Available online at www.sciencedirect.com
SCIENCE DIRECT®
Phytochemistry 65 (2004) 1721–1732
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

# Sub-cellular proteomic analysis of a *Medicago truncatula* root microsomal fraction

Benoît Valot, Silvio Gianinazzi, Dumas-Gaudot Eliane *

*UMR 1088 INRA/CNRS 5184/UB Plante-Microbe-Environment, INRA/CMSE, BP 86510, 21065 Dijon cedex, France*

Received 28 January 2004; received in revised form 2 April 2004
Available online 6 May 2004

**Abstract**

Since the last decade, *Medicago truncatula* has emerged as one of the model plants particularly investigated in the field of plant–microbe interactions. Several genetic and molecular approaches including proteomics have been developed to increase knowledge about this plant species. To complement the proteomic data, which have mainly focused on the total root proteins from *M. truncatula*, we carried out a sub-cellular approach to gain access to the total membrane-associated proteins. Following the setting up of the purification process, microsomal proteins were separated on 2-DE. Ninety-six out of the 440 well-resolved proteins were identified by MALDI-TOF peptide mass fingerprinting. A high percent (83%) of successful protein identification was obtained when using *M. truncatula* clustered EST database for queries. During the purification process, the enrichment in membrane-associated proteins was monitored on 2-D gels. The membrane location of microsomal proteins was further confirmed using PMF identification. This study reports a fractionation process for characterizing microsomal root proteins of *M. truncatula*, which could be an interesting tool for investigating the molecular mechanisms involved in root symbioses.


*Keywords:* *Medicago truncatula*; 2-D electrophoresis; MALDI-TOF mass spectrometry; Membrane-associated proteins; Clustered EST database

## 1. Introduction

In the recent past, new amendable plant models were looked for in order to investigate particular biological situations that cannot be achieved with *Arabidopsis thaliana*. This is particularly evident when studying root–microbe symbiotic interactions (Oldroyd and Geurts, 2001; Stougaard, 2001). In this context, legume species have been proposed as models because they formed beneficial root symbioses with both nitrogen fixing bacteria and mycorrhizal fungi (Barker et al., 1990; Harrison, 1997). Moreover, legumes offer unique research opportunities in areas of basic and applied plant biology (Cook, 1999). Among leguminous plants, including soybean, alfalfa, pea, two species have emerged: *Lotus japonicus* and *Medicago truncatula* (Cook, 1997). They were chosen because of their small diploid genome, autogamus nature, short generation time, and prolific seed production (Barker et al., 1990; Handberg and Stougaard, 1992; Cook, 1997; Jiang and Gresshoff, 1997; Cook, 1999). *M. truncatula* can be readily transformed using *Agrobacterium tumefaciens*, regenerated into fertile plant (Trieu et al., 2000) and shows a high degree of synteny with important cultivated legumes (Frugoli and Harris, 2001). An international sequencing project of this plant species has generated, by the first may 2003, 190,000 *M. truncatula* Expressed Sequence Tags (ESTs), available online at the Institute for Genomic Research (http://www.tigr.org/tdb/tgi/mtgi/), which constitutes the basis for genetic and functional genomic studies, including proteomics.

Since the first development of two-dimensional electrophoresis (2-DE) of proteins (O'Farrel, 1975), this approach has been very often used as a current technique for protein display. The introduction of immobilized pH gradients (IPG) for the first dimension (Görg, 1988) and the recent development of mass spectrometry for protein identification (Corthals et al., 2000), have

* Corresponding author. Tel.: +33-380693424/3126; fax: +33-380693753.
*E-mail address:* dumas@epoisses.inra.fr (D.-G. Eliane).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.04.010

opened the way towards high throughput protein identification as defined by the term of proteomics (Wilkins et al., 1996). The increase interest in this strategy become obvious with regards to the number of published papers based on proteomics (from 191 papers in 1999, the number has reached 2403 in 2003). Proteomes have been described so far for the model plant *M. truncatula* for different organs/tissues (Mathesius et al., 2001; Watson et al., 2003), as well as for symbiotic root interactions (Bestel-Corre et al., 2002). However, all these studies were directed to total proteins.

Sub-cellular proteomics, defined as the analysis of the expressed proteins of purified individual cell compartments has emerged as an interesting tool to complement total proteins data (Jung et al., 2000). In plant, as reviewed by Heazlewood and Millar (2003), various studies have been aimed at characterizing the organelle proteomes including chloroplasts, mitochondria, peroxisomes and cell wall. In the case of biological membranes, various researches were directed to identify membrane proteins of either microsomes (Prime et al., 2000) or plasma membranes (Santoni et al., 1998; Santoni et al., 1999) of *A. thaliana*, germinating seed endoplasmic reticulum (Maltman et al., 2002) as well as of peribacteroid membranes from root nodules (Saalbach et al., 2002; Wienkoop and Saalbach, 2003).

As strictly hydrophobic proteins are not yet amenable by 2-DE analysis (Seigneurin-Berny et al., 1999), the aim of the present work was to gain access to proteins associated with total membrane structures, i.e., microsomes. This was achieved by combining a sub-cellular fractionation process to 2-DE separation. The proteomic data we generated will be further applied to study membrane-associated proteins involved in particular root biological processes. Following refinements in protein extraction and 2-DE separation, 96 microsomal proteins were identified by Matrix-Assisted Laser Desorption/Ionisation-Time Of Flight (MALDI-TOF) peptide mass fingerprinting (PMF) using clustered EST database for queries. The membrane location of the microsomal proteins was determined with regard to the purification enrichment process, the putative assigned function (Journet et al., 2002) together with data comparison with the published *M. truncatula* root reference map (Mathesius et al., 2001). In addition, the database search strategy will be discussed.

## 2. Results and discussion

### 2.1. Microsomal proteins separation by 2-DE

To optimise the 2-DE microsomal protein separation, several technical adjustments concerning the protein extraction, solubilization and set up of electrophoretic parameters were performed (data not shown). Three different extraction methods based on phenol (Bestel-Corre et al., 2002), acetone and chloroform/methanol processes (Ferro et al., 2000) were compared for preparing the microsomal extract. For solubilizing the extracted proteins, buffers were supplemented with thiourea (Rabilloud, 1998) and TBP (Herbert et al., 1998) because of their known properties for improving solubility and separation of membrane proteins. Different detergents were tested such as a mix of CHAPS and Triton X-100 (Rabilloud et al., 1997) or ASB14 combined with either Tris (Molloy et al., 1999) or Triton X-100 (Chevallet et al., 1998). The last point considered for improving the microsomal fraction resolution was the sample loading mode. The three methods tested were the active rehydratation (Islam and Landers, 1998), the cup sample loading at the anodic or at the cathodic end of the strips (Rabilloud et al., 1994). The parameters were evaluated with regard to the number of detected spots together with a reduced protein precipitation into the gels. By eliminating strictly hydrophobic proteins (Seigneurin-Berny et al., 1999), the chloroform/methanol extraction process was the most satisfying protocol leading to well-resolved microsomal proteins. The best separations were obtained using the mix of CHAPS and Triton X-100 as detergents, combined with the loading of the protein samples at the anodic end of the strips. A more limited precipitation was observed with this loading method in comparison to the cathodic one. Moreover, a net increase in the number of basic spots was observed in contrast to the active rehydratation as

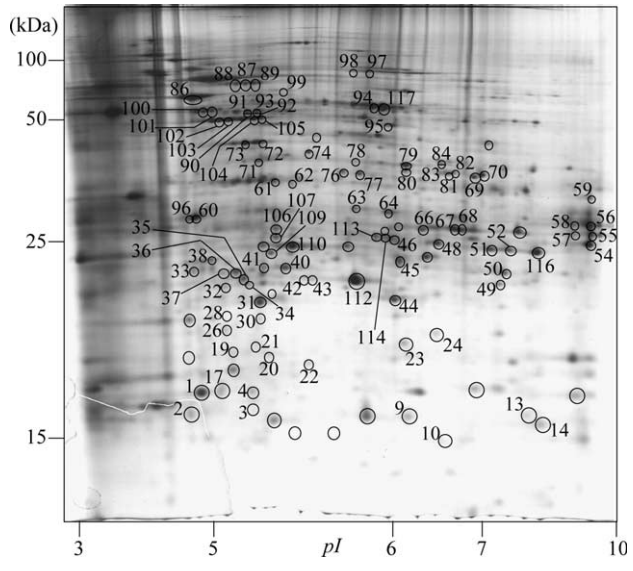


Fig. 1. Silver-stained 2-D gel of the microsomal fraction. Eighty micrograms of proteins was loaded at the anodic end. Circled spots indicated the location of the proteins sampled on a micropreparative Coomassie blue-stained gel further analyzed by MALDI-TOF MS. The numbers closed to the circled spots correspond to proteins that give a hit and which are listed in Table 1.

described recently by Barry et al. (2003). An example of the microsomal extract separation is shown in Fig. 1.

