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Proteomic profiling of rice roots from a super-hybrid rice cultivar and its parental lines

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Abstract

Heterosis is a common phenomenon in which the hybrids exhibit superior agronomic performance than both parents such as biomass production, grain yield, and stress tolerance. Although heterosis breeding is one of the main techniques in rice breeding, the molecular mechanisms responsible for this basic biological phenomenon are not well understood. To further get an insight into the molecular mechanisms of rice heterosis, comparative root proteomic analysis between a super-hybrid rice *LYP9* and its parental cultivars *9311* and *PA64S* at seedling stage were performed. Total proteins were extracted and subjected to two-dimensional gel electrophoresis. The scatter plots analysis results showed that the *LYP9's* expression profiles were more similar to *9311* than *PA64S*. A total of 11 differentially expressed protein spots were detected and identified by matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry, corresponding to 10 unique proteins. These proteins were identified to be involved in several cellular processes, including metabolism, secondary metabolism, Transcription regulation, energy metabolism, signal transduction, disease and defense. Moreover, based on our present data, the differentially expressed proteins could work together to play a major role in the induced defense response. In addition, we observed that phenylalanine ammonia-lyase, Os12g0440200 and putative PrMC3 proteins had higher expression in *LYP9* than its parents. These proteins can be strongest candidates for further functional research.

Keywords: Heterosis; Two-dimensional gel electrophoresis; Proteome; Rice roots.
Abbreviations: 2-DE, two-dimensional gel electrophoresis; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization-time of flight/time of flight mass spectrometry; NCBInr protein database, National Center for Biotechnology Information nonredundant protein database; DEPs, differentially expressed protein spots.

Introduction

Heterosis is a common phenomenon in which the hybrids exhibit superior agronomic performance than the two parents such as biomass production, grain yield, and stress tolerance. Heterosis in rice was first reported by Jones (1926) who observed marked increase in culm number and grain yield in some F_1 hybrids in comparison to their parents. Since then, many scientists have endeavored to improve yield of rice plants by mean of crossing breeding. Despite the fact that hybrid vigor has been exploited in agricultural crops, the molecular mechanisms responsible for this basic biological phenomenon are not well understood. With the development of functional genomics, it is possible to examine gene expression profiles between the hybrid and its parents and thus to explore the possible molecular mechanisms for heterosis. Recently, the comparative gene expression profiling between the hybrid and its parents has been investigated in maize (Stupar et al., 2008), Arabidopsis (Vuylsteke et al., 2005), wheat (Sun et al., 2004), and rice ((Wei et al., 2009). These studies have indicated that the hybridization between two parents can lead to the changes in the expression of different genes, which may responsible for heterosis. However, the changes on the levels of gene expression do not necessarily reflect the changes in the abundance of proteins. Therefore, the differential protein expression between the hybrid and its parents will be important for us to understand the possible molecular mechanisms for heterosis. Rice is not only an important crop worldwide but also a model plant for monocots because of its relatively small genome size. A superhybrid rice, *LYP9*, from a cross between 9311 (*Oryza stavia L. ssp. Indica*) and *PA64S* (mixed background of *Indica, Japonica*, and *Javanica*), is a successful rice cultivar with 20-30% more yield per hectare than its parents crops in cultivation and is widely cultivated in southern China (Lu and Zhou, 2000). More importantly, both parent genomes, *9311* and *PA64S*, have been sequenced, thus providing invaluable information and available genetic resources for further functional studies (Yu et al., 2002; Goff et al., 2002). In rice, root systems serve many important functions, including anchorage of the plant, uptake of water and nutrients, synthesis of amino acids and hormones, and secretion of organic acids, enzymes, and alkaloids. Previous studies demonstrated that hybrid rice has also been found to have stronger and more active root system, and the root system was proposed to be used as a model trait for investigating the molecular basis of heterosis (Virmani et al., 1982; Ekanayake., et al., 1985; Chang et al., 1982). Two-dimensional electrophoresis analysis among *9311*, *PA64s*, and *LYP9* revealed that there were significant differentially expressed proteins in leaves, embryos and endosperms (Xie et al., 2006; Wang et al., 2008; Zhang et al., 2012). However, until now there is no report about the differences in the proteomic profiling of rice roots between *LYP9* and its parents. In this study, we report research into the differences in proteome

profiles of roots between *LYP9* and its parents at seedling stage. The proteomic analysis in this study reveals that the proteins involved in metabolism, secondary metabolism, Transcription regulation, energy metabolism, signal transduction and disease/defense may be responsible for heterosis in rice. The molecular insights provide by this study might help to better understand the possible molecular mechanism involved in rice heterosis .

