Advances in Biological Research 3 (5-6): 237-241, 2009
ISSN 1992-0067

In vitro and *In vivo* Identification of Variation in Protein Expression in *Artemisia vulgaris* L.

S. Pradeep Kumar and B.D. Ranjitha Kumari

Stress Physiology and Medicinal Plant Biotechnology,
Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India

Abstract: Proteomics has become an important approach for investigating cellular processes and network functions. Significant improvements have been made during the last few years in technologies for high-throughput proteomics, both at the level of data analysis software and mass spectrometry hardware. This paper describes an efficient method to identify the variation of protein expression in *in vitro* and *in vivo* plants of *Artemisia vulgaris*. Leaf samples were collected from both micropropagated plants raised in *in vitro* and *in vivo* plants. This study shows that 59 µg of protein is present in *in vitro* leaf samples [50 µl]. Where as *in vivo* plants have 58 µg of proteins in 50 µl of leaf sample. We also found 20 clear protein bands, in which protein bands of Molecular weight 64, 48 and 37 kDa were significantly increased in *in vivo* and where as 79, 53 and 28 kDa proteins were significantly increased *in vitro* plant samples. The intensity of protein bands was high in *in vitro* sample compared to *in vivo* protein samples. This is the first report on protein identification and variation in *in vitro* and *in vivo* plants of *Artemisia vulgaris*.

Key words: *Artemisia vulgaris* · Bradford method · Image analysis · MALDI-TOF-MS · Protein Identification

INTRODUCTION

Artemisia vulgaris L. (mugwort) belongs to the family *Asteraceae* and is a tall aromatic perennial herb, which grows 1-2m tall. Mugwort contains volatile oils, sesquiterpene lactones and flavonoids used for insecticidal, antimicrobial and antiparasitical properties. In traditional medicine, this plant is being widely used for the treatment of diabetes, epilepsy, depression, insomnia and anxiety stress [1].

All parts of the plant are antihelmintic, antiseptic, antispasmodic, carminative, cholagogue, digestive, expectorant, nervine, purgative and stimulant. The essential oils of the plant were reported to exhibit 90% mosquito repellency against *Aedes aegypti*, a mosquito that transmits yellow fever [2]. A paste or powder of the leaves is applied over skin diseases [3]. In recent years, there has been an increased interest in *in vitro* techniques, which offers powerful tools for germplasm conservation and the mass multiplication of many threatened plant species [4].

Arabidopsis thaliana has become the plant model organism of choice for which a systems level understanding of complex cellular processes seems to be within reach [5]. Global analysis of the system components [DNA, RNA, proteins, metabolites] is now possible, although at different analytical depth at present. High-quality genome sequence information is available for *Arabidopsis* [6] and based on this information Gene Chips have been developed to analyses the transcriptional activity of most predicted genes. The analysis of all proteins [proteome] and all metabolites [metabolome], however, continues to pose significant challenges. Proteins and metabolites are more diverse and biochemically heterogeneous, which precludes the application of a single standardized procedure for their analysis [7].

MALDI-TOF MS has the potential to address the most challenging issues of species and adulterant identification and product consistency, which also having the potential to support breeding programs and rapid plant screening for phytochemical quantity and quality. The homology-based searching with MALDI-TOF data from a pea protein resulted in the successful identification of a protein that previously had been sequenced only in Arabidopsis. Development of improved high-throughput

Corresponding Author: B.D. Ranjitha Kumari, Department of Plant Science, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India

proteomics techniques has shifted attention to 'protein profiling', which attempts to identify all proteins that are present in a cell. Protein profiling in high-throughput mode is relatively simple and provides a snapshot of the major protein constituents of the cell [8]. With the advent of proteomics and mass spectrometry [MS], systematic identification of proteins has become possible, as demonstrated in several studies in different organisms [9], including plants [10].

Analysis of a complete proteome remains a challenge despite significant advances in mass spectrometry technology and peptide fractionation tools. Such a challenge can best be tackled by a community effort. Integration of data from different sources will increase the information to expand proteome coverage. Genome annotation based on peptide identification in particular requires an open source Platform to collect and integrate MS/MS data. The Peptide Atlas Platform [11] has the potential to develop into such an open source platform that will also serve the *Arabidopsis* community.

