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Separation of ovule proteins during female gametophyte cellularization of *Pinus tabuliformis* using 2D-DIGE

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Abstract

Female gametophyte cellularization is an important process in ovules development of *Pinus tabuliformis* Carr., which is regulated and characterized by multiple proteins. To separate the differential expressed proteins in female gametophyte cellularization, 2D-DIGE were used with its high efficiency and repeatability. The ovule proteins were extracted in prophase and anaphase of the process with TCA-acetone and rinsed with ice-cold acetone at least five times. The protein concentration of the sample solutions was adjusted to 1.5 $g \cdot L^{-1}$ - 2.5 $g \cdot L^{-1}$, and the pH of the solutions was kept at 8.5 before labeling. A total of 800 proteins were observed on the 2D-DIGE gel using the optimized protocol. Ninety-one differential expressed proteins were separated with 2D-DIGE, while only 62 were separated with 2D-E. Eleven proteins were specific to prophase and 14 proteins were specific to anaphase. During the cellularization process, 45 proteins were up-regulated and 21 proteins were down-regulated. This study established an 2D-DIGE protocol for proteomics research on the development of *P. tabuliformis* ovules, and provided a technical reference for 2D-DIGE in tissues that are rich in secondary metabolites like *P. tabuliformis*.

Introduction

Pinus tabuliformis Carr. is one of the primary species used in afforestation throughout China, and is also important to the study of gymnosperm ovule development. *Pinus tabuliformis* ovule development takes approximately three years and includes the following stages, 1) ovule primordia differentiation, 2) female gametophyte formation, 3) free nucleus division, 4) female gametophyte cellularization, 5) egg cell fertilization and 6) seed formation. In prophase during female gametophyte cellularization, cell walls begin forming around a few free nuclei, and in anaphase all of the free nuclei are surrounded by walls, thus completing the cellularization process (Zhou et al., 2006). During this period a large number of regulatory genes impact ovule development. Because the cell walls in the female gametophyte were constructed in the cellularization process, a differential proteomic study on female gametophyte cellularization could directly explore the functional proteins associated with cell wall formation. In previous research, proteins associated with ovule development were found using a classical 2D-E method in lychee (Li et al., 2006), garlic (Winiarczyk et al., 2009), rice (Agrawal et al., 2009) and other angiosperms. However, the 2D-E method the lacks of reproducibility and has a low sensitivity to low-abundance proteins, which reduces the detection of differential expressed proteins (Renaut et al., 2014). By running two samples on one gel, an internal control is provided for a two-dimensional difference in-gel electrophoresis (2D-DIGE) (Ünlü et al., 1997). The advantages of improved sensitivity and accuracy provided by the ability to separate more than one sample on a single gel have added a new dimension to the traditional 2D-E (Marouga et al., 2005). In pollen of wild type and mutant tomato more than 1,800 spots were detected by using 2D-DIGE, which was more than double the number of spots observed in 2D-E, thus the 2D-DIGE method is better for the detection of low abundance proteins (Sheoran et al., 2009).

2D-DIGE has been used to study proteomics of plant development such as the molecular mechanism of phloem cell wall formation (Hotte and Deyholos, 2008), embryonic development (Shi et al., 2010), the differences between zygotic and somatic embryos (Rode et al., 2011), and the stress response mechanisms on salt (Yang et al., 2013), cold (Kosova et al., 2013) and viruses (Wu et al., 2013). However, this approach needs high-purity protein samples, because contaminants can interfere with the protein-dye labeling process (Hannigan et al., 2006). Research showed that it is not possible to recommend a single protocol for the extraction of all plant proteins (Fido et al., 2004). About the free nucleuis cellularization, there are few reports on endosperm in rice (Xu et al., 2008 and 2010; Yu et al., 2012), which has simple components and contains few substances interfere the electrophoresis. So far, there are no proteomic studies on female gametophyte development of *P. tabuliformis* with 2D-DIGE. This study established a viable 2D-DIGE method for proteomics research on ovules of *P. tabuliformis* and separated the differential expressed proteins in prophase and anaphase during female gametophyte cellularization.

Results

The effects of different protein sample treatments on CyDye labeling

To increase the efficiency of protein and dye labeling, impurities (DNA, salt and other plant secondary metabolites) were reduced by rinsing the samples with ice-cold acetone. In this experiment we compared the effect of rinsing five times

Table 1. The effect of protein extraction protocol in dye labeling.