Results and discussion

Characteristics of the root proteome map of LYP9 and its parents

2-DE was employed in this study for protein separation. As a traditional method in proteomic analysis, 2-DE has been criticized in many aspects, including the sensitivity of staining methods, the reproducibility of parallel experiments, and the limitations of the proteins with extremely acidic and basic p*I* values, and high hydrophobicity (Gorg., 2004). The objective of this study was to identify differentially expressed protein spots (DEPs) between *LYP9* and its parents at seeding stage. To obtain more accurate date for determination of differential proteomes, especially in analyzing the gel spots from 2-DE of rice root proteins, we have conducted strictly parallel experiments. Proteins were first separated by isoelectric focusing (IEF) on a nonlinear gradient ranging from pH3.5 to 10. After IEF, proteins were separated according to their molecular masses using SDS-PAGE and stained with Coomassie brilliant blue (CBB) G250. On the other hand, although the quantitative differences in the spot volume may bring some useful information on protein expression, the image data of 2-DE are not easily and universally analyzed quantitatively. In this study, we only focused on these spots that differentially expressed between *LYP9* and its parents. The image data thus provided a high quality to ensure the accuracy of the comparison analysis. More than 500 protein spots were reproducibly detected by Imagemaster 2D Platinum software (GE healthcare) on 2D gel maps for each cultivar. The representative 2-DE maps are shown in Fig. 1, in which a high similarity of protein profiles was observed between *LYP9* and its parents, there were significant differences between them in expression patterns of some proteins. Scatter plots of 2-DE analysis of the three cultivars are presented in Fig.2. This results indicated that the protein expression profiles of *LYP9* is more similar to *9311* (correlation coefficients=0.913) than *PA64S* (correlation coefficients=0.872). The result is consistent with the fact that *LYP9* is more like *9311* than *PA64S* in their phenotypic appearances (Tab.1). Quantitative image analysis revealed a total of 11 protein spots that changed their intensities significantly ($p<0.05$) by more than 2-fold between *LYP9* and its parents (Fig. 1). Eight typical regions are enlarged in Fig. 3. DEPs between the hybrid and its parents were categorized into 6 expression patterns: *PA64S* (P)＞*LYP9* (L)＞*9311* (N) (spots 1, 7, 9), L＞N＞P (spots 2, 3, 8), L＞N=P (spot 4), L=N＞P (spot 5), N＞L＞P (spot 10) and N＞P＞L (spot 11). We noticed that a large portion of DEPs in the hybrid expressed at a higher level than those of its one or both parents, which suggested that protein expression patterns in *LYP9* could be important for rice root heterosis (Tab.2).

Identification and categorization of differentially expressed proteins

A total of 11 DEPs were detected and picked up between *LYP9* and its parents. To further understand the function of DEPs, these DEPs were identified by MALDI-TOF/TOF MS in MASCOT database searching (Tab.2). All protein sequences detected and identified were searched against gene ontology tool (http://www.geneontology.org) for functional classification. The identified 11 protein spots were identified as 10 individual proteins and classified into six groups according to function, viz. metabolism (30%), secondary metabolism (20%), Transcription regulation (10%), energy metabolism (20%), signal transduction (10%), disease and defense (10%) (Fig.4). As would be expected, 50% (5 of 10) proteins in this experiment were involved in metabolism and energy production/regulation. Proteins grouped under metabolism include those involved in the metabolism of amino acids, carbohydrate, lipid, coenzyme and proteins related to secondary metabolism. Two identified proteins were associated with energy production, and play roles in oxidation-reduction process. The remaining functional groups contained proteins involved in Transcription regulation, signal transduction, disease and defense.

