Proteomics of *Medicago truncatula*

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Related Websites:

http://semele.anu.edu.au/2d/2d.html
http://www.chez.com/elianet
http://www.dijon.inra.fr/pme/
http://www.mtproteomics.com/
http://www.noble.org/PlantBio/MS/index.html
http://www.pierroton.inra.fr/genetics/2D/Proteomevert/

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Abstract

Proteomics has evolved greatly in the past few years and the number of researchers utilizing proteomics to investigate plant biology has substantially increased. The predominant aim of this chapter is to aid *Medicago* researchers in initiating proteomics experiments. The chapter consists of a brief literature review of *Medicago* proteomics, followed by protocols used in the authors' labs. Further optimization of the protocols to suit ones individual needs may be required.

1. Introduction to proteome analysis

Legumes, particularly soybean and alfalfa, are economically important crops with an estimated US annual value of $17 billion and $7 billion respectively (USDA-NASS, 2005). Though substantial research is being conducted using soybean and alfalfa, *Medicago truncatula* has gained popularity as a model legume because of its small genome size, self-fertility, rapid generation time, large number of mutants, large expressed sequence tag libraries, and genomic sequencing (Bell et al., 2001; Cook, 1999; Trieu et al., 2000). Both custom and commercial transcriptome based approaches have been established in *Medicago* research (See chapter "Transcriptomics"), however transcript profiles do not always provide a complete story due to limited correlations in transcript and protein levels (Gygi et al., 1999). The lack of correlation is not surprising considering the important roles of post translational modifications, controlled proteolysis, protein sorting, and protein-protein interactions on the regulation of active enzyme levels. Thus, proteomics has become a critical complement to mRNA data and a better systems biology view of plant and legume biology.

Several plant proteomics reviews have been published (Canovas et al., 2004; Rossignol, 2001), including sub-cellular proteomics (van Wijk, 2004). A more recent three-part review discusses proteomics of dicot plants in great detail (Agrawal et al., 2005a; Agrawal et al., 2005b; Agrawal et al., 2005c). A large number of groups have published protein reference maps for specific *Medicago* tissues that include roots, stems, flowers, seed pods and cell suspension cultures (Lei et al., 2005; Mathesius et al., 2001; Watson et al., 2003). Additional organ specific proteome analyses have been reported for specific stages of seed filling and seed development (Gallardo et al., 2003) and for somatic embryogenic tissue culture cells (Imin et al., 2004).

A major factor that accentuates *Medicago truncatula* as a model plant is its ability to associate with nitrogen fixing rhizobium bacteria and mycorrhizal fungi. Unlike in *Arabidopsis,* this enables the study of plant symbioses in *Medicago*. Proteomics of roots inoculated with mycorrhizal fungi *Glomus mosseae* or nitrogen fixing bacteria, *Sinorhizobium meliloti* (Bestel-Corre et al., 2002), and the study of *Sinorhizobium meliloti* and *Medicago truncatula* symbiosome membrane protein profile (Catalano et al., 2004) have also been published. The effect on *Medicago* roots upon infection by a pathogen, *Aphanomyces euteiches* has been analyzed (Colditz et al., 2004).

The following sections describe the commonly utilized protocols for protein extraction from various tissues, 2-dimensional gel electrophoresis (2-DE), staining, detection, difference gel electrophoresis (DIGE) and the relatively new Multi-dimensional protein identification technology (Mud-PIT) followed by protein identification by mass spectrometry. However, this chapter describes only the protocols and if the readers want to know more about the techniques, there are several reviews that discuss the technical aspects, tools and hurdles of plant proteomics (Hirano et al., 2004; Rose et al., 2004). There are also reviews that discuss the popularity (Rabilloud, 2002) and drawbacks (Gygi et al., 2000; Hamdan and Righetti, 2002; Lilley et al., 2002; Ong and Pandey, 2001) of two-dimensional gel electrophoresis (2-DE), which is currently the most widely used technique in legume proteomics.

2. Protein extraction

2.1. Cell suspension cultures (method for parallel extraction of 12 Samples) (Lei et al., 2005)

Cell culture systems, though considered simplified model systems, have been widely used to study biochemistry of *Medicago* (Baier et al., 1999; Daniell and Edwards, 1995; Edwards and Dixon, 1991; Gana et al., 1998; Guo et al., 1994; Ni et al., 1996; Otvos et al., 2005; Shorrosh et al., 1994; Steward et al., 1999; Suzuki et al., 2005). Cell culture systems are often used in experiments that require large quantities of a homogeneous cell-type that can be subjected to various conditions and multiple samples can be obtained from these conditions. Experiments like this have been performed in the author's lab (Lei et al.; Nagaraj et al., Manuscripts in preparation). A representative 2-DE gel of cell suspension culture is shown in Figure 1.

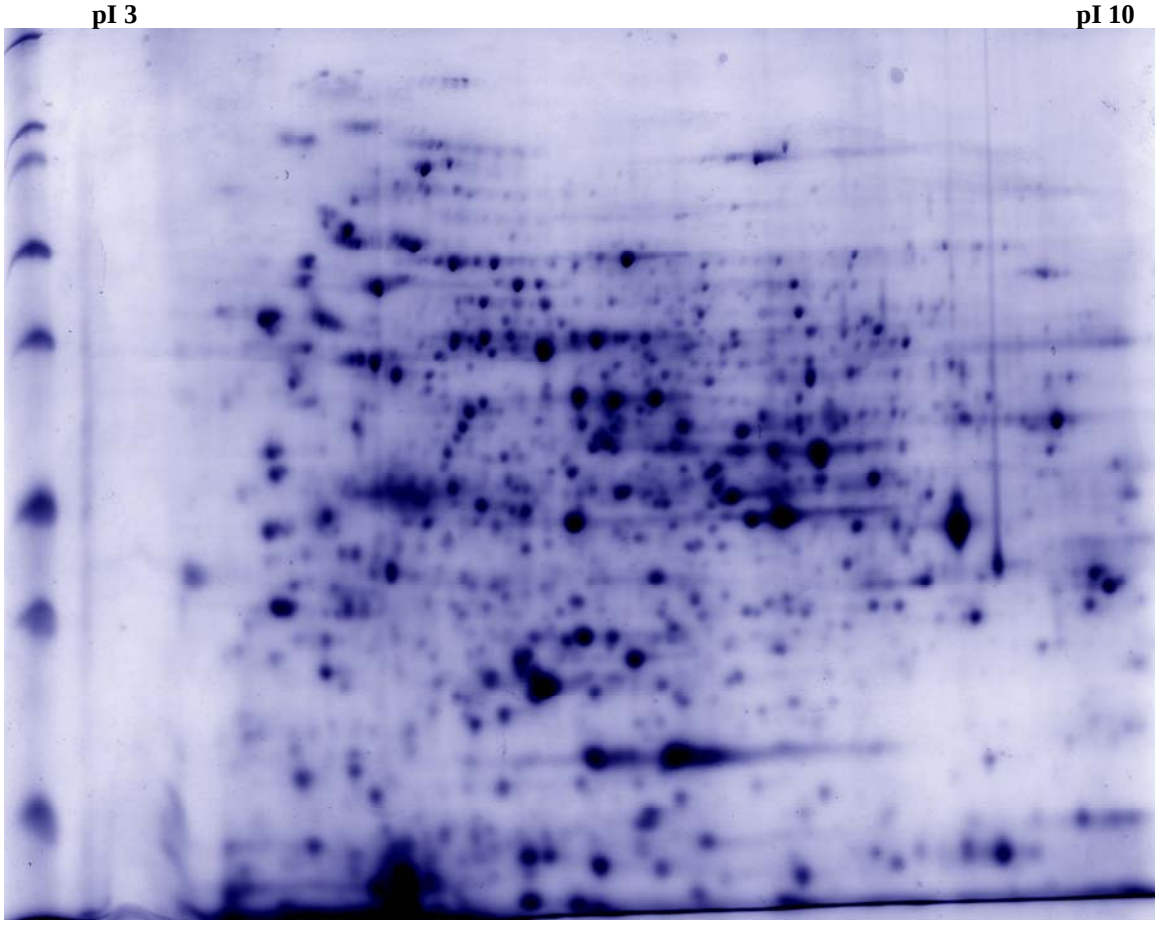


Figure 1. Representative 2-DE gel illustrating the resolution of *M. truncatula* proteins extracted from liquid cell suspension cultures. The sample was collected following treatment with a biotic elicitor. The harvested sample was homogenized, proteins were extracted and separated by 2-DE. Molecular weight markers are present on the left of the 2-DE and are composed of MARK 12 unstained standard from Invitrogen.

1. Grind approximately five grams of frozen cell tissue in a mortar (pre-chilled with liquid nitrogen).
   i. Add liquid nitrogen, grind slowly* as it boils off, and then grind faster when the tissue is "wet" with liquid nitrogen. Repeat the grinding step for a total of three times.
   *Grinding faster might cause splashing of the samples.

2. Add a small amount of liquid N_2, and swirl to break loose the ground tissue. Tissue should form a lose mass.
   i. For extracting proteins from multiple samples, place mortar in dry ice until ready for next step.
   ii. Repeat steps (1) and (2) with the remaining samples.

3. Transfer ground tissue to a 4°C mortar and add 8 mL of Tris extraction buffer.
   i. Transferring samples to a 4°C mortar will accelerate thawing and helps in homogenization.
   ii. Wait a minute or two for buffer to thaw. Grind until tissue and buffer are thoroughly mixed.
   iii. Prepare Tris Extraction Buffer:
   40 mM Tris-HCl (pH 9.5)
   50 mM $MgCl_2$
   2% PVPP
   125 U endonuclease/mL
   1 mM PMSF
   iv. Tris Stock = 1 M at pH 9.5 (stored at 4°C). FW: 121.4.
   $MgCl_2$ Stock = 1 M $MgCl_2.6H_2O$. FW: 203.31.
   PMSF Stock = 100 mM in acetone (made fresh). FW: 174.2*.
   * Dissolve 34.84 mg in 2 mL.

   v.

Tris Extraction Buffer	mL stock sol per 100 mL	mL stock sol per 125 mL	mL stock sol per 150 mL
Tris-HCl Stock (pH 9.5)	4.0 mL	5.0 mL	6.0 mL
$MgCl_2$ Stock	5.0 mL	6.25 mL	7.5 mL
2% PVPP	2.0 g	2.5 g	3.0 g
125 U endonuclease/mL	Lot dependant		
PMSF Stock	1.0 mL	1.25 mL	1.5 mL

4. Pour homogenate into 13 mL polypropylene centrifuge tube, vortex, and sonicate.
   i. Vortex for 1 minute, set on ice for 1 minute.
   ii. Sonicate for 10 pulses (Each Pulse = 2 sec). Set on ice for 3 minutes. Set sonicator (Model 150 V/T ultrasonic homogenizer, Biologics Inc.) to Power Output = 50 and Pulse = 70%.
   iii. Repeat (a) and (b) for a total of two times.

5. Centrifuge at 5,000 g for five minutes (6,500 rpm in SM-24 rotor) to pellet debris and recover the supernatant (~7-8 mL).

6. Precipitate protein by adding 4.33 mL of 37.5% TCA plus 1.0% β-mercaptoethanol, bring to a final volume of 13 mL using 37.5% TCA, incubate on ice for 45 minutes, and centrifuge at 14,000 g for 15 min.

7. Wash protein pellet 3 times in 80% acetone containing 0.05% β-mercaptoethanol.
   i. Break up protein pellet using small metal spatula.
   ii. Sonicating in a water bath for five minutes will help disrupt the pellet*.
   *Avoid heating of samples by checking intermittently and adding ice to the sonicator bath, if necessary.

8. Air dry protein pellet in laminar flow hood until all the liquid is gone but the pellet still looks damp*. Store protein samples at –80°C.
*Over drying the pellet will cause problems (lower recovery) in the solubilization step.

2.2. Green tissues

Differentiated green tissues (leaves) are typically more problematic during 2-DE analysis than cell cultures because they contain high levels of salts, chlorophyll and other molecules (polyphenolics, polysaccharides, lignins, pigments, etc.) that interfere with isoelectric focusing. The relatively large abundance of photosynthetic proteins also dominate the gels and prevent the visualization of lower abundant proteins due to the limited dynamic range of common visualization stains. In addition, plant cells contain many proteases. One extraction method used to overcome some of these problems is precipitating with trichloroacetic acid (TCA) in acetone. Homogenizing the sample in 10% TCA dissolved in acetone almost immediately inactivates proteases and precipitates proteins while providing a means of delipidating membranes and releasing membrane associated proteins. This procedure also allows interfering substances to be washed from the precipitated proteins and provides a clean sample for isoelectric focusing. Please note that a strong resolubilizing buffer must be used to ensure efficient resolubilization of the precipitated protein Be careful not to overdry the sample before resolubilization. Complete drying removes residual water trapped within the protein pellet and makes resolubilization considerably more challenging. Representative 2-DE gels of leaf and stem tissues are shown in Figure 2 (A and B).

1. Grind frozen tissue very finely in liquid nitrogen with a mortar and a pestle pre-chilled with liquid nitrogen.

2. Transfer to a polypropylene tube containing chilled (4°C) 10% TCA in acetone plus 0.07% β-mercaptoethanol and vortex. The size of the tube will be determined by your sample. For older leaves and Coomassie staining, use 2 g of tissue and 25 ml tubes. An equivalent amount of protein can be obtained from 0.5 g of younger leaves. Thirteen ml tubes are large enough for this amount of tissue. For very small samples, eppendorf tubes are probably large enough.

3. Precipitate protein for at least 45 min at -20° C.

4. Centrifuge at 14,000 x g, 15 min, 4°C. Remove supernatant without disturbing the pellet.

5. Add pre-chilled (4°C) 90% acetone containing 0.07% β-mercaptoethanol to wash protein pellet. Centrifuge at 14,000 x g, 15 min, 4°C. Wash a total of 3 times. Make sure the pellet is washed well - break up protein pellet using small metal spatula with pointed tip if necessary. The additional washes with acetone help remove residual traces of TCA that have a negative impact on isoelectric focusing.

6. After the last wash, leave a small amount of 90% acetone on the pellet. Resuspend the pellet in the acetone and move it to an eppendorf tube. Rinse the large tube with more acetone and add that to the sample in the eppendorf. Spin the sample in a 4°C microfuge, 14,000 x g, 5 min, to pellet the protein. Remove the acetone.

7. Air dry protein pellet in laminar flow hood. The acetone must be removed from the sample, but do not over dry the pellet. This will cause problems (lower recovery) in the resolubilization step. Dry until the pellet looks crisp around the edges and comes loose from the side of the tube when the tube is thumped. The interior of the pellet should still look damp.

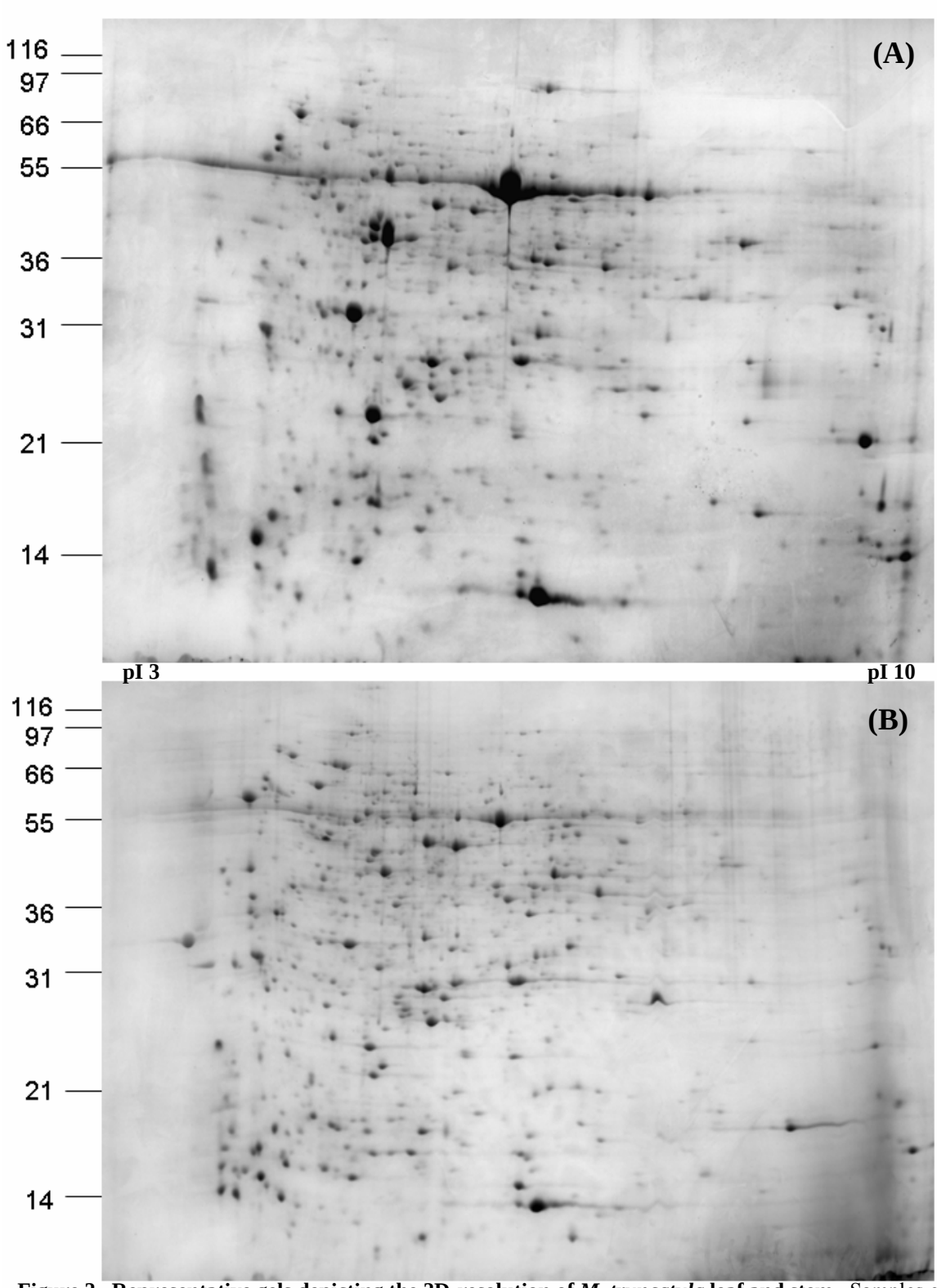


Figure 2. Representative gels depicting the 2D-resolution of *M. truncatula* leaf and stem. Samples of leaf tissue (A) and stem tissue (B) were harvested and processed for 2-DE separation. The protein samples were focused using 3-10 linear IPG strips for the 1st dimension, electrophoretically separated on a 12% acrylamide gel, and stained with Coomassie.

