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RESEARCH ARTICLE

A proteome study of the proliferation of cultured *Medicago truncatula* protoplasts

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A proteome study of the first five days of *Medicago truncatula* protoplast cultures was done to investigate molecular changes taking place during protoplast proliferation. A total of 1556 protein spots were analysed, of which 886 protein spots showed significant ($p<0.005$) changes in abundance at some time during the first five days of protoplast culture. Of the 886 significantly changing protein spots, 89 proteins were identified by MALDI-TOF MS. The majority of the identified proteins were part of four main cellular processes that may be involved in protoplast proliferation: energy metabolism, defence or stress response, secondary metabolism and protein synthesis and folding. The accumulation pattern of these proteins indicates extensive changes in the energy metabolism of the cells, accompanied by the activation of stress response pathways and modifications of the cell wall. In addition, seven PR10-like (pathogenesis related) proteins were identified. The accumulation pattern of these seven PR10-like proteins suggests that they could have a developmental role during protoplast proliferation.

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1 Introduction

Plants are well known for their ability to regrow a lost organ such as a leaf and even develop new organs such as root nodules. This ability to regenerate has been used in plant tissue culture using tissue explants and protoplasts. Explant and protoplast cultures are widely used to create plantlets for agricultural purposes [1]. In addition, plant tissue culture is used to gain a better understanding of how organ formation takes place. Homogeneous cell populations of protoplast cultures are also used in biochemical studies. Although protoplasts are used for a wide range of developmental studies and biochemical analysis [2–6], the molecular events that occur with protoplast proliferation are unknown. Studies of protoplast cultures have examined the influence of hormones [7–11], and 'stress' factors [11]. Most of these studies provide information about the physiology of protoplast cultures, but little is known about the proliferation process. Protoplast proliferation can be seen as the dedifferentiation of the protoplast to a stem cell-like state after which the cell starts dividing. Recent studies have provided some information about the molecular events that occur during the dedifferentiation process [12–14]. For example, it was shown that the dedifferentiation of somatic cells to a stem cell-like state is accompanied by chromatin reorganization [15, 16]. Moreover, dedifferentiation has also been connected to stress and auxin responses, whereby the wound response activated by the isolation of protoplasts initiates cellular reorganization [14].

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Abbreviations: CCoAOMT, caffeoyl-CoA O-methyltransferase; **ROS,** reactive oxygen species

Medicago truncatula mesophyll protoplasts are able to form somatic embryos *via* callus culture [17]. With this optimized procedure, it was shown that *M. truncatula* protoplasts in culture regenerate their cell wall and undergo divisions during the first week of culture. In this paper, we present a 2-DE and MS-based proteomic study of the first five days of protoplast cultures to investigate protein profiles during proliferation. Moreover, two different genotypes of *M. truncatula* were analysed. An embryogenic (2HA) line and a non-embryogenic (A17) line were used for the comparison of the protein accumulation patterns. It is known that protoplast culture of 2HA protoplasts results in the development of somatic embryos 70 days after culture initiation, while, the A17 tissue does not develop somatic embryos [18].

2 Materials and methods

2.1 Growth conditions for plants

M. truncatula cultivar Jemalong plants were grown in a controlled growth chamber under the following conditions: 23°C day and 19°C night temperature, with an 80% relative humidity and a 12 h day length with a photon flux density of 40–60 μmol m^{-2} s^{-1} before protoplast isolation was initiated. An embryogenic (2HA [19]) line and a non-embryogenic (A17) line of *M. truncatula* were used.