Several studies have reported on a successful reversible coupling of a MALDI source to an ESI tandem quadrupole/TOF mass spectrometer [12]. The possibility to routinely use two different ionization techniques (MALDI and ESI) in combination with MS/MS will increase the success rate of protein identification because each ionization method is generally quite selective for different peptides. Using both ionization principles will improve the "coverage" of the proteins and therefore the success of identification. Moreover, MALDI is less sensitive to salts and detergents thus reducing the need for "sample cleanups." In addition, a prototype of a new tandem mass spectrometer (MALDI-TOF-TOF) was reported, possibly improving both speed and sensitivity [13].

MATERIALS AND METHODS

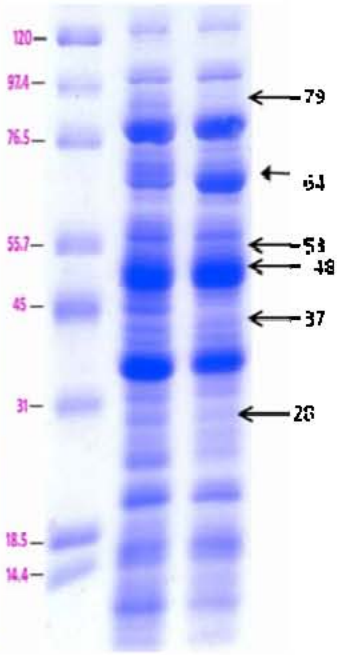
Seed Collection and Germination: *A.vulgaris* L. seeds were collected from Johnny's selected seeds, USA located at Winslow, Maine. Seeds of both *in vitro* and *in vivo* plant leaf samples were used for the study. The seeds were surface disinfected with 10% dettol solution for 5 min, followed by rinsing three to five times in sterile distilled water. The seeds were than surface sterilized with 0.1% [w/v] aqueous mercuric chloride [$Hgcl_2$] for 3 min and finally rinsed with autoclaved distilled water [four times]. Surface sterilized seeds were inoculated in Murashique and Skoog's [14] germination (MS)] medium [15]. Cultures were initially incubated in darkness for 5-7 days at a temperature of 23°C to facilitate germination. Later they were transferred to photoperiodic conditions and maintained for another 28-30 days for seedling growth. In similar way *in vivo* leaf samples were taken for present study.

Quantification of Proteins by Bradford Method: Leaf samples were collected from both *in vitro* and *in vivo* plants. Total proteins were analyzed by assay method [16]. Assay materials including color reagent [Coomassie Brilliant Blue G-250], Protein standard BSA [Bovine Serum albumin] and absorbance reading was measured at 595nm in light spectrophotometer. Bradford is recommended especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. In this method we used 100µl sample volume using 5 ml color reagent. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality.

Separation of Protein by SDS-PAGE: One gram Leaf samples were collected from both *in vitro* and *in vivo* plants, Proteins were separated by SDS-PAGE method (17) using a Hoefer Scientific Instrumentation apparatus with a discontinuous buffer system. Proteins present in leaf samples were Separated based on molecular mass. The protein components were resolved on a 12% gel, with a standard protein mixture for mol. wt. reference, at 50 v for 1h and 100 v for a further 2h. The gel was stained with Coomassie Brilliant Blue G-250 to visualize the separated proteins. Resolved components were blotted by a semi-dry electro blotter onto nitrocellulose paper Fig. 1.

Image Analysis [Lark] and In-gel Protein Digestion: The blotted protein gel was used for Image analysis for *in vitro* and *in vivo* leaf sample proteins. It shows 20 clear protein bands and intensity of protein bands were high in *in vitro* sample compared to *in vivo* protein samples. The experiments were repeated three times and only the protein bands which are differentially expressed were excised and subjected to in-gel digestion process according to the guidelines given in the In-Gel Tryptic Digestion Kit [Pierce, IL, USA] was identified by MALDI-TOF-MS (Fig. 1).

MALDI-TOF-MS Analysis: The differentially expressed protein samples used for MALDI-TOF MS analysis was performed on a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Bio-systems, Foster City, CA). A vital component of higher-throughput method



(1) (2)

Fig. 1: Artemision vuigaris Plant
Fig. 2: in vitro and in vivo protein profile

development is sample preparation prior to MALDI-TOF MS analysis. Ideally, the sample extraction, handling and spotting methods should be simple, reproducible, rapid and automated.