### 2.2. Comparison of total, cytosolic and microsomal polypeptides profiles

On analytical silver-stained gels, approximately 630 intense spots were well resolved for both the total and cytosolic fractions (Fig. 2). In comparison, only 440 spots were detected for the microsomal extract (Fig. 1). While the 2-DE profiles of the total and cytosolic fractions were very similar, the microsomal fraction behaved with a clearly different polypeptide profile. These differences were related to a decrease in the acidic spots together with an increase in the basic ones. In addition, while most of the spots of the total and cytosolic fractions were displayed within the 4–7 isoelectric point (p*I*) range, the microsomal spots were uniformly distributed over the complete p*I* range (3–10). A more detailed image analysis comparison between total and cytosolic fractions revealed that four proteins cannot be visualized in the 2-DE profiles of the cytosolic fraction. These particular proteins (spots 102, 103, 104, and 117), which were subtracted by the ultracentrifugation step, could therefore correspond to microsomal proteins detectable into the total fraction. This is further supported by the fact that proteins with similar electrophoretic parameters were revealed in the microsomal extract showing a net increase in their respective volume. In contrast, protein overlapping based on image analysis comparison was hardly possible due to the great differences in the polypeptide profiles between total and microsomal fractions.

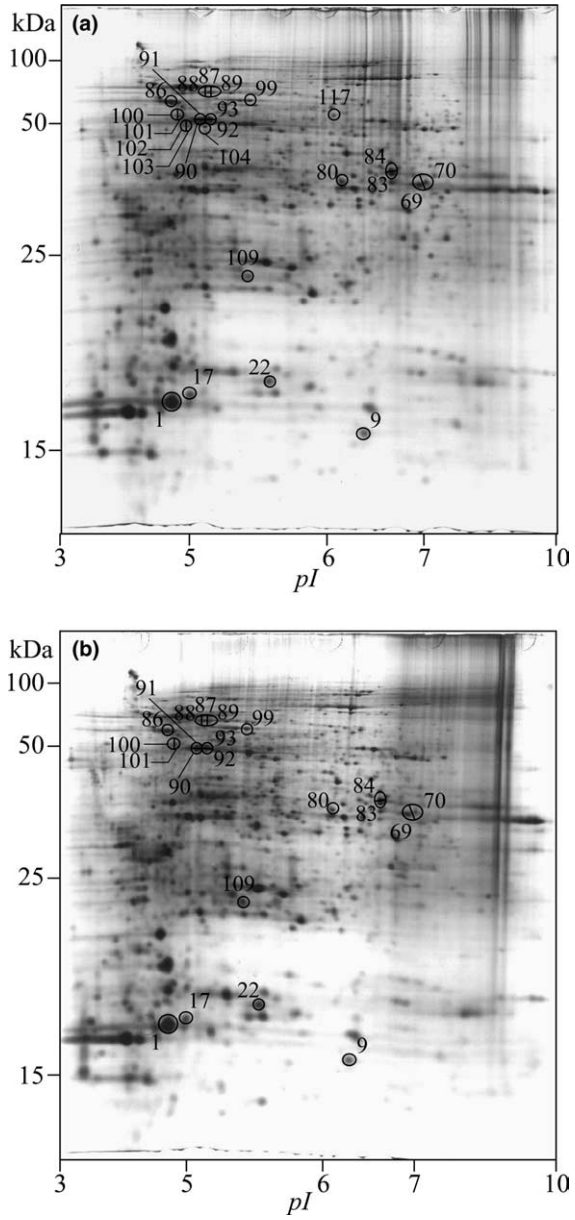


Fig. 2. Silver-stained 2-D gels of the total (a) and cytosolic (b) fractions. One hundred micrograms of proteins was loaded at the cathodic end of 18 cm 3–10 NL IPG strips. Numbers assigned to the circled spots correspond to potentially identical spots detected into the microsomal fraction (Fig. 1).

### 2.3. Identification by PMF of the most abundant proteins of the microsomal fraction

The micropreparative Coomassie blue-stained gel of the microsomal fraction, revealed more than 250 proteins, among which, 115 were characterized by their electrophoretic parameters and numbered (data not shown). Following gel excision, these proteins were submitted to MALDI-TOF mass spectrometry analyses. Among them, 96 proteins matched to proteins of known sequences deduced from the two clustered EST database of *M. truncatula* (Table 1). All the identified proteins have displayed a minimum of 5 matched peptides, with a maximum of 32 matches that was obtained for spot 86. The mean number of peptide matches was 12.8. In addition, a good coverage of the identified proteins by the matched peptides was obtained, with a mean of 44.5%. In complement to the fact that matched peptides belonged to the intense peaks of the mass spectra, and to high MOWSE score levels, these results bring further support to our identifications.

Confidence for identification is also very often related to the similarity of calculated and theoretical p*I* and $M_r$ (Link et al., 1997). In this study, 71% of the identified proteins showed a difference between calculated and theoretical p*I* and $M_r$ lower than 10%. A good congruence of the p*I* was observed for 12.5% of the identified proteins, among which the majority behaved with calculated $M_r$ lower than the theoretical ones. This could be due to the proteolysis of the terminal region during protein processing or to a false assignation of the first methionine during the automatic translation process. In contrast, 13.5% presented similar $M_r$. In this case, the

Table 1
Peptide mass fingerprinting analyses of M. truncatula microsomal proteins and results of sequence analyses