The 11 proteins spots identified in this study are derived from 10 different genes or gene families. Further analysis indicated that two spots (spots 2 and 3) representing the phenylalanine ammonia-lyase were found to be different both in their M_r and p*I* (Fig. 3). These isoforms, if correctly identified, could represent post-translationally modified form of the same protein.

Analysis of differentially expressed rice root proteins between LYP9 and its parents

Previous studies indicated that gene expression differed between hybrids and their parents in rice, which may be responsible for hybrid vigor (Xie et al., 2006; Wang et al., 2008; Wei et al., 2009; Song et al., 2010; Zhang et al., 2012). In this paper, 2-DE was firstly employed to detect differential proteome expression of seedling root between *LYP9* and its parents, and 10 proteins showed differential expression between the hybrid and its parents. These proteins might be involved in the complex mechanisms of rice heterosis, and are anticipated to be useful in obtaining a better understanding of the molecular mechanisms of rice heterosis. The metabolism-associated group was the major group that changed between *LYP9* and its parents. Metabolisms of materials and energy, e.g., glycolysis, tricarboxylic acid cycle, pentose phosphate pathway, and amino acid synthesis, are essential processes in all kinds of plant tissues. Among protein metabolism-related proteins, phenylalanine ammonia-lyase (PAL) (spots 2, 3) and putative PrMC3 (spot 8) are markedly up-regulated in hybrid than in both female and male parents. PAL is a key regulatory enzyme in phenylpropanoid metabolism. The pathway produces a large amount of phenolic compounds, which are the precursor molecules of isoflavonoid phytoalexins, suberins and lignins, both of which were involved in plant defense reactions (Li et al., 2001). PrMC3, which is a member of a family of proteins that all contain a Ser hydrolase motif (GXSXG) and have similarity to lipases and esterases of prokaryotic origin (Walden et al., 1999). It has been reporter that the protein is induced under chilling stress in rice roots (Lee et al., 2009). In addition, we observed that the expression level of 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (MetE), S-adenosylmethionine synthetase (SAM) and Glucanase in *LYP9* was between two parents. MetE (spot 1) catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine resulting in methionine formation, which further catalyzed into S-adenosylmethionine by SAM. Interestingly, we also observed SAM (spot 6) in hybrid expressed at a higher level than *PA64S*

Table 1. Root traits of hybrid *LYP9* and its parents at seedling stage.

Root traits	RFW (g)	RDW (g)	TRN	MRL(cm)
9311	0.43±0.16[a]	0.036±0.013[a]	23±3.9[a]	13.8±1.2[a]
PA64S	0.07±0.02[b]	0.006±0.002[b]	9±2.2[b]	9.7±2.0[b]
LYP9	0.44±0.08[a]	0.043±0.012[a]	25±3.3[a]	13±2.0[a]

Note: Each value was the average of five replicates. Superscript letters show the statistical significance ($p<0.05$) between *LYP9* and its parents. "TRN" means taproot number, "MRL" means maximum root length, "RFW" means root fresh weight, "RDW" root dry weight.