Times of rinsing	Number of spots	Number of Spots in high MW	Number of Spots in low MW	Relative volume of certain standard spots		
				3601	3602	4901
Two times rinsing	339	55	64	12438.488	36070.048	14418.168
Five times rinsing	533	102	24	44011.592	43862.720	17013.216

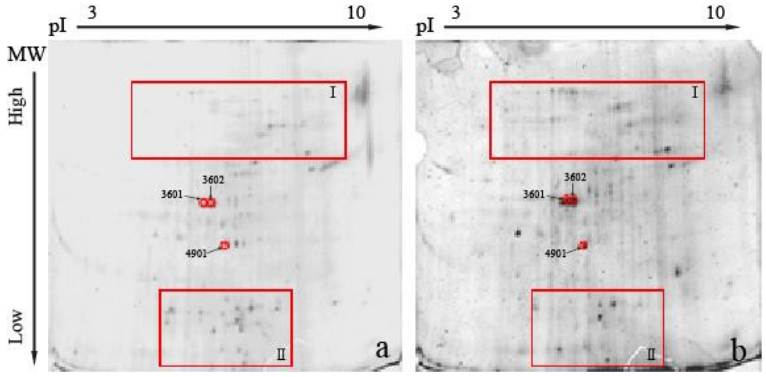

Fig1. The effect of the rinsing times in dye labeling. The protein samples were washed with ice-cold acetone repeated twice and five times respectively, Cy2 were used to detect the spots. The total number of protein spots was 533 with five times rinsing sample (a) while the number was 339 with twice (b). The number of spots in low MW (zone Ⅱ) was 64 with twice rinsing while 24 spots with five times. In high MW proteins (zone Ⅰ), the total spots number was 102 with five times rinsing, while 55 with twice. The relative volume of most of the protein spots, such as 3601, 3602 and 4901 were all larger with five times rinsing than with twice.

with two times which was used in Jiang's protocol (Jiang et al., 2006). The results of this comparison are shown in Figure 1 and further details are provided in Table 1. The total number of protein spots was 533 with five times rinsing sample while the number was 339 with twice. The number of spots in low molecular weight (MW) was 64 with twice rinsing while 24 spots with five times. In high MW proteins, the total spots number was 102 with five times rinsing, while 55 with two times. Although increase of rinsing time may lose several low molecular weight proteins, the combining rate of protein-dye was much higher and more protein spots were observed on the gel. The fresh weight of the ovule increased rapidly from dormancy to maturity and the proteins within the ovule changed, thus a large difference was seen in the protein concentration even when using the same extraction protocol. The results are shown in Figure 2 and further details are provided in Table 2. The total number of protein spots was 472 by using 1.5 $g \cdot L^{-1}$ protein sample; the number was 540 by using 2.5 $g \cdot L^{-1}$ protein sample; and 275 by using 0.8 $g \cdot L^{-1}$ protein sample. There were few difference between protein samples at the concentration of 2.5 $g \cdot L^{-1}$ and 1.5 $g \cdot L^{-1}$. However, the total number of protein spots by using 0.8 $g \cdot L^{-1}$ protein sample was much fewer than the other two treatments.A pH 8.0 and pH 8.5 were selected in dye labeling, and SDS-PAGE was used for protein visualization. Three bands were chosen for the analysis of results. Results from the SDS-PAGE showed that a pH of 8.5 is optimum pH for dye labeling in protein solution from ovules for Cy2, Cy3 and Cy5. Additionally, pH has a great influence on the efficiency of Cy3, but was not as important for Cy2 and Cy5. The results are shown in Figure 3 and further details are given in Table 3.

Differential proteins in ovules during female gametophyte cellularization

The ovule proteins at two development stages (prophase and anaphase of female gametophyte cellularization) were successfully separated using both 2D-E and 2D-DIGE (shown in Fig. 4 and Fig. 5). Differential expressed proteins were found after three repetitions. Data analysis with PDQuest™ software led to a resolution of more than 800 protein spots per gel in both 2D-E and 2D-DIGE. Ninety-one differential expressed proteins were observed using 2D-DIGE and 62 protiens were observed using 2D-E on female gametophyte cellularization. Among the 91 proteins that were observed using 2D-DIGE, 11 were expressed special in prophase and 14 proteins were specially expressed in anaphase. Additionally, in anaphase 45 proteins were up-regulated and 21 proteins were down-regulated comparing with the prophase.