| Spot | Database entry | Peptides matched | Coverage/score | Identification | pI/$M_r$[a] calculated (theoretical) | Classes of function[b] | Previously identified[c] | TM domain[d] |
|---|---|---|---|---|---|---|---|---|
| 1 | MtC00227 | 6 | 43%/5.14e + 3 | PR10 | 4.9/16.7 (4.95/16.7) | XII.A | 79 | – |
| 2 | MtC60333 | 5 | 29%/3.04e + 3 | Cytochrome *c* oxidase subunit Vb | 4.8/16.2 (5.9/16.4) | V | – | – |
| 3 | MtC00115 | 6 | 37%/1.38e + 3 | Glycine-rich DNA binding protein | 5.24/16.2 (6.52/18.7) | VIII | 83 | – |
| 4 | MtC10007 | 12 | 76%/3.88e + 7 | Putative fruit-induced CSF-2 protein | 5.24/16.8 (5.4/17.4) | XII.C | – | – |
| 9 | MtC00016 | 7 | 36%/5.42e + 3 | Nucleoside diphosphate kinase | 6.05/15.9 (5.98/18.6) | V | 57 | – |
| 10 | MtC10444 | 9 | 70%/1e + 4 | Probable NADH-ubiquinone oxidoreductase | 6.36/15.2 (6.73/12.2) | V | – | – |
| 13 | MtC10323 | 7 | 59%/4.13e + 3 | Hypothetical protein | 7.89/16.1 (8.02/13.7) | XII.C | – | – |
| 14 | MtC10115 | 9 | 42%/6.56e + 3 | Hypothetical protein | 8.11/15.7 (10.17/18) | XII.C | – | 68–87 $S = 2406$ |
| 17 | MtC10531 | 5 | 38%/626 | Probable PR 10 | 5.04/16.9 (5.11/17.2) | XII.A | 81/82 | – |
| 19 | MtC10319 | 14 | 54%/9.37e + 5 | Hypothetical ATP synthase D chain mitochondrial | 5.12/18.5 (5.44/20.9) | III | – | – |
| 20 | MtC10319 | 16 | 61%/2.79e + 5 | Hypothetical ATP synthase D chain mitochondrial | 5.37/18.3 (5.44/20.9) | III | – | – |
| 21 | MtC10319 | 12 | 49%/2.69e + 4 | Hypothetical ATP synthase D chain mitochondrial | 5.3/18.9 (5.44/20.9) | III | – | – |
| 22 | MtC00261 | 6 | 30%/2.67e + 3 | Peroxiredoxin | 5.62/17.9 (5.51/20.7) | XII.A | 440 | 66–68 $S = 687$ |
| 23 | MtC10091 | 7 | 32%/2.25e + 3 | Probable developmentally regulated plasma membrane polypeptide | 6/18.9 (4.93/23.8) | XII.C | – | – |
| 24 | MtC00416 | 8 | 50%/7.14e + 4 | Probable ethylene-regulated protein | 6.32/19.4 (6.47/17.9) | XII.B | – | 130–157 $S = 858$ |
| 26 | MtC10319 | 11 | 49%/2.26e + 4 | Hypothetical ATP synthase D chain mitochondrial | 5.12/19.4 (5.44/20.9) | III | – | – |
| 28 | MtC10319 | 14 | 59%/2.57e + 5 | Hypothetical ATP synthase D chain mitochondrial | 5.12/20.2 (5.44/20.9) | III | – | – |
| 30 | MtC10319 | 16 | 66%/2.34e + 5 | Hypothetical ATP synthase D chain mitochondrial | 5.3/20.2 (5.44/20.9) | III | – | – |
| 31 | MtC10319 | 19 | 72%/1.67e + 6 | Hypothetical ATP synthase D chain mitochondrial | 5.32/21.3 (5.44/20.9) | III | – | – |
| 32 | MtD09954 (TC88421) | 12 | 55%/3.57e + 6 | Protein of unknown function UPF0172 | 5.07/22.4 (5.66/23.5) | XII.C | – | – |
| 33 | MtC00544 | 8 | 31%/2.35e + 3 | Putative NADH-ubiquinone oxidoreductase | 4.85/23.7 (5.41/23.8) | V | – | – |
| 34 | MtD09954 (TC88421) | 10 | 51%/1.65e + 5 | Protein of unknown function UPF0172 | 5.22/22.5 (5.66/23.5) | XII.C | – | – |
| 35 | MtC00127 | 8 | 25%/2.19e + 3 | Probable ferritin | 5.2/23 (6.12/32.1) | VI | – | – |
| 36 | MtC20131 | 12 | 48%/1.57e + 6 | Ferritin | 5.15/23.6 (5.66/28.1) | VI | – | – |
| 37 | MtC20131 | 9 | 32%/2.05e + 5 | Ferritin | 5.06/23.5 (5.66/28.1) | VI | – | – |
| 38 | MtD07835 (TC76949) | 8 | 50%/2.63e + 4 | Chalcone–flavanone isomerase | 4.99/24.7 (4.99/23.6) | VI | – | – |
| 40 | MtC00127 | 13 | 40%/1.31e + 5 | Probable ferritin | 5.5/23.9 (6.12/32.1) | VI | – | – |
| 41 | MtC00127 | 10 | 28%/2.74e + 4 | Probable ferritin | 5.35/24 (6.12/32.1) | VI | – | – |
| 42 | MtC00067 | 7 | 34%/1.3e + 4 | Probable 1,4-benzoquinone reductase/TRP repressor binding protein | 5.6/23.1 (6.2/24) | XII | – | 155–174 $S = 1001$ |
| 43 | MtC10046 | 5 | 28%/2.56e + 3 | Probable 1,4-benzoquinone reductase/TRP repressor binding protein | 5.64/22.9 (7.11/24.2) | XII | – | 154–173 $S = 1061$ |
| 44 | MtC10461 | 15 | 84%/7.87e + 7 | Probable Cop-coated vesicle membrane protein P24 precursor | 5.95/21.4 (5.71/23.6) | IV | – | 173–192 $S = 819$ |
| 45 | MtC10834 | 8 | 38%/1.13e + 4 | Putative disease resistance protein 206-like | 5.99/24.5 (6.15/23.9) | XII.A | – | – |
| 46 | MtC10528 | 15 | 58%/1.24e + 7 | Putative carnitine operon protein caie-like | 5.94/26.8 (6.32/29.3) | V | – | – |
| 48 | MtC10528 | 18 | 61%/5.46e + 8 | Putative carnitine operon protein caie-like | 6.34/26.3 (6.32/29.3) | V | – | – |
| 49 | MtC10528 | 5 | 27%/3.46e + 3 | Putative carnitine operon protein caie-like | 7.43/22.7 (6.32/29.3) | V | – | – |
| 50 | MtC50199 | 8 | 21%/3.35e + 3 | Probable ubiquinol-cytochrome *c* reductase iron–sulfur subunit precursor | 7.53/23.6 (9.53/33.7) | V | – | 238–257 $S = 1078$ |
| 51 | MtC10996 | 9 | 30%/2.09e + 4 | Outer mitochondrial membrane porin | 7.25/25.7 (9.06/38.7) | III | – | 136–158 $S = 1156$ |
| 52 | MtC10996 | 12 | 38%/7.44e + 5 | Outer mitochondrial membrane porin | 7.6/25.7 (9.06/38.7) | III | – | 136–158 $S = 1157$ |
| 54 | MtC20367 | 18 | 61%/2.2e + 10 | Prohibitin | 9.09/26.4 (9.29/32.8) | XI | – | 37–58 $S = 1210$ |
| 55 | MtC10204 | 14 | 55%/2.35e + 7 | Outer plastidial membrane protein porin | 9.14/27.5 (8.84/31.6) | III | – | 131–153 $S = 1051$ |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 56 | MtC91290 | 10 | 50%/4.13e + 6 | Hypothetical porin | 9.1/28.7 (6.78/34.9) | III | – | 148–168 $S = 849$ |
| 57 | MtC10204 | 12 | 53%/3.64e + 6 | Outer plastidial membrane protein porin | 8.8/27.5 (8.84/31.6) | III | – | 131–153 $S = 1051$ |
| 58 | MtC91290 | 9 | 43%/1.11e + 6 | Hypothetical porin | 8.72/28.5 (6.78/34.9) | III | – | 148–168 $S = 849$ |
| 59 | MtC00519 | 10 | 25%/1.38e + 3 | Putative NADH-ubiquinone oxidoreductase | 9.1/33.2 (9.4/45.1) | V | – | 72–92 $S = 1518$ |
| 60 | MtC10091 | 10 | 25%/1.38e + 36 | Probable developmentally regulated plasma membrane polypeptide | 4.8/29.2 (4.93/23.8) | XII.C | – | – |
| 61 | MtC30120 | 22 | 58%/3.96e + 8 | Probable annexin | 5.34/34.1 (5.44/35.7) | X | – | – |
| 62 | MtC30078 | 10 | 37%/3.36e + 3 | Putative seed storage protein | 5.43/34 (5.54/38.9) | V | – | – |
| 63 | MtC60249 | 13 | 60%/3.34e + 4 | Bacterial transferase hexapeptide repeat protein | 5.77/30.2 (6.06/29.2) | V | – | – |
| 64 | MtC60249 | 14 | 60%/3.06e + 4 | Bacterial transferase hexapeptide repeat protein | 5.91/29.8 (6.06/29.2) | V | – | – |
| 66 | MtC00173 | 6 | 20%/3.23e + 3 | Adenylate kinase | 6.18/27.8 (7.75/29.3) | V | 684 | – |
| 67 | MtC00629 | 15 | 42%/2.28e + 4 | Vacuolar ATP synthase subunit E | 6.54/27.9 (8.59/31.8) | III | – | – |
| 68 | MtC00629 | 17 | 48%/3.86e + 5 | Vacuolar ATP synthase subunit E | 6.66/27.7 (8.59/31.8) | III | – | – |
| 69 | MtC00021.2 | 12 | 43%/1.85e + 5 | Glyceraldehyde-3-phosphate dehydrogenase | 6.89/34.6 (6.58/39.6) | V | 160 | – |
| 70 | MtC00030 | 14 | 44%/9.65e + 4 | Probable glyceraldehyde-3-phosphate dehydrogenase | 7.07/34.9 (6.96/39.1) | V | 161 | – |
| 71 | MtC00744 | 8 | 24%/1.03e + 3 | Glutamine synthetase MtGSb | 5.22/36.8 (5.38/39.2) | V | – | – |
| 72 | MtC00417 | 9 | 32%/8.23e + 4 | Probable protein disulfide isomerase precursor | 5.24/40 (5.38/40.4) | IX | + | – |
| 73 | MtC10470 | 9 | 28%/1.97e + 4 | Actin/actin like | 5.2/39.8 (5.75/47.5) | II | – | 391–412 $S = 1388$ |
| 74 | MtC60189 | 23 | 61%/1.5e + 10 | Vacuolar-ATPase subunit C | 5.47/37.8 (5.67/42.9) | III | – | 309–327 $S = 828$ |
| 76 | MtC30206 | 8 | 21%/1.31e + 3 | Putative hypothetical protein | 5.71/33.5 (6.55/33.2) | XII.C | – | – |
| 77 | MtC30206 | 8 | 21%/1.31e + 3 | Putative hypothetical protein | 5.84/33.2 (6.55/33.2) | XII.C | – | – |
| 78 | MtC20220 | 8 | 40%/3.04e + 4 | 26S Proteasome regulatory subunit S12 | 5.79/34.9 (5.85/34.8) | IX | – | – |
| 79 | MtC00445 | 9 | 51%/3.85e + 6 | ER-associated hsp40 co-chaperone | 6.05/36.3 (5.77/28.2) | IX | – | 8–29 $S = 1054$ |
| 80 | MtC00021.1 | 9 | 20%/2.25e + 5 | Glyceraldehyde-3-phosphate dehydrogenase | 6.06/35.2 (8.27/41.5) | V | 190 | – |
| 81 | MtC00021.1 | 12 | 38%/7.16e + 6 | Glyceraldehyde-3-phosphate dehydrogenase | 6.38/35.1 (8.27/41.5) | V | + | – |
| 82 | MtC00021.1 | 9 | 19%/3.09e + 5 | Glyceraldehyde-3-phosphate dehydrogenase | 6.55/35.1 (8.27/41.5) | V | + | – |
| 83 | MtC00100 | 8 | 26%/6.07e + 4 | Probable fructose-bisphosphate aldolase | 6.38/36.9 (7.21/43.4) | V | 159 | – |
| 84 | MtC00100 | 12 | 39%/1.26e + 6 | Probable fructose-bisphosphate aldolase | 6.38/36.5 (7.21/43.4) | V | 162 | – |
| 86 | MtC10403 | 32 | 66%/2.36e + 17 | Probable protein disulfide isomerase precursor | 4.9/58.2 (5.02/58.8) | IX | 3 | – |
| 87 | MtC20140 | 27 | 58%/2.01e + 11 | Vacuolar ATP synthase catalytic subunit A (alfa) | 5.27/68.1 (5.16/68.8) | III | 269 | – |
| 88 | MtC20140 | 27 | 56%/1.16e + 11 | Vacuolar ATP synthase catalytic subunit A (alfa) | 5.33/68.1 (5.16/68.8) | III | 269 | – |
| 89 | MtC20140 | 23 | 51%/3.19e + 9 | Vacuolar ATP synthase catalytic subunit A (alfa) | 5.37/68.1 (5.16/68.8) | III | 269 | – |
| 90 | MtC00268 | 24 | 59%/7.8e + 11 | ATP synthase β-chain, mitochondrial precursor | 5.19/49.1 (5.8/59.9) | III | 5, 714 | – |
| 91 | MtC00268 | 20 | 51%/2.5e + 11 | ATP synthase β-chain, mitochondrial precursor | 5.19/49.7 (5.8/59.9) | III | 5, 714 | – |
| 92 | MtC00268 | 26 | 62%/6.37e + 11 | ATP synthase β-chain, mitochondrial precursor | 5.22/49.1 (5.8/59.9) | III | 5, 714 | – |
| 93 | MtC00268 | 21 | 55%/4.92e + 10 | ATP synthase β-chain, mitochondrial precursor | 5.22/49.8 (5.8/59.9) | III | 5, 714 | – |
| 94 | MtC30030 | 25 | 56%/2.37e + 8 | Probable cytochrome *c* reductase-processing peptidase subunit | 5.86/52.3 (6.45/59.6) | IX | 241 | – |
| 95 | MtD04868 (TC77343) | 9 | 25%/1.07e + 5 | Biotin/lipoyl attachment protein | 5.97/44.2 (9.14/49.8) | XII.C | – | – |
| 96 | MtC10091 | 5 | 35%/1.14e + 3 | Probable developmentally regulated plasma membrane polypeptide | 4.85/29.2 (4.93/23.8) | XII.C | – | – |
| 97 | MtC00504 | 17 | 34%/4.64e + 6 | Probable NADH-ubiquinone oxidoreductase 75 kDa subunit | 5.86/77.6 (7.11/80.8) | V | – | – |
| 98 | MtC00504 | 19 | 38%/3.57e + 8 | Probable NADH-ubiquinone oxidoreductase 75 kDa subunit | 5.82/77.6 (7.11/80.8) | V | – | – |

