26.1

Tuber borchii Vittad. Mycelial Hyphae; Protein Expression Profile and Protein Identification

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The *Tuber borchii* Vittad. ectomycorrhizal fungus is an hypogeous ascomycete that forms highly specialised symbioses with the fine roots of gymnosperms and angiosperms. Owing to the truffle central relevance, as ectomycorrhizal partners, in the ecosystem biodiversity preservation of boreal and temperate forests, and to the well known organoleptic properties of their fruit bodies, interest in the biology of the *Tuber* genus members is growing rapidly. However, in spite of the researcher enthusiasm, very few information are currently available about the molecular and cellular processes which take place during the truffle life cycle. In attempt to extend our knowledge in this research field, efforts were directed to resolve and characterise the protein pattern of truffle secondary mycelial hyphae, and, despite the several difficulties encountered, *Tuber borchii* electropherograms and some protein spot identifications were obtained. The public database lack of truffle genomic sequences, except for very few EST-sequences, make protein identification difficult. Actually, the numerous MALDI-ToF MS and the ESI-Ion trap MS/MS acquired spectra, despite their good quality, did not lead to any identification when the software search was carried out in SwissProt, NCBInr and EST others databases. The cross-specie identification failing further emphasizes the biological peculiarity of this hypogeous symbiont. The presented MS positive results were achieved exploiting our own EST-sequence database that we recently set up from tuber fruit-body cDNA libraries. Such database immediately resulted to be an invaluable tool in our truffle proteomics investigations.

26.2

Proteomic Strategy for Investigation of Cytokinin Action in *Arabidopsis thaliana*

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Plant growth is accomplished by orderly cell division and tightly regulated cell expansion. Coordinate control of plant growth is regulated by both external stimuli and internal mechanisms. The internal components of plant signalling are generally mediated by chemical growth regulators (phytohormones). Cytokinins (CKs) represent a class of plant-specific hormones that play a central role during the cell cycle and influence numerous developmental programs.

A highly sensitive proteomics-based approach for the investigation of Arabidopsis thaliana seedlings with CK overproduction was applied. Low protein concentration was loaded on an IPG strip in the first dimension. Sensitive fluorescent staining was used for protein detection after SDS-PAGE. Spots of interest were excised and in-gel digested. We used both MALDI-MS and LC-MS/MS techniques for peptide analysis.

Plantlets overproducing CKs showed significant differences in protein quantity compared to controls. Due to low protein concentration no MS-spectra were obtained for many samples provided by MALDI-MS. However, most of these proteins were successfully identified using LC-MS/MS method.

Our results showed the effective application of different methods for protein identification. In our case the combination of MALDI-MS and LC-MS/MS increased the number of identified proteins to over 90% of all analysed samples. Our comparative proteomic approach enables us to elucidate CK role(s) in cellular function directly via protein elements. We identified new protein candidates potentially involved in CK action that have not been studied so far.

26.3

Two-dimensional Gel Electrophoresis and Subsequent Protein Identification via MALDI-MS/MS; a Successful Approach to Unravel the Abiotic Stress Responses in a Non-model Organism (*Musa* spp.)

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Bananas and plantains (*Musa* spp.), with an annual production of about 100 million tons, are important throughout the developing countries of the (sub)tropics both as a subsistence and export crop. Through breeding and farmer selection, varieties are available with different degrees of tolerance towards (a)biotic stresses. Proteomic research in non-model plants is often hampered by the lack of routine sample preparation procedures and the dependence of protein identification on orthologous proteins. Banana is a difficult plant species since it contains extremely high amounts of interfering metabolites and its genome is poorly characterized. Using a 2DE protocol developed for small amounts of tissue (Carpentier *et al., Proteomics* 2005, in press) and cross species MS/MS identification (Witters *et al.,* RCMS 2003), we investigate the influence of sucrose preculture on the proteome of meristematic cells in a drought and cryopreservation tolerant and a susceptible cultivar. We have shown that a two-week preculture on 0.4M sucrose is essential for the acquisition of tolerance towards the severe dehydration prior to cryopreservation (Panis *et al., Plant Science* 1996). Up till now, we were able to classify 40 proteins that were significantly up-regulated and 19 proteins that were down-regulated due to the sucrose treatment. We classified the abiotic stress response proteins within different categories and put forward some intra- and inter-categorical protein interactions. Sucrose up-regulated categories are: (i) energy (35%), (ii) protein destination (20%), (iii) disease and defense (13%), (iv) signal transduction (10%), (v) transporters (5%), (vi) transcription (5%), (vii) secondary metabolism (5%), (viii) metabolism (3%) and (ix) cell structure (3%). Sucrose down-regulated categories are: (i) metabolism (39%), (ii) storage (39%) and (iii) protein synthesis (22%). This work proves that quantitative differential proteome analysis is also successful for recalcitrant non-model crops and sheds new light on sugar metabolism and the adaptation towards dehydration tolerance.