2.2 Protoplast isolation and culture

Two- to five-month old *M. truncatula* plants were used as a source for the protoplast isolation. Protoplast isolation was done essentially according Rose and Nolan [17] with the additional modifications of using PES-5$^-$ medium (0.45 M mannitol, 7.0 mM $CaCl_2 \cdot 2H_2O$ and 3.0 mM MES buffer) for the plasmolysis and leaving out the vacuum infiltration step. The source plants were kept in the dark the night before use. About 0.3 g of the youngest, fully expanded leaves from different plants were harvested. The leaves were sterilized for 40 s in 70% v/v ethanol and for 10 min in 1% sodium hypochlorite and the leaves were rinsed three times for 3 min with milliQ water. The sterile leaves were cut into 2 mm squares which were put in a 100-mL flask containing 6 mL of PES-5$^-$ medium, and left for 60 min in the dark for plasmolysis. The PES-5$^-$ medium was replaced with 8 mL of enzyme medium PES-5 (PES-5$^-$, 0.5% w/v Cellulase Onozuka RS (DUCHEFA Biochemie, The Netherlands), 0.5% w/v Macerozyme R-10 (SERVA Electrophoresis, Germany), 0.025% v/w Pectolyase Y-23 (MP Biomedicals) and 0.5% w/v BSA). The leaf pieces were incubated for 3.5 h on a 60 rpm rotary shaker at 27°C. the released protoplasts were kept at 4°C for 5–15 min, and each dish was filtered through a 40 μM nylon mesh filter into a 15 cm centrifuge tube. One-third of the volume of 80% Percoll solution, precooled to 4°C, containing 0.45 M mannitol solution was added and mixed well. Then 1 mL of P1 medium [20] was layered carefully on the top and the protoplast suspension was centrifuged at 80 × g for 15 min. The green bands (containing the protoplasts) were collected in a sterile centrifuge tube and resuspended in 12 mL of P1 medium and centrifuged at 100 × *g* for 10 min. The supernatant was removed and each tube was made up to 8 mL with P1 medium. The number of protoplasts were counted with a haemocytometer. The tubes were then centrifuged at 100 × *g* for 10 min. The supernatant was removed and washed again before culturing the protoplasts. The protoplasts were cultured at a density of 0.8×10^6 cells/mL, as described previously by Imin *et al.* [21]. A microscopic analysis of the protoplasts was done, using an Olympus IX50 inverted microscope, to study the development of protoplasts in culture.

2.3 Protein extraction and 2-DE

The protein extraction was done using a TCA precipitation method. The protein concentration was determined by using a Bradford assay. For each analytical gel, 200 μg of soluble proteins were separated on a 24 cm long, pH 4–7 IPG strips (GE Healthcare) and further separated on precast 12–14% SDS–PAGE gels (GE Healthcare). The protein spots on the gel were visualized by silver staining for quantification. Because the protoplast material was limiting, we identified the differentially displayed proteins from two time points, 0 and 72 h, as almost all the proteins were present on at least one of those gels. For these identifications, 1 mg of protein from both 0 and 72 h cultures were separated and were visualized by CBB staining. All the procedures were done as previously described by Imin *et al.* [21].

2.4 Scanning and gel analysis

After staining the proteins the gels, were scanned using a UMAX Astra 2400 scanner at a resolution of 600 dpi, true colour, and the image was stored as a TIF file. The scanned image of the gel was analysed by using ImageMaster software (GE Healthcare). The percentage volume (%vol) was calculated for each protein spot and used for protein spot quantification. For this, the total sum of spot volumes was considered as 100%. While there were differences in spot numbers between gels, all the gels contained the same amount of protein and we found a high correlation between the % spot volume and the absolute spot volume within and between gels (usually $r^2 = >0.99$ and $p<1 \times 10^{-20}$ within gels (data not shown). Gels of three biological repeats were analysed for each selected time point and genotype. A linear restricted maximum likelihood (REML) analysis using GenStat 7 (VSN International) was done to calculate which differentially expressed protein spots showed statistically significant changes in response to time point, genotype, and interaction of genotype and time point ($p<0.005$).

2.5 In-gel digestion

Protein spots were excised with a clean surgical blade from CBB stained 2-DE gels. The excised spots were placed in clean Eppendorf tubes or a 96-well tray. The spots were destained with four washes of 120 μL 50% ACN/25 mM NH_4HCO_3, pH 7.8. After destaining, the spots were dehydrated in 120 μL of 100% ACN for 10 min and air-dried and digested with porcine trypsin (Promega) as follows: 40 μL of 15 ng/μL porcine trypsin in 250 μL of NH_4HCO_3 was added to the spot and incubated for 60 min. The excess of trypsin solution was removed and the spots were immersed in 40 μL of 25 mM NH_4HCO_3. The spots were then incubated overnight (16 h). Using $ZipTip_{\mu\text{-}C18}$ (Millipore), the tryptic peptides were desalted and concentrated. The peptides were first acidified to 1% TFA after which the peptides subjected to MALDI-TOF-TOF analysis were eluted with 50% ACN/0.1% TFA onto a MALDI-TOF target plate that was spotted with 0.5 μL of matrix solution (8 mg/mL CHCA in 70% ACN/ 0.1% TFA).