Protein Identification

Database Searching: The protein were successfully identified by MALDI spectra and were used directly for database searches using the software MS-Fit, [18] developed at the University of California at San Francisco MS Facility to match known proteins or translated open reading frames in databases at the National Center for Biotechnology Information [NCBI] and SWISS-Prot. For more abundant spots (>25 kD), usually >25 peptide masses were obtained by MALDI-TOF and a very good coverage of the full length proteins was typically found (30 to 60%) within the specified 50-ppm mass accuracy. In the first round of data base searching, the maximal molecular mass was restricted to 120 kD to avoid hits of polyproteins or very large proteins and no miscleavage was allowed. Mass accuracy was set at 15 ppm [thus, for a 1-kD peptide, the maximum allowed difference between the measured and theoretical peptide masses was defined as 0.015 D] and minimally four matching peptides were required.

RESULTS

Quantification of Proteins: To investigate the expression of proteins regulated during *in vitro* and *in vivo* studies. Total proteins were analyzed by Bradford assay method. This study shows the presence 59 µg of protein in 50 µl *in vitro* leaf sample and 58 µg of proteins in 50 µl *in vivo* of leaf sample.

SDS-PAGE and Image Analysis: *In vitro* and *In vivo* protein profiles were resolved and image analysis shows 20 clear Protein bands from the leaf samples (Fig 1 and Table 1). The intensity of protein bands was high in *in vitro* sample compared to *in vivo* protein samples. The protein bands of Molecular weight 64,48 and 37 kDa were significantly increased in *in vivo*, where as 79,53 and 28 kDa proteins were significantly increased *in vitro*.

Table 1: Image analysis of *in vitro* and *in vivo* leaf samples

Bands	*In vitro* (Intensity)	*In vivo*(Intensity)
1	822.12	801.01
2	944.01	914.13
3	897.84	862.02
4	5126.23	5021.11
5	946.76	877.13
6	977.94	901.01
7	1231.12	1221.01
8	999.02	879.47
9	15525.13	14241.23
10	787.94	433.87
11	846.94	644.03
12	877.96	788.35
13	988.35	899.32
14	1222.45	1024.63
15	864.98	857.09
16	946.54	345.85
17	879.24	343.98
18	985.47	346.65
19	964.78	541.423
20	978.98	348.556

Table 2a: Database results for Rubisco

Mass Value	Sequence Coverage(%)	Score	Accession no.	Name of the Protein
64 kDa	76	107	gi/1552335	Ribulose-1,5-bis-phosphate carboxylase

Table 2b: Database results for L-ascorbate peroxide

Mass Value	Sequence Coverage [%]	Score	Accession no.	Name of the Protein
28 kDa	51	387	gi /07890	L-ascorbate peroxide