26.4

Extracellular Matrix Proteome of Chickpea (*Cicer arietinum*) Illustrates Pathway Abundance, Novel Protein Functions, and Evolutionary Perspective

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Extracellular matrix (ECM) is a dynamic system that serves as the first-line mediator in cell signaling to perceive and transmit extra- and inter-cellular signals during cell-cell communications, cellular development and also in response to environmental and pathogenic factors. Although ECM is a conserved compartment ubiquitously present throughout evolution, a compositional variation does exist amongst different organisms. It is essentially composed of carbohydrate polymers, proteins, phenolics and lipids. Increasing evidence suggest that ECM proteins are linked to numerous molecules with various functions but the complexity of ECM proteins and their role in cell signaling is poorly understood. In an attempt to understand the function of ECM proteins, we have developed a proteome from ECM enriched fraction of a crop legume, chickpea using 2-DE gel electrophoresis. 131 ECM proteins were identified by MALDI-TOF mass spectrometry, ion-trap MS-MS and genomic database searches and functionally categorized into 11 different classes. Our data shows involvement of the ECM in different important metabolic pathways. We also report the comparative biology and evolution of ECM proteome at organismal level. This is a comprehensive overview of the chickpea ECM proteome, which would provide a basis for future comparative proteomic efforts for this important legume crop. We report here evidence for new ECM proteins of unknown functions vis-à-vis the presence of many known cell wall proteins of prokaryotes and other higher eukaryotes. In addition, we report the presence of unexpected proteins with known biochemical activities, which have never been associated with ECM. This is one of the first report of ECM proteome in any plant whose genome is yet to be sequenced.

26.5

Out on a Limb; Forging a New Trail in Forest Research with Proteomics

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With the completion of the first tree genome sequence, from *Populus trichocarpa* (black cottonwood), forestry genomics is moving rapidly to advance biological knowledge and potentially aid future tree breeding programs. Treenomix is a forestry genomics research program based at the University of British Columbia. This program combines a variety of genomic tools with the application of high throughput proteomic techniques to investigate forest health and wood formation. The focus is on two economically important Canadian tree species, spruce and poplar, in addition to *Arabidopsis*, which is a model of relevant plant biology and readily available for the optimization of new techniques. We have employed a suite of proteomics technologies to monitor protein expression profiles within these biological systems. These investigations include profiling of vascular (wood-forming) tissues and an examination of the natural responses of these plants to insect attack. Gel-based investigations of spruce tissues have achieved a rate of protein identification exceeding 70% in the absence of a sequenced genome through the use of large EST resources developed within the project. Gel-free studies have increased the throughput of profiling experiments and have allowed the investigation of very low abundance proteins such as transcription factors. In addition, quantitative profiling of posttranslational modifications (phosphorylation) has been possible through a combination of techniques (isotopic labeling and precursor ion scanning). The results presented showcase the pliability of the various proteomics technologies currently available in their application to a multifaceted research program.

