehydrogenase (MDH), existing widely in the mitochondria and bacterial membranes, could oxidize oxaloacetate to malate resulting in malic acid accumulation, thus increasing the resistance to acid and aluminium toxins in plants (Ferraris et al., 2015; Rozova et al., 2015). Protein spots 265 and 669 might be up-regulated MDH in this study, demonstrating that the mutant caused an advantageous variation and enhanced the energy metabolism after space flight. In addi-

tion, protein spots 270, 1030 and 994, 493 were associated with the nucleoside diphosphate kinase and ATP synthase, respectively, which are key enzymes involved in the energy metabolism process (Roberts et al., 1997). Interestingly, these four up-regulated proteins further suggested an enhanced energy metabolism in mutants compared with the controls. Protein spot 985 included the fructose-bisphosphate aldolase, which is involved in the starch biosynthesis of the chloroplast, and is the so-called entry-enzyme of photosynthesis and PO_4^{3-} recycling in the calvin cycle (Cieśla et al., 2014). The up-regulated expression of the fructose-bisphosphate aldolase suggested that photosynthesis could be also stimulated in hot pepper mutants induced by space flight.

Additionally, space environment could induce other changes of protein spot. For example, protein spot 301 might include the ascorbate peroxidase (APX) whose traits were associated with scavenging hydrogen peroxide in the ascorbic acid-glutathione (AsA-GSH) system (Asada, 1992). The ascorbate peroxidase mainly uses ascorbic acid as an electron donor to timely eliminate H_2O_2 for ensuring the normal physiological functions in plants (Caverzan et al., 2012). Pignocchi et al. (2006) proved that over-expression of APX results in an increased sensitivity to ozone and antioxidant protection. Therefore, an up-regulated APX of space-induced mutants indicates that mutants have the ability of improving the antioxidant enzyme activities and the high level of antioxidant metabolism, thus improving plant stress resistance. This result agrees with that reported by Wan (2008).

Overall, the present research represents the study of variation in hot pepper after space mutagenesis using proteomic. Proteins of hot pepper mutants were greatly changed after space induction. Therefore, our results will contribute to the research of the mutagenic mechanisms at the proteome level, and provide an effective way for new hot pepper breeding.

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REFERENCES

Asada, K. (1992). Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. *Physiologia Plantarum* 85: 235-241.

Caverzan, A., G. Passaia, S.B. Rosa, C.W. Ribeiro, F. Lazzarotto & M. Margis-Pinheiro (2012). Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* 35: 1011-1019.

Cieśla, M., J. Mierzejewska, M. Adamczyk & A.K. Farrants (2014). Fructose bisphosphate aldolase is involved in the control of RNA polymerase III-directed transcription. *Biochimica et Biophysica Acta* 1843: 1103-1110.

Conesa, A. & S. Gotz (2008). Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. *International Journal of Plant Genomics* 2008: 619832.

Feller, U., S.J. Crafts-Brandner & M.E. Salvucci (1998). Moderately High Temperatures Inhibit Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (Rubisco) Activase-Mediated Activation of Rubisco. *Plant Physiology* 116: 539-546.

Ferraris, D.M., R. Spallek, W. Oehlmann, M. Singh & M. Rizzi (2015). Structures of citrate synthase and malate dehydrogenase of *Mycobacterium tuberculosis*. *Proteins-structure Function & Bioinformatics* 83: 389-394.

Hasse, D., A.M. Larsson & I. Andersson (2015). Structure of *Arabidopsis thaliana* Rubisco activase. *Acta Crystallographica* 71: 800-808.

Jorrín-Novo, J.V., J. Pascual, R. Sánchez- Lucas, C. Lenz & L. Valledor (2015). Fourteen years of plant proteomics reflected in Proteomics: Moving from model species and 2DE-based approaches to orphan species and gel-free platforms. *Proteomics* 15: 1089-1112.

Li, C.J., Q.H. Lin & Q.F. Zhang (2001). Progress of the studies on glutamine synthetase in higher plants. *Journal of Biology* 18: 1-3.

Li, G.G., M. Liu, P.S. Wang, J. Zhang, H. Xie & Y.H. Guo (2000). Effects of Space Conditions On Mutation and Inheritance of Tomato. *Space Medicine & Medical Engineering* 13: 114-118.

Liu, L.X., H.J. Guo, L.Z. Zhao, J.Y. Gu & S.R. Zhao (2007). Achievements in the past twenty years and perspective outlook of crop space breeding in China. *Journal of Nuclear Agricultural Sciences* 21: 589-592.