2.6 Protein identification

Protein identification was done either by MALDI-TOF or by MALDI-TOF-TOF analysis. MALDI-TOF-TOF analysis was performed with an Applied Biosystem 4800 Proteomics Analyser (at the John Curtin School of Medical Research, Australian National University, Canberra) with TOF/TOF optics in MS mode. The spectra were acquired in reflectron mode over the m/z range of 800–3500 Da. The instrument was then switched to MS/MS mode, where the 25 strongest peptides from the MS scan were isolated and fragmented and their masses and intensities were measured. A near-point external calibration was applied and gave a typical mass accuracy of ~50 ppm or less. Automated MALDI-TOF-MS analyses were performed at the Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia) on a TofSpec 2E mass spectrometer (Micromass, Manchester, UK) using trypsin autodigestion peaks at 842.51 and 2211.10 Da for internal calibration. The mass spectra generated by MALDI-TOF and MALDI-TOF-TOF analyses were used to search the MTGI (*M. truncatula* Gene Index at TIGR, http://www.tigr.org/) database (release date 25 January 2005, containing 226 923 EST sequences), MSDB (ftp://ftp.ncbi.nih.gov/repository/MSDB/msdb.nam, Release date 31st August 2006) and the IMGAG protein database of sequence data from BAC sequences (downloaded from http://www.medicago.org/genome/downloads.php, release date 17 July 2006). All the searches were done with the MASCOT-demon 2.1.0 software (Matrix-Science). The search parameters for MALDI-TOF-TOF mass spectra included a maximum of one miss-cleavage, a peptide tolerance of 100 ppm, allowance of Oxidation (M) modifications, and a peptide charge of 1^+, 2^+ and 3^+. In addition, an MS/MS ion search was done with an MS/MS tolerance of 0.8 Da. Good matches were classified as those having a MOWSE score higher than 44 (threshold). The search parameters for the single peptide matches included no miss-cleavage, no allowance of any modifications, and a peptide charge of 1^+, a peptide tolerance of 50 ppm unless *de novo* sequences of at least eight amino acids were obtained. The search parameters for MALDI-TOF mass spectra include no miss-cleavages, a peptide tolerance of 100 ppm, allowance of Oxidation (M) modifications, and a peptide charge of 1^+. Good matches were classified as those having a MOWSE score higher than 66 (threshold). To avoid false positives, an additional BLAST search at NCBI (http://www.ncbi.nlm.nih.gov/) was done to confirm the right ORFs for all the matches. The identified proteins were then searched against the UniProt (http://www.ebi.uniprot.org/index.shtml) database to find out if their function was known.

3 Results

3.1 Culture analysis

A microscopic analysis was done to study the development of the protoplasts during their growth in culture. The protoplasts were round shaped when first placed in culture. After 2–3 days of culture, the shape of the protoplasts changed into an oval shape. After 5–7 days of culture, the protoplasts began to divide (data not shown). Microscopic analysis did not show any difference between A17 and 2HA protoplasts during these stages, although their ability to form embryos differs dramatically at later stages of culture [18].

3.2 2-DE analysis

On the basis of microscopic analysis, a time course proteomic analysis was made during the first five days of the protoplast culture. Protoplasts that were isolated from an embryogenic (2HA) and a non-embryogenic (A17) line of *M. truncatula* were used for the protoplast culture. Samples of protoplasts were taken at the start of culture (0 h), after 12, 24 and then every 24 h up to 120 h of culture.

The protoplasts were cultured in agarose droplets. Attempts to isolate the protoplasts from the agarose droplets did not result in successful isolation of viable cells. Therefore, the agarose droplets were harvested with the protoplast samples. This resulted in very low protein yields, but was the only way to purify proteins from protoplasts without damaging them.

Total proteins were isolated from the protoplast samples and ran on 2-DE gels and the gels were silver stained. Global analysis showed changes in the protein accumulation profile over time (Supporting Information 1). However, fewer differences were observed between A17 and 2HA 2-DE gels (see Section 3.4). Protein spots that were detected in a minimum of three biological repeats of one time point were further studied. A total of 1556 protein spots were statistically analysed, of which 886 protein spots showed significant ($p<0.005$) changes either by time point, genotype or interaction of time point with genotype, and most of them by time point. These 886 protein spots were further analysed.