8. The protein pellet can be stored in the freezer at this step.

9. Resolubilize the protein pellet in an appropriate volume of solubilization buffer (See 3.1). A rule of thumb for determining the amount of solubilization buffer needed is to add 0.5-1 ml buffer/g starting material, but this depends on the protein concentration of your starting material (older tissue usually has a lower protein concentration per gram dry weight) and whether you plan to silver or Coomassie stain the gel.

10. Pellet insoluble material @ 14,000 x g for 10 min, 10°C. The urea may precipitate if you spin at 4°C.

11. Transfer supernatant to new test tube and check protein concentration using the Bradford assay (See 3.1.a - 6).

12. Proteins can then be resolved by 1D or 2D gel electrophoresis as described below (See 3.1).

2.3. Total protein extraction from developing and mature seeds (Gallardo et al., 2003)

Total protein extracts were prepared from *M. truncatula* developing seeds collected on a batch of 20 plants grown in pots at 22/19°C day/night temperatures, under a 16-h photoperiod at 220 μE/m^2/s light intensity with 60-70% relative humidity. Individual flowers were tagged on the day of flower opening (i.e. 1 day after pollination, dap) over a one-month period. Pods were harvested from 12 days after pollination until maturity (Table 1). The developing seeds were collected on Petri dishes placed on ice to prevent any dehydration, weighed (Sartorius ISO 9001 Scale, Quality Control Services, Portland, OR), and rapidly frozen in liquid nitrogen. To minimize the effects of heterochrony it is advisable to harvest grains from the central part of the pod only. Between 110 and 400 mg (corresponding to an equivalent of 65 seeds) of fresh immature seeds were collected per stage (Table 1). Seed samples were ground in liquid nitrogen using mortar and pestle. The powder was homogenized and stored at -80°C until protein extraction.

Because *M. truncatula* seed development is associated with important physiological changes (increase in seed dry weight, water loss, see Table 1), total proteins were extracted from immature seeds by using 20 μl, per mg of seed dry matter, of a thiourea/urea lysis buffer (Gallardo et al. 2003). Total proteins were extracted from mature seeds with 50 μl, per mg of seed weight, of the same buffer (Le Signor et al., 2005). All extractions were carried out at 4°C in 1 ml of the thiourea/urea lysis buffer (Table 1) containing 7M PlusOne urea (Amersham Biosciences, Orsay, France), 2M thiourea (Sigma, St Quentin Fallavier, France), 62 mM PlusOne CHAPS (3-[3-cholamidopropyl-dimethylammonio]-1-propane-sulphonate, Amersham Biosciences), 1% (v/v) carrier ampholytes (pharmalyte) pH 3 to 10 (Amersham Biosciences), 0.24% (v/v) Triton X-100 (Sigma). This extraction buffer also contained nucleases (60 Units DNase I from Roche and 5.8 Kunits RNase A from Sigma), 21 mM Tris-HCl, 16.5 mM Trizma base (Sigma), and the protease inhibitor cocktail complete Mini from Roche Diagnostics GmbH (Mannheim, Germany). After stirring the samples for 30 min at 4°C, 8 mM dithiothreitol (PlusOne DTT, Amersham Biosciences) was added. The protein extracts were stirred for 20 min at 4°C, then centrifuged (20,000g, 10 min, 4°C). The supernatant was submitted to a second clarifying centrifugation as above. The final supernatant corresponded to the total protein extract. Protein concentration was measured according to Bradford (1976) by using the Bio-Rad (Marnes-la-Coquette, France) Protein Assay compatible with the reducing and denaturing agents used to prepare all protein extracts. Bovine serum albumin was used as a standard. A representative gel of developing seed proteins is shown in Figure 3.

Table 1. Seed fresh weight used to extract total proteins in 1 ml of the thiourea/urea lysis buffer. Correspondence between seed fresh weight (FW) and seed dry weight (DW) at specific stages of seed development is indicated.

Stage	FW (mg/seed)	DW (mg/seed)	FW (mg) equivalent to 1 mg DW	FW (mg) for 1 ml of extraction buffer
12 dap	1.71±0.21	0.21±0.02	8.14	**407**
14 dap	3.86±0.27	0.60±0.04	6.43	**322**
16 dap	4.84±0.5	0.89±0.16	5.44	**272**
20 dap	6.38±0.44	1.91±0.21	3.34	**167**
24 dap	7.22±0.08	2.80±0.06	2.58	**129**
36 dap	10.47±0.6	4.79±0.43	2.18	**109**
Dry mature	4.35±1.02	3.46±0.42	1.26	**25**

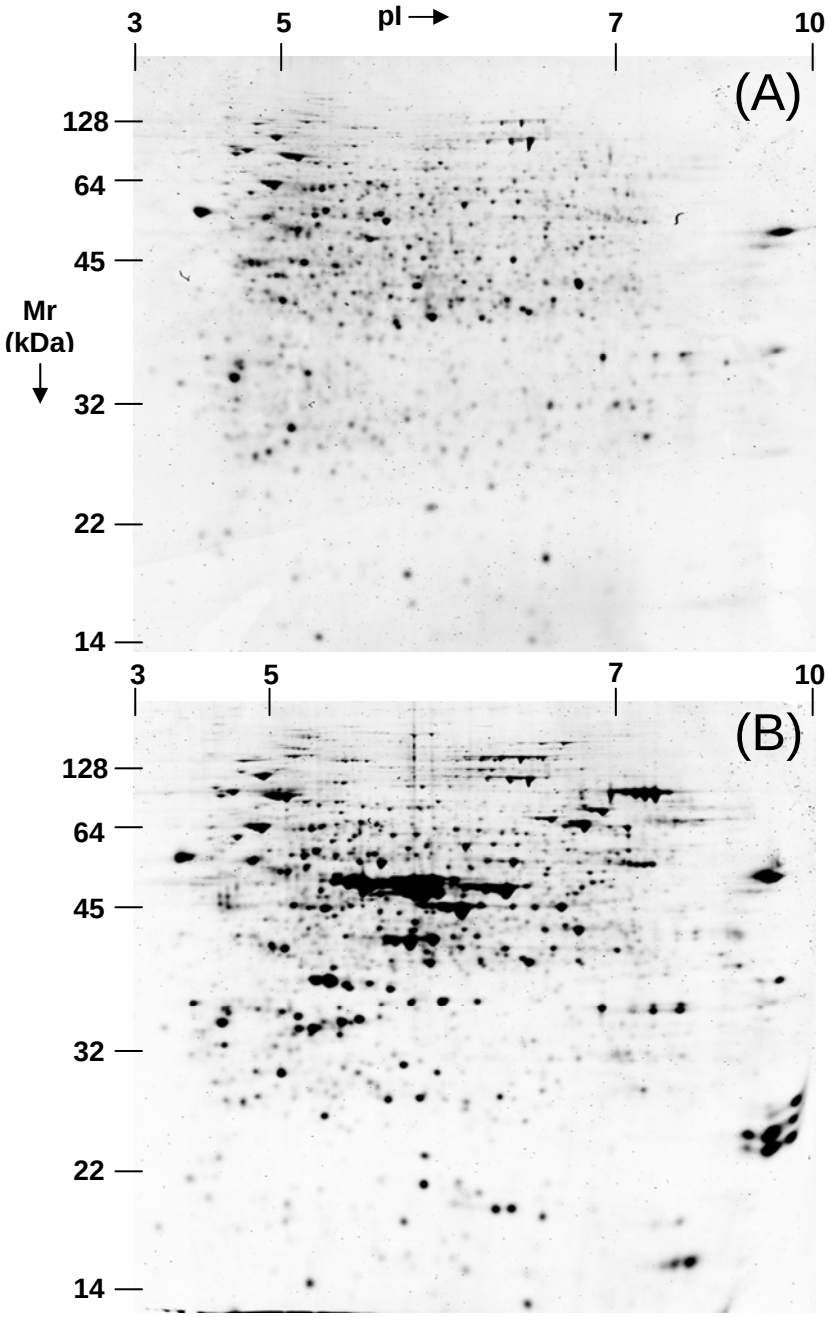


Figure 3. Representative gels depicting the 2D-resolution of *M. truncatula* developing seeds. Samples of seeds harvested 12 dap (A) and 16 dap (B) are shown. The protein samples were focused using 3-10 nonlinear IPG strips for the 1st dimension, electrophoretically separated on a 10% acrylamide gel, and stained with Coomassie Brilliant Blue G-250. Image acquisition was done using the Odyssey Infrared Imaging System.

2.4 Protein extraction from roots and nodules (Mathesius et al., 2001; 2003)

Most parts of the root are highly vacuolated any contain contaminants that interfere with protein extraction, for example phenolics and flavonoids. We have found that extraction preceded by a precipitation method with tricholoacetic acid (TCA) and acetone leads to a clean protein preparation. The protein yields are approximately 0.1% of fresh weight in roots, and up to ten times more in less vacuolated tissues like root tips and nodules.

A typical protein extraction protocol will take about a day.

1. Harvest tissue quickly into liquid nitrogen to prevent and protein breakdown. This is particularly important if you want to look at post translational modifications, for example phosphorylation (see 2.9). Store at -80°C until needed.

2. Prepare the following solutions on the day of the extraction and store at -20°C:
   - 10% TCA in acetone, including 0.07% (w/v) dithiothreitol (DTT)
   - 100 % acetone, including 0.07% (w/v) DTT

3. Prepare Solubilization Buffer (Table 2) on the day, or beforehand and keep frozen at -80 °C (for up to 3 months) until the day of the extraction.

Table 2. Solubilization buffer:

Ingredient	Add for a final volume of 10 ml	Final concentration	Comments
Urea	5.4 g	9 M	Solubilizes proteins
DTT	0.1 g	1 % (w/v)	Breaks S-S bonds
CHAPS	0.4 g	4 % (w/v)	Detergent
Biolyte Amphotytes, pH 3-10	100 µL		Help disaggregating proteins
Tris base	0.042 g	35 mM	Buffer
EDTA	100 µL of 0.5 M stock	5 µM	Protease inhibitor, removes Ca^{2+} ions
PMSF	100 µL of 100 mM stock (in ethanol)	1 µM	Protease inhibitor, very toxic
Purified water	Up to 10 ml		

Additional comments:
This solution will be very concentrated. Only add the water at the end because there are a lot of dry ingredients. They will dissolve better if you hold the tube into a sonic bath for a few minutes with shaking, or place on a rotating wheel for a few minutes.

If making a stock solution and freezing at -80°C, do not add the protease inhibitors to the frozen stock, add fresh on the day of use to defrosted solution. We have found these protease inhibitors quite sufficient, although other ones could be used. Generally it doesn't make a difference to add them or leave them out because the TCA will inhibit most proteases.

4. Have ready a bucket of dry ice and a bucket of water ice, liquid nitrogen, cleaned and pre-cooled mortar and pestle, spatula and centrifuge tubes.

5. Grind tissue (optional with a bit of fine glass powder), in liquid nitrogen in a clean mortar and pestle. Thorough grinding is a very important step. Usually we grind tissue for at least 5 minutes until a fine white powder appears.

6. Scrape the ground tissue out of the mortar with a pre-cooled spatula into a pre cooled (best on dry ice) Eppendorf tube or ultracentrifuge tube (needs to be acetone resistant, for example Beckman Polyallomer tubes).

7. Add approximately 10 times volume of the pre-cooled TCA/acetone solution to the ground tissue. Vortex. Keep on dry ice. The TCA/acetone mixture will usually inhibit most of the proteases. It also precipitates the proteins into a pellet, and removes contaminants that dissolve in the acetone, e.g. phenolics. Keeping the samples cold is important to improve precipitation and inhibit protein breakdown.

8. Securely close the tube and sonicate in a sonicator (for example, we use a Branson Ultrasonifier). Place the tubes on water ice during this step because the sonication will produce heat. For this step both probe sonicators (which use a metal rod sticking into the sample) or a cup sonicator (in which the closed tube sits in a cup and sound waves are produced from the outside) are OK. Cup sonicators are better to prevent contamination of the samples. Place the tubes on ice in the cup and sonicate 5-6 times for 10 seconds each, with 30 second breaks in between. Replace ice in between as it will melt. The sonification is important to disrupt the tissue even more than what can be achieved with mortar and pestle.

9. Return tubes onto dry ice and leave for approximately 1 hr (this step can be longer, even over night).

10. Centrifuge the tubes either in a cooled bench centrifuge (e.g. in the cold room) at 14,000 rpm or in an ultracentrifuge at 14,000 – 17,000 rpm. Ultracentrifugation is not necessary, but often better if the samples are large and won't fit into an Eppendorf tube).

11. Remove the supernatant and discard. Keep the pellet. Add a volume of pre-cooled acetone (with DTT), again about 10 times the volume of the pellet, more is also fine. Flick the pellet or stir briefly with a clean glass rod to wash the TCA out. Keep on dry ice for at least 30 min (again this step can be extended to over night if the experiment needs to be interrupted).

12. Centrifuge as in 9.

13. Remove the supernatant. Repeat steps 10 and 11.

14. After removing the second volume of acetone, dry the pellet. Either leave the tubes open on the bench (at room temperature is fine at this stage as the TCA treatment should have inhibited proteases) for at least 1 hr. Alternatively, dry the pellet for no more than 5 min in a rotary evaporator (on low heat setting), or leave tube open in the

freezer over night. The acetone should evaporate. Avoid over-drying the pellet as it will be difficult to resuspend in the next step.

15. If you used an ultracentrifuge so far, transfer the pellet into an Eppendorf tube, otherwise leave in Eppendorf tube. Suspend the pellet in a small volume of Solubilisation Buffer (see Table 2.). The volume depends on the size of the pellet. We usually start with a volume slightly bigger than the pellet size. The sample should be as concentrated as possible. It will look like this mixture will stay almost dry, but after rigorous vortexing (several minutes) and sonication in a sonic bath (add some ice to the water) the pellet will solubilise. To help resuspension the tube can be left at room temperature over night, but this is not necessary. Once the solution looks homogenous, spin for 5 min at 14,000 rpm in a bench centrifuge. The proteins should go into solution. Collect the supernatant into a clean Eppendorf tube. Repeat this step, i.e. add a little more solubilisation buffer to the pellet, vortex and centrifuge again, combine both supernatants. Keep the pellet if you are interested in extracting some less soluble proteins from it, e.g. membrane proteins. In that case, it is best to boil the pellet in a solution containing SDS and separating proteins on a 1D gel. Looking at membrane proteins for subsequent 2D gel analysis is not recommended (this is because the strong detergents, e.g. SDS, needed to extract membrane proteins will interfere with the isoelectric focusing). See paragraph 2.6 for protocols on membrane protein extraction.

16. Measure the protein concentration of the solution. We use a Bradford protein assays but others are fine as well. We use a protein dilution range from 0.1 to 1 mg of bovine serum albumin (BSA), dissolved in the same solubilisation buffer, as a standard. Usually protein concentrations are between 1 - 5 mg/ml.

17. At this stage the protein solution can be kept for several months at -80°C, or can be used immediately for subsequent 1 or 2D gel electrophoresis.

2.5 Protein extraction from soil-grown roots inoculated or not with micro-organisms

To sample roots from plants that have been grown in a soil consisting of any kind of substrate, the roots are carefully removed from the soil mix by immersing pots in tap water. Then, roots are gently rinsed to eliminate any remaining soil particles, first with running tap water, and secondly with deionized water. At this stage, part of the root system may be checked for microbial infections (*i.e.* mycorrhizal, bacterial, pathogenic, nematode infections etc….) with an appropriate test. The remaining root system is weighed and either immediately frozen in liquid nitrogen and stored at -80°C until protein extraction or directly submitted to the selected protein extraction process.