Discussion

Ovules of *P. tabuliformis* contain large amounts of polyphenols, lipids and other secondary metabolites, which severely restrict protein-dye labeling, thus sophisticated purification techniques are needed. Furthermore, the ovules of *P. tabuliformis* at early development stages were too small to obtain, which leads to the limited quantity of samples (Zhou et al., 2006). Preparation of good-quality protein extracts is critical for successful electrophoretic analysis (Zienkiewicz et al., 2014). Protein samples labeled with CyDye dyes requires even higher quality samples than the 2D-E method. Salts and other contaminants that may result in an inefficient labeling should be removed after sample preparation (May et al., 2012). There is one exception found in the study of protein expression in grape berry, where numerous tissue samples were used to increase the ration of protein to dyes thus making the samples acceptable for analysis with 2D-DIGE. Although 870 total spots were detected, horizontal streaks and vertical gaps were caused by multiple secondary metabolites in the final 2D gel (Di Carli 2010). In our study, rinsing the samples five times with acetone effectively reduced secondary metabolite interference and better effect of 2D gels were obtained (Fig. 1). Although proteins can be isolated using 2D-E, deviation of pouring, running and analyzing the gel decreases the

Table2. Total number of spots and the relative volume of certain protein spots.

The protein concentration in labeling system	Total number of spots	Relative volume of certain standard spots					
		3902	3903	3904	4301	4901	8701
1.5 g·L^{-1} protein sample labeled with Cy3	487	127299	36381	8787	39	31531	2784
1.5 g·L^{-1} protein sample labeled with Cy5	472	144188	35778	8286	41	24170	8401
0.8 g·L^{-1} protein sample labeled with Cy3	275	170163	12438	-	-	36070	3304
2.5 g·L^{-1} protein sample labeled with Cy5	540	247436	44011	15386	262	43862	2427

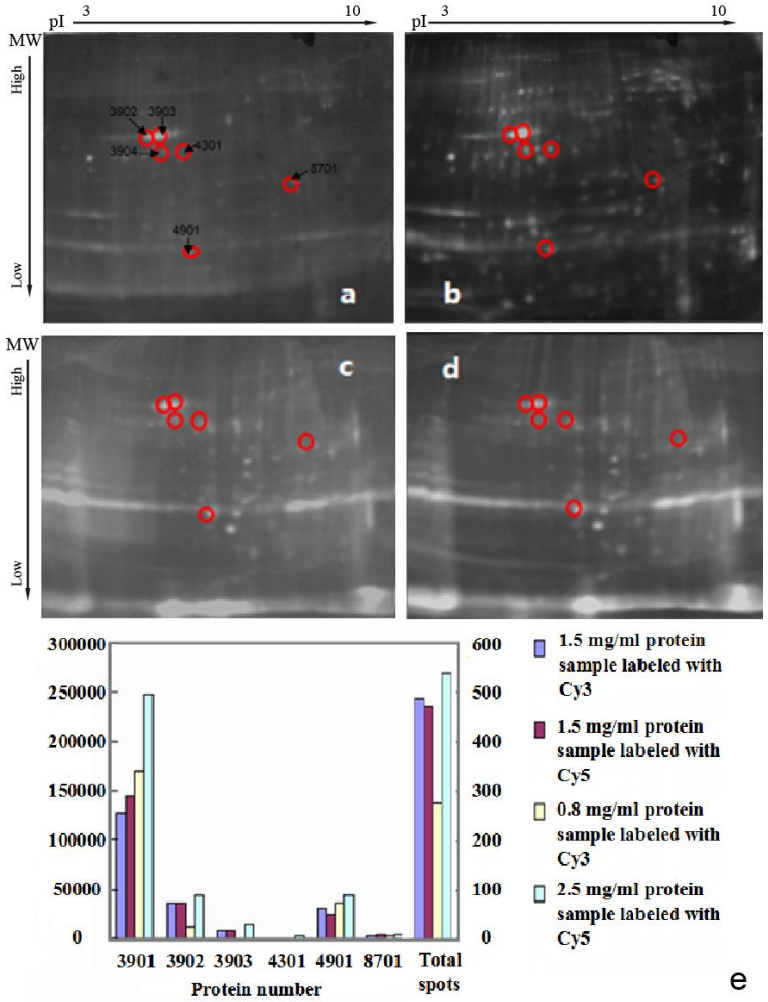

Fig 2. The effect of protein concentration of sample solution on labeling 0.8 g·L^{-1} protein sample labeled with Cy3 (a), and 2.5 g·L^{-1} labeled with Cy5 (b), 1.5 g·L^{-1} protein samples labeled with Cy3 (c) and Cy5 (d), all sample solution had 50 μg protein. The 1.5 g·L^{-1} protein samples labeled with Cy3 and Cy5 respectively were used to adjust PMT to make the spots value in other gel reliable. The total number of protein spots was 472 and 487 by using 1.5 g·L^{-1} protein sample and 540 by using 2.5 g·L^{-1} protein sample while the number was 275 by using 0.8 g·L^{-1}. In most of the protein spots, the relative volume of protein increased with the protein concentration.