(*continued on next page*)

Table 1 (*continued*)

| Spot | Database entry | Peptides matched | Coverage/score | Identification | pI/$M_r$[a] calculated (theoretical) | Classes of function[b] | Previously identified[c] | TM domain[d] |
|---|---|---|---|---|---|---|---|---|
| 99 | MtC00348 | 10 | 28%/7.14e + 5 | Probable 2,3-bisphosphoglycerate-independent phosphoglycerate mutase | 5.48/62.5 (5.43/60.6) | V | 389, 390 | – |
| 100 | MtC10407 | 28 | 68%/2.4e + 13 | Vacuolar ATP synthase subunit B | 4.93/50.1 (5.29/56.7) | III | 671 | – |
| 101 | MtC10407 | 25 | 55%/2.1e + 11 | Vacuolar ATP synthase subunit B | 4.98/50.4 (5.29/56.7) | III | 671 | – |
| 102 | MtC60672 | 20 | 72%/4.95e + 6 | Insulinase-like peptidase, familly M16 | 5.03/46.2 (5.16/41.9) | IX | + | 202–222 $S = 1376$ |
| 103 | MtC60672 | 16 | 60%/1.89e + 5 | Insulinase-like peptidase, familly M16 | 5.08/46.3 (5.16/41.9) | IX | + | 202–222 $S = 1376$ |
| 104 | MtC40075 | 16 | 40%/4.02e + 7 | Probable mitochondrial processing peptidase α subunit precursor | 5.23/46.5 (5.74/55) | IX | 393 | – |
| 105 | MtC40075 | 18 | 54%/5.16e + 7 | Probable mitochondrial processing peptidase α subunit precursor | 5.31/46.7 (5.74/55) | IX | 393 | – |
| 106 | MtC10055 | 6 | 33%/2.6e + 4 | Probable lectin | 5.44/27.9 (5.35/30.9) | XII | – | – |
| 107 | MtC10055 | 8 | 37%/3.99e + 5 | Probable lectin | 5.44/26.9 (5.35/30.9) | XII | – | – |
| 109 | MtC30069 | 11 | 52%/4.22e + 3 | Proteasome subunit α type 2 | 5.4/25.3 (5.51/25.6) | IX | 372 | – |
| 110 | MtC00696 | 17 | 47%/3.05e + 4 | ATP synthase subunit | 5.54/26 (8.83/31.6) | III | – | – |
| 112 | MtC00067 | 8 | 46%/4.11e + 4 | Probable 1,4-benzoquinone reductase/TRP repressor binding protein | 5.77/22.8 (6.2/24) | XII | – | 155–174 $S = 1001$ |
| 113 | MtC20060 | 13 | 55%/1.06e + 6 | Hypothetical protein | 5.89/26.8 (6.17/29.8) | XII.C | – | – |
| 114 | MtC20060 | 10 | 41%/3.36e + 4 | Hypothetical protein | 5.94/26.8 (6.17/29.8) | XII.C | – | – |
| 116 | MtC10996 | 11 | 35%/1.47e + 5 | Outer mitochondrial membrane porin | 8.07/25.5 (9.06/38.7) | III | – | 136–158 $S = 1157$ |
| 117 | MtC30030 | 16 | 25%/1.02e + 6 | Probable cytochrome *c* reductase-processing peptidase subunit | 5.93/51.6 (6.45/59.6) | IX | 241 | – |