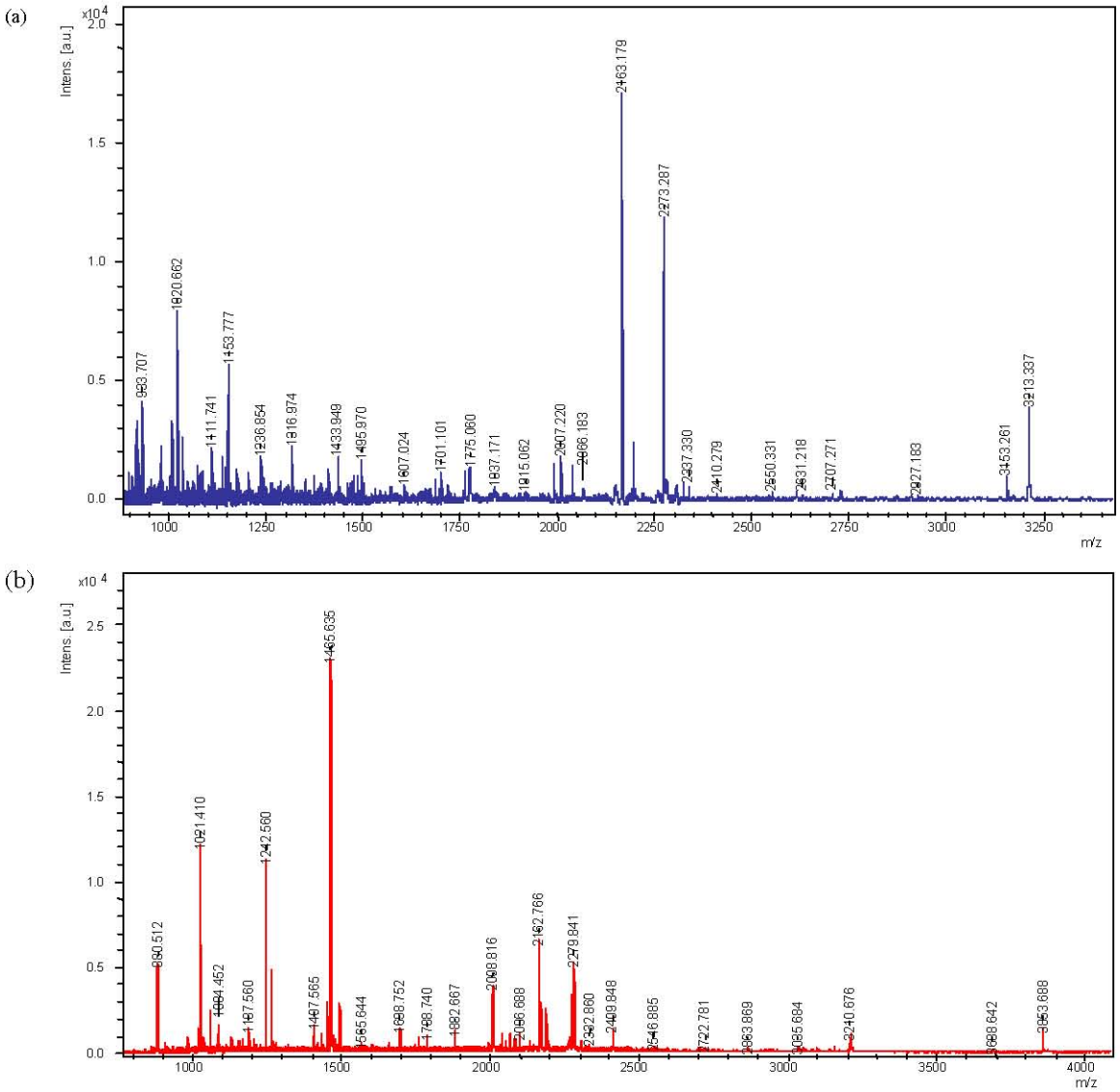


Fig. 2a,b: a: MALDI-TOF-Mass Spectrum of RuBisCo [64.5 kDa]
b: L-ascorbate peroxide [28 kDa]

MALDI-TOF-MS Analysis: Among the differentially expressed proteins 64 kDa protein was identified by MALDI-TOF-MS as Ribulose Bisphosphate Carboxylase (Figure 2 and Table 2a). RuBisCo is present in chloroplast and mainly involved in carbon dioxide fixation. Among the differentially expressed proteins, 28 kDa proteins were identified by MALDI-TOF-MS as L-ascorbate peroxide.

Protein Identification

Database Searching: In the MALDI-TOF MS/MS analysis, one up-regulated protein and one down-regulated protein were successfully identified with significant hits [$P < 0.05$] in MASCOT probability analysis (Table 2a). This shows the mass value of 64 kda and sequence coverage of 76% and name of the protein identified is Ribulose Bisphosphate Carboxylase [RuBisCo]. Another protein identified (Table 2b) shows the mass value of 28 kda and sequence coverage of 51% and name of the protein identified as L-ascorbate peroxide.