comparability of results because the same protein may be found in different locations and relative volumes in different gels. Less differential expressed proteins would be obtained because of this shortcomings of 2D-E. In our study, ninety-one differential expressed proteins were obtained during female gametophyte cellularization, the number were larger than using 2D-E. More differential expressed proteins for mass spectrometry identification were obtained in the *P. tabuliformis* female gametophyte cellularization by 2D-DIGE than 2D-E. Our results showed that the optimized 2D-DIGE protocol is a reliable method in the study of female gametophyte cellularization in *P. tabuliformis*.With ovule development, the ovule fresh weight increased rapidly, which may have led to a large difference in protein concentration of solutions extracted from the same quantity of samples. The protein concentration may influence protein-dye labeling. In our study, the results showed that the efficiency of protein-dye labeling was significantly lower when the protein concentration of the solution was 0.8 g·L^{-1} (Fig. 2a). When the protein concentration of the solution was increased to 1.5 g·L^{-1} the labeling efficiency improved (Fig. 2c and d). When the protein concentration was increased to 2.5 g·L^{-1}, the labeling efficiency remained the same as 1.5 g·L^{-1} (Figure 2b). Therefore, the protein concentration of the extraction solution should be adjusted to over 1.5 g·L^{-1} to improve the labeling results. The pH value of the protein solution may also influence the labeling efficiency. The instructions of the CyDyes kit states that the suitable pH value of the labeling solution is between 8.0 to 8.5; however, in our study the labeling efficiency of the solution at pH 8.5 was higher than

Table 3. Relative volume of bands in different pH value of protein solution on dye labeling.

Bands in Red boxes	Cy2 labeling at pH 8.0	Cy2 labeling at pH 8.5	Cy3 labeling at pH 8.0	Cy3 labeling at pH 8.5	Cy5 labeling at pH 8.0	Cy5 labeling at pH 8.5
1	12868	11398	-	24005	20004	21382
2	234080	245930	52215	266020	268730	281200
3	162700	168980	78268	213140	262620	284840

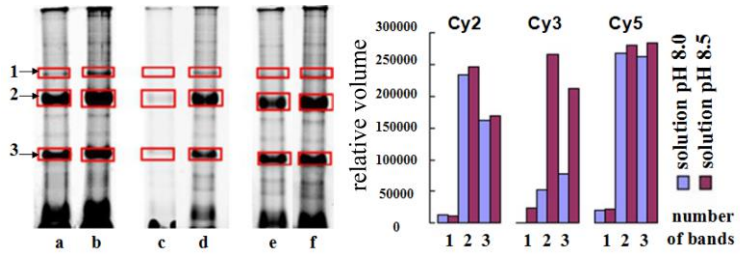

Fig 3. The effect of pH value of protein solution on dye labeling lane a, c, e were Cy2, Cy3, Cy5 labeling under buffer system of pH 8.0 respectively; b, d, f were Cy2, Cy3, Cy5 labeling under buffer system of pH 8.5. There was little difference in relative volume at certain pH labeled with Cy2, Cy3, Cy5. The relative volume of all three bands at pH 8.5 (lane b, d and f) were larger than at pH 8.0 (lane a, c and e). For Cy3 (lane c, d), the differences of the relative volume were larger than Cy2 (lane a, b) and Cy5 (lane e, f).