Products and Buffers

- It is strongly recommended to use high-grade quality electrophoresis products
- Extraction buffer: see Table 3
- Biophenol pH 8.0 Tris-buffered (Biosolve or any other chemical company)
- Cold 0.1 M ammonium acetate in methanol (kept at - 20°C)
- Cold methanol and cold acetone (kept at - 20°C)
- Solubilization buffer (Table 4) [modification of O'Farrell's lysis buffer (O'Farrell, 1975)]

Extraction of total and soluble proteins

This protocol has been adapted from Bestel-Corre *et al.,* (2002) as following:

1. Crush roots in liquid nitrogen (N_2). Routinely, 1g (fresh weight) of *M. truncatula* roots is crushed in a mortar cooled with liquid N_2. Once the root sample has become a very thin powder, transfer it into a second cold mortar (4°C) and homogenize with cold extraction buffer (1/10 w/v of fresh root material) (Table 3)
2. Transfer the resulting suspension to centrifuge tubes (be careful to choose tubes resistant to organic solvents)
3. Immediately add under hood, an equal volume of biophenol pH 8.0 Tris-buffered. Mix thoroughly for 30 min on a shaker in a cold room
4. Centrifuge for 30 min at 12 000 g (4°C). Collect the phenolic phase and add one volume of the extraction buffer. Repeat the shaking step as in 3 and then centrifuge for 30 min at 12 000 g (4 °C)
5. Collect the phenolic phase and precipitate the proteins overnight at -20°C with 5 volumes of 0.1 M ammonium acetate in methanol
6. The next day, pellet the proteins by swing centrifugation at 16 000 g for 30 min (4 °C). Rinse the pellet with methanol (3x 1ml) and then acetone (3x 1ml) (kept at -20°C) and dry under nitrogen gas at room temperature
7. Dissolve the pellet 4 h at 20 °C in O'Farrell's lysis buffer (Table 4) and centrifuge for 30 min at 170 000 g (20 °C). Supernatant can be either directly analyzed or stored at - 80°C for further analyses.

Measure of protein content in samples

This step is crucial for further 2D-PAGE analysis since the determination of the amount of proteins effectively solubilized and applied to gels is required to evaluate 2D protein maps accurately and quantitatively. The protein content of the supernatant is determined by Bradford's assay (Bradford, 1976) with the modifications suggested by Ramagli and

Rodriguez (1985). This allows protein quantification even in samples containing components that usually interfere with the reactive of Bradford's assay.

Table 3: Extraction buffer (100ml) for total and soluble proteins

Tris 0.5 M	6.055 g
Deionized water	sqf 50 ml
Sucrose 0.7 M	23.9 g
KCl 0.1M	0.75 g
Thiourea 10mM	0.076 g
EDTA 5 mM	50 mM 10 ml
β-mercaptoethanol 2%*	2 ml
PMSF 2 mM in DMSO*	18 mg (1 ml DMSO)
MilliQ water *	sqf 100 ml

* To be added just before using the solution

Table 4: **Solubilization buffer for soluble proteins***

Urea 9 M	5.4 g
CHAPS 4% (w/v)	0.4 g
Triton X-100 0.5% (v/v)	50 µl
DTT 20 mM	0.154 g
IPG buffer 3-10 1.2% (w/v)	200 µl
MilliQ water	sqf 10 ml

*can be stored at -20°C

2.6 Extraction and solubilization of *M. truncatula* root membrane proteins by chloroform/methanol (Valot *et al.,* 2004 and 2005, adapted from Ferro *et al.,* 2000)

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 the case of biological membranes, various studies were directed to identify membrane proteins of either microsomes (Prime *et al.,* 2000) or plasma membranes (Santoni *et al.,* 1998 & 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 to 2-DE analysis (Seigneurin-Berny et al., 1999), we have developed a protocol to gain access to proteins associated with total membrane structures of *M. truncatula* roots, ie. microsomes. This was achieved by combining a sub-cellular partitioning process to 2-DE separation (Valot *et al.,* 2004) and that has recently been validated for the study of the late stages of arbuscular symbiosis (Valot *et al.,* 2005).

1. Sample roots (at least 10g of fresh material or of material stored at – 80°C)
2. All steps are carried out in a cold room
3. Crush with liquid nitrogen and add the extraction buffer E_1 (Table 5) (2 ml of E_1 buffer/g of fresh material)
4. Cell walls, nuclei and most of the mitochondria will be precipitated by two successive steps of centrifugation 20 min at 12000 g and 16000 g (Figure 4A)
5. The supernatant corresponds to the "**total protein fraction"** from which 2 ml will be reserved for a subsequent phenolic extraction
6. The remaining volume is centrifuged one hour at 100000 g. The second supernatant corresponds to the "**cytosolic fraction"**, that will be, like the total fraction, submitted later to a phenolic extraction (Figure 4A)
7. The pellet, containing the whole cell membranes, corresponds to the "**microsomal fraction"** (Figure 4A)
8. This pellet is solubilized in 3 ml of the E_1 extraction buffer and centrifuged 1h at 100000 g (Figure 4A)
9. The resulting pellet is suspended in 0.1ml of E_1 extraction buffer

This microsomal fraction is then extracted according to the protocol of Ferro *et al.,* (2000) modified as following:

10. Add the microsomal fraction very carefully to 0.9 ml of a mix of cold $CHCl_3/CH_3OH$ (C/M) (6/3 v/v). Mix carefully with a pipette, leave on ice 30 min but shake with a vortex every 5 min
11. Centrifuge 30 min at 15000 g 4 °C: a very thin layer of proteins will be visible at the interface of the two phases that corresponds to the fraction of proteins insoluble in the organic mix and will be called "**insoluble C/M fraction**" (Figure 4B)
12. Collect the two phases that contain the proteins solubilized in the organic mix solvent corresponding to the **"soluble C/M fraction"** (Figure 4B)
13. Both fractions are dried under vacuum
14. Suspend the **insoluble C/M fraction** in 200 µl of the isofocalisation buffer I_2 (Table 6)
15. Lipids will be eliminated by a 30 min centrifugation at 170000 g
16. Protein amounts will be determined according to Schaffner and Weissmann's method (1973)

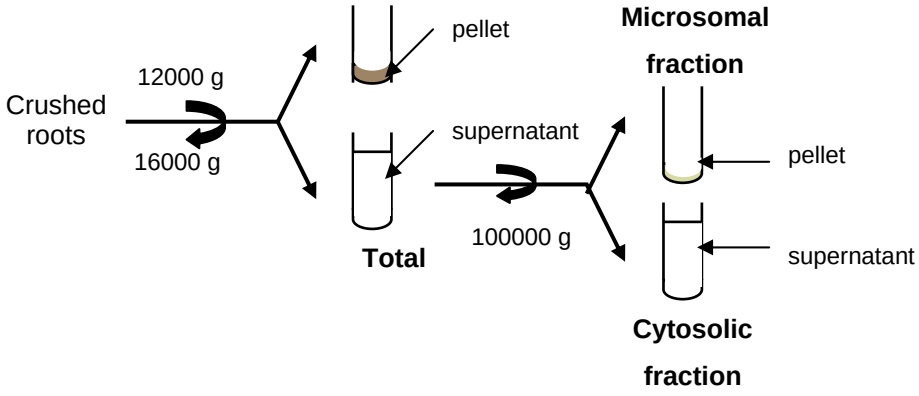
17. Samples can be kept at -20°C

18. The **"soluble C/M fraction" can be solubilized** either in 30 to 50 µl of the isofocalisation buffer I_3 (Table 7) and proceed further as for the "**insoluble C/M fraction"** or in 50 µl of the extraction buffer E_1

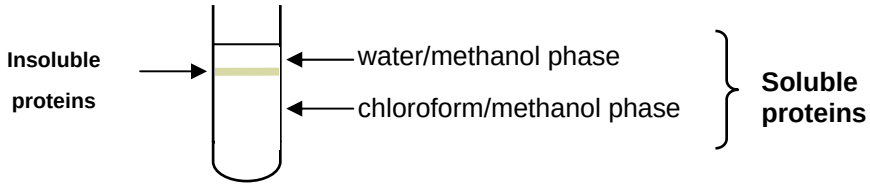
19. The protein amount is determined by the method of Bradford (1976) modified by Bearden (1978) in presence of 0.01%Triton X100 with BSA as standard. Add 4 volumes of cold acetone to precipitate proteins overnight. They can be kept at -20°C until electrophoresis.

Figure 4: Schematic representation of purification and extraction of root microsomal proteins

A-The different fractions collected during purification of microsomes



B-Scheme of protein separation with the chloroform/methanol extraction of the microsomal fraction



2.7 Plasma membrane proteins

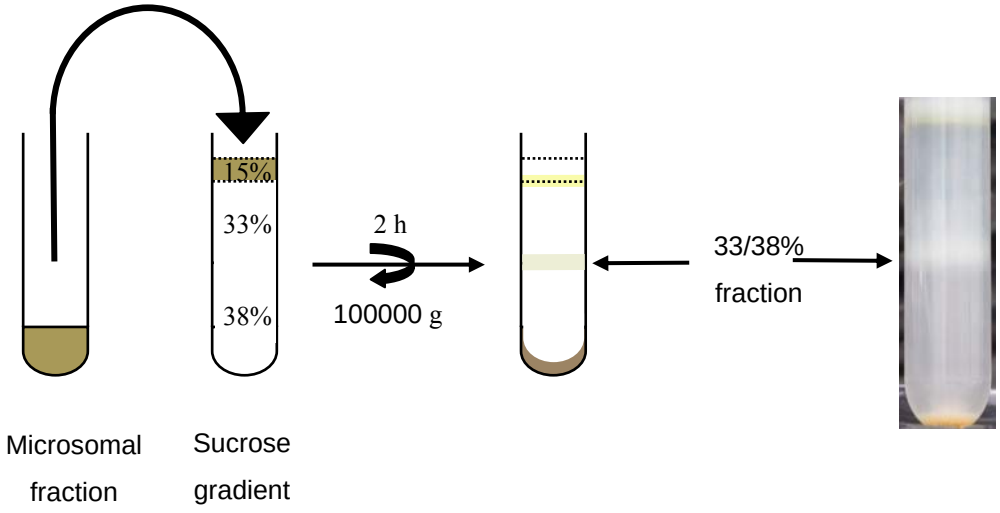
Plasma membrane protein purification is performed starting from a microsomal fraction of root proteins (see 2.6.10). The various membranes are separated by a discontinuous gradient of sucrose. The purification process is followed by measuring several enzymatic activities, selected as markers of the different types of membranes (see below) (Valot *et al.*, 2006).

1. Prepare a microsomal fraction from hundred grams of roots, crush in liquid N_2 and suspend in 150 ml of extraction buffer E_3 (Table 8). After two successive centrifugations at 12000 and 16000 g for 20 min each, the supernatant is then centrifuged at 120000 g for 1 hour
2. The pellet, corresponding to microsomes is re-solved in 600 µl of a sucrose buffer (Table 9) and homogenized with a potter

Membrane separation is performed on discontinuous sucrose gradient (Figure 5). The protocol of Hodges et al (1972) has been modified as following

3. The discontinuous sucrose gradient is formed by 2 successive layers of a solution containing 3.5ml of 38% (W/W) and 3 ml of 33% (W/W) of sucrose in the sucrose buffer S_1 (Table 9). An aliquot (60 µl) is reserved to follow the purification. The remaining volume is layered on the sucrose gradient
4. Centrifuge at 120,000 g for 2 hours in a swing rotor. Membranes form rings at the 15/33% and 33/38% interfaces and a pellet can be detected at the end of the 38%phase (picture on the right in Figure 5)
5. The 33/38% interface, corresponding to a fraction enriched in plasma membrane, is taken off with a syringe with a bevelled needle. Sample the two other interfaces in order to follow the purification process
6. Wash the various fractions with 4 volumes of buffer S_2 (Table 10)
7. Centrifuge at 140,000 g for 1 hour and re-suspend the fraction into 80 to 350 µl of preservation buffer P_1 (Table 11)
8. The protein amount is determined by the method of Bradford (1976) modified by Bearden (1978) in presence of 0.01%Triton X100 with BSA as a standard. Fractions should be kept at-80°C before analysis.

Figure 5: The different steps of plasma membrane purification by sucrose gradient. The fraction containing the plasma membrane is sampled at the 33/38% interface. On the right: a picture showing a separation after centrifugation (Valot, unpublished result).



Enzymatic assays

Marker enzymes for plant cell membranes must be assayed in the 3 fractions obtained after sucrose gradient partitioning. The K^+, Mg^{2+}-ATPase activity sensitive to SW26 was assayed as a marker for the PM, whereas the insensitive one was used to follow the other membranes (Blein *et al.,* 1986). The pyrophosphatase, inosine diphosphatase, NADH-cytochrome c reductase insensitive to antimycin A and cytochrome c oxidase were used as markers for the tonoplast, the Golgi apparatus, the endoplasmic reticulum and mitochondria, respectively (Joyce *et al.,* 1988, Bomhoff, G. H. and Spencer 1977). Assays should be performed on 3 independent sucrose-partitioning experiments in a sufficient number of replicates.

Table 5: Extraction buffer E_1

Tris-HCl pH 7.5	0.5 M
KCl	0.1 M
EDTA	5 mM
PMSF	1 mM

Table 6: Isofocalisation buffer I_2

Urea	7 M
Thiourea	2 M
Triton X-100	0.1% (v/v)
CHAPS	4% (w/v)
TBP	2 mM

Table 7: Isofocalisation buffer I_3

Urea	7 M
Thiourea	2 M
Triton X-100	0.5% (v/v)
ASB14	2 % (w/v)
TBP	2 mM

Table 8: Extraction buffer E_3

Tris-MES pH 8	50 mM
Sucrose	0.5 M
EDTA	20 mM
DTT	0.1 mM
PMSF	1mM

Table 9: Sucrose buffer S_1

Tris-MES pH 7.2	1 mM
$MgSO_4$	1 mM
Sucrose	15 % (w/w)

Table 10: Sucrose buffer S2

Tris-MES pH 7.3	10 mM
Sucrose	250 mM
ATP	1 mM
EDTA	1 mM
DTT	1 mM
PMSF	1 mM

Table 11: Preservation buffer

Tris-MES pH 6.5	10 mM
Sucrose	250 mM
$MgCl_2$	2 mM

2.8. Combined extraction of RNA and proteins

Functional genomics, i.e. the systematic elucidation of coding sequences in the genome, has mainly been investigated at transcript or protein levels separately. However, in most cases, there is little correspondence between these two components (Chen *et al.,* 2002, Gygi *et al.,* 1999). This is mainly due to post-translational modifications that affect protein behaviour and the fact that both levels are measured independently in separate sample extracts. Few biological systems are sufficiently synchronised or amenable to direct comparative transcriptome/proteome analyses, and they are mainly limited to isolated cell cultures (Chen *et al.,* 2002). In the case of complex situations involving two or more organisms, synchronisation of developmental processes is often difficult to establish. The root-fungus symbiosis represented by arbuscular mycorrhiza associations is one example of such a case. To circumvent the problems inherent to a limited amount of biological material, we have developed a protocol in which proteins can be analyzed from the same root sample as a mRNA population, in order to make simultaneous proteome and transcriptome profiling possible. This protocol was validated to analyze the early stages of the AM symbiosis (Dumas-Gaudot *et al.,* 2004) (Figure 6).