[a] Calculated p*I* and $M_r$ (kDa) were determined using image Master 2D Elite (Amersham Biosciences) and standard proteins (Bio-Rad) co-electrophoresed with the root extracts. Theoretical *pI* and $M_r$ (kDa) were calculated from the protein sequence by using Proteinprospector software.

[b] The class of function corresponds to the manual annotation of the EST clustering *M. truncatula* database as described by Journet et al. (2002): I Cell wall, II Cytosketon, III Membrane transport, IV Vesicular trafficking, secretion and protein sorting, V Primary metabolism, VI Secondary metabolism and hormone metabolism, VII Chromatin and DNA metabolism, VIII Gene expression and RNA metabolism, IX Protein synthesis and post-translational regulation, XI Cell division cycle, XII Miscellaneous, XII.A. Defence and cell rescue, XII.B. Abiotic stimuli and development, XII.C. Unknown function, and XIII No homology.

[c] The numbers correspond to the spot identifying the same protein in the root proteome reference map described by Mathesius et al. (2001). + indicates that the proteins have been identified in common with Mathesius et al. (2001) on a different area of the gel.

[d] The possible transmembrane domains, defined in *4.10*, were characterized by the number of the residues surrounding the TM domain and the associated score ($S =$ ).

shift of the p*I* was mainly defined by a higher theoretical p*I*. This difference could correspond to a post-translational modification such as phosphorylation, known to decrease the p*I* (Gooley and Packer, 1997). Finally, only 3% showed no congruence of p*I* and $M_r$.

### 2.4. Classification of the identified proteins and sequence analysis

The 96 identified proteins were classified according to their different biological functions based on the same categories as Journet et al. (2002): 28% belonged to membrane transport, 25% to primary metabolism, 13.5% to unknown function, 11.5% to protein synthesis and processing, 6.2% to secondary metabolism, 5.2% to miscellaneous and 4.2% to defence and cell rescue. For categories corresponding to cytoskeleton, vesicular trafficking, RNA metabolism, signal transduction, cell division cycle and abiotic stimuli and development, only one identification was obtained.

The most abundant class identified was the membrane transport proteins and corresponded mainly to the different subunits of the mitochondrial ATP synthase complex (12 spots) and the vacuolar ATPase (8 spots). The other protein belonging to this class was the porin family with 8 hits.

The second most abundant class we obtained corresponded to proteins involved in primary metabolism. Among these, we identified seven proteins (spots 2, 10, 33, 50, 59, 97 and 98) known to be localized on the inner mitochondrial membrane and involved in the electron transfer and proton translocation in the respiratory chain pathway (Harnisch et al., 1985; Van der Pas et al., 1991).

Concerning the third class of proteins of known function, i.e., the protein synthesis and processing, among the 11 proteins belonging of this class, six matched to a subunit of the mitochondrial processing peptidase (spots 94, 102–105 and 117) known to be localized on the inner mitochondrial membrane in plant (Duby and Boutry, 2002).

Among the proteins associated with the biological membranes, the intrinsic proteins are defined by one or more transmembrane (TM) regions (Fasman, 1989). In our study, 21 identified proteins showed one possible TM region (Table 1). Among them, 7 fitted to different isoforms of the outer mitochondrial porin, involved into the transfer of small solutes (Benz, 1994).

### 2.5. Use of clustered EST database for PMF identification

PMF identifications are based on the comparison between the masses of the peptide mixtures generated by enzymatic digestion of a gel spot measured by MS and those of theoretical digests of proteins contained in database (Shevchenko et al., 1996). In two previous studies directed as well to the identification of *M. truncatula* root proteins (Mathesius et al., 2001; Watson et al., 2003), the PMF approach led to percentages of successful identification of 37% and 43%, respectively. In the present work, 83% of protein identifications were successful. This improved success rate in performing PMF may be partly explained by the fact that this study was focused on a well-defined sub-cellular compartment, i.e., the microsomal fraction of root proteins. However, the improvement could also be related to the use of clustered EST database for queries.

In Mathesius et al. (2001), the PMF search was performed on the *M. truncatula* ESTs contained in the NCBI database. In Watson et al. (2003), the PMF search was carried out on *M. truncatula* EST database and extended by a search into the SwissProt and NCBInr protein database. On contrary, we used a different searching strategy by querying for PMF search in clustered EST database, in which each clusters correspond to the alignment of different EST sequences derived from the same gene. Consequently, predicted proteins display longer sequences than that can be obtained from a single EST. As an illustration, the advantages of this approach are shown for two proteins identified in the 3 studies (Table 2). The validity of PMF identification is strongly based on the greater numbers of matched peptides with a good accuracy (Rappsilber and Mann, 2002). The protein disulfide isomerase was identified with at least three times more of matched peptides and the mitochondrial ATP synthase beta chain with twice to three times more of matched peptides compared to a *Sorghum bicolour* protein (Watson et al., 2003) and an EST sequence of *M. truncatula* (Mathesius

Table 2
Example of protein identification query improvements by PMF using clustered EST database

| Protein identified | Spot[a] | Number of peptides matched for identification in | | |
|---|---|---|---|---|
| | | This study[b] | Mathesius et al. (2001)[b] | Watson et al. (2003)[b] |
| Protein disulfide isomerase precursor | 86 | 32 | 6 | 10 |
| ATP synthase β-chain | 90–93 | 20–26 | 5–9 | 13[c] |

[a] The number corresponds to the spot defined in Fig. 1 and Table 1.
[b] For multiple identifications of the same protein, the numbers represent the minimum and the maximum of matched peptides.
[c] Indicates that the hit was obtained with an heterologous protein.

et al., 2001), respectively. This difference in matched peptides was obtained without changing the accuracy parameters. Indeed, these two proteins were identified with a maximum of 20 ppm for peptide mass accuracy.