26.6

Hiding Behind Hydrophobicity; Transmembrane Segments in Mass Spectrometry

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Proteomics of membrane proteins is essential for the understanding of cellular function. However, mass spectrometric analysis of membrane proteomes has been less successful than the proteomic determination of soluble proteins. To elucidate the mystery of transmembrane proteins in mass spectrometry, we present a detailed statistical analysis of experimental data derived from chloroplast membranes. This approach was further accomplished by the analysis of the *Arabidopsis thaliana* proteome after *in silico* digestion. We demonstrate that both the length and the hydrophobicity of the proteolytic fragments containing transmembrane segments are major determinants for detection by mass spectrometry. Based on a comparative analysis, we discuss possibilities to overcome the problem and provide protocols to shift the hydrophobicity of transmembrane segment-containing peptides to facilitate their detection.

26.7

Membrane Proteomics of *Arbuscular mycorrhizal*

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The *Arbuscular mycorrhizal* (AM) fungi, belonging to the Glomeromycota, undergo symbiosis with most terrestrial plants. The resulting structure formed between plant roots and fungi is called a mycorrhiza. By increasing root spatial exploitation of the soil through its extraradical mycelium, the AM fungus improves plant acquisition of mineral nutrients whilst in turn, it benefits from plant carbohydrates. Root colonisation is characterised by the hyphal growth within the host cortical tissue, with differentiation of branched haustoria, the arbuscules, in which the fungus is surrounded by a host-derived plasma membrane, called the periarbuscular membrane. The arbuscules are thought to be the main sites for nutrient exchanges between the 2 symbionts. To gain access to the membrane proteins differentially expressed in mycorrhiza, we are carrying comparative proteome analyses of Medicago truncatula roots inoculated or not with an AM fungus. Methods included separation of extrinsic membrane proteins, comparative 2DE and identifications by MALDI-TOF and/or ESI-MS/MS analyses. To focus on more intrinsic membrane proteins possibly involved at the symbiotic interface (arbuscules), we also targeted plasma membrane proteins, which were analyzed using 2 different methods. Firstly, following trypsic digestion, peptide separation and analysis were performed with 2D LC-MS/MS. Secondly, SDS-PAGE separations were combined with LC-MS/MS analyses. Taken together, these sub-cellular proteomic approaches allowed to identify numerous *M. truncatula* root proteins. Moreover, most of the proteins identified in response to the fungal colonization have not been previously reported as regulated during the symbiosis, bringing new informations to understand the functioning of such interactions.

26.8

A Comparative Proteomics Approach to Identifying Salt and Drought Tolerance Genes in Plants

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Drought and salinity are the major limitations to agricultural production worldwide. The identification of candidate genes for marker-assisted selection could greatly improve the efficiency of breeding for increased drought and salt tolerance. We applied a comparative proteomics approach to discover the genes and pathways that are crucial for drought and salt tolerance. Proteome response of rice, wheat, sugar beet, and *Suaeda aegyptiaca* and *Elymus elongatum* to salt and/or drought stresses were studied using two dimensional electrophoresis coupled with mass spectrometry. We identified several tolerance candidate proteins and pathways involved in redox regulation, oxidative stress tolerance, cytoskeleton remodeling, osmotic homeostasis, signal transduction, and protein synthesis and folding. Comparative proteome analysis of these species and the strategies for maximizing the success of such applications will be discussed.

26.9

Rearrangement in the Chromatin Parts under Influence of Non-thermal Electromagnetic Radiation

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Gene function is subjected to the effects of surrounding chromatin. The nature of these effects may be epigenetic occurring in some cell, but under influence of some external factors these processes can be disrupted. Inside the nucleus there are three structural compaction of DNA. Regions of dense heterochromatin masses scattered throughout the interphase nucleus. Only recently has some understanding of the mechanisms of its formation and propagation been achieved. Heterochromatin is divided into constitutive heterochromatin, containing satellite DNA and facultative heterochromatin, inactive in certain cell lineage but expressed in other lineages. Heterochromatin is involvement in epigenetic silencing phenomena including repression along extended regions of chromosomes and the inactivation of whole chromosomes. The potential of heterochromatin to silence of nearby genes, a phenomenon known as position effect variegation, has been both puzzling and attractive for scientist since its discovery. In the presented work we study changes in melting parameter of chromatin having different localization in nucleus: euchromatin and heterochromatin under influence of coherent non thermal electromagnetic irradiation on wheat seeds during germination. The EHF generator with the range of working frequencies 37,5–53,5 GHz was used as a source of monochromatic radiation of mm-waves. The irradiation was carried out in a distant zone of radiation of the generator. Our experimental data allows us to expect that influence of coherent non thermal electromagnetic irradiation on wheat seeds during germination lead to the significant changes in some part of heterochromatin and in result to increasing of preservation system of living organisms.