3.3 Time point analysis

Silver-stained gels from protoplast proteins of different time points were compared to identify candidate proteins that may be involved in the proliferation of protoplasts. Table 1 shows the number of detected protein spots for each time point. Moreover, it shows the high number of protein spots that changed between each time point. In addition, there were a large number of proteins that disappeared or were newly accumulated.

To make it easier to compare the different time points of protoplast proliferation, the analysed time of protoplast proliferation was divided into three phases: (i) 0–24 h, an early phase, (ii) 24–72 h, an intermediate phase and (iii) 72–120 h, a late phase. Eight categories of protein spots were recognized, whose protein accumulation pattern suggests that they have a possible role during the events associated with protoplast proliferation. Each category consists of a group of protein spots that have a similar accumulation pattern during a phase of protoplasts proliferation (Table 2).

3.4 Comparison of A17 with 2HA protoplasts

Of the 886 differentially displayed proteins spots, 27 differed significantly ($p<0.005$) between A17 and 2HA protoplasts during the time of analysis. Of these 27 protein spots seven protein spots only accumulated in A17 protoplasts and three protein spots only accumulated in 2HA protoplasts. The other 17 protein spots that accumulated in both A17 and 2HA protoplast had similar accumulation patterns, but showed higher or lower relative accumulation levels. In addition, 117 proteins showed a significant interaction between time point and genotype, *i.e.* the differential expression in each genotype depended on the time point of analysis.

3.5 Protein identification

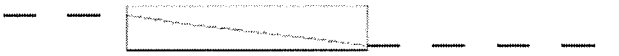
For protein identification, 135 proteins were excised from CBB stained 2-DE gels and submitted to MS analysis, resulting in the identification of 89 protein spots (Fig. 1, Supporting Information 2). Most of the differentially displayed proteins were either not visible at all on CBB-stained gels or were very faint, so that protein identification was not possible. Both the lack of a fully sequenced genome [22] and the low abundance of proteins are common limiting factors in protein identification [23]. Due to the very low protein yield from plant protoplasts, partially due to the high water content of the surrounding agarose, we could not obtain more biological material for the identification.

The identified proteins were grouped according to their functional classes, following Bevan *et al.* [24] (Table 3). Most of the identified proteins had a role in energy metabolism, with 49 of the 89 identified protein spots in this class. Sixteen of the identified proteins had some role in the synthesis and folding of proteins, and another seven play a role in the defence or stress response of the plant. Among the identified proteins, six were found to have a role in the primary metabolism and three proteins had a role in the secondary metabolism. Only three proteins each were identified as having a role in the cell structure or transcription. One protein identified as the embryo-abundant protein EMB had an unclear classification. One protein spot was identified as an unknown protein, and remains unclassified (Table 3). Of the proteins that were differentially expressed between A17 and 2HA, we identified six proteins with a significant interaction between time point and genotype, but none with a significant genotype-only effect. These six proteins included an actin isoform B, a chloroplast inorganic pyrophosphatase, pyrophosphatase kinase, plastidic aldolase, glutamine synthetase and cysteine synthetase (Table 3).

Table 1. Analysed protein spots. The table shows the number of analysed spots and the number of protein spots that changed significantly ($p<0.005$) for each time point. In addition, it shows the number of protein spots that show an up- or down-regulation in accumulation compared to the previous time point, and the number of protein spots that disappeared or newly accumulated compared to the previous time point. For the up- or down-regulated and the new or disappearing spots only the significantly changing spots were analysed. All the numbers are the average of six repeats (three repeats for A17 protoplasts and three repeats for 2HA protoplasts) for each time point

	0 h	12 h	24 h	48 h	72 h	96 h	120 h
Total number of detected spots (1556 over all time points)	1101	997	941	558	795	594	598
Number of significantly changing spots ($p<0.005$. 886 over all time points)	614	540	478	254	429	268	312
Increased (new)		114 (75)	122 (63)	100 (43)	264 (213)	109 (49)	149 (114)
Decreased (absent)		351 (147)	206 (124)	262 (264)	92 (37)	204 (205)	92 (69)
Total change		465	328	362	356	349	241