DISCUSSION

Proteomics has already become an important tool for drug discovery and for the analysis of yeast and *Escherichia coli* protein expression patterns. More

recently, advances in protein and metabolite technologies are now making global studies of these parameters more accessible. Integrating data from transcriptomics, proteomics and metabolomics will allow for a more precise knowledge of how changes in gene expression lead to changes in metabolism. In particular, proteomics offers great potential for studying mechanisms of post-translational regulation as well as biosynthetic pathways.However, it has not been widely applied in plant biology. This study demonstrates the potential of proteomics in the field of plant sciences. This potential will become more evident once the full genome sequences of *Arabidopsis*,Rice, or other plant genomes are available. The total proteome of higher plants is estimated to consist of ~21,000 to 25,000 proteins [19,20]. Although membrane protein complexes are labile and insoluble in common buffers used in protein electrophoresis, they were well resolved by BN/SDS-PAGE. When combined with biotin surface tagging and nano LC-MS/MS sequencing, several new membrane-associated proteins were identified. Considering their sequence similarity to known proteins, we estimated that more than 60% of the identified proteins are either directly membrane-associated or have a functional relationship to membrane activities. In the present study we demonstrates the abundance of 20 protein spots were as 3 up-regulated proteins *in vitro* and 3 up-regulated proteins in *in vivo* plant samples were identified. In which we had identified 64 kDa protein as Ribulose Bisphosphate Carboxylase [RuBisCo] and 28 kDa protein identified as L-ascorbate peroxide .This is the first report of protien indentification in *Artemisia vulgaris* plant.

REFERENCES

1. Walter, H.L., P.F. Memory, Elvin-Lewis, 2003. Medical botany. In: plants affecting human health, 2nd edn.Wiley, Jersey, pp: 345.
2. Ram,.P.R. and M.N. Mehrotra, 1995. Compendium of Indian medicinal plants, publication and information directorate, CSIR, New Delhi, 74: 1985-1989.
3. Kapoor, L.D., 2000. CRC Handbook of Ayurvedic medicinal plants .CRC Press, Boca Raton, pp: 53.
4. Murch, S.J., S. Krishna Raj and P.K. Saxena, 2006. Phytomaceuticals: Mass production, standardization and conservation. Sci Rev Alternative Med, 4: 39-43.
5. Provart, N.J. and P. McCourt, 2004. Systems approaches to understanding cell signalling and gene regulation. Current Opinion in Plant Biology, 7: 605-609.
6. Arabidopsis Genome Initiative, 2000. Analysis of the genome sequence of the fiowering plant Arabidopsis thaliana. Nature, 408: 796-815.
7. Bino, R.J., R.D. Hall and O. Fiehn, 2004. Potential of metabolomics as a functional genomics tool. Trends in Plant Sciences, 9: 418-425.
8. Yates, III. J.R., 2004. Mass spectral analysis in proteomics. Annual Review of Biophysics and Biomolecular Structure, 33: 297-316.
9. Tyers, M. and M. Mann, 2003. From genomics to proteomics. Nature, 422: 193-197.
10. Millar, A.H., L.J. Sweet love, P. Giege and C.J. Leaver, 2001. Analysis of the Arabidopsis mitochondrial proteome. Plant Physiol., 127: 1711-1727.
11. Desiere, F., E.W. Deutsch and A.I. Nesvizhskii, 2007. Integration with the human genome of peptide sequences obtained by high throughput mass spectrometry. Genome Biology, 6: 745-749.
12. Shevchenko, A., A. Loboda and K.G. Standing, 2000. MALDI quadrupole time-of-flight mass spectrometry: a powerful tool for Proteomics research. Anal.Chem. 72: 2132-2141.
13. Quiniou, M.L., A.J. Yates and P.R. Langridge-Smith, 2000. Laser photo-induced dissociation using tandem time-of-flight mass spectrometry. Rapid Commun Mass Spectrum, 14: 361-367.
14. Murashique,T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant, 15: 473-497.
15. Becwar, M.R., R. Nagamani and S.R. Wann, 1988. Initiation of embryogenic cultures and somatic embryo development in lobolly pine (*pinus taeda*); Can. J.Forest.Res, 20: 810-817.
16. Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of Protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.
17. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
18. Parker, K.C., J.I. Garrels, W. Hines, E.M. Butler, A.H. McKee, D. Patterson and S. Martin, 1998. Identification of yeast proteins from two-dimensional gels: Working out spot Cross-contamination Electrophoresis, 19: 1920-1932.
19. Bouchez, D. and H. Hofte, 1998. Functional genomics in plants. Plant Physiol, 118: 725-732.
20. Meinke, D.W., J.M. Cherry C. Dean, S.D. Rounsley and F.M. Koornnee, 1998. *Arabidopsis thaliana*: A model plant for genome analysis. Science, 282: 679-682.