### 2.6. Protein overlapping between microsomal and other plant proteomes

In order to determine whether the microsomal proteins have also been detected into a total fraction, each protein identified in the present work was looked for into the *M. truncatula* root reference map (Mathesius et al., 2001). Based on their identification, 32 proteins were at first sight found in common to both studies (Table 1), among which, 25 and 21 could be localized on the 2-DE gels corresponding to either total or cytosolic fractions (Fig. 2). In fact, spots 102, 103, 104 and 117, only detected into the total fraction (see Section 2.2.), corresponded to subunits of mitochondrial processing peptidases known to be localized in the inner mitochondrial membrane (Duby and Boutry, 2002). When the p*I* and $M_r$ were also taken into account, five proteins with different electrophoretic parameters were identified in this analysis. The 9 subunits of both vacuolar (spots 87, 88, 89, 100 and 101) and mitochondrial (spots 90, 91, 92 and 93) ATP synthase could be detected in both the total and cytosolic fractions. ATP synthase subunits, which formed respectively the V1 or F1-ATPase, are usually classified into the membrane transport category. Their occurrence in the cytosolic fraction could represent the soluble part of these peripheral membrane proteins (Merzendorfer et al., 1997; Capaldi and Aggeler, 2002). Among the 32 proteins, we also identified three spots (3, 83 and 84) previously reported as being specific of the leaf plasma membrane of *A. thalania* (Santoni et al., 1998). The same abundance levels into plasma membrane and cytosolic extracts were reported by Santoni et al. (1998) for the nucleoside diphosphate kinase, the protein disulfide isomerase, the phosphoglycerate mutase and the glyceraldehyde-3-phosphate dehydrogenase, a feature that we also reported (spots 9, 69, 70, 72, 80–82, 86 and 99). It seems unlikely that these cytosolic proteins identified in two independent studies (Santoni et al., 1998; the present work), are contaminants of purification. They could represent a pool of soluble proteins linked to membranes.

When we compared our data to those of plant plasma membrane studies (Santoni et al., 1998; Santoni et al., 1999), three proteins (spots 61, 67 and 68) were found in common. They corresponded to the vacuolar ATP synthase subunit E and the annexin. Eighteen microsomal proteins of *A. thaliana* callus were previously reported by Prime et al. (2000), among which five proteins (spots 31, 86, 100, 101 and 117) have also been identified in the present work.

The mitochondrial membranes are generally considered to account for a large contaminating part of the microsomal preparation (Larsson et al., 1988). Our microsomal protein identifications were compared to mitochondrial proteome studies (Kruft et al., 2001; Heazlewood et al., 2003). Thirty-three over 96 belonged to true mitochondrial proteins. They corresponded principally to the F0F1-ATP synthase complex (12 spots), the different respiratory chain complex (7 spots), the transport (8 spots) and the folding of the mitochondrial proteins (6 spots) as described in Section 2.4. All these mitochondrial proteins have a membrane location (Heazlewood et al., 2003).

## 3. Concluding remarks

This sub-cellular study was aimed at characterizing microsomal root proteins of the model plant *M. truncatula*. The combination of specific conditions for extraction, solubilization and sample loading has notably improved the protein separation and spot resolution over the complete p*I* range. Moreover, a searching strategy based on PMF performed on clustered EST database has led to a substantial increase in the percentage of successful protein identification. The comparison with total, cytosolic and microsomal 2-DE gels revealed the net enrichment of the microsomal extract in new proteins. Protein identification by MALDI-TOF analyses has shown that these microsomal proteins were either known to have a possible membrane location or to display a TM domain.

The present study constitutes the preliminary approach towards a better characterization of proteins associated with biological membrane structures such as those involved for example in the nitrogen fixing and/or mycorrhizal symbioses.

## 4. Experimental

### 4.1. General

IPG strips and IPG buffer were purchased from Amersham Biosciences (Uppsala, Sweden). All other electrophoretic reagents and Coomassie Blue G-250 were obtained from Bio-Rad (Hercules, CA). The chemicals for staining procedures, iodoacetamide and DTT were from Sigma (St. Louis, MO).

### 4.2. Biological material and growth conditions

Seeds from *M. truncatula* Gaertn. *cv* Jemalong J5 were surface-sterilized and germinated at 27 °C in the dark on 0.7% sterile agar (Bestel-Corre et al., 2002). Two days-old seedlings were transplanted into 400 ml

pots containing a sterile mix (2:1 v/v) of terragreen (Agsorb, Oil Dry Corporation, IMC Imcore) and Epoisses soil (clay loam). Plants were grown under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night, 60% relative humidity, 220 μEinstein $m^{-2}$ $s^{-1}$ photon flux density). Three plants per pot were grown for 5 weeks; one replicate consisted in three pots. They were watered with demineralized water and, once a week, with a nitrogen-enriched nutrient solution (Dumas-Gaudot et al., 1994). At harvest, pots were immersed in tap water to carefully remove the roots from the soil mix. Roots were then gently rinsed to eliminate any remaining soil particles, first with running tap water, and then with deionized water. Root systems were weight, frozen in liquid nitrogen and stored at −80 °C until protein extraction. Two independent experiments were performed.

### 4.3. Microsome preparation by using differential centrifugation

Ten grams of *M. truncatula* roots were ground with liquid $N_2$ and homogenized in 20 ml of lysis buffer (0.5 M Tris–HCl, pH 7.5, 0.1 M KCl, 5 mM EDTA and 1 mM PMSF). Walls, nuclei, plastids and mitochondria were removed by two successive centrifugations (12,000*g* and 16,000*g* for 20 min at 4 °C). The supernatant, representing the total fraction, was then centrifuged at 100,000*g* for 1 h at 4 °C (L7-55 Ultracentrifuge, Beckman Coulter, Villepinte, France), leading to a second supernatant which corresponded to the cytosolic fraction. The corresponding pellet, consisting in the microsomal fraction, was washed with lysis buffer (3 ml), centrifuged (100,000*g* for 1 h at 4 °C) and then resuspended into 0.1 ml of lysis buffer.

### 4.4. Phenolic extraction of the total and cytosolic fractions

Proteins from the total and cytosolic fractions were phenol-extracted (Bestel-Corre et al., 2002). At the final step of the extraction process, the proteins were resuspended into 200 μl of 9 M urea, 4% w/v CHAPS, 0.5% v/v Triton X-100, 100 mM DTT and 2% v/v IPG buffer, pH 3–10 (Amersham Biosciences). Lipids and nucleic acids were removed by a 30 min ultracentrifugation step at 170,000*g* (Airfuge, Beckman Coulter). The protein content of the supernatant was quantified by the modified Bradford method described by Ramagli and Rodriguez (1985) using BSA as a standard. Samples were stored at −20 °C before electrophoresis.

### 4.5. $CHCl_3$/MeOH extraction of the microsomal fraction

Proteins were extracted according to the procedure of Ferro et al. (2000), with the following modifications. The microsomal fraction (0.1 ml) was slowly added to 0.9 ml of a cold mixture of $CHCL_3$/MeOH (6:3). Following complete homogenisation, the mix was stored on ice and vortexed each 5 min during 30 min. After 30 min of centrifugation (15,000*g*, 4 °C), a small pellet was visible at the interface between the two phases. These two phases were carefully removed, the pellet was dried and then resuspended in 200 μl of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.1% v/v Triton X-100, 2 mM TBP and 2% v/v IPG buffer, pH 3–10. The protein content was determined with the method of Schaffner and Weissmann (1973) using BSA as a standard. Samples were stored at −20 °C before electrophoresis.

### 4.6. Two-dimensional gel electrophoresis

Precast 18 cm nonlinear, pH 3–10, IPG strips (Amersham Biosciences) were rehydrated overnight with 350 μl of either (i) 8 M urea, 2% v/v CHAPS, 20 mM DTT, 2% v/v IPG buffer, pH 3–10, and bromophenol blue for the total and cytosolic fractions, or (ii) 7 M urea, 2 M thiourea, 4% w/v CHAPS, 0, 2 mM TBP, 2% v/v IPG buffer, pH 3–10, and bromophenol blue for the microsomal one. For analytical separations, 100 μg of total or cytosolic proteins were loaded at the cathodic end of the strips and focused at 20 °C for 50 kV h using a gradually increasing voltage. For microsomal proteins, 80 μg were loaded at the anodic end of the strips and allowed to focus at 20 °C for 57 kV h. Ten microlitres of standard proteins (2-D, Bio-Rad) were co-electrophoresed with samples in order to determine the $M_r$ and p*I* of the polypeptides separated in the gels. For the micropreparative analysis, 600 μg of proteins were loaded and the focusing was extended to 71 kV h. After focusing, strips were either stored at −80 °C or immediately equilibrated (Görg et al., 1987). Strips were then transferred onto homemade 12%, pH 8.8, SDS–polyacrylamide gels (Hoefer DALT, Amersham Biosciences). Electrophoresis was run at 10 °C for 1 h at 35 V and then at 80 V until the dye front reached the bottom of the gels.