Table 2. Categories for protein accumulation patterns. The protein spots were categorized according to their accumulation pattern depending on the phase of culture. The boxed area of the accumulation pattern represents the characteristics of the category. Outside the boxed area the dashed line indicates that accumulation pattern of the proteins may vary between the different accumulation patterns. For each category, the number of spots that follow the accumulation pattern is given

Category	Accumulation pattern	Spot number
1		114
2		73
3		172
4		194
5		96
6		176
7		94
8		84
	0 h 12 h 24 h 48 h 72 h 96 h 120 h	

3.6 PR10-like proteins

The acidic low molecular weight range of the gel showed consistent up-regulation, up to a 40-fold increase in five days, among the most abundant protein spots during the first five days of protoplast culture (Supporting Information 1). In this area, 11 protein spots were identified, of which seven were members of the PR10-like protein family. The accumulation pattern of each of the seven PR10-like proteins was unique (Fig. 2). However, they were consistently strongly up-regulated, up to five-fold, after 48 h of protoplast culture. Before 48 h, PR10-like proteins could be divided into two groups: (i) a group which accumulated from 0 h (p00183, p00184, p00186 and p00197), and (ii) a group whose accumulation started later (p01143, p01263 and p01627). Two different PR10-like proteins were identified, PR10 and Ppgr2. Two isoforms of PR10 were identified, PR10-1 (p01627) and disease resistance protein Pi49 (p00197 and p01143). Among Ppgr2, three isoforms were identified (p00183, p00184, p00186 and p01263) of which p00184 and p01263 represent two isoforms of the same *Ppgr2* gene. All of the Ppgr2 proteins are part of the group of proteins whose accumulation suggests that they were important for the process of protoplast proliferation.

4 Discussion

Even though protoplasts have been used for developmental and biochemical studies [2, 3, 5, 6], it is poorly understood which genes and proteins play a role during the development of protoplast culture. In this study, an investigation was made of the proteins that are differentially accumulated and might therefore have a role during protoplast development in culture.

A microscopic analysis of protoplast cultures showed a change in protoplast shape from spherical to oval during 2–3 days after initiation of protoplast culture. Previous studies relate this change in shape to the regeneration of the cell wall [25]. This is supported by a 2.5-fold up-regulation of caffeoyl-CoA O-methyltransferase (CCoAOMT) (p01186) from 0 to 96 h in both genotypes, after which the expression decreased. The CCoAOMT enzyme is involved in the synthesis of lignins that are used for cell wall modification and lignification and is induced under wounding and fungal stress situations [26]. The up-regulation of CCoAOMT during protoplast culture suggests that CCoAOMT may be involved in primary cell wall formation, although other enzymes for cell wall synthesis, *e.g.* cellulose synthase, could not be identified. Cellulose synthase is a large protein complex located in the