26.10

Proteome Analysis of Cold Stress Response in *Arabidopsis thaliana*; a Quantitative Proteomic Study Using Shotgun Technology

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The comparative analysis of protein patterns from different samples or treatments is an important component in proteome studies to understand the underlying biological system. In this expression proteome study we used wild type plants from *Arabidopsis thaliana* (Col 0) to investigate the cellular responses to cold stress. Two approaches have been used; a 2D-gel method and an LC-MS based strategy. Plants were germinated under short day conditions for three weeks then half of the plant material was subjected to cold stress conditions (6 °C) and the other half kept at 20 °C. After one week cold stressed plants were transferred back to control conditions. *Arabidopsis thaliana r*osette leaves were harvested at he end of each week and directly frozen in liquid nitrogen. Proteins were precipitated according Damerval *et al*., (*Electrophoresis* 1986, *7,* 52–54) and dissolved in lysis buffer without primary amines such as DTT and ampholytes. The dissolved proteins were either run via 2D gel electrophoresis or reduced, alkylated and digested with trypsin for analysis via LC-MS. Preliminary results show subtle changes in protein regulation across many systems, and a variety of types of response have been observed. Kinase F4H5–18 is the most down regulated protein in cold shock, and this appears to change little after one week of recovery at 20 °C. Carbonic anhydrase is down regulated in cold shock, but returns to normal after one week of recovery. The photosystem II oxygen evolving complex protein is down regulated under cold shock, but appears to overcompensate by up regulating during the recovery phase.

26.11

Variations in Peroxidase due to Environment Change

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Plants are good natural sources of an important group of proteins, peroxidases. These are haemoproteins with the enzyme classification number PODs, E.C. 1.11.1.7. They have the ability to catalyze the oxidation of a large variety of substrates through a reaction with hydrogen peroxide. They are widely used in clinical biochemistry and in enzyme immunoassays. Some novel applications of peroxidases include treatment of wastewater containing phenolic compounds, synthesis of various aromatic chemicals and removal peroxide from materials such as foodstuffs and industrial wastes. Horseradish root tubers are commonly employed as a commercial source for peroxidase production. However, other cultivated species may provide PODs exhibiting similar or better properties, especially recombinant species. Any climate variation may affect the activity of peroxidases present in different parts of a plant. In this research we compared the peroxidase activity in two groups of plane trees located at various environment conditions in a northern Iranian city, Rasht. It was found that peroxidase activity was remarkably higher in plane leaves collected from trees grown in polluted area of Rasht, when compared to the peroxidase activity in the same tree grown in an area of Rasht, where the car traffic was minimal. The results indicated that the heavy stress applied by toxic gases in polluted air induces a high rise in the activity of peroxidase. As the activity is directly related to the peroxidase content of the leaves, we concluded that the waste leaves of plane trees collected from highly polluted area could be a good source of peroxidase and a possible replacement for horseradish peroxidase (HRP). We also studied the characteristics of this peroxidase and its kinetics and found many similarities with horseradish peroxidase.

26.12

Tracing Altered Metabolic States in the Thylakoid Membrane

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Two-dimensional electrophoresis based on isoelectric focusing with immobilized pH gradients in the first dimension and SDS-PAGE in the second dimension is the standard method of protein separation in proteomic approaches. However, despite recent developments, isoelectric focusing is still not the method of choice for separation of hydrophobic membrane proteins. A promising alternative is blue-native polyacrylamide gel electrophoresis (BN-PAGE) capable of separating native, catalytically active membrane protein complexes (*e.g.* Schägger, *Methods Cell. Biol.* 2001, 65, 231–244).