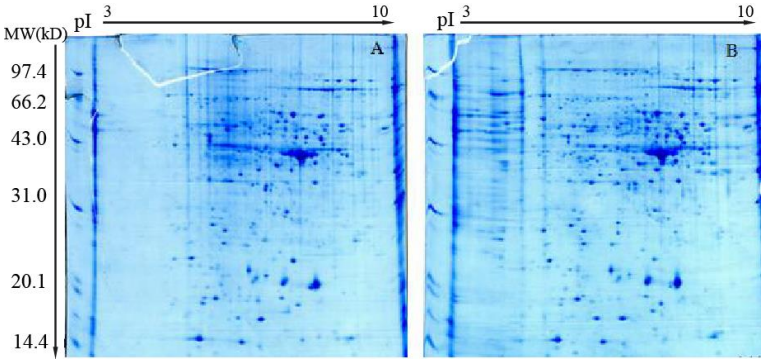

Fig 4. 2D-E images of the ovule proteins of *P. tabulaeformis* at different cellularization stages The images were acquired in two different gels with blue silver staining. (A) was the sample in prophase of female gametophyte cellularization, and (B) was the sample in anaphase. After three repetitions, more than 800 protein spots were detect, and 62 differential expressed proteins were found.

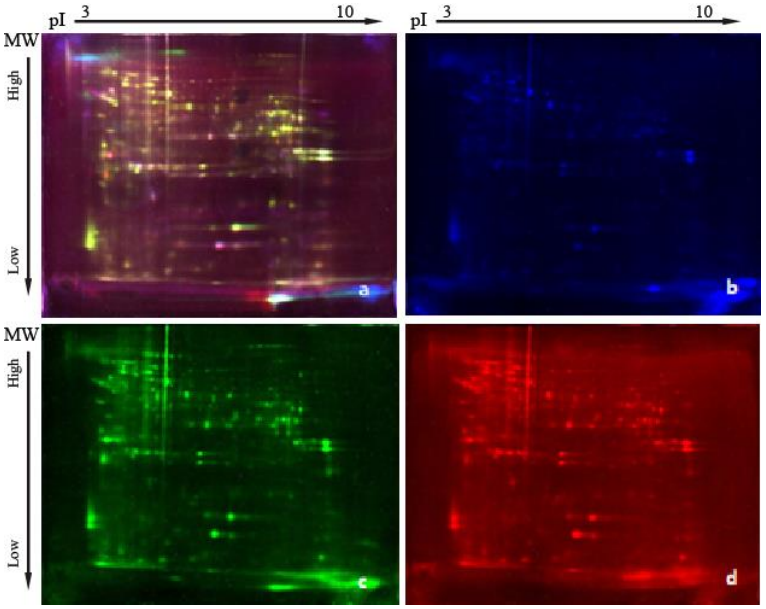

Fig 5. 2D-DIGE images of the ovule proteins of *P. tabulaeformis* at different cellularization stages The images were acquired in one gel under different lasers, internal standard (b) labeled with Cy2 displayed in blue, proteins in prophase (c) labeled with Cy3 displayed in green, and proteins in anaphase (d) labeled with Cy5 displayed in red, differential expressed proteins could be found inthe multi-channel synthesizer image (a). Ninety-one differential expressed proteins were found among the total of 800 proteins spots after three repetitions.

that at pH 8.0 (Fig. 3). Consequently, the pH value of the protein solution of the ovules should be adjusted to 8.5 before labeling to insure adequate protein labeling. In this study, among the 91 differential expressed proteins seperated in the female gametophyte cellularization of *P. tabuliformis*, 11 proteins were found only in prophase and disappeared as cellularization was accomplished. Also, 21 proteins were down-regulated in anaphase. Generally, in prophase of female gametophyte cellularization, cell walls start to form after free nucleus division has stopped. This process is similar to the cellularization of rice endosperm at three to six days after fertilization (Xu et al., 2008). It is possible that the 11 protiens found only in prophase and the 21 down-regulated proteins in anaphase may associated with cell growth, division, and central carbon metabolism and morphogenesis such as cytoskeleton proteins and their associated proteins, according to our results. Forty-five proteins were up-regulated and 14 proteins were identified that are unique to anaphase cellularization in the female gametophyte. Normally, walls surrounded all free nuclei and some small vacuoles were generated in the new cells in anaphase. Some storage substances, such as lipids and storage proteins, began to accumulate in the cells. The results of our study suggest that the 45 up-regulated proteins and 14 unique proteins play a role in the synthesis of storage substances. Xu and colleagues (2010) has also indicated that some proteins implicated in storage substance synthesis, such as starch in rice, were up-regulated at the end of endosperm development. Because the differential expressed proteins were formed in the ovule in the phase of the female gametophyte cellularization, we cannot entirely exclude the role of these proteins on integument development, even if most of the integument proteins were eliminated as backgrounds because the physiological changes in the integument were much smaller than in the female gametophyte at this stage. Further research, such as mass spectrometry identification, needs to be conducted to confirm the function of these proteins.In summary, we established an available 2D-DIGE technique for proteomics research on *P. tabuliformis* ovules and maybe on the tissues that are rich in secondary metabolites. The differential expressed proteins merit further investigation to reveal the molecular regulated mechanisms in the cellularization of the female gametophyte.