### 4.7. Staining procedure and image analysis

2-DE analytical gels were silver stained (Mathesius et al., 2001). Images were digitalized using a Sharp JX-330 scanner (Amersham Biosciences) *via* the Labscan 3.1 software. Two independent plant repetitions were performed. For each experiment, duplicate gels were realized for the three fractions. The averaged gels of each fraction, containing only spots detected in the two experiments, were compared between them using polypeptides profiles and apparent $M_r$/p*I* calculated by the Image Master 2D Elite software (Amersham Biosciences). Spot detection was performed with the following parameters: sensibility 9048, operator size 47,

noise factor 5 and background 1. Spot volumes were calculated following background subtraction performed using the non-spot mode with a factor of 45. For spots potentially represented in at least two fractions, comparisons were based on their volume. Differences were confirmed by the statistical bilateral Student test with a probability of 0.1.

Micropreparative gel was stained with Coomassie blue G-250 (Bio-Rad) (Mathesius et al., 2001). Following extensive washing of the gel with water, spots were excised into small pieces with tips, dried and stored at room temperature before mass spectrometry analyses.

### 4.8. In gel enzymatic digestion

Proteins from spots were washed until destained in 100 μl of (1v/1v) acetonitrile and 50 mM $NH_4HCO_3$, pH 8. Gel pieces were then dried during 30 min under vacuum centrifugation. After rehydration into 10 μl of 50 mM $NH_4HCO_3$, pH 8, containing 0.5 μg of porcine trypsine (Promega, France), samples were incubated overnight (16–18 h) at 37 °C.

### 4.9. MALDI-TOF mass spectrometry

Peptide digests were crystallized with α-cyano-4-hydroxycinnamic acid as a matrix. Peptide mass fingerprints were obtained using a MALDI-TOF MS (voyager DE super STR, Applied Biosciences, Framingham, MA), equipped with a $N_2$ laser (337 nm, 3 Hz, 3 ns impulsion). Mass spectra were acquired in reflectron mode with a delay extraction time of 130 ns. The trypsin autodigestion peaks at 842.509 and 2211.104 were used for internal calibration.

### 4.10. Database search and sequence analysis

The PMF search were performed on two clustered EST *M. truncatula* database available online (http://medicago.toulouse.inra.fr/Mt/EST/DOC/MtB.html). The first, named MtC, contained 6350 clusters defined from three root EST libraries (24347 ESTs) of a Genoscope project (http://www.cns.fr/). The clustering process has been previously described in Journet et al. (2002). The second, named MtD, was obtained using the same process on the *M. truncatula* ESTs (approximately 180000 ESTs) available at the Institute for Genomic Research (http://www.tigr.org/tdb/tgi/mtgi/). It contained 21400 clusters defined from EST libraries corresponding to different *M. truncatula* tissues.

The protein prospector software (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) were used for PMF search with the peptide masses ranging from 500 to 5000 Da. For peptide matching, a minimum of four peptides matches, a maximum of one miscleavage, and peptide modifications by carboxyamidomethylcysteine, methionine sulfoxide, and pyro-glutamic acid or acetylated N-terminal residue, were accepted. The maximum tolerance for peptide mass matching was limited to 20 ppm. The confidence in the peptide mass fingerprinting matches was based on the MOWSE score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum.

The putative TM domains were searched in the TMpred server (http://www.ch.embnet.org/software/TMPRED_form.html) within a range 19–25 residues. They were taken into account when they were previously reported in literature or when they were located in a protein region covered by the PMF.

## Acknowledgements

The authors are grateful to Celine Henry (INRA, Jouy-en-Josas, France) who performed mass spectrometry and to Dr. Ghislaine Recorbet (INRA, Dijon, France) for critical reading of the manuscript. Benoît Valot was financially supported by a MENRT (Ministère de l'Education Nationale et de la Recherche Technique) grant.

## References

Barker, D.G., Bianchi, S., Blondon, F., Dattee, Y., Duc, G., Essad, S., Flament, P., Gallusci, P., Genier, G., Guy, P., Muel, X., Tourneur, J., Denarie, J., Huguet, T., 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium* – legume symbiosis. Plant Mol. Biol. Rep. 8, 40–49.

Barry, R.C., Alsaker, B.L., Robison-Cox, J.F., Dratz, E.A., 2003. Quantitative evaluation of sample application methods for semi-preparative separations of basic proteins by two-dimensional gel electrophoresis. Electrophoresis 24, 3390–3404.

Benz, R., 1994. Permeation of hydrophilic solutes through mitochondrial outer membranes –review on mitochondrial porins. Biochim. Biophys. Acta 1197, 167–196.

Bestel-Corre, G., Dumas-Gaudot, E., Poinsot, V., Dieu, M., Dierick, J.F., van Tuinen, D., Remacle, J., Gianinazzi-Pearson, V., Gianinazzi, S., 2002. Proteome analysis and identification of symbiosis-related proteins from *Medicago truncatula* Gaertn by two-dimensional electrophoresis and mass spectrometry. Electrophoresis 23, 122–137.

Capaldi, R.A., Aggeler, R., 2002. Mechanism of the F1F0-type ATP synthase, a biological rotary motor. Trends Biochem. Sci. 27, 154–160.

Chevallet, M., Santoni, V., Poinas, A., Rouquie, D., Fuchs, A., Kieffer, S., Rossignol, M., Lunardi, J., Garin, J., Rabilloud, T., 1998. New zwitterionic detergents improve the analysis of membrane proteins by two-dimensional electrophoresis. Electrophoresis 19, 1901–1909.

Cook, D.R., 1997. Model legumes get the Nod. Plant Cell 9, 175–281.

Cook, D.R., 1999. *Medicago truncatula* – a model in the making! Curr. Opin. Plant Biol. 2, 301–304.

Corthals, G., Gygi, S., Aebersold, R., Patterson, S.D., 2000. Identification of proteins by mass spectrometry. In: Rabilloud, T. (Ed.), Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods. Springer-Verlag, Berlin, pp. 197–232.

Duby, G., Boutry, M., 2002. Mitochondrial protein import machinery and targeting information. Plant Sci. 162, 477–490.

Dumas-Gaudot, E., Asselin, A., Gianinazzi-Pearson, V., Gollotte, A., Gianinazzi, S., 1994. Chitinase isoforms in roots of various pea genotypes infected with arbuscular mycorrhizal fungi. Plant Sci. 99, 27–37.

Fasman, E.D., 1989. Prediction of Protein Structure and the Principles of Protein Conformation. Plenum press, New York.

Ferro, M., Seigneurin-Berny, D., Rolland, N., Chapel, A., Salvi, D., Garin, J., Joyard, J., 2000. Organic solvent extraction as a versatile procedure to identify hydrophobic chloroplast membrane proteins. Electrophoresis 21, 3517–3526.

Frugoli, J., Harris, J., 2001. *Medicago truncatula* on the move. Plant Cell 13, 458–462.

Gooley, A.A., Packer, N.H., 1997. The importance of protein co- and post-translational modification in proteome project. In: Wilkins, M.R., Williams, K.L., Appel, R.D., Hochstrasser, D.F. (Eds.), Proteome Research: New Frontiers in Functional Genomics. Springer, Berlin, pp. 65–86.

Görg, A., 1988. Two-dimensional electrophoresis. Electrophoresis 9, 531–546.

Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J.R., Hanash, S.M., Somerlot, L., 1987. Horizontal two-dimensional electrophoresis with immobilized pH gradients in the first dimension in the presence of non ionic detergent. Electrophoresis 8, 45–51.