Following to mild solubilization with digitonin, we have applied BN-PAGE to thylakoid membranes as first separation dimension and Tricine SDS-PAGE as second dimension. This technique enabled us to identify integral membrane proteins from the green algae Chlamydomonas reinhardtii by peptide mass fingerprinting and matrix assisted laser desorption/ionization-mass spectrometry (MALDI-MS) (Rexroth *et al*., *Electrophoresis* 2003, 24, 2814–2823).

Even extremely hydrophobic subunits of the photosystems with 5–11 transmembrane helices were identified, which could not be accessed by in-gel digestion in previous studies. In addition to well-known proteins of photosynthesis, a dimer of the chloroplast ATP synthase was found for the first time (Rexroth *et al*., *Biochim. Biophys. Acta* 2004, 1658, 202–211). In recent work we have compared different metabolic states of Chlamydomonas reinhardtii. The green algae was cultivated under photoautotrophic, photomixotrophic or heterotrophic conditions. Variation of selected proteins and membrane protein supercomplexes was quantified by an isotope labelling technique with N-15 in combination with MALDI-MS. The response of Chlamydomonas reinhardtii to altered culture conditions was a variation of the assembly of photosystems and light harvesting complexes showing so called state-transitions. Furthermore, the abundance of the dimer of chloroplast ATP synthase was changed.

26.13

Characterization of Regulatory Genes in the Secondary Meristem of Populus by *in Situ* Protein Localization

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Trees maintain their stem cells population for hundreds of years, with continuous production of biomass as a result. However, the genetic mechanisms for regulation of growth in secondary meristems are essentially unknown. We aim to find and characterize the genes involved in this process in order to understand if increased productivity can be reached by manipulation of the pool of stem cells, the xylem and phloem mother cells or the differentiating cells. The goals will be achieved by functional analysis of potential target genes. Currently, the tools for this type of analysis include RNAi knock-down and overexpression under cambium-specific promoters. This study aims to introduce a complementary approach based on *in situ* protein localization, which allows for higher resolution and analysis of protein activity on the cellular and sub-cellular level. Antibodies to the target proteins will be produced in using a sophisticated design procedure, allowing selection of epitopes that will ensure specificity to the marker protein. Thin tissue sections of the meristem will be stained by immunohistochemical methods. More than 200 transgenic trees from a commercial partner through a collaboration with SweTree Technologies (www.swetree.com).

26.14

Using Proteomics Approach to Help the Rice Genome Annotation

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All sequenced genomes have contained a large proportion of genes with no obvious homolog in the databases. They have been called orphans or LH (Low Homolog) genes. They are often dismissed as artifacts of the gene prediction software or as a transient phenomenon that will disappear as more genomes are sequenced. However, despite the growing databases, the LH gene's number continues to expand, and some believe it is time to take them more seriously.

At least one third of rice genes show no obvious homolog in *Arabidopsis*. The number of LH genes came from a non-redundant set of 19,079 full-length cDNAs to avoid the problem of *ab initio* gene predictions. Large scale proteomics work is applied here to independently confirm that these LH genes are real. The protein confirmation is done through mass spectrometry methods, preceded by 2D-gel electrophoresis (2D-MS) or liquid chromatography (LC-MS)for separation purposes. Similar efforts were expended on both separation technologies. Of the 3276 nr-KOME cDNAs with a proteomics confirmation, 9.2% are confirmed in 2D-MS ($p < 0.05$), and 95.5% are confirmed in LC-MS (score>2400). The net confirmation rate is 20.6% for HH (High Homolog) genes and 10.6% for LH genes. Although it is not obvious, this is consistent with the mRNA expression data.

We will show that these LH genes are by-products of a massive ongoing process of evolution by gene duplication. Although some LH genes are likely to be dead, many others are evolving lineage-specific functions.