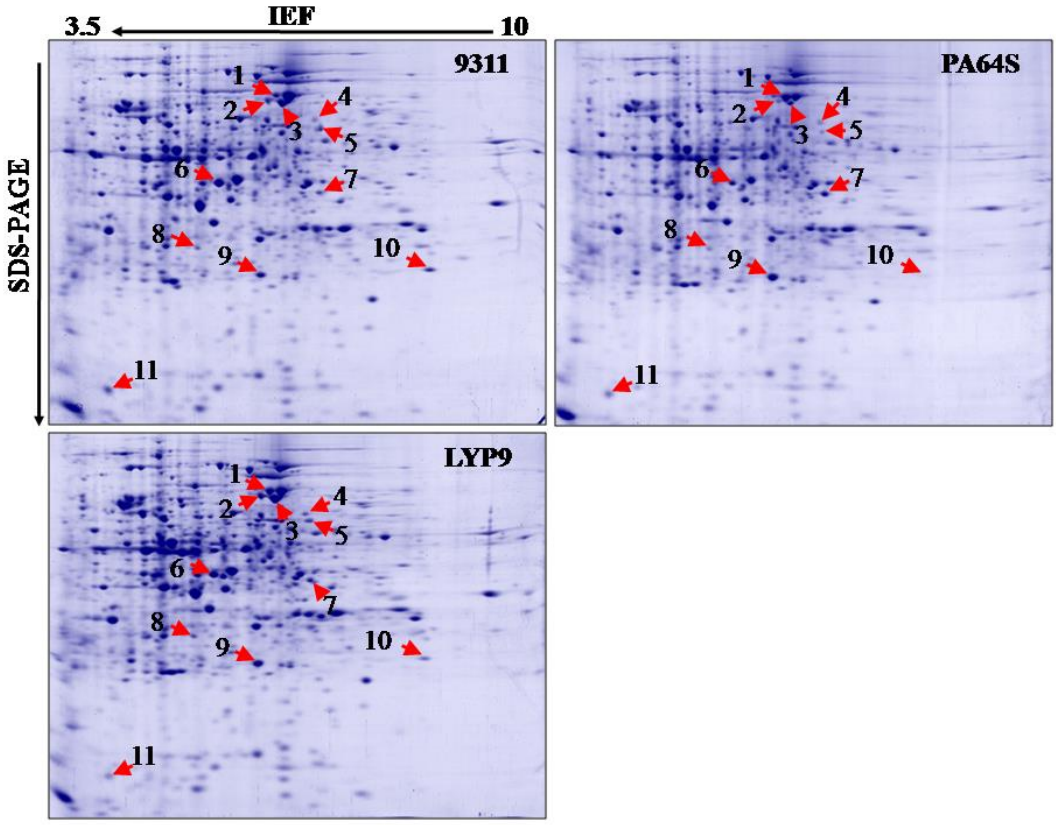

Fig 1. Protein expression profiles in roots of *LYP9* and its parents. The protein spots visualized using coomassie brilliant blue staining, and 11 differentially expressed proteins showing significant volume changes between *LYP9* and its parents are labeled on the 2D gel. The spots numbering scheme was based on what is described in Figure 3 and Table 2.

but lower than *9311*. SAM catalyzes the synthesis of S-adenosylmethionine that serves as a methyl group donor in transmethylation of proteins, nucleic acids and a precursor in the biosynthesis of polyamines, biotin, and nicotianamine (Roeder et al., 2009). S-adenosylmethionine also is the precursor of phytohormone ethylene, which takes part in many important cellular processes in plants such as cell differentiation, cell expansion. Ethylene is produced at a faster rate in rapidly growing and dividing cells, and this might explain the RFW, RDW and TRN in *LYP9* are higher than its parents. Meanwhile, under stress conditions, SAM could possibly increase the lignifications by providing the methyl for the polymerization of lignin monomers and reinforce the cell walls to alleviate the damages caused by pathogen attacks (Yan et al., 2005). Yang et al. (2006) reported that the expression of SAM was up-regulated in cold stressed *LYP9* seedlings. Glucanase (spot 9), is commonly found in plants and there is abundant evidence that it is involved in the plants' defense mechanisms against pathogen infection (Simmons et al., 1992). As shown in Tab.2, about half of the identified protein spots (spots 4, 5, 7, 10, 11) belong to unknown or hypothetical proteins. In order to obtain more information about the unknown proteins, their protein sequences were used as queries to search for homologues by BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/) tool. The identified corresponding homologues classified into different functional groups are listed in Tab.2. Spot 4 was identified as Os12g0440200 which is similar to Zn-finger protein. In this study, we found that Os12g0440200 was up-regulated in *LYP9*, indicating the basic genetic processes including replication, repair, recombination, transcription and translation were impacted. The identified protein involved in signal transduction is Os03g0844100 (spot 7), which is similar to Pti1 kinase-like protein, a serine/threonine protein kinase that is specifically phosphorylated *in vitro* by Pto and is involved in the hypersensitive response (Zhou et al., 1995), putative transcription factors that are similar to the tobacco ethylene-responsive element-binding proteins (Zhou et al., 1997; Ohme-Takagi et al., 1995). Spot 5 and spot 10 were identified as hypothetical protein OsI_01694 and OSJNBa0018J19.13, respectively. These two proteins are all involved in energy metabolism. Spot 11 was identified as Os12g0555000, which is similar to Probenazole-inducible protein (PBZ1).