First day

1. Take 1g of roots from plantlets freshly harvested or stored at -80°C
2. Crush the roots in a cold mortar with liquid N_2 into a very thin white powder
3. Pour this material in a N_2-cooled tube, wash the mortar with little liquid N_2 to collect all the root material and leave the liquid N_2 to evaporate without letting the powder dry
4. Add 2 ml of NTES buffer (Table 12) per g of roots
5. Shake with a vortex until obtaining a very viscous liquid
6. Add 1 volume of phenol/chloroform/isoamylic alcohol (25/24/1) and shake again with a vortex until obtaining a whitish liquid. Sample 4 X 1000 µl in 4 Eppendorf tubes
7. Centrifuge at 12000 g during 15 min at 20°C
8. Collect the supernatant (400 to 500 µl). Avoid taking the intermediary phase and keep the phenolic phase on ice for the protein extraction
9. Repeat the steps 6 to 9 twice
10. Keep the supernatant (about 200 µl) to proceed to a chloroform extraction of RNA and put aside the phenolic phases 2 and 3 at – 20°C
11. Centrifuge 12000 g for 10 to 15 min at 20°C
12. Collect the aqueous phase and add 0.05 v acetic acid (1 M) et 0.7 cold ethanol 96% (kept at -20°C)
13. Mix carefully and precipitate nucleic acids overnight at – 20°C

Second day

1. Centrifuge at 12000 g for 35 min at 4°C
2. Throw away the supernatant and suspend the pellet in 200 µl DEPC water
3. Shake the tubes up side down and leave on ice 5 min
4. Add 1 volume of LiCL (4M) to precipitate the RNA, mix carefully and leave 4-5 hours in a cold room (4°C)
5. Centrifuge 12000 g 35 min at 4°C
6. Throw away the supernatant and suspend the pellet in 200 µl DEPC water

7. Shake the tubes and leave on ice for 10 min
8. Precipitate the RNA overnight at –20°C with 0.1 volume sodium acetate (3M) and 3 volumes of cold ethanol 96% (kept at -20°C)

Third day

1. Centrifuge at 12000 g for 35 min at 4°C
2. Throw away the supernatant and re-suspend the pellet in 200 µl of cold ethanol 75 % (kept at -20°C)
3. Centrifuge at 12000 g for 35 min at 4°C
4. Dry the pellet (about 1h) and suspend in 50 µl of DEPC water (leave about half a day and mix from time to time)
5. Leave overnight at -20°C (may also be kept at -80°C)

Fourth day

Check the RNA quality on an agarose gel before its quantification with a spectrophotometer (Sambrook and Russell, 2001)

Protein extraction

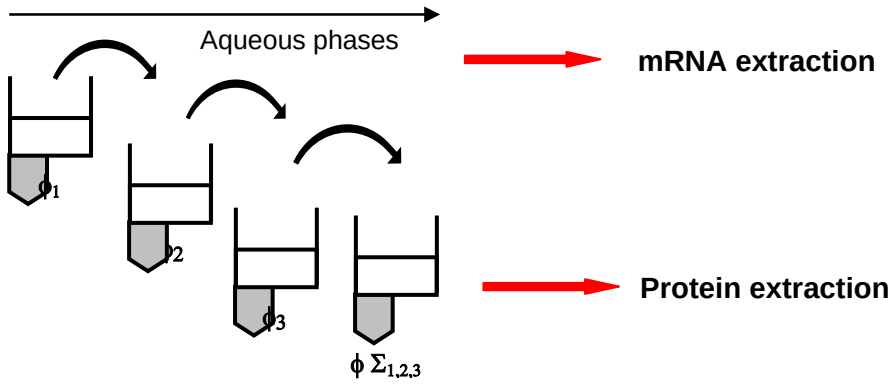
1. Pool all the phenolic phases (ϕ 1 to 3) and complete with phenol up to 10ml
2. Homogenize in 10 ml of extraction buffer (Table 3) and proceed as previously described (see paragraph 2.5). Proteins can be then analyzed by 2-DE.

Table 12: NTES buffer for RNA extraction

	Initial concentration	Final Concentration	per ml
NaCl	5 M	150 mM	30 µl
EDTA	0.5 M	5 mM	10 µl
Tris HCL pH 9	1 M	50 mM	50 µl
SDS	10 %	5 %	500 µl
DEPC water			410 µl

*May be prepared in advance in Eppendorf tubes. Add extemporaneously some β-mercapto-ethanol (10 µl.10^{-1} ml NTES buffer)

Figure 6: Schematic representation of the protocol for extracting RNA and proteins simultaneously.



2.9 A batch procedure for the enrichment of phosphorylated proteins from *Medicago truncatula* protein extracts using Metal Oxide/Hydroxide Affinity Chromatography (MOAC)

This protocol for denaturing protein extraction and subsequent phosphoprotein enrichment can be applied to a variety of plant materials, such as leaves, roots, nodules, seeds or whole seedlings. After enrichment and SDS-PAGE, different methods can be applied for the detection and visualization of phosphoproteins, eg western blotting or fluorescent phosphoprotein specific dyes (ProQ Diamond). This procedure has been optimized for plant tissues and successfully applied for the identification and determination of phosphorylation sites in *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (Wolschin and Weckwerth 2005; Wolschin, Wienkoop et al. 2005).

General precautions

- prepare protein samples freshly
- do not freeze proteins or store in aqueous solution
- in case storage is inescapable store as dried pellets overnight
- worked with chilled solutions (4°C)
- use protease and protein phosphatase inhibitors
- be careful with inhibitors as they are very toxic
- work under the fume hood for the denaturing extraction procedure

Protease inhibitors and protein phosphatase inhibitors

- Plant Protease Inhibitor Cocktail (Sigma, Taufkirchen) contains :
  AEBSF, 1.10-Phenanthroline, Pepstatin A, Leupeptin, Bestatin and E-64
- Benzamidine and EDTA (both 1mM) can be additionally used as protease inhibitors as not included in the cocktail
- Protein Phosphatase inhibitors such as Sodium fluoride (60mM), sodium orthovanadate (1mM) and Mikrocystin (0.3µM) are known to be compatible with the enrichment

Preparation of biological material

1. freeze plant material in liquid nitrogen
2. grind to a fine powder (you can add a spatula tip of quartz sand)
3. store at -80°C until usage
4. weigh 1g of frozen powder into 15ml Falcon tube
5.

Denaturing Protein Extraction

Extraction

1. add 7.5ml chilled Phenol (TE buffer saturated Phenol) to the frozen plant material
2. add 2.5ml chilled Denaturing Extraction Buffer (DEB, Table 13)
3. vortex mixture thoroughly and make sure no frozen clots are left, keep chilled
4. incubate on a rotator at 4°C (cold room) for 30min
5. centrifuge at 4°C and 3,250g for 10min (pre-cool the centrifuge)
6. you will get a four phase system: supernatant (phenolic phase), solid interphase, aqueous phase and bottom precipitate

7. remove the supernatant (phenolic phase) to a pre-chilled and clean tube (preferably 50ml Falcon tube)
8. do not include the interphase or aqueous phase

Table 13. Denaturing Extraction Buffer (DEB)

50mM	HEPES KOH pH7.5
40%	Sucrose
1%	β-Mercaptoethanol
60mM	Sodium fluoride
1mM	EDTA sodium salt
1mM	Sodium orthovanadate
1mM	Benzamidine
50µL/10mL	Sigma Plant Protease Inhibitor Cocktail
0.3µM	Mikrocystin

(solubilise HEPES and sucrose first, adjust pH, add inhibitors and fill up to desired volume)

Variations

In case you expect a lot of interfering compounds (eg seeds), you can wash the phenolic phase with additional 2.5ml DEB for 10 min at 4°C or you can also increase the amount of DEB Buffer in the first step (use 5ml instead of 2.5ml). Depending on the certain material you need to try out what works best.

Acetone Precipitation

1. precipitate proteins out of the phenolic phase by adding five volumes (30-40mL) of ice-cold acetone at -20°C overnight (16h)
2. centrifuge at 4°C and 3,250g for 10min
3. remove the supernatant
4. crush the pellet with ice-cold methanol (the pellet is very sticky and chewy)
5. centrifuge at 4°C and 3,250g for 10min
6. wash the pellet for a total number of 2-3 washing steps
7. dry for 20min at room temperature
8. solubilise each pellet in 0.8ml Phosphoprotein Loading Buffer (Table 14) by vortexing
9. (Since it is a denaturing extraction, make sure you vortex very thoroughly to renature the proteins.)
10. you might need to clarify the solution by transferring the supernatant to a 2mL tube and spin insoluble compounds down (13,350g, 4°C, 2min)
11. determine the protein concentration with the Bradford assay
12. one gram of leaf powder yields approximately 0.5mg of protein

Metal oxide/hydroxide affinity chromatography (MOAC)

Preparation of the original protein sample

1. use 1mg protein in 1.5ml PLB per enrichment preparation (80mg aluminium hydroxide)
2. save around 100µl of this original protein sample to monitor the success of your enrichment
3. continue to work chilled. However, you will find that it is difficult to keep the PLB at 4°C, since urea is precipitating.

Table 14. Phosphoprotein Loading Buffer (PLB)

8M	Urea
30mM	MES

200mM	Sodium glutamate
200mM	Potassium aspartate
20mM	Imidazole
0,25%	CHAPS

+ Inhibitors (see above)
(Adjustment of the pH with HCl to 6.1. Do not use phosphoric acid to adjust the pH!)

Equilibration of the matrix

1. weigh 80mg aluminium hydroxide (Sigma, Taufkirchen) in a 2ml cup
2. add 1.5ml PEB, mix on vortex
3. centrifuge at 13,350g and 4°C for 2min
4. remove the supernatant

Binding phosphoproteins to the matrix

1. add 1.5ml of the prepared protein solution (0.7mg/ml) to the equilibrated matrix
2. allow phosphorylated proteins to bind to the matrix by shaking (rotator) at 4°C for 30min
3. remove unbound protein (flow-through, FT) with a centrifugation step (13,350g, 4°C, 2min) as supernatant
4. save the flow-trough to monitor the success of the enrichment

Washing of unbound protein

1. add 1.5mL PEB to the loaded matrix
2. do not vortex (!)
3. mix manually by flipping your fingertip against the bottom of the tube until the matrix is dispersed (2-3 times)
4. centrifuge at 13,350g and RT for 2min
5. wash unspecifically bound proteins for a total number of five washing steps

Elution

1. elute using 800µL freshly prepared Phosphoprotein Elution Buffer (Table 15) by shaking for 30min (RT)
2. centrifuge at 13,350g and RT for 2 min
3. transfer the supernatant to a Schott GL14 glass test tube (Cat. number 23 175 11 59)

Table 15. Phosphoprotein Elution Buffer (PEB)

8M	urea
500mM	potassium pyrophosphate

(Adjustment to the pH of 9.0 with H_3PO_4.)

Protein Precipitation (Wessel and Flugge, 1984)

(Use 50µL of the retained sample solution, 100µL of the flow-through and the complete eluate of 800µL for the precipitation.)

General protocol:

1. add 4 volumes of Methanol and mix until homogeneity of turbidity
2. add 1 volume of chloroform and mix until homogeneity of turbidity
3. add 3 volumes of ddH_2O and mix until homogeneity of turbidity
4. spin for 3,250g for 10 min at RT

5. carefully remove upper phase with a Pasteur pipette, do not touch the interphase
6. add 3 volumes of methanol and mix until homogeneity of turbidity
7. spin for 3,250g for 10 min at RT
8. carefully remove solution
9. dry pellet at room temperature for 20min
10. resuspend every pellet in 40L singly concentrated SDS sample buffer
11. heat the glass test tube in the burner flame for a few seconds
12. spin the solution down and load the proteins on a gel
13. Adjust your loadings onto the gel so that you have similar amounts of total protein from every sample.

Positive and negative controls for the detection of phosphoproteins

Common molecular weight markers can be used as positive and negative controls for the staining procedure. The commercially available molecular weight markers often contain ovalbumin, which is phosphorylated at four serine residues (Swiss Prot entry P01012). Standards for threonine- or tyrosine-phosphororylated proteins are also available (eg EGF-stimulated cell lysate from Upstate). For an immuno staining it is very handy to use the appropriate phosphoamino acid as antibody inhibitors. Pre-incubate the inhibitor and antibody, before giving it to the membrane. Cross reactivity and unspecificity - especially of secondary antibodies - can be easily detected in this manner.

Protein detection

Perform multiplexed staining to monitor the success of the enrichment. Firstly, Phosphoproteins are detected with the Pro-Q Diamond Phosphoprotein gel stain (Steinberg, Agnew et al. 2003; Schulenberg, Goodman et al. 2004). Coomassie staining and if necessary silver staining are accomplished afterwards. If not otherwise stated 80mL of staining and washing solutions were used. Alternatively, Western Blotting can be performed.

Detection of phosphoproteins with the Pro-Q Diamond Gel Stain (Molecular Probes, Eugene)

1. remove excess running buffer after SDS-PAGE by washing 15min with ddH_2O
2. incubate for 15min in fixation solution (Table 16 A)
3. renew solution and perform fixation overnight
4. (This step is considered to be important for the specificity of the staining.)
5. remove fixation solution by three 10 min incubations in ddH_2O
6. stain for 2h in 50mL Pro-Q Diamond gel stain
7. protect the trough during staining and destaining from light
8. destain for 3h overall, renew the destain solution every hour (Table 16 B)
9. substitute the destain solution with ddH_2O before imaging
10. There are different ways of imaging the Pro-Q stain. If there is no appropriate fluorescent scanner available the smaller UV excitation maximum at 380nm can be used in standard UV-Geldocs. You will need an additional broad range amber filter. Also, you can use the Dark Reader (Clare Chemicals, Dolores).

Table 16 (A)

Phosphostain Fixation Solution	
50%	methanol
10%	acetic acid
40%	bidest water

(B)

Phosphostain Destain Solution	
20%	acetonitrile
5%	1M sodium acetate stock pH 4 (pH adjusted with acetic acid)
75%	bidest. water

Coomassie staining (Garfin 1990)

1. rinse gel with ddH_2O and stain for 45min (Table 17A)
2. incubate in destain solution (Table 17B) until background is satisfactorily reduced

Table 17 (A)

Coomassie Staining Solution	
0.1% (w/v)	Coomassie Brilliant Blue G-250
50%	methanol
10%	acetic acid

(B)

Coomassie Destain Solution	
10%	methanol
5%	acetic acid

Silver staining (Blum et al. 1987)

1. rinse the gel with water and perform fixation overnight (Table 18A)
2. wash the gel for 30min with 30% Ethanol and three times with ddH_2O for 5min
3. put in 50mL Sensitising Solution (Table 18B) for 10min
4. three 30s rinses with ddH_2O
5. stain in 50mL of Silver Staining Solution (Table 18 C)for 20min
6. quickly rinse in ddH_2O (10s) to remove excess Staining Solution
7. visualize spots using 50mL Developing Solution (Table 18D)
8. stop with 5% acetic acid as soon as background staining becomes observable
9. this silver stain is reported to be MS compatible.

Table 18. (A)

Silver Fixation Solution	
30%	ethanol
5%	acetic acid
65%	ddH_2O

(B)

Silver Sensitising Solution
20mg sodium thiosulfate
In 100mL bidest. water
(saving 2mL for developing solution)

(C)

Silver Staining Solution
200mg silver nitrate
in 100mL ddH_2O

(D)

Silver Developing Solution	
3g	sodium carbonate
2mL	Sensitising Solution
50µL	formaldehyde

in 100mL ddH_2O

3. Protein separation

3.1. 2D gel electrophoresis

a) Resolubilization, rehydration and isoelectric focusing (i.e., 1st dimensional separation (12 Samples) (Lei et al., 2005)

1. Rehydrate pellets in 1 mL of IEF solubilization buffer containing 9 M Urea, 3% CHAPS, 2% Triton X-100, 20 mM DTT, 1 % ampholytes.

i. IEF solubilization buffer per 17.5 mL

Urea	9.45 g
CHAPS	525 mg
Triton X-100	350 µL
DTT	54 mg
Ampholytes	175 µL

ii. Break up protein pellet using small metal spatula.
iii. Degas protein solution in water bath sonicator for five minutes. Be sure the sample doesn't heat up while in the sonicating water bath.
iv. Place the samples on orbital shaker, set to slow speed, for ~ 1 hour.
v. Vortex occasionally to assist resolubilization.

2. Transfer to 1.5 mL microfuge tube.
i. Break-up protein pellet with a blue pestle. Alternatively, sonicating in a water bath for five minutes will help disrupt the pellet.
ii. If using a pestle, ensure that no protein chunks stick to the pestle.

3. Degas in water bath for 5 minutes followed by sonication for 30 minutes. Check and prevent heating of the samples by adding ice to the sonicator bath.

4. Allow proteins to solubilize for at least an hour with occasional mixing.

5. Pellet insoluble material by centrifugation at 14,000 g for 10 minutes at 10°C*.
*Occasionally, Urea might crystallize if centrifugation is performed at 4°C.

6. Protein concentration is determined by Bradford's method, using the Bio-Rad kit or any other method as per the lab's procedures.
i. Make BSA standards at 5 µg, 10 µg, 15 µg, 20 µg, and 25 µg using a 1µg/µl BSA stock solution.
ii. Add 2 µL of IEF solubilization buffer to each BSA standard to correct for urea and detergent in the protein sample.
iii. Aliquot 2 µL of sample to three cuvettes, *i.e.* the measurements should be performed in triplicate.
iv. Mix 50 mL of Bradford's reagent (1 part of reagent to 4 parts of water).
v. Add 1.0 mL of Bradford's reagent to each sample and standard.
vi. Wait for 5 minutes and measure absorbance at 595 nm.

7. Centrifuge the sample again to remove any residual insoluble material and dilute protein with additional resolubilization buffer as necessary to obtain a final concentration of 2.5µg/µl. Add bromophenol blue to a final concentration of 0.001%.

8. Rehydrate IPG strips passively (ie no voltage applied), or actively at 30V/strip for at least 11 hours.

9. Add wicks moistened with water and begin IEF focusing. The length of time needed for IEF depends on the strip length, strip pH range, and sample concentration. Read the instructions which come with your specific system and make sure the dye front reaches the anode. In the author's lab, IEF focusing is performed at 500 V for 1 hour, 1000 V for 1 hour, and 8000 V for a total of 76,500 volt-hours (Vhrs).

i. For troublesome samples it sometimes helps to add more ampholytes, up to 0.8-2.0% for Amersham strips, to the resolubilization solution.

ii. Also, the strips can start to dehydrate with prolonged focusing; in that case it can help to change the wicks or to pause focusing and add additional buffer to the strips (~50µl for 11 cm strips and 100µl for 24cm strips).