Lv Haixia, Z.Y., L.H. Wang, W.L. Wei, D.H. Li, X.Y. Zhang & X.R. Zhang (2013). Effects of Space-induced Mutation and Analysis of Polymorphism by AFLP Molecular Marker in Sesame. *Acta Agriculturae Boreali-Sinica* 28: 227-233.

Lv, J.Y., W.N. Qi, D. Yang, S.N. Zhang & J.F. Gao (2009). Study on mutation lines of wheat SP2generation under spaceflight. *Journal of Northwest A&F University* 123-126.

Masalkar, P.D. & D.M. Roberts (2015). Glutamine synthetase isoforms in nitrogen-fixing soybean nodules: Distinct oligomeric structures and thiol-based regulation. *Febs Letters* 589: 215-221.

Materska, M. & I. Perucka (2005). Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L.). *Journal of Agricultural and Food Chemistry* 53: 1750-1756.

Meng, F., M. Peng, H. Pang & F. Huang (2014). Comparison of photosynthesis and leaf ultrastructure on two black locust (*Robinia pseudoacacia* L.). *Biochemical Systematics & Ecology* 55: 170-175.

Miernyk, J.A. (2014). Seed proteomics. *Journal of Proteomics* 1072: 389-400.

Perucka, I. & W. Oleszek (2000). Extraction and determination of capsaicinoids in fruit of hot pepper *Capsicum annuum* L. by spectrophotometry and high-performance liquid chromatography. *Food Chemistry* 71: 287-291.

Pignocchi, C., G. Kiddle, I. Hernandez, S.J. Foster, A. Asensi, T. Taybi, J. Barnes & C.H. Foyer (2006). Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. *Plant Physiology* 141: 423-435.

Roberts, J.K, S. Aubert, E. Gout, R. Bligny & R. Douce (1997). Cooperation and Competition between Adenylate Kinase, Nucleoside Diphosphokinase, Electron Transport, and ATP Synthase in Plant Mitochondria Studied by 31P-Nuclear Magnetic Resonance. *Plant Physiology* 113: 191-199.

Rozova, O.N., V.N. Khmelenina, K.A. Bocharova, I.I. Mustakhimov & Y.A. Trotsenko (2015). Role of NAD+-Dependent Malate Dehydrogenase in the Metabolism of *Methylomicrobium alcaliphilum* 20Z and *Methylosinus trichosporium* OB3b. *Microorganisms* 3: 47-59.

Wan, X.Y. (2008). Comparative proteomics analysis reveals an intimate protein network provoked by hydrogen peroxide stress in rice seedling leaves. *Molecular & Cellular Proteomics Mcp* 7: 1469-1488.

Wan, X.Y. & J.Y. Liu (2008). Comparative proteomics analysis reveals an intimate protein network provoked by hydrogen peroxide stress in rice seedling leaves. *Molecular & Cellular Proteomics* 7: 1469-1488.

Wang, X.C., P.X. Fan, H.M. Song, X.Y. Chen, X.F. Li & Y.X. Li (2009). Comparative proteomic analysis of differentially expressed proteins in shoots of *Salicornia europaea* under different salinity. *Journal of Proteome Research* 8: 3331-3345.

Xie, L., X. Wang, M. Peng, F. Meng, Y. Zhou, L. Chen, L. Liu, Y. Gao & Y. Guo (2014). Isolation and detection of differential genes in hot pepper (*Capsicum annuum* L.) after space flight using AFLP markers. *Biochemical Systematics and Ecology* 57: 27-32.

Xu, X., T. Yan, Q. Zhao, Q.G. Shen & C.D. Liu (1997). The effects of space circumstance on tropism growth and metabolic process of asparagus seedlings. *Acta Biophysica Sinica* 13: 660-664.

Yang, Z.F. & C.R. Guo (2008). Review of space Mutation Breeding and its application in pepper breeding. *Journal of Shanxi Agricultural Sciences* 36: 21-25.

Zhang, H.J. (2008). Study on the Glutenin Macropolymer of Spring Wheat with Same HMW- GS. *Chinese Agricultural Science Bulletin* 24: 172-175.

Zhang, S.N. & J.Y. Lv (2009). SDS-PAGE Analysis of High Molecular Weight Glutenin Subunits in SP3 from Spaceflight ARRIED Wheat. *Journal of Nuclear Agricultural Sciences* 23: 193-196.

Zhang, Y.W., W.B. Ren, M. Liu, J.G. Han, B. Shu & W. Lu (2004). Study on Peroxidase and Esterase Isoenzymes and the Ultrastructure of the Space Induced Mutant Leaves of Sainfoin (*Onobrychis viciaefolia* Scop.). *Acta Agrestia Sinica* 12: 223-226.