Materials and Methods

Plant materials

Dynamic ovulate strobilus in prophase and anaphase of female gametophyte cellularization were cut off and collected from *P. tabuliformis* trees growth in the campus of Beijing Forestry University. The ovules of the strobilus were carefully picked out in the dissecting microscope and immersed into liquid nitrogen, and stored at -80 ºC.

Protein sample preparation

The ovules were grinded in liquid nitrogen, then mixed with 20% TCA in cold acetone and kept at least 2 h at –20 ºC to precipitate the proteins, The mixture was centrifuged for 40 min at 15000 *g* at 4 ºC. The pellets were rinsed with ice-cold acetone and centrifuged again at 4 ºC. To compare the rinsing effect, this step was repeated twice and five times respectively. The pellets were finally freeze-dried. The dried pellets were resuspended in labeling buffer and incubated for 30 min at room temperature. The pH of protein samples were adjusted to 8.0 and 8.5 for comparison in 2D-DIGE experiment. The protein concentration was determined by Bradford using BSA as the standard, and adjusted to 0.8 $g \cdot L^{-1}$, 1.5 $g \cdot L^{-1}$ and 2.5 $g \cdot L^{-1}$ respectively.

The CyDye labeling

Protein extracts and the pooled internal standard were labeled prior to electrophoresis with the CyDyes™ (GE Healthcare, UK). Each extract was labeled at the ratio 400 pmol CyDye for 50 mg of protein, vortexed, and incubated on ice for 30 min. The reactions were quenched by addition of 1 μL of 10 mM lysine, vortexed, and incubated on ice for 10 min in the dark. An equal volume of lysis buffer was added. The samples were vortexed and incubated on ice for a further 15 min in the dark.

SDS-PAGE

The PROTEAN II xi Cell vertical slab electrophoresis tank (BIO-RAD) was used for SDS-PAGE. Concentration of the separating gel was 13% and the concentration of stacking gel was 5%. This step was performed at 25 ℃ in 5 mA/gel for 20 min and 30 mA/gel for 1 h.

2D-E

Immobiline DryStrips (GE Healthcare, pH 3-10, 24 cm) were rehydrated overnight with the protein extracts. Referring to Jiang (2006), The IEF was carried out on an Ettan IPGphor (GE Healthcare) with the following settings: gradient steps of 50 V for 0.5 h, 200 V for 1 h, 2000 V for 1 h, 4000 V for 2 h, and a constant step at 8000 V for 7 h at 20 ºC with a maximum current setting of 50 mA/strip in an IPGphor IEF unit (GE Healthcare). After the IEF, the IPG strips were equilibrated for 15 min in equilibration buffer supplemented with 1% w/v DTT. A second equilibration step of 15 min with the same equilibration buffer, containing now 2.5% w/v iodoacetamide, was carried out afterwards. The IPG strips were then sealed with 0.5% agaros in SDS running buffer on top of gradient gels 13% w/v acrylamide and 0.1% *N*,*N*'-methylenebisacrylamide. The gels were poured between low fluorescent glass plates. The SDS-PAGE step was performed at 25 ºC in Ettan Dalt II tanks (GE Healthcare) at 2.5 W/gel for 24 h.

Blue silver staining

The 2D-E gels were washed with Milli-Q water briefly, stained with the staining solution (0.12% w/v Coomassie brilliant blue G-250, 10% w/v $(NH_4)_2SO_4$, 10% v/v phosphoric acid, 20% v/v methanol) overnight, and transferred into neutralization buffer (0.1 mol/L Tris) for 1-3 min, Then the gels were washed with 25% (v/v) methanol for less than 1 min.

Image capture and data analysis

Cy2-, Cy3- and Cy5-labeled protein images were produced by excitation of gels at 488, 532 and 633 nm, respectively, and emission at 520, 590 and 680 nm, respectively, using Typhoon Variable Mode Imager 9400 (GE Healthcare) at a resolution of 50 μm. The PMT was adjusted to make the spots value reliable. Data was performed with PDQuest™ 2-D Analysis image analysis software for protein points identification, background cancellation, point matching and differences analysis. Standard spots were numbered by

PDQuest™ software. The relative volume was used to quantify the signal intensity according to the software.

Acknowledgments

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