10. The focused strips can be stored at –20^{o}C overnight or immediately equilibrated for the 2nd dimension gel. Overnight to over-the-weekend storage does not have any negative effects and freezing might even help break-up the IEF gel matrix, thereby increasing the efficiency of transfer of proteins to the 2nd dimension gel matrix.

b) Equilibration and second dimensional separation (12 Samples)

1. Equilibrate strips in 7 M urea, 375 mM Tris (pH 8.8), 3% SDS, 10% glycerol, and 50 mM DTT for 10 minutes. This will aid in the reduction of cysteine bonds.

2. Equilibrate strips in 7 M urea, 375 mM Tris (pH 8.8), 3% SDS, 10% glycerol, and 100 mM iodoacetamide for 10 minutes. This will aid in the alkylation of the reduced cystines.

3.

Equilibration buffer	per 150 mL*
Urea	63.1 g
1.5M Tris (pH 8.8)	37.5 mL
SDS	4.5 g
Glycerol	15 mL
DTT	0.58 g to 75 mL
IAA	2.79 g to 75 mL

*Use 6 mL for each strip.

4. Pipette marker proteins (2 µl silver stain, 15 µl for Commassie if using Mark 12 markers) onto a paper wick. Let the wick air dry for ~5 minutes and coat with 1% agarose.

5. Place IEF strip on top of a 1.5 mm thick 10% acrylamide-bis or acrylamide-PDA gel.

i.

10% acrylamide-bis Gel	per 12 gels
30% acrylamide-bis	396.0 mL
1.5M Tris (pH 8.8)	300.0 mL

Water	486.0 mL
10% SDS	12.0 mL
10% APS	9.0 mL
TEMED	0.9 mL

ii. Place 1X run buffer (25mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) on top of the gel to help prevent bubble formation at the strip-gel interface. Note 10X running buffer can be purchased (Bio-Rad) and diluted prior to analysis.

iii. Keep strips wet with equilibration buffer to facilitate their sliding into place.

iv. Once the strip is in place, remove excess run buffer by tilting gel cassette/ and wick with Kimwipe or pipette.

v. Place the marker protein wick onto the gel next to but not touching the strip.

6. Seal strip in place by overlaying with 1% agarose in 1X running buffer containing 0.001% Bromophenol blue. Note: 1% agarose solutions need to be warmed prior to overlaying. This is commonly done by heating the solution in a microwave or on a warming plate.

7. Run gels at 25 mA per gel or per the manufacturer's recommended voltage/amperage settings.

8. When dye front is 1 cm from bottom of the gel (~15.5 hours), stop the electrophoretic separation. Remove the gel from the glass being careful not to break the gel. A helpful procedure to remove the gel from the glass plates is to float the gels off the glass plates in a large dish of water. The gel can then be stained with Commassie, fluorescent stain or silver stain (See 3.2)

Separation conditions for seed proteins

Gel strips of 24 cm forming an immobilized non-linear 3 to 10 pH gradient (Immobiline DryStrip, Amersham Biosciences) were used for isoelectrofocusing. These strips allowed a good resolution of the seed proteins that mostly range in pI from 5 to 7 with some highly basic proteins located at around pH 8 (e.g. basic chains of legumins). Twenty µl of the various protein extracts (corresponding to about 300 µg proteins) were added to 440 µl of rehydration buffer containing 7M Plus One urea, 2M thiourea, 65mM PlusOne CHAPS, 20mM PlusOne DTT, 0.5% pharmalyte, 2% (v/v) Triton X-100, and a trace of bromophenol blue (Sigma). Following strip rehydration for 7h at 20°C in the presence of the protein extract, IEF was performed in the IPGphor system (Amersham Biosciences) for 7 h at 50 V, 1 h at 300 V, 2 h at 3500 V and 7 h at 8000 V. Equilibrated gel strips (Görg et al., 1987) were placed on top of vertical 10% (w/v) polyacrylamide gels in a denaturing solution containing 1% (w/v) low-melting agarose, 0.4% (w/v) PlusOne SDS (Amersham Biosciences), 0.15M BIS-Tris (Sigma), and 0.1M HCl. For each seed sample analyzed, 2D gels were made in triplicate and from two independent protein extractions. Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad, Hercules, CA, USA) according to Mathesius et al. (2001). Image acquisition was done using the Odyssey Infrared Imaging System (LI-COR Biosciences, GmbH, Germany), at 700 nm with a resolution of 169 µm. This scanning system allows a highly sensitive detection of proteins stained with Coomassie Blue in 2D gels.

3.2. Gel staining

a) Coomassie staining (adapted from Anderson et al., 1991)

1. Stain gels with Coomassie Brilliant Blue G-250 (CBB G-250).

i. Fix gels for ~4 hours in 8 L* of 40% methanol and 2% phosphoric acid.
*The quantity mentioned here is for 12 gels and is for using with an automated DoDeca stainer large (BioRad)

ii.

Fixing Solution	per 8.0 L
Methanol	3200 mL
Phosphoric acid	160.0 mL

Add MilliQ water to make up the volume to 8.0 L.

iii. Equilibrate gels for 12 hours in 8 L of 34% Methanol, 12% ammonium sulphate, and 2% phosphoric acid.

iv.

Equilibrating Solution	per 8.0 L
Methanol	2720 mL
Phosphoric acid	160 mL
Ammonium sulphate	960 g

Add milliQ water to a final volume to 8.0 L.

v. Stain the gels with 8 L of CBB G-250 for 3-4 days*. The final concentration of G-250 is 1g/L or 0.1% in solution of 34% Methanol, 12% ammonium sulphate, and 2% phosphoric acid (solution 2).
*Shorter staining times are optional, however longer staining times provide enhanced sensitivity and dynamic range.

vi.

Staining Solution	per 8.0 L
CBB G-250	8.0 g*
Methanol	2720 mL
Phosphoric acid	160 mL
Ammonium sulphate	960 g

*Dissolve 8 g of CBB G-250 in 2000 mL of methanol (stock: 4 mg/mL). Stir overnight. To this, add the remaining volume (720 ml) of methanol, phosphoric acid, ammonium sulphate and make up the volume to 8.0 L using milliQ water.

vii. Drain CBB G-250 from the staining containers and replace with 10% methanol. Shake for 1 hour to remove residual CCB G-250 on the walls of the container.

viii. Drain and add ~10 L of H_2O, plus 2 Kimwipes*. Change Kimwipes and allow destaining overnight.
*Kim wipes are added to absorb the Commassie and speed up the destaining process. This addition may be a source of contamination during mass spectrometry analysis.

ix. The following morning add 8.0 L of 5% acetic acid and equilibrate for 1 hour.

x. The gels can be stored at 4^{o}C after scanning (see 4. Gel Analysis).

xi. Alternative method: Neuhoff et al., 1985. Electrophoresis 6:427-448.

b) Silver staining

Silver staining is a sensitive staining method, but the disadvantages of its use are that after silver staining gels cannot be blotted, proteins cannot be identified subsequently by mass spectrometry and the silver staining is not very quantitative. Silver ions complex to Glu, Asp and Cys residues in proteins. Formaldehyde under alkaline conditions reduces the Ag^{+} to Ag, which is visible as a dark brown to black spot on the gel. Silver does not uniformely bind to all proteins, some proteins are not stained at all

with silver and appear as "negative" spots on the clear (even clearer than the gel background), and typically different spots on a silver stained gel have slightly different shades of brown. While comparing the same protein across several gels is semiquantitative, it is not a reliable method for quantitative proteomics. Silver staining is useful for staining proteins sensitively to optimize 2D separation and gel running.

Prepare all solutions just before use (or extemporaneously) and with highest grade chemicals, all in ultrapure water.
Solutions:

i. Fixation solution: 10 % (v/v) acetic acid, 40 % (v/v) ethanol, 50 % ultrapure water.
ii. Sensitiser: 30 % (v/v) ethanol, 4.1 % (w/v) sodium acetate, 0.275 % (w/v) potassium tetrathionate, and 0.5 % (v/v) glutaraldehyde.
iii. Silver stain: 0.2 % (w/v) silver nitrate, 0.062 % (w/v) HEPES, 0.07 % (v/v) formaldehyde.
iv. Developer: 3 % (w/v) potassium carbonate, 0.0012 % (w/v) sodium thiosulfate, 0.025 % (v/v) formaldehyde.
v. Stop solution: 5 % (w/v) Tris base, 2 % (v/v) acetic acid.

Method:
For successful silver staining, use only high purity fresh chemicals. Especially formaldehyde and glutaraldehyde solutions must be made fresh. We never use stock solutions but make up all solutions just before use.
It is easier to stain in photographic trays on an orbital shaker (50 rotations per minute) at room temperature under a fume hood.

i. Directly after SDS-PAGE, fix gels three times, for 30 min each, in fixative solution.

ii. Transfer to sensitiser for 16 h, avoid evaporation.

iii. Wash the gel at least six times in ultrapure water for 30 min each Be sure to wash several times to remove glutaraldehyde).

iv. Incubate gels with silver staining solution for 2 h in the dark. To wash off the silver, quickly rinse for 10 s in ultrapure water. If this washing step is done for any longer, the silver will disassociate from the protein spots.

v. Develop gels for 5 to 7 min and stop the development by replacing the developer with stop solution. The development should be stopped when no more new spots become visible and before the background of the gel becomes dark. Timing is important because gels of different runs need to be comparable in staining.

vi. Leave the gels in stop solution for no longer than 20 min to avoid colour changes in the silver stain.

vii. Wash gels in distilled water several times, scan and store sealed in plastic pouches in a few mL of 1 % methanol to help prevent microbial contamination. Gels can be stored at room temperature or at 4 °C for many years.

Note:
When silver staining results in only faintly stained spots, the formaldehyde solution might be old. In this case, re-sensitize and re-stain the gel in silver solution with new or a larger volume of formaldehyde.

4. Gel analysis

4.1. Comparative software of single stained gels –Nonlinear Progenesis

1. Scan gel images using a UMAX Power Look 1100 (UMAX technologies) or an equivalent scanner/imager.
   i. Scanner Settings: Grayscale, 14-bits/channel, 300 dpi.
   ii. Assign a name to the image to be scanned. Double click on slide icon.
   iii. Preview and set the frame to be scanned.
   iv. Scan. This will save image as the name provided before preview.
   v. Remember to reset name before next image, or data will be lost.
   vi. If desired, perform auto levels and crop images using photoshop.

2. Open a new experiment in 2D Expression/Progenesis (Nonlinear Dynamics) by selecting gels to be analyzed.

3. Spot Detection.
   i. Detection Settings: Sensitivity = 9,850
      Noise factor = 15
      Operator size = 45
      Background = 1
   ii. Set to automatic splitting.
   iii. Spot filter settings: Area = 60
      Peak height = 15,000
      Circularity = 0.2
      Volume = 200,000

4. Manually edit gels for artifact spots, undetected spots, and mis-split spots.
   i. Use a pen size ~8.
   ii. Zoom in (1=1) and manually edit.
   iii. Use high contrast for low level spots.
   iv. Use the brightness/contrast slide bars to help find splits in the saturated spots.

5. Select a reference gel based on the gel with the best focusing and most spots, or based on the most representative gel within a sample.

6. Spot Matching.
   i. Examine gels for high volume spots that will serve as user seeds. These spots will help the matching software find real matches.
   ii. Warp to user seeds.
   iii. Make sure selected user seeds actually helps the spot patterns match up.
   iv. Run matching.
   v. Examine matching for missed matches and mismatches.

7. Manual editing of matching.
   i. Select high abundance spots with computer selected matched as user seeds.
   ii. If you set a user seed and it negatively impacts the spot overlay, then undo the user seed.
   iii. After computer matching, set additional user seeds if necessary, then manually match remaining spots.

8. Background subtraction.
   i. Mode of non-spot (set to 45).

9. Normalization
   i. Total spot volume multiplied by 100.

4.2. Comparative software of single stained gels – Image Master

Normalization of spot volumes between seed developmental stages (K. Gallardo).

Image analysis was carried out with the ImageMaster 2D Platinum software (Amersham Biosciences) according to the instruction manual. Protein spots were selected for quantitative analyses if they were consistently visible in three 2-D gels for at least one stage. After spot matching across the different gels, spot volumes were collected and manually normalized to profile individual spot quantity during seed development. Because this process is associated with many changes at the protein level (e.g. storage proteins, which account for up to 70% of the total nitrogen in mature legume seeds, accumulate in abundance) the probably most reliable scaling procedure, in each 2D gel, is a normalization of the volume (i.e, abundance) of each spot to the volume of a set of housekeeping proteins consistently accumulated during seed development. Such internal reference proteins were selected by comparing qualitatively silver and Coomassie blue stained 2-D gels across the developmental stages (Gallardo et al., 2003). Ten of them were identified by mass spectrometry as corresponding to calcium ATPase, RuBisCO subunit binding-proteins, Heat shock proteins, glucose-6-phosphate isomerase, putative aminoaldehyde dehydrogenase, and putative TPR-repeat protein (TC91689). Table 19 shows the experimental characteristics of these reference proteins in 2D gels from total protein extracts of developing seeds. Interestingly, most of them are known to play housekeeping roles in the cell such as maintenance of pH, Ca2+ homeostasis, protein folding, or glycolysis, suggesting a function for these proteins throughout seed development. Following spot volume normalization, differences in the abundance of each spot among the different samples was analyzed by ANOVA (one-way analysis of variance) and a Student-Newman-Keuls test using the SAS software package (SAS Institute, 1999).

Table 19. Experimental characteristics of the protein spots identified by mass spectrometry and used as internal references to normalize spot volumes across the different gels.

Putative protein name	Tentative consensus sequence from TIGR[1]	pI	Mr (kDa)
Molecular chaperone Hsp90-2	TC85438	5.0	89
Heat shock 70 kDa protein	TC85718	4.6	80
Type IIB calcium ATPase MCA5	TC85483	5.8	83
F1 ATPase	TC85540	5.3	58
Putative TPR-repeat protein	TC91689	5.8	75
Putative aminoaldehyde dehydrogenase	TC77827	5.4	60
RuBisCO subunit binding-protein (alpha)	TC85910	4.7	62
RuBisCO subunit binding-protein (beta)	TC76847	5.3	64
(two spots)		5.3	65
Glucose-6-phosphate isomerase	TC77538	5.9	64

[1]The Institute for Genomic Research, http://www.tigr.org/

4.3. Difference Gel Electrophoresis (DIGE)

One major drawback of comparative proteomics by 2D gel analysis is the matching of 2D gels. A recent technique to improve matching of gels from different samples is DIGE (Difference Gel Electrophoresis; Ünlü et al., 1997, Figure 7). This technique is designed to make it easier to compare gels of different biological samples, for example a mutant and wild type tissue (M vs. WT). The main difference conventional 2D gel electrophoresis is that the two samples to be compared are run on the same gel; thereby the same protein from each sample should migrate to exactly the same spot. To distinguish the proteins from the two samples, they are labeled – before the 1D separation – by different fluorescent dyes (Cy3 and Cy5, similar to labeling RNA in a microarray). The intensity of fluorescence at each of the wavelengths for Cy3 and Cy5 is measured and the ratio gives an indication of the abundance of protein X from M or WT ("within-gel matching").

Like in any other biological experiment, one needs to run biological repeats of samples (i.e. separately grown biological material, separately extracted and separated on different 2D gels). Each pair of repeats of M vs WT would be run on a separate gel. There will therefore still be gel-to-gel variation that makes matching between gels difficult. To improve between-gel matching, each gel also contains a third sample. This is a mixture of all samples of the whole experiment (e.g. M and WT of each biological repeat). This mixture is labeled with a third dye (Cy2) and run on each gel. Therefore, this internal control should be representing the same proteins on each gel and can be used to compare protein patterns between gels. It also serves as an internal control for the statistical analysis of the protein abundance difference between the different samples.

The CyDyes have an NHS ester group which reacts with the epsilon amino group of lysine residues of proteins. The dyes are called "minimal dyes" because only enough dye is used to label approximately 1-2 % of the available lysine residues (about 1 residue per protein). All three dyes have approximately the same size so that they add the same mass to each protein (~500 Da). They do not change the charge of the proteins, do not interfere with 2DE or with mass spectrometry. For spot picking, it is best to counter stain the gels with Coomassie Brilliant Blue.