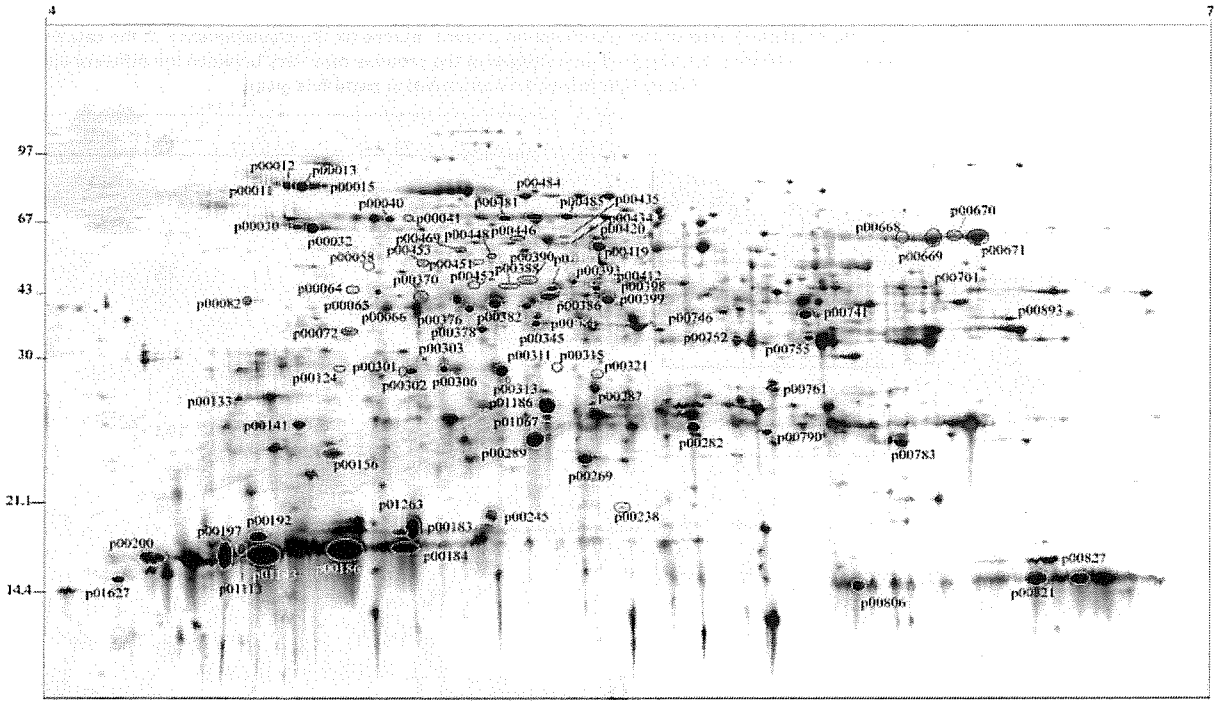


Figure 1. 2-DE proteome map of *M. truncatula* 2HA protoplasts 72 h after the start of culture. Identified protein spots are labelled. First-dimension IEF was done on a 24 cm IPG strip with a linear pH gradient 4 to 7 loaded with 200 μg of soluble proteins. The pH range (4–7) of the IPG strips is shown above the gel, and the position of protein molecular weight markers, MW (kDa), is shown to the left of the gel.

plasma membrane, which might have prevented its isolation [27]. To our knowledge, cellulose synthase has not been identified from proteomics studies in *M. truncatula* previously.

It has been suggested that freshly isolated protoplasts contain all the differentiated features of the donor tissue [3]. This suggests that freshly isolated protoplasts have to dedifferentiate to a less differentiated cell that is able to undergo cell division.

The analysis of the proteome of protoplast culture up to 120 h showed a large number of protein spots that changed in accumulation. Previously, it was suggested that this large number of protein spots that are changing could result from cellular reprogramming [14, 28]. Because the majority of the 886 changing proteins remain unidentified, we are unable to suggest what causes the cellular reorganization. However, Fehér *et al.* [14] suggested that stress has a role in cellular reorganization. Thus, the cellular reorganization could be initiated by the isolation of the protoplasts, during which the cells were wounded to remove the cell wall.

The accumulation of 27 protein spots with a genotype effect and 117 proteins with an interaction between genotype and time point differed between A17 and 2HA during protoplast proliferation. While most of these proteins could not be identified due to their low abundance, we identified six proteins (Table 3) with differing expression. An actin isoform, glutamine synthase and phosphoglycerate kinase initially showed higher expression in A17, followed by higher expressionin 2HA in the later time points. A chloroplast inorganic pyrophosphatase, cysteine synthase and a plastidic aldolase showed differences that varied with time and whose consequences are not clear. A study by Imin *et al.* [29] studying the differences in protein expression of later time points of embryogenesis between the A17 and 2HA found differences in expression of thioredoxin H, vicilin, RuBisCo subunits and several chaperone proteins, all of which were either not identified or not found to be differentially expressed at the early stages of protoplast differentiation examined in this study.

Among the identified proteins that were considered to be of importance during protoplast proliferation, four main cellular processes were recognized that may be involved in the proliferation and initiation of cell division of protoplasts: proteins involved in energy metabolism appear to be down-regulated during protoplast proliferation, except during the early phase (0–24 h). Proteins involved in the defence/stress response and in secondary metabolism appear to be up-regulated during the intermediate (24–72 h) and late phase (72–120 h) of protoplast proliferation. However, they were also up-regulated during the early phase of protoplast proliferation. The last group involved in protoplast proliferation are the proteins that have a role in protein synthesis and