Table 2. Differentially expressed root protein spots between *LYP9* and its parents identified by MALDI-TOF-TOF MS. The spot number from the 2-D gel, the accession number, putative protein identification, functional classification, and differential expression pattern between *LYP9* and its parents for each protein identity are presented

Spot No.[a]	Protein name	Accession NO.	Mr (Da)/p*I*[b]	Protein score	Functional categories	Expression patterns[c]
1	5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase	gi|108862990	85/5.93	139	Secondary metabolism	P＞L＞N
2	phenylalanine ammonia-lyase	gi|82496	76/6.11	118	Metabolism	L＞N＞P
3	phenylalanine ammonia-lyase	gi|82496	76/6.07	130	Metabolism	L＞N＞P
4	Os12g0440200	gi|115488414	157/8.42	66	Transcription regulation	L＞N＝P
5	hypothetical protein OsI_01694	gi|125525704	49/6.26	149	Energy metabolism	L＝N＞P
6	S-adenosylmethionine synthetase	gi|100801534	43/5.83	122	Secondary metabolism	N＞L＞P
7	Os03g0844100	gi|115456539	41/6.15	72	Signal transduction	P＞L＞N
8	putative PrMC3	gi|51090388	35/5.61	110	Metabolism	L＞N＞P
9	Glucanase	gi|13249140	35/5.92	91	Metabolism	P＞L＞N
10	OSJNBa0018J19.13	gi|38346452	17/5.37	17	Energy metabolism	N＞L＞P
11	Os12g0555000	gi|115489014	17/4.88	81	Disease and defence	N＞P＞L

Note: [a] Spot numbers match those marked in Fig. 1. [b] Mr, theoretical relative molecular mass in kD; p*I*, theoretical isoelectric point. [c] Protein expression patterns were summarized; N, L, and P stand for *9311*, *LYP9*, and *PA64S*, respectively.

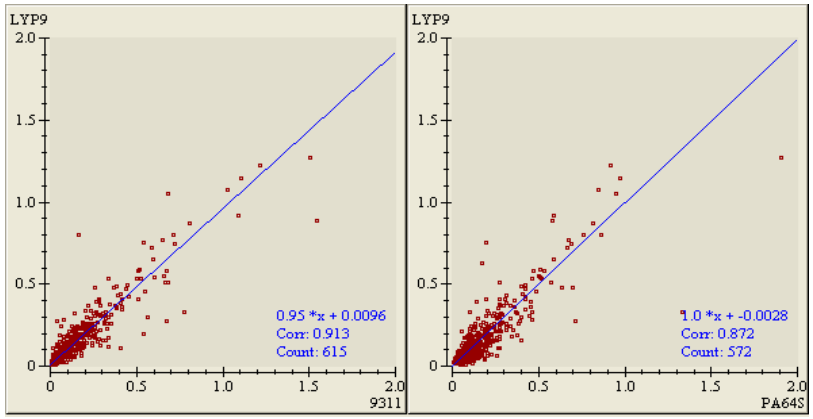

Fig 2. Scatter plots of 2-DE analysis of total proteins between *LYP9* and its parents.
Note: The gel maps were analyzed by ImageMaster 2D Platinum Trial software taking the volume fraction as the spot value type, and the x-axis indicates the abundance of the proteins from one 2-DE map, and the y-axis indicates those from the other 2-DE map.