Handberg, K., Stougaard, J., 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. Plant J. 2, 487–496.

Harnisch, U., Weiss, H., Sebald, W., 1985. The primary structure of the iron–sulfur subunit of ubiquinol-cytochrome *c* reductase from *Neurospora*, determined by cDNA and gene sequencing. Eur. J. Biochem. 149, 95–99.

Harrison, M.J., 1997. The arbuscular mycorrhizal symbiosis: an underground association. Trends Plant Sci. 2, 54–60.

Heazlewood, J.L., Howell, K.A., Whelan, J., Millar, A.H., 2003. Towards an analysis of the rice mitochondrial proteome. Plant Physiol. 132, 230–242.

Heazlewood, J.L., Millar, A.H., 2003. Integrated plant proteomics – putting the green genomes to work. Funct. Plant Biol. 30, 471–482.

Herbert, B.R., Molloy, M.P., Gooley, A.A., Walsh, B.J., Bryson, W.G., Williams, K.L., 1998. Improved protein solubility in two-dimensional electrophoresis using tributyl phosphine as reducing agent. Electrophoresis 19, 845–851.

Islam, R.K.C., Landers, T., 1998. A new approach to rapid immobilised pH gradient IEF for 2-D electrophoresis. Sci. Tools 3, 14–15.

Jiang, Q., Gresshoff, P.M., 1997. Classical and molecular genetics of the model legume *Lotus japonicus*. Mol. Plant–Microbe Interact. 10, 59–68.

Journet, E.P., Tuinen, D.v., Gouzy, J., Crespeau, H., Carreau, V., Farmer, M.J., Niebel, A., Schiex, T., Jaillon, O., Chatagnier, O., Godiard, L., Micheli, F., Kahn, D., Gianinazzi-Pearson, V., Gamas, P., 2002. Exploring root symbiotic programs in the model legume *Medicago truncatula* using EST analysis. Nucleic Acids Res. 30, 5579–5592.

Jung, E., Heller, M., Sanchez, J.C., Hochstrasser, D.F., 2000. Proteomics meets cell biology: the establishment of subcellular proteomes. Electrophoresis 21, 3369–3377.

Kruft, V., Eubel, H., Jansch, L., Werhahn, W., Braun, H.P., 2001. Proteomic approach to identify novel mitochondrial proteins in *Arabidopsis*. Plant Physiol. 127, 1694–1710.

Larsson, C., Widell, S., Kjellbom, P., 1988. Preparation of high-purity plasma membranes. Methods Enzymol. 148, 558–568.

Link, A.J., Robison, K., Church, G.M., 1997. Comparing the predicted and observed properties of proteins encoded in the genome of *Escherichia coli* K-12. Electrophoresis 18, 1259–1313.

Maltman, D.J., Simon, W.J., Wheeler, C.H., Dunn, M.J., Wait, R., Slabas, A.R., 2002. Proteomic analysis of the endoplasmic reticulum from developing and germinating seed of castor (*Ricinus communis*). Electrophoresis 23, 626–639.

Mathesius, U., Keijzers, G., Natera, S.H.A., Weinman, J.J., Djordjevic, M.A., Rolfe, B.G., 2001. Establishment of a root proteome reference map for the model legume *Medicago truncatula* using the expressed sequence tag database for peptide mass fingerprinting. Proteomics 1, 1424–1440.

Merzendorfer, H., Graf, R., Huss, M., Harvey, W.R., Wieczorek, H., 1997. Regulation of proton-translocating V-ATPases. J. Exp. Biol. 200, 225–235.

Molloy, M.P., Herbert, B.R., Williams, K.L., Gooley, A.A., 1999. Extraction of *Escherichia coli* proteins with organic solvents prior to two-dimensional electrophoresis. Electrophoresis 20, 701–704.

O'Farrel, P.H., 1975. High resolution two-dimensional electrophoresis. J. Biol. Chem. 250, 4007–4021.

Oldroyd, G.E.D., Geurts, R., 2001. *Medicago truncatula*, going where no plant has gone before. Trends Plant Sci. 6, 552–553.

Prime, T.A., Sherrier, D.J., Mahon, P., Packman, L.C., Dupree, P., 2000. A proteomic analysis of organelles from *Arabidopsis thaliana*. Electrophoresis 21, 3488–3499.

Rabilloud, T., 1998. Use of thiourea to increase the solubility of membrane proteins in two-dimensional electrophoresis. Electrophoresis 19, 758–760.

Rabilloud, T., Adessi, C., Giraudel, A., Lunardi, J., 1997. Improvement of the solubilization of proteins in two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 18, 307–316.

Rabilloud, T., Valette, C., Lawrence, J.J., 1994. Sample application by in-gel rehydration improves the resolution of 2-dimensional electrophoresis with immobilized ph gradients in the first-dimension. Electrophoresis 15, 1552–1558.

Ramagli, L.S., Rodriguez, L.V., 1985. Quantification of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. Electrophoresis 6, 559–563.

Rappsilber, J., Mann, M., 2002. What does it mean to identify a protein in proteomics. Trends Biochem. Sci. 27, 74–78.

Saalbach, G., Erik, P., Wienkoop, S., 2002. Characterisation by proteomics of peribacteroid space and peribacteroid membrane preparations from pea (*Pisum sativum*) symbiosomes. Proteomics 2, 325–337.

Santoni, V., Doumas, P., Rouquie, D., Mansion, M., Rabilloud, T., Rossignol, M., 1999. Large scale characterization of plant plasma membrane proteins. Biochimie 81, 655–661.

Santoni, V., Rouquie, D., Doumas, P., Mansion, M., Boutry, M., Degand, H., Dupree, P., Packman, L., Sherrier, J., Prime, T., Bauw, G., Posada, E., Rouze, P., Dehais, P., Sahnoun, I., Barlier, I., Rossignol, M., 1998. Use of a proteome strategy for tagging proteins present at the plasma membrane. Plant J. 16, 633–641.

Schaffner, W., Weissmann, C., 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56, 502–514.

Seigneurin-Berny, D., Rolland, N., Garin, J., Joyard, J., 1999. Differential extraction of hydrophobic proteins from chloroplast envelope membranes: a subcellular-specific proteomic approach to identify rare intrinsic membrane proteins. Plant J. 19, 217–228.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Boucherie, H., Mann, M., 1996. Linking genome and proteome by mass spectrometry: large-scale identification of yeast proteins from two dimensional gels. Proc. Natl. Acad. Sci. USA 93, 14440–14445.

Stougaard, J., 2001. Genetics and genomics of root symbiosis. Curr. Opin. Plant Biol. 4, 328–335.

Trieu, A.T., Burleigh, S.H., Kardailsky, I.V., Maldonado-Mendoza, I.E., Versaw, W.K., Blaylock, L.A., Shin, H., Chiou, T., Katagi, H., Dewbre, G.R., Weigel, D., Harrison, M.J., 2000. Transformation

of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*. Plant J. 22, 531–541.

Van der Pas, J.C., Rohlen, D.A., Weidner, U., 1991. Primary structure of the nuclear-encoded 29.9 kDa subunit of NADH: ubiquinone reductase from *Neurospora crassa* mitochondria. Biochim. Biophys. Acta 1089, 389–390.

Watson, B.S., Asirvatham, V.S., Wang, L.J., Sumner, L.W., 2003. Mapping the proteome of barrel medic (*Medicago truncatula*). Plant Physiol. 131, 1104–1123.

Wienkoop, S., Saalbach, G., 2003. Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. Plant Physiol. 131, 1080–1090.

Wilkins, M.R., Pasquali, C., Appel, R.D., Ou, K., Golaz, O., Sanchez, J.C., Yan, J.X., Gooley, A.A., Hughes, G., Humphery-Smith, I., Williams, K.L., Hochstrasser, D.F., 1996. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Bio/Technology 14, 61–65.