The workflow for DIGE is as follows:

- Extract proteins as usual, but elute into a different buffer (see below).
- Split samples into different tubes: sample to be labeled with C3 or C5 and a tube containing the mixture of all samples
- Label proteins
- Run straight away on 1D and 2D
- Scan gels on a laser scanner immediately after second dimension is finished
- Generate gel analysis files and analyse using software
- Counter-stain gels with coomassie and keep for later mass spectrometry

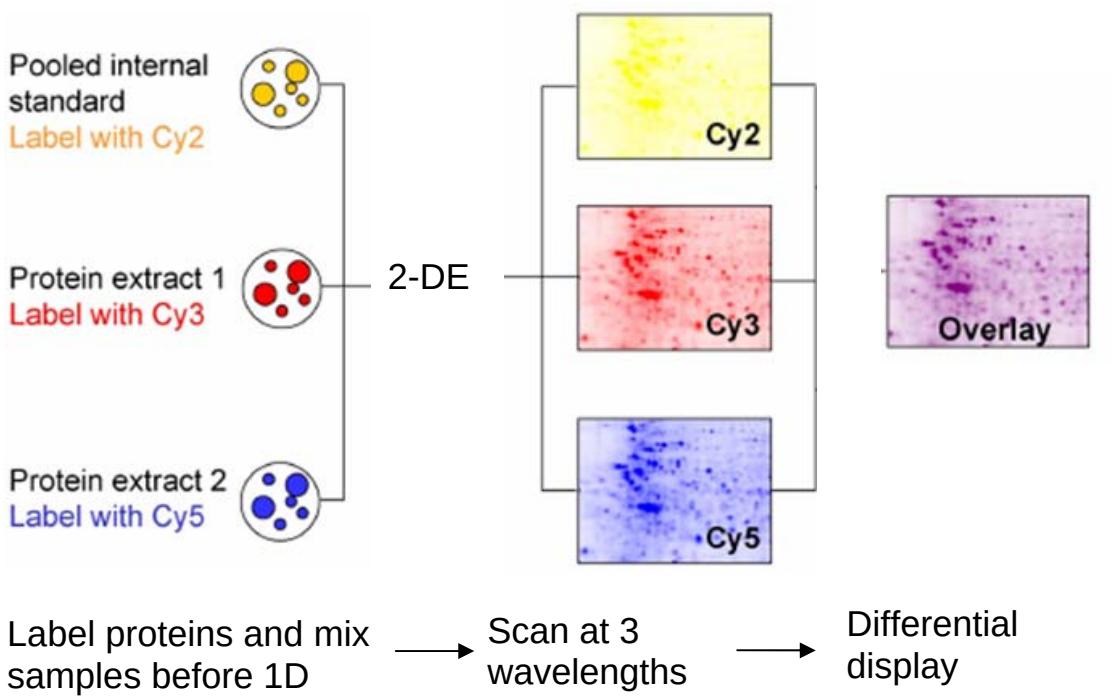


Figure 7: **Principle of DIGE.** Three samples are labeled with Cy Dyes before separation by 1D and 2D electrophoresis. Adapted from Amersham Biosciences Ettan DIGE User Manual.

Protein extraction of proteins for DIGE-labelling

Protein extraction should proceed as normal, until the last step, in which proteins need to be solubilised in a buffer not containing any DTT and ampholytes. The recommended buffer contains 30 mM Tris, 7M urea, 2M thiourea, and 4% (w/v) CHAPS. After solubilizing the proteins in this buffer, the pH needs to be adjusted to between 8-9 (ideally 8.5) to allow the labeling reaction to occur. The pH can be adjusted with HCl or NaOH, depending on pH of the sample. The easiest thing is to spot a tiny drop of sample onto some pH paper. Measure protein concentration of the samples and set aside 100 µg protein from each sample in a separate Eppi tube, and mix an additional 50 µg of each sample into a separate tube for the internal control. Adjust volumes of the sample with solubilization buffer to make them approximately equal.

To label the proteins, the CyDyes first need to be diluted into anhydrous, fresh dimethylformamide (add 5 µL DMF to each tube, this will give a 1 mM solution). Keep dyes dark and at -20 ºC at all times until use. Once the DMF is added the dyes will keep for 3 months at -20 ºC. We found that a ratio of 200 pmol of dye to 100 µg protein is optimal.

After adding the appropriate dye to each sample, put tubes on ice for 1 h in the dark.

Add 1 µL of 10 mM lysine to stop the reaction. Leave on ice for 10 min. Samples are ready to be mixed together and loaded.

Setup for gel loading

A typical setup of a DIGE experiment would be (assuming four biological repeats of samples and a dye swap between Cy3 and Cy5):

M = sample of mutant
WT = sample of wild type
Mix = mixture of all mutant + all wild type samples of all repeats
Cy2 = Cy2-labelled etc

Gel 1: M1 Cy3 + WT1 Cy5 + Mix Cy2
Gel 2: M2 Cy3 + WT2 Cy5 + Mix Cy2
Gel 3: M3 Cy5 + WT3 Cy3 + Mix Cy2
Gel 4: M4 Cy5 + WT4 Cy3 + Mix Cy2

After loading the samples onto 1D strips and running the first and second dimension (see 3.1) the gels are then scanned at three wavelengths as shown below, resulting in an overlay image (Figure 8)

Cy 2: Excitation at 488 nm, emission at 520 nm (40 nm bandwidth)
Cy 3: Excitation at 532 nm, emission at 580 nm (30 nm bandwidth)
Cy 5: Excitation at 633 nm, emission at 670 nm (30 nm bandwidth)

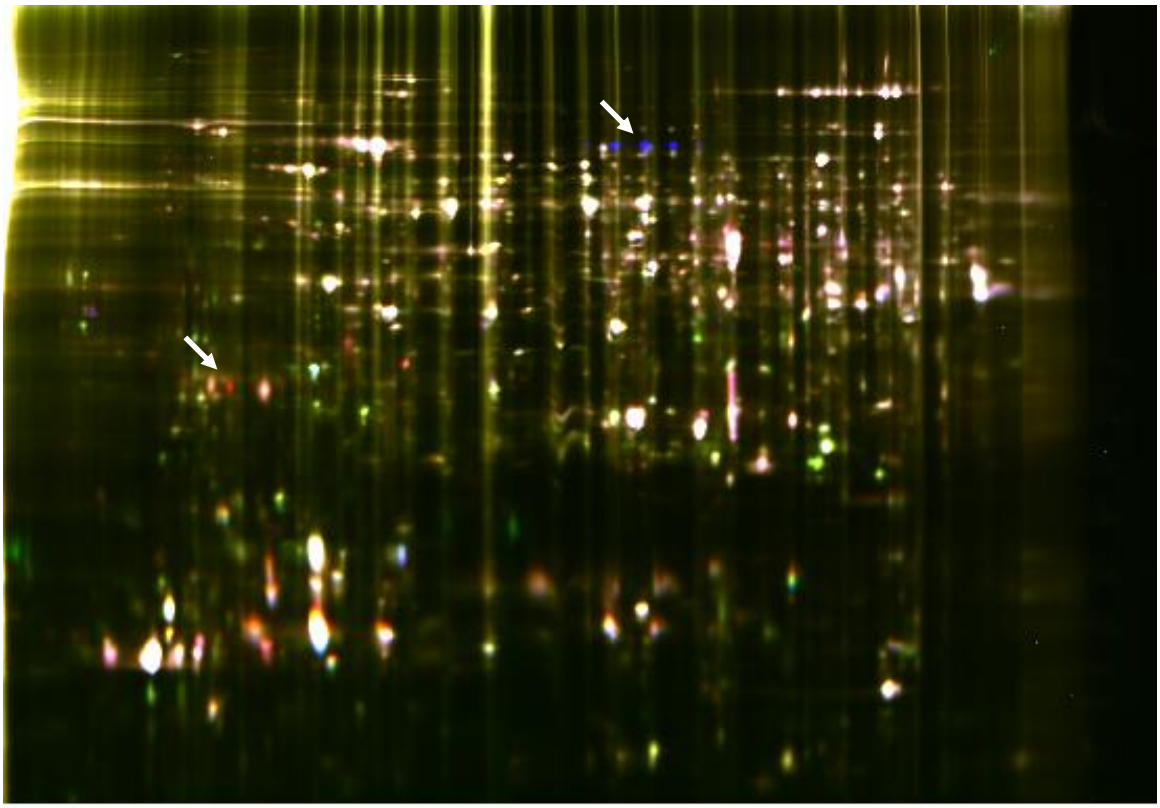


Figure 8: Gel overlay of three samples in one gel, imaged at three different wavelengths (false colour image). White spots are similarly abundant in all three samples, blue, red and green spots (arrows) are more abundant in one of the three samples. The gel shown as example of *M. truncatula* root proteins.

The gels are cropped, named with the correct extensions for analysis, the spots matched (Figure 10), normalized (Figure 9), compared (Figure 11) and statistical analysis (Figure 12) done to test whether differences in spot abundance are significant over the four biological repeats.

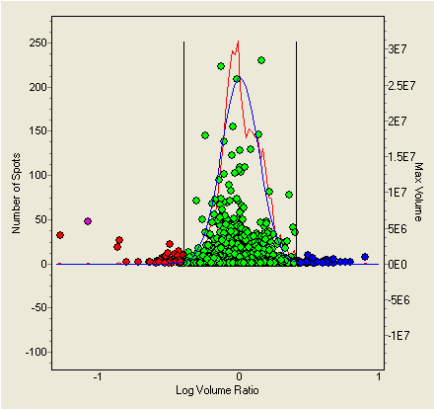


Figure 9: Distribution of spots from a DIGE experiment and correction of spot volumes to a normal distribution (to allow statistical analysis by ANOVA). The log volume ratio is the ratio of spot abundance between test and control. Green spots are not significantly different in the two samples, red spots are down-regulated in one of the samples, and blue spots are up-regulated in the same sample, compared to the control.

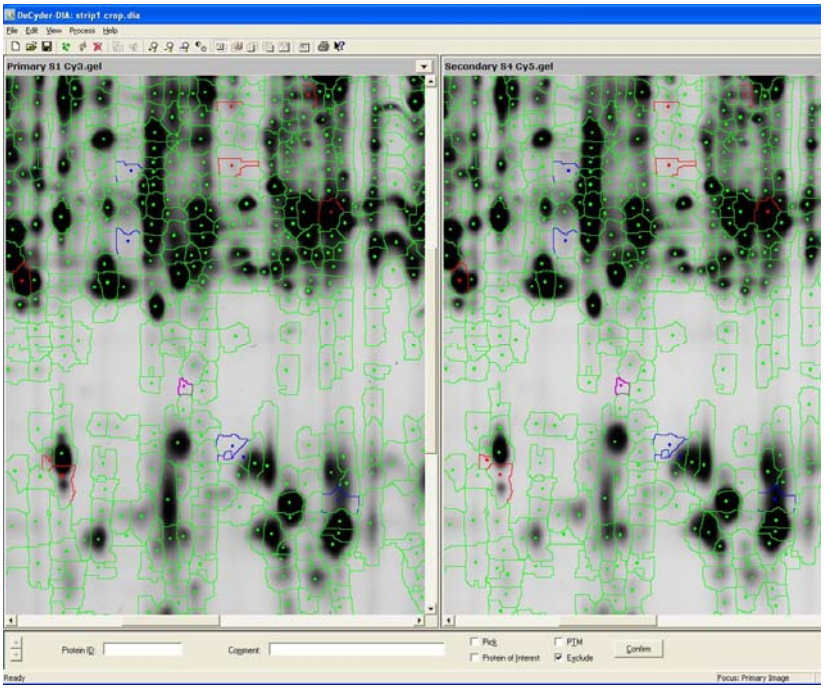


Figure 10: Matching of spots between gels. All green-circled spots are detected and matched between the gels. Red spots are down-regulated in one of the gels, blue ones up-regulated. Any spot that is clicked by the mouse will have a purple circle.

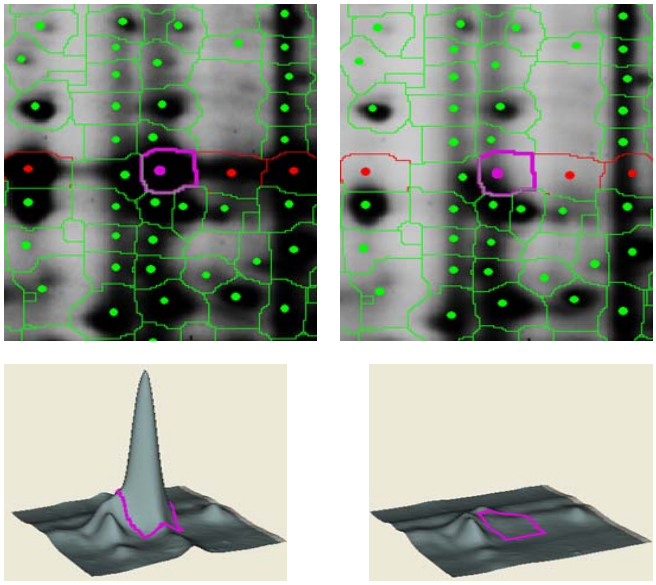


Figure 11: Differential display of spot volumes between two gels. The purple spot is marked on the gel by clicking with the mouse, and will be displayed as a "Mountain Plot" below. (the red marked spots are differentially displayed between the gels, the green ones are similar).

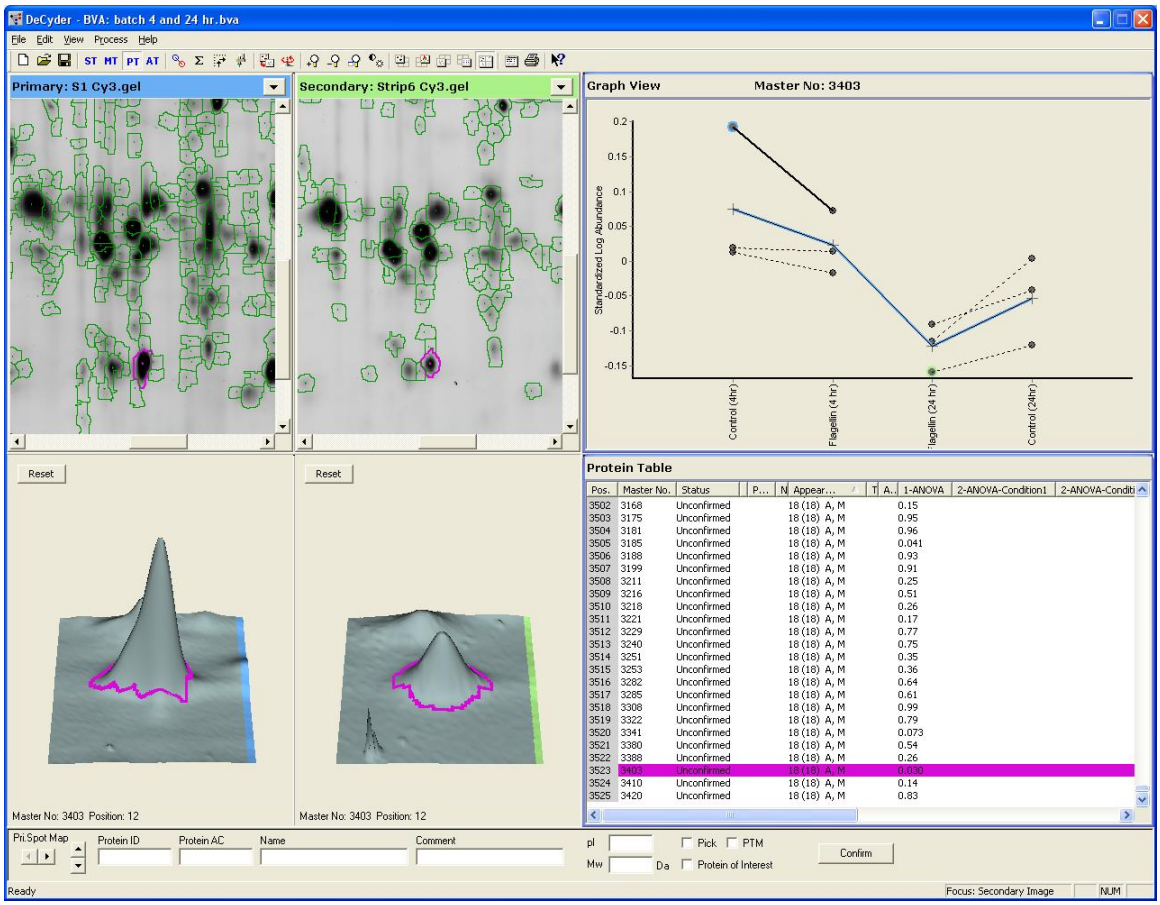


Figure 12: Statistical analysis of spot differences. Gels are shown in the top left. Mountain plots of the marked spots are shown in the bottom left. A list of all proteins and the p value for their significance of expression is on the right bottom. A plot of the spot abundance across all repeat gels (in this case 3) is shown on the top right (the blue line is the average spot volume across the three gels. In this case there were 4 different treatments (shown along the x-axis).

With all experiments, it is necessary to check the computer-generated matches by eye and correct them, if necessary.

All protocols for protein labeling, scanning etc can be found in the Amersham Biosciences DIGE user manual.

5. Protein identification

5.1. Tryptic digestion (Lei et al., 2005)

Comments — Wear clean gloves. Work in a laminar flow hood if possible, to avoid contamination. Great care should be taken not to touch gels, allow hairs to fall on gels, or allow wools to come into contact with gels prior to staining or digestion. All these can contribute protein contaminants (keratins) which will appear and interfere with the final protein identification.

1. Excise gel bands or spots. Manual excision of spots can be achieved using a pipette style spot picker (The Gel Company, San Francisco, CA) and automated excision can be performed using commercial equipment (Investigator Propic robotic workstation, Genomics Solutions, Ann Arbor, MI).

2. It is highly recommended that a positive and negative control be conducted in parallel to all analyses. The positive control can be a marker or known protein while the negative control should be a spot/band from a blank region of the gel.