26.15

Proteome Analysis of Barley Seeds

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Barley (*Hordeum vulgare*) is an important cereal crop in northern Europe grown both for the feed and malting industries. Hence, there is a high interest to gain deeper insight into the determinants of nutritional quality at the molecular level in order to improve the assessment of new traits.

In the GABI-SEED II project, barley is used as a model plant to explore gene expression networks determining seed traits. In an Genetical Genomics approach, gene expression profiling and marker-based fingerprinting of related lines are used to identify trait-related genes by treating gene expression as inherited characters. By using a range of genetic, biochemical, molecular and bioinformatic tools we want to characterize barley introgression lines for marker-assisted breeding on the molecular level as well as to identify quantitative trait loci (QLT's).

In our sub-project we focus on the analysis of the content and composition of ripe seed proteins and investigate introgression lines presenting a wild barley genome (*Hordeum spontaneum* HS213) within a modern breeding line background (*H. vulgare* cv. Brenda). Aim of this protein profiling is the identification of proteins showing significant variability between the lines in order to link the results of this analysis with data gained by the transcriptional and metabolite profiling in order to calculate QLT's.

In the analysis, the water soluble fraction of proteins is investigated. Spots with at least 2-fold altered expression between BRENDA and the line under investigation are identified by MALDI-TOF and subsequent database searches. In addition, ESI-MS/MS is currently established in the laboratory and will be used for the *de novo* sequencing of spots not identified by peptide mass fingerprinting.

26.16

Effects of Copper (Cu^{2+}) on Chlorophyll, Proline, Protein, and Abscisic Acid Level of Sunflower (*Helianthus annuus* L.) Seedlings

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This study was designed to examine the effects of copper chloride ($CuCl_2$) on the level of chlorophyll ($a+b$), proline, protein and abscisic acid in sunflower (*Helianthus annuus* L.) seedlings. One-week-old seedlings were grown at copper concentration of 0.4, 0.5 and 0.6 mM for ten days in Hoagland solution at room temperature. It was observed that the level of chlorophyll ($a+b$) and total protein ($p < 0.05$ or $p < 0.01$) remarkably decreased as copper concentration increased to 0.6 mM, although the levels of proline and ABAin the leaves of plant were increased a dose-depended behavior. The same trends were also observed with the level of ABAof stems and roots. Copper has dose-depended effects on chlorophyll, proline, protein and abscisic acid level of sunflower (*Helianthus annuus* L.) seedlings. Thus, we assumed that copper levels increases above some critical points seedling growths get negative effects. This assumption is in line with previous findings.

26.17

The Proteomic Comparison of the Rice with and without the Transformation of Genomic DNAs from *Echinochloa crusgalli*

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The transformation of distal related genomic DNAs into plant was proposed a novel technique to breed new cultivars. Through transformation of genomic DNAs of *Echinochloa crusgalli* (C4 plant) to R207, a new rice line, restorer line RB207, has been successfully developed and stabilized to 7 generations. In spite of the phenotypes of this variant line apparently different from its receptor, the molecular bases are obvious lack. Herein, we have systematically studied the differential proteomes from the tissues of *Echinochloa crusgalli,* R207 and RB207, to inquire about a reasonable explanation of phenotypic changes in RB07. The technique of 2DE was employed to separate the proteins from leave and embryo of *Echinochloa crusgalli,* R207 and RB207, respectively. The different spots on 2DE were identified by MALDI-TOF-MS or LC-ESI-MS/MS. In leaf, 953, 1084 and 1091 silver stained spots were detected, whereas in embryo, 986, 883 and 892 spots were found from *Echinochloa crusgalli,* R207 and RB207, respectively. Compare of these 2DE images, few unique different spots were verified between *Echinochloa crusgalliand* RB207, however, a number of unique different spots were confirmed between the two rice cultivars, 38 leaf spots and 39 embryo spots, respectively. Furthermore the spot identification with mass spectrometry revealed no direct linkage between *Echinochloa crusgalland* RB207, either in leave or in embryo, suggesting that no barnyard genes are expressed in the new rice cultivar. With approximately 75% identification rate, total spots of 27 in leaf and 31 in embryo were confirmed to be unique different rice proteins.