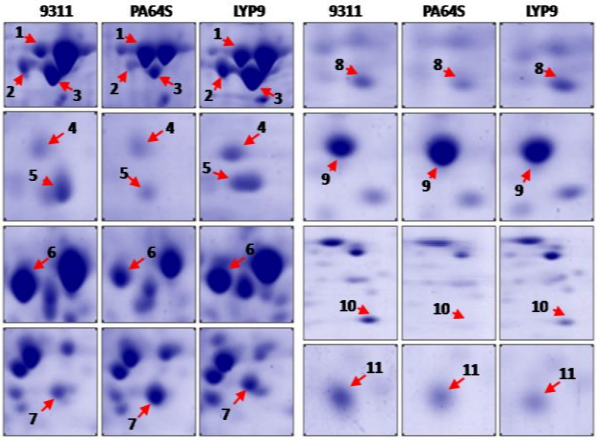

Fig 3. Differential expression of proteins among *9311*, *PA64S* and *LYP9*. Total proteins were extracted from the rice roots and resolved by two-dimensional polyacrylamide gel electrophoresis maps. The corresponding portions of the 2-DE gels were cropped for comparison. Arrows indicate the positions of protein spots showing differential expression.

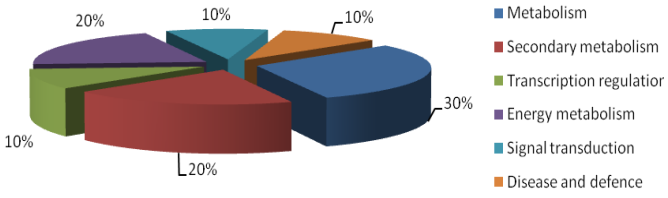

Fig 4. Pie chart showing the distribution of the proteins identified between *LYP9* and its parents according to their functions. Six protein groups were categoried based on putative functions.

PBZ1, a rice PR10 (pathogenesis-related protein 10) protein, was induced by probenazole (Oryzemate, 3-allyloxy-1, 2-benzisothiazole-1, 1-dioxide) an effective fungicide against rice blast disease, and has important function in disease resistance in rice (Midoh and Iwata, 1996). It should be noted that Os12g0555000 has lower expression in the hybrid than its parents. The results suggested that as compared with its parents, the hybrid had a better anti-disease system to protect cell against viral-induced damage and environmental stresses.

Materials and Methods

Rice culture conditions

LYP9 and its parental lines (*9311* and *PA64S*) were used as the experimental material. Rice seeds were sterilized with 10% H_2O_2 for 10 min and 70% ethanol for 5min, followed by a thorough washing in distilled water. The sterilized seeds were soaked in distilled water and germinate at 30℃ in an incubator for 48h. Rice seedlings were grown hydroponically in a greenhouse in Hoagland nutrient solution under the natural light. The pH of the solution was daily adjusted to 5.5, and the entire nutritive solution was changed weekly. After culturing for 25 days, whole rice roots were harvested, weighed, and immediately frozen in liquid nitrogen and then stored in a –80°C freezer until protein extraction.

Root traits measurement

Roots were carefully harvested to assure the removal of the whole root system and minimize losses. The root system were dried in an oven at 120°C for 0.5 h, then 80°C for 24 h, for dry weight determination. Four root traits were characterized including taproot number (TRN), maximum root length (MRL), root fresh weight (RFW), root dry weight (RDW). Statistical analysis of the differences in root traits was performed by using SPSS software.

Extraction of rice root proteins

About 2 g root samples were ground in a prechilled mortar containing 10% w/w polyvinylpolypyrrolidone (PVPP) of sample weight. The powder sample was homogenized in 10 mL of ice-cold Mg/NP-40 extraction buffer containing 0.5 mM Tris-HCl, pH 8.3, 2% v/v NP-40, 20 mM $MgCl_2$, 2% v/v β-mercaptoethanol (β-ME), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% w/v PVPP. The mixture was sonicated in an ice bath for 10 min and shaken on ice for 30 min. After centrifugation at 12,000×g for 15 min at 4°C, proteins in the supernatant were precipitated by adding four volumes of cold acetone containing 10% w/v trichloroacetic acid (TCA) and 0.07% v/v β-ME at –20°C for at least 3 h. The precipitated proteins were washed with ice-cold acetone containing 0.07% v/v β-ME. The treatment was repeated until the supernatant was colorless. Pellets were vacuum-dried and stored at –80°C.