3. Wash twice with 50 µl of 18 Mohm water for approx 15 minutes.

4. Wash sufficient times with 50 µl of a 50%/50% solution of acetonitrile/50mM ammonium bicarbonate to remove all stain from gel plug. Each wash should be approximately 30 minutes in length. Two washes should be sufficient for moderately intensity bands.

5. Wash and dehydrate with 50 µl (spot) to 100 µl (band) of acetonitrile until gel plugs turn opaque and dramatically shrinks in size, generally 20 minutes.

6. Remove all liquid from opaque gel plugs.

7. Prepare trypsin solution using sequence grade, modified bovine or porcine Trypsin (Roche/Promega). Prepare stock trypsin solution by dissolving 20 to 25 mg in 200 µl of 1mM HCl so that final concentration is between 100 - 125 ng/µl.

8. Prepare working trypsin solution by diluting stock solution 1 to 10 with 25 mM ammonium bicarbonate + 1% acetonitrile such that final concentration = 10 to 12.5 ng/µl. Note the pH difference in stock solution (~2.9) versus working solution (pH~8.0). Trypsin activity is pH dependent. At low pH trypsin has low activity and can be stored for approximately 2 weeks. Trypsin working solutions must be used shortly after increasing pH to 8, i.e. 2 hrs. If not, autolytic digestion products are observed at higher levels than desirable.

9. Rehydrate dried gel plug/bands in approximately 10 µl trypsin for 20 minutes on ice. This allows the trypsin to infuse into the gel plug for "in-gel" digestion.

10. Remove excess trypsin solution by washing twice with 25mM ammonium bicarbonate to remove excess trypsin and to lower the autolytic peak abundances.

11. Add 10 to 20 µl of 25 mM ammonium bicarbonate to sample vial to ensure proper hydration of the gel plugs/bands during digestion at elevated temperatures.

12. Digest at 37^{o}C for 4 to 6 hrs. Overnight digestions are possible but yield higher levels of autolytic trypsin products which may decrease sensitivity for low level protein digests. Overnight digestions should only be considered for intense Coomassie stained gel plugs/bands.

13. Stop digestion by adding 25 µl of 10 % formic acid (note pH change). Allow to stand for 15 minutes.

14. Recover supernatant — one can sample this solution directly for peptide mass mapping or continue extracting additional peptides. We suggest further extraction.

15. Extract gel plug/band twice with 25µl of 50%/50% acetonitrile/50mM ammonium bicarbonate for 15 minutes to recover additional peptides. Pool with supernatant from step 14.

16. Extract with 25 µl to 50 µl of 100% acetonitrile. Pool with supernatant from step 14.

17. Concentrate to 5 µl or dryness in vacuum centrifuge. Concentration to dryness is more convenient but may result in some loss of peptides which will not resolubilize following precipitation due to drying.

18. Dried peptides can be stored at -20^{o}C or -80^{o}C for long periods of time (1yr).

19. Immediately prior to mass analysis dissolve dried peptides in 50%/50% acetonitrile/1% formic acid. Alternatively 0.1% trifluoroacetic acid can be used but TFA can reduce mass spectral ionization efficiency through ion pair formation.

5.2. Mass Spectral analysis via nano-LC/QTOF/MS/MS

Over the past decades, the rapid development of mass spectrometry (MS) has made it an indispensable tool in biological research. The introduction of soft ionization techniques such as electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI) has made it possible to characterize relatively large and labile biomolecules including peptides and proteins using MS. MS-based protein identification typically involves two different steps: (1) analysis of peptides generated by proteolyic digestion; and (2) database searching. Digestion of protein can be achieved using various proteases, but the most popular one has been trypsin which hydrolyzes the C-terminal side of lysine and arginine (unless the subsequent amino acid in the sequence is a proline). Peptides produced by tryptic digestion normally possess two basic sites, *i.e*., the basic lysine or arginine residual at the C terminal and the basic amine group at the N terminal. This allows peptides to be protonated efficiently and hence enhances ionization of mass spectral sensitivity. Multiple protonation sites also result in the production of multiple charged species in ESI although not readily in MALDI due to fundamental differences in the energetics of these two techniques. The multiple charges render one important advantage, i.e. the peptides can be easily distinguished from impurities which are typically singly charged. This is especially useful when peptides need to be selected for tandem mass spectrometry (MS/MS) analysis. Currently, the authors use an ABI QSTAR pulsar *i* (Q-TOF) for protein identifications. This protocol describes the use of the QSTAR Q-TOFMS coupled to a nano liquid chromatography system (LC packings) to analyze peptide mixtures generated by tryptic digestion. The resultant data is then compared to theoretical peptide fragments for protein sequences housed in databases using the search engine MASCOT for protein identification. The nano LC system consists of an autosampler (Famos), a valve switching device (Switchos) and a gradient pump system (Ultimate). Peptides are first loaded onto a trap column on Switchos for desalting and concentrating. The trap column is then switched in-line with the analytical column by the Switchos for the separation of peptides. Peptides eluting from the column are electrosprayed directly onto the QSTAR and positive-ions analyzed by both MS and tandem MS analyses (Figure 13).

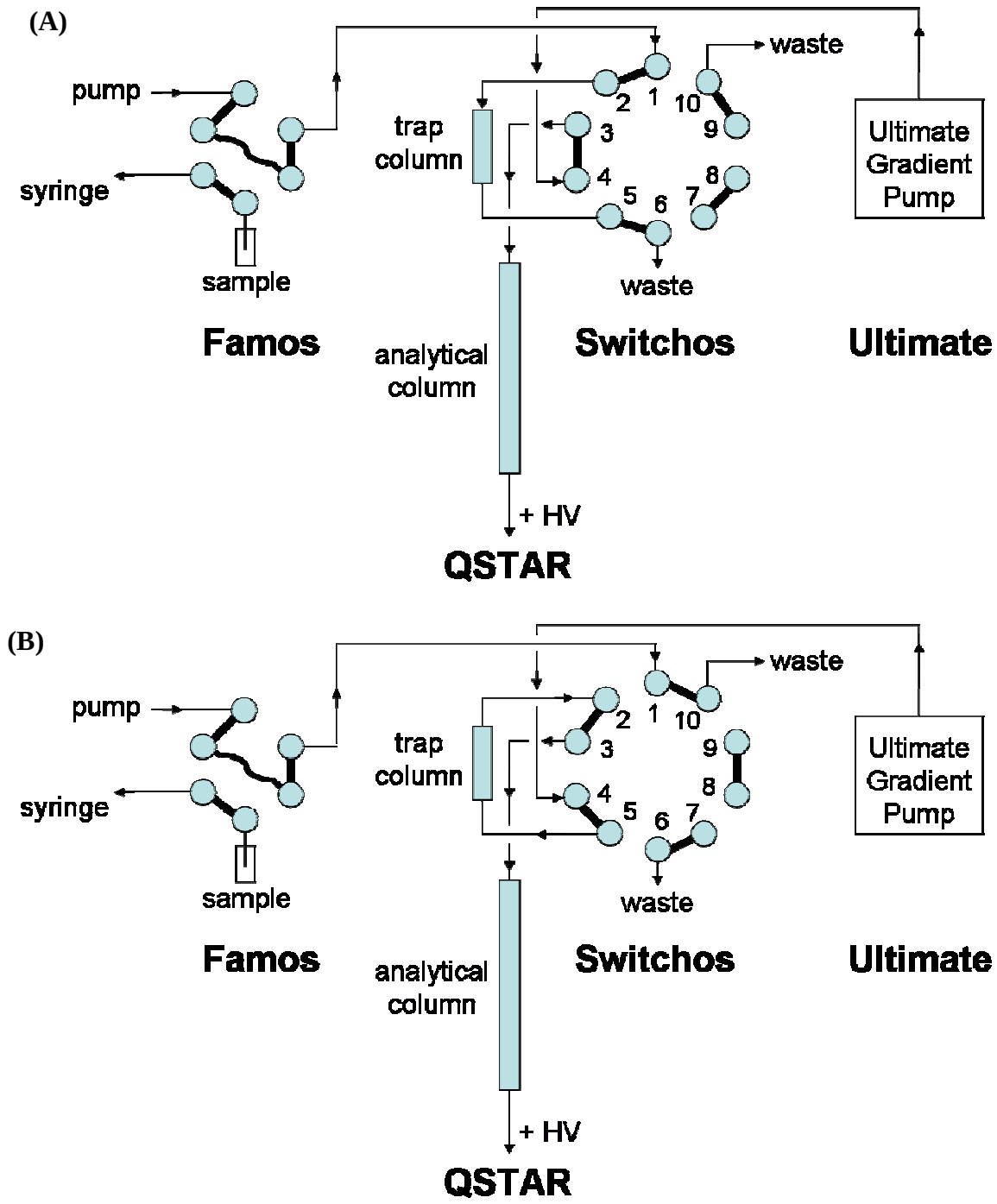


Figure 13. Configurations of nano LC system.
(A) In load position, peptides are transported to the trap column for desalting and concentration. The trap column is not in-line with the analytical column. (B) In separation position, the trap column is now in-line with the analytical column for separation.

1. Degas solvents (solvent A: 5% acetonitrile with 0.1% formic acid; solvent B: 95% acetonitrile with 0.08% formic acid) using helium for 10 minutes. Pressure should not exceed 20 psi. Use only HPLC grade solvents.

2. Fill pump heads with 50% isopropanol using a 20cc syringe. This will dissolve salts that may deposit on the pump heads.

3. Open the drain valve and purge pumps by pressing the *PURGE* button. First purge pump A and then pump B on the Ultimate until no air bubbles come out. Close the drain valve. The Switchos also needs to be purged.

4. Inspect the injection syringe for air bubbles. Air bubbles of accumulate after long idle periods of time. Remove air bubbles by pressing *wash* button on the panel of the autosampler. If air bubbles still exit, take the syringe out and manually remove the bubbles. Incorrect sample volume may result if there are air bubbles in the syringe and the tubing.

5. Check to make sure the trap column is in load position or the sample will be transported directly to the waste bottle. If it is not in load position, use the switch at the back of the Switchos to adjust the trap position.

6. Check all waste bottles (one for Switchos and two for Ultimate) to avoid overflow during the run, especially during the weekend.

7. Turn on all pumps and check trap column backpressure. Under the current setup, the normal back pressure reading for trap column is about 85 psi.

8. Set autosampler (Famos) to serial mode so that the Analyst software can communicate with it.

9. Restart the computer to clear any programs that may run in the background and free up the memory.

10. Launch the Analyst software.

11. Activate the hardware profile (LC-MS). Three icons (MS, Famos and Ultimate) will show up in yellow at the low right corner.

12. Equilibrate the LC system with the method that you will use later to acquire data. The equilibration washes the injection syringe and begins to pump solvents through the column to equilibrate it.

13. Click *tune* in the left column (the navigator) and then *manual tuning*. Open the manual tuning file by clicking the *file opening* icon and select method *Manual tuning.dam*.

14. Begin the manual tuning by clicking the start button. The purpose of manual tuning is to obtain an optimal baseline. Adjust the electrospray needle position to optimize the intensity of baseline. Under the current flow rate and mobile phase compositions, the baseline should be between 5,000 to 20,000 cps. Do not spray directly onto the orifice. This will not only reduce the signal intensity but also clog the orifice more quickly over time.

15. After manual tuning, exit the *tune* mode by clicking the T icon on the tool bar. Calibrate the TOF with 250 fmol [Glu]-Fibrinopeptide B (50 fm/µL, 5µL pickup, Sigma cat#: F-3261). Acquire both TOF-MS and tandem MS data using the existing acquisition method “glufib calibration.dam”.

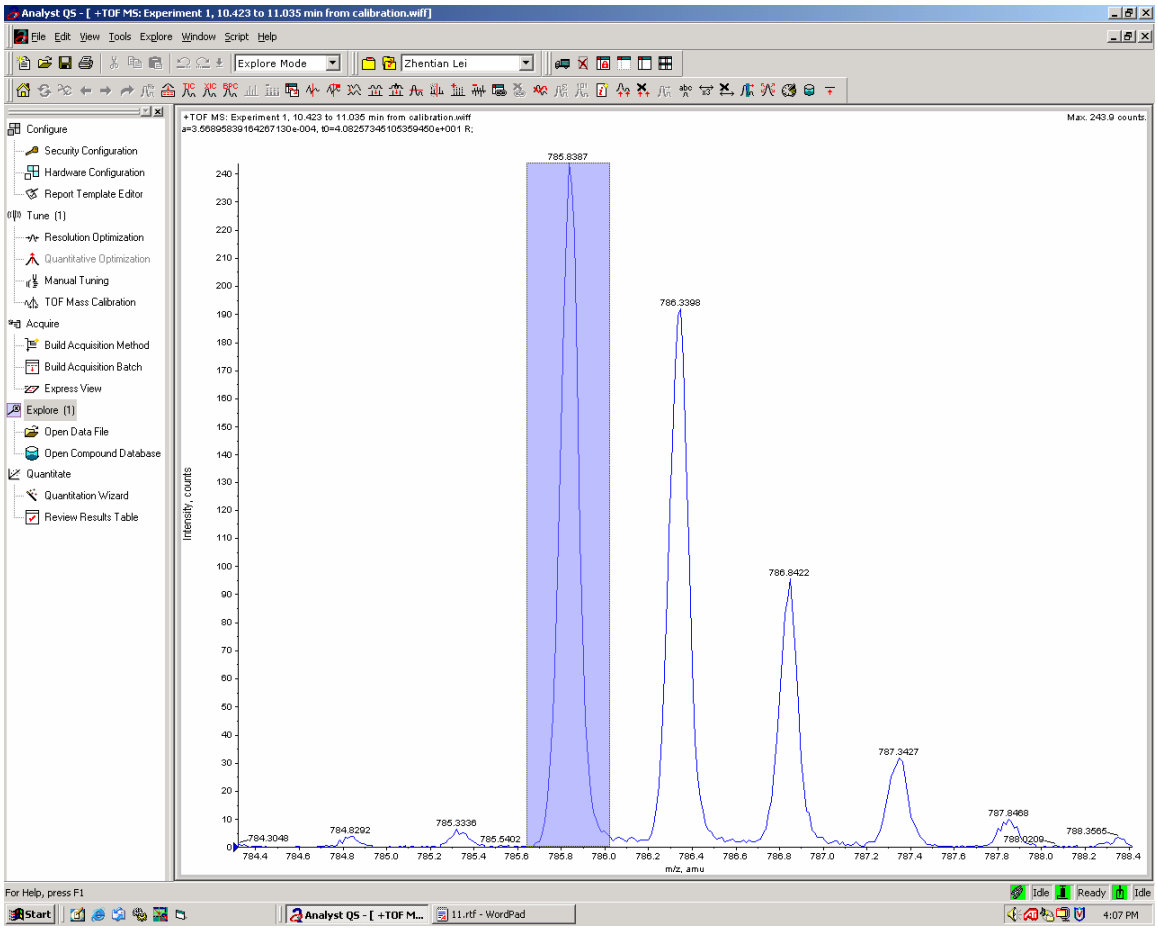


Figure 14. Mass spectrum peak selection for TOF MS calibration.
Screen shot showing the selection of a peak for calibration of the TOF MS. The description is provided in 16.

16. After data acquisition, open the data file. Zoom in the data in the region of m/z 785 and then drag across the C_{12} isotope peak. Hold down the *Shift* key and double click the X-axis to zoom out. Keep the *Shift* key depressed and zoom in another region of m/z 785. Drag across the C_{12} isotope peak as shown in Figure 14.

17. Right click anywhere on the spectrum and a pull-down menu pops up. Select *Re-Calibrate TOF* from the menu to open the calibration window. Follow the steps outlined in the Figure 15. First enter the theoretical molecular weight of peptide (step 1, 524.2256 for $[M+H]^{+2}$ and 785.8384 for $[M+H]^{+1}$), then click *Calculate new calibration* (step 2), *Calibrate spectrum* (step 3) and *Entire file* (step 4). Make sure that *Set as default* and *Overwrite current file* are checked or the new calibration will not be used in the future data acquisition.

18. Build a batch file in which you can enter sample names, vial positions, acquisition methods and the folder/path where you want your data stored. Save the batch file or submit it directly. The current acquisition method employs a 60 minutes gradient (flow 200 nl/min, solvent B increases from 5% to 45 % in 40 minutes and then to 95% for 5 minutes) and uses the IDA (information dependent acquisition) feature for tandem MS experiments. You can edit the current method file or build your own method, but should save it

as a different name. Do not overwrite the existing method. A good starting point for building your own method is: electrospray voltage: 2400 v, mass scan range (m/z): 100 – 1500, charge state for parent ion selection: 2 to 5, intensity threshold for tandem MS: 10 counts/s, precursor ions exclusion: 90s using a window of 6 amu to minimize the redundancy in tandem mass spectra following IDA data acquisition.

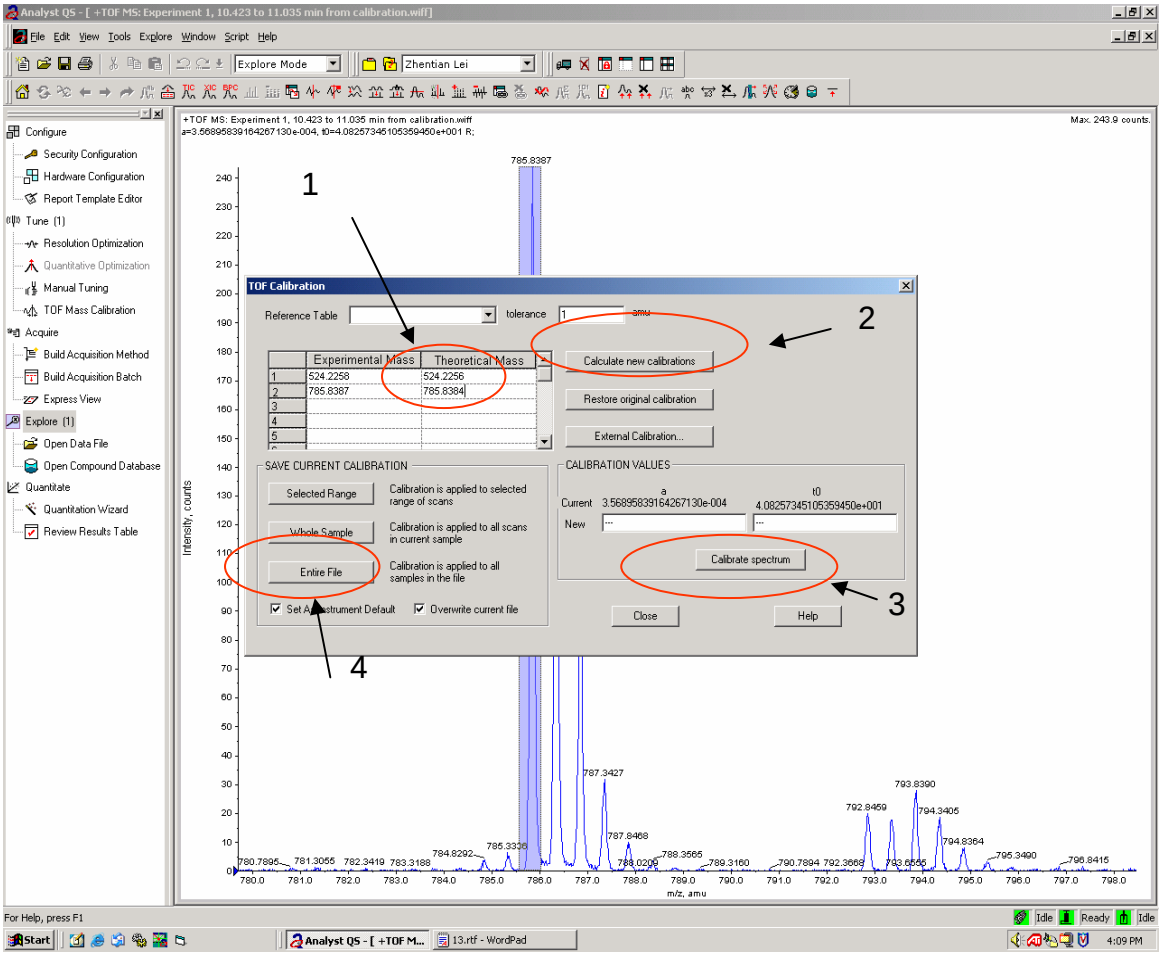


Figure 15. Calibration of TOF MS.
A screen shot showing the example of calibration step of TOF MS. The red circles and the numbered arrows depict the steps described in 17.

19. Submit the batch file by clicking the *submit* button. It should automatically begin analyzing samples. If it does not start, click the *start* button on the tool bar to manually activate the data acquisition process. During data acquisition, do not use the computer to do other work such as checking email, writing paper or searching the internet, as we have found that these activities can interfere with the instrument's communications and efficiency. One hour after all samples are acquired, the system automatically goes idle to stop the Ultimate.

20. The sizes of LC-MS/MS data files are large (15-25 MB) and they can take up the hard drive space quickly. Move your data files to the common network drives, CDs or DVDs for long term storage.

5.3. Database searching

A public Mascot server can be assessed through the Matrix Science website (http://www.matrixscience.com/search_form_select.html). This web-based public server is free and allows one to upload mass spectra data files and perform the database searches on-line. However, there are some limitations on the web-based Mascot server: (a) maximum of 2 simultaneous searches per user, (b) maximum file upload 5 Mb (note our LC-MS/MS data are typically in the range of 15-25 MB), (c) maximum of 300 spectra in a single MS/MS search, and (d) custom databases can not be searched. In addition, users are requested not to submit more than one very large search at a time as the search can take a long time. Very large search refers to the search, in which no enzyme specificity is given, or more than 5 variable modifications of amino acids are used, or the search is against a large EST database. An in-house Mascot server does not have these limitations, but requires the purchase of the licensed Mascot server. The current version of Mascot server requires computers to have high speed Intel and AMD processors, at least 2GB RAM, and 200 GB IDE hard drive. Mascot currently does not support other CPU processors. After installing the software, one can then create the custom database.

a. Database Maintenance

1. Database maintenance is not a routine task and only performed when you have a new sequence database or new sequences to add to the current local sequence database. To create a new Mascot searchable sequence database, go to *Programs*, click *Mascot*. The local mascot homepage will appear. Click the *Database Maintenance* button and the database maintenance window comes up as shown in Figure 16.

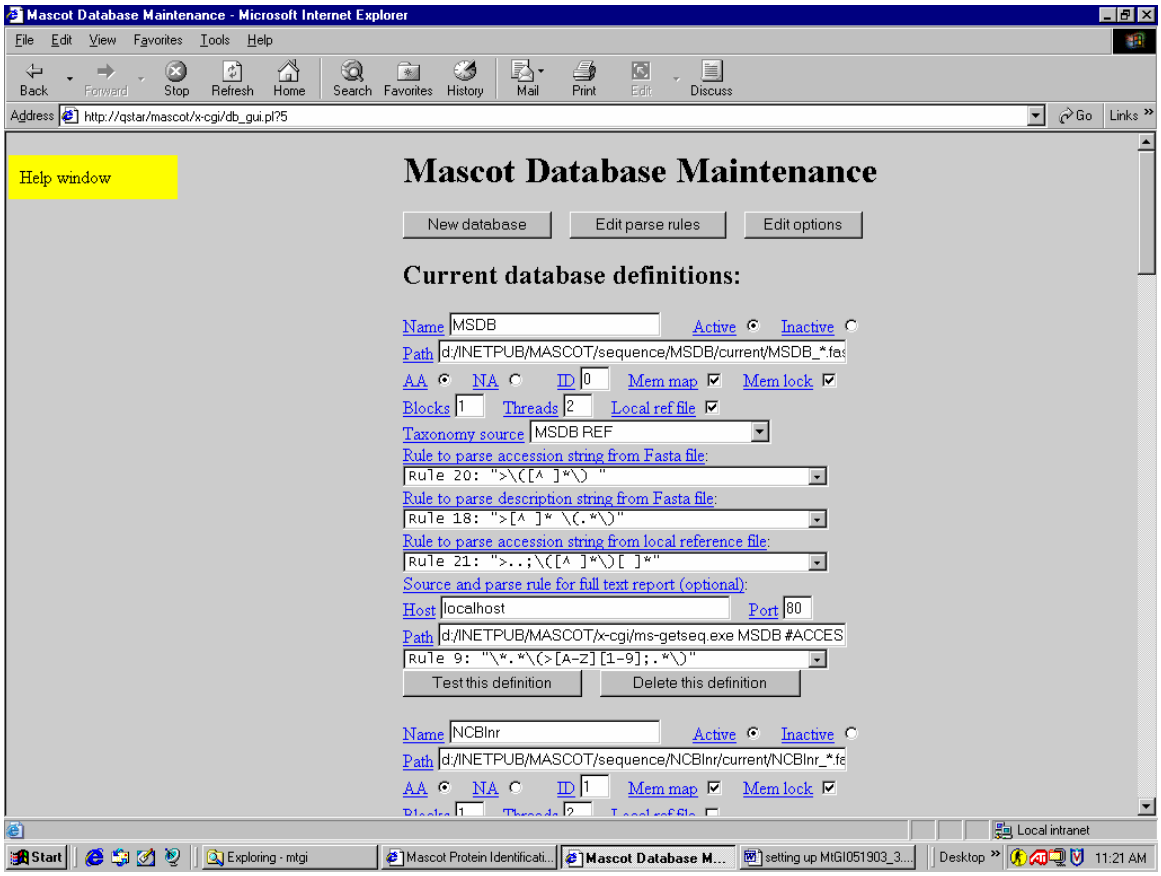


Figure 16. Mascot database maintenance page.

2. Click *New Database* button if you need to create one. Type in the name of the new database, path, select AA if it is a protein database or NA if it is a nucleotide database. Choose appropriate rules for different type of database. For FASTA databases, use rule 6 or other appropriate rule to parse the accession number and rule 7 or other appropriate rule to the description from the FASTA file.

3. Click *Test This Definition* button at the bottom of the page. Mascot will then compress the first 5 and the final 5 sequences. If no problem (meaning no red in test result) is encountered, you can then click *Apply* to begin compiling the new local database.

b. Database Search

MASCOT is a probability-based search engine that uses mass spectrometry data to identify proteins by searching sequence databases (Perkins et al., 1999). It can perform peptide mass fingerprinting (PMF), sequence query and MS/MS ion searches. MASCOT Daemon is an automation script that allows one to query multiple data files through a batch search.

1. To use the Mascot Daemon, go to *Programs* and select *Mascot Daemon*. It opens the Mascot Daemon menu as shown in Figure 17.

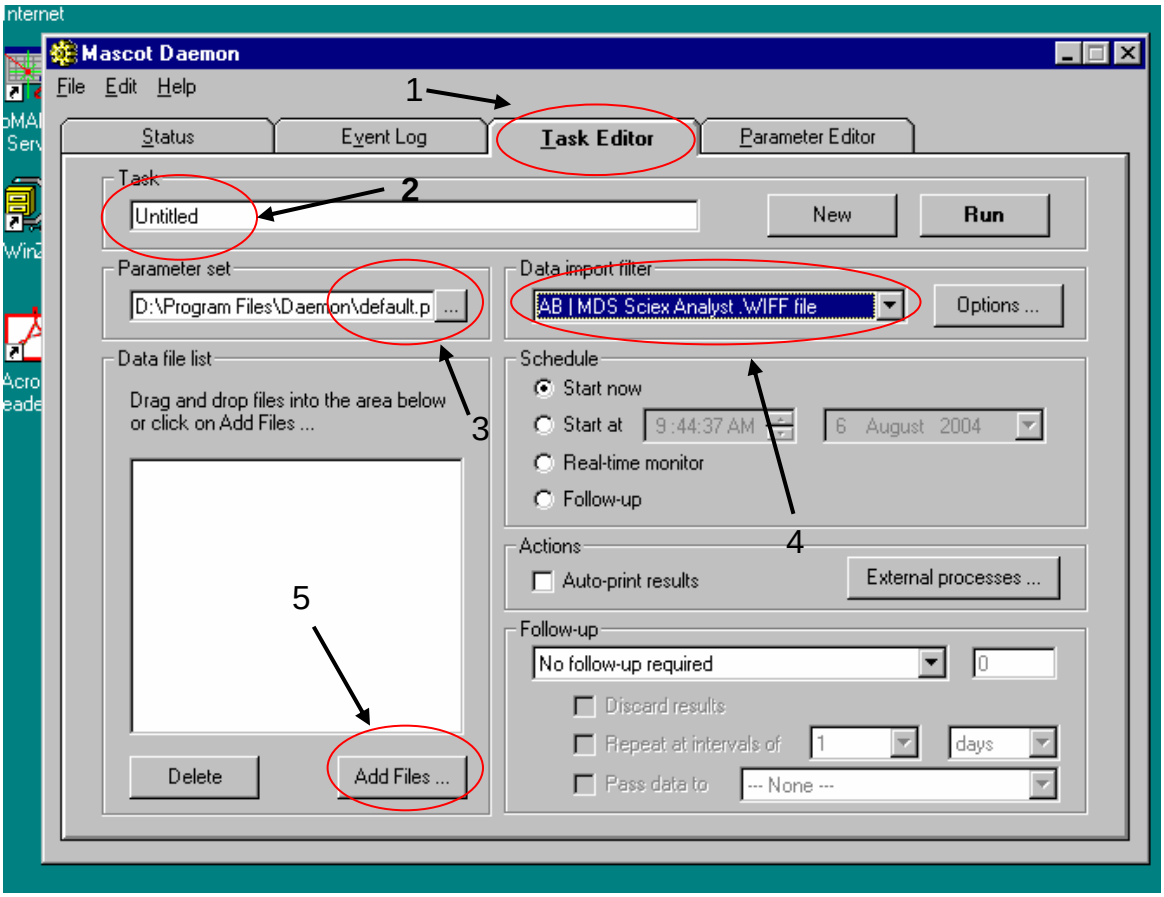


Figure 17. Steps for setting up Mascot Daemon.
The protocol describing the initial steps to setup a Mascot search is shown and the individual steps are circled and indicated by numbered arrows.

2. First click *task editor* (step 1) to open the search menu. Enter the name of the task (step 2); select the database which you want to search (step3). We currently have MSDB, NCBInr, dbEST, Swiss-prot and several custom local databases. The legume specific protein database is named "legprot" which was compiled mostly from *Medicago truncatula*.

3. Select "ABI MDS Sciex Analyst. WIFF File" in the *Data Import Filter* tab (step4). If data file format rather than WIFF format is selected for QSTAR data files, the search will fail.

4. Add mass spectral data files you want to search into the *Data file list* window by clicking the *Add File…* button (step 5). Browse and select files and click *Ok* to add them into the search list.

5. Click *Run* button to begin the search (step 6). While searching is in progress, do not use the computer for other work.

6. When the search is finished, click *Status* to view and print the search results. The results can be opened by clicking on individual search results.

6. Concluding remarks

Proteomics is a fast changing field and new protocols are being established frequently. Most procedures depend on high quality ingredients and careful experimentation to achieve good quality gels. Especially procedures for pure protein samples are important for all subsequent steps and will require optimization in any individual lab.
So far, the diversity of proteins in an organism make it impossible to visualize all proteins at once. Compared to transcriptome analysis, which can reliably track the expression of tens of thousands of genes, proteomics has been limited in the number of proteins that are resolvable and possible to identify. Altogether, several thousand proteins have been identified from *M. truncatula*, and this number will rise. Any user of proteomics will have to keep the advantages (e.g. ability to visualize protein isoforms, post translational modifications, and ability to quantify the "real" gene expression) and disadvantages (technical limitations) in mind.

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7. Abbreviations

1D	One dimensional
2-DE	Two dimensional electrophoresis
2D-LC	Two dimensional-liquid chromatography
AA	Amino acid
AM	arbuscular mycorrhizal
amu	Atomic mass unit
APS	Ammonium persulphate
ASB14	amidosulfobetaine-14
BSA	Bovine serum albumin
C terminal	Carboxy terminal
C/M	chloroform/methanol
CBB G-250	Commassie Blue G-250
cc	Cubic centimeters
CHAPS	3-[3-(Cholamidopropyl)dimethylammonio]-1-proanesulfonate
cm	centimeter
cps	Counts per second
DIGE	Difference gel electrophoresis
DMSO	dimethylsulfoxyde
dpi	Dots per inch
DTT	Dithiothreitol
EDTA	ethylene diaminetetraacetic acid
ESI	Electrospray ionization
EST	Expressed sequence tags
fm	Femto moles
g	Gram
g	Gravity
H_2O	Water
HPLC	High performance liquid chromatography
hrs	Hours
IAA	Iodoacetamide
ICAT	Isotope-coded affinity tagging
IDA	Information-dependent acquisition
IEF	Isoelectric focusing
IPG	immobilised pH gradient gel
iTRAQ	Isobaric tags for relative and absolute quantification
L	Liter
LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
M	Molar
m/z	Mass/charge
mA	Milli amperes
MALDI-TOF MS	Matrix assisted laser desorption/ionization-time of flight mass spectrometry
MB	Mega bytes
mg	Milli gram
$MgCl_2$	Magnesium chloride
ml	Milli liter
mm	Milli meter

mM	Milli molar
Mohm	Mega ohm
MP	Multiplexed proteomics
mTorr	Milli torr
Mud-PIT	Multidimensional protein identification technology
N terminal	Amino terminal
N_2	Nitrogen
NA	Nuclei acid
ng	Nano gram
nl	Nano liter
nm	nanometer
NTES buffer	NaCl-Tris HCl EDTA SDS buffer
PAG	Plant and animal genome conference
PMF	Peptide mass fingerprinting
PMSF	Phenylmethylsulphonylfluoride
psi	Pounds per square inch
Q-TOF	Quadropole-TOF
rpm	Revolutions per minute
SDS	Sodium dodecylsulphate
TBP	tributyl phosphine
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
TFA	Trifluoroacetic acid
Tris-MES	Tris-morpholinoethanesulfonic acid
U	Units
v	Voltage
yr	Year
µg	Micro gram
µl	Micro liter

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