2-DE experiments and gel staining

The 2-DE protocol was performed by O'Farrell (1975).With some modifications. IEF gel was carried out in glass tubes, 2.0 mm inner diameter and 17 cm in length. The bottom of tube was sealed with parafilm. The 4 tubes of IEF gels were made as follows :1.44 g urea, 0.35 mL of acrylamide solution (28.38% w/v acrylamide and 1.62% w/v bisacrylamide), 0.60 mL 10% v/v NP-40, 0.85 mL ddH_2O, 0.15 mL ampholine (pH3.5-10:pH5-8=1:4).To polymerize the gel, 5.60 μL 10%v/v ammonium persulfate and 1.40 μL N, N, N', N'-tetramethylethylenediamine (TEMED) were added into the gel mixture. The tube was filled with the gel solution up to 17 cm and overlaid with overlay solution (5 M urea). After the gel polymerization, this overlay solution was removed. Each sample (200 μg protein) was loaded. IEF was performed at 200V, 300V, 400V, 500V, 600V for 0.5 h per step, and 800V for 16.5 h, and then 1000V for 4h. The cathode buffer was 20 mM NaOH and the anode buffer was 10 mM H_3PO_4. Upon completion of IEF, the gel was extruded using a syringe and then rinsed with Double distilled water. The focused gel was put into a 10 mL centrifuge tube with 5 mL of an equilibration buffer that contained 10% v/v glycerol, 2.5% w/v SDS, 125 mM Tris-HCl (pH 6.8), 5% v/v β-ME and then shaken gently for 20 min. The second dimensional electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). The focused gel was transferred onto 10.0%w/v SDS-PAGE self-cast gels. Electrophoresis was carried out at 20 mA per gel for 30 min and 15 mA pel gel until the dye had reached the bottom of the gel. The gels were stained by the method of Candiano et al (2004). Before dying, gels were fixed with 50%v/v methanol and 5% v/v acetic acid for at least 3 h, and then the gel transferred into staining solution (0.12% w/v CBB G-250, 10% w/v ammonium sulfate, 10% w/v phosphoric acid, 20% v/v methanol) overnight with gentle shaking. After staining, the gel was decolorized with distilled water.

Image acquisition and data analysis

The gels were scanned using ScanMaker 8700 (Microtek) at a resolution of 1000 dots *per* inch. Data were analyzed using Image Master 2D Platinum Version 5.0 Analysis Software (Amersham Pharmacia). The abundance of each protein spots was estimated by the percentage volume (%Vol). Only those with significant and reproducible changes were considered to be differentially accumulated proteins.

MALDI-TOF/TOF MS analysis and identification of proteins

Protein spots with differential expression patterns on gels were manually excised from gels, washed three times with ultrapure water, and digested with sequencing-grade trypsin (Promega). Mass spectra analysis was performed on an Utraflex III MALDI-TOF/TOF MS (Bruker, USA). All spectra of proteins were submitted to database searching using online MASCOT program (http://www.matrixscience/com) against NCBInr databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the corresponding protein identify.

Metabolic pathway identification for proteins

The functional information of the identified proteins was extracted from Gene Ontology Database (http://www.geneontology.org), and the pathway was searched from GenomeNet (http://www.genome.ad.jp/kegg). The functional categories were decided according to Bevan et al (1998).

Conclusion

Based on the observations described above, we firstly employed to detected differential proteome expression of seedling root between *LYP9* and its parents, making it plausible to explore the molecular mechanism related to the heterosis of hybrid rice. With our preliminary results, we observed that a large portion of DEPs in the hybrid expressed at a higher level than those of its one or both parents, which are involved in a wide range of cellular processes, *e.g.* metabolism, secondary metabolism, Transcription regulation, energy metabolism, signal transduction. Moreover, the differentially expressed proteins could work together to play a major role in the induced defense response.

These results indicate that: compared with its parents, *LYP9* have an extraordinary capacity to perceive changes in their external environment and adapt rapidly to maximize opportunity and minimize risk. This plasticity depends on the evolution of diverse mechanism to regulate cellular homeostasis. So that is why *LYP9* had a wide adaptation range of region, high yield, high quality and disease resistance. Deeper proteomic analysis may help us to better understand the mechanism of heterosis and give reference for molecular breeding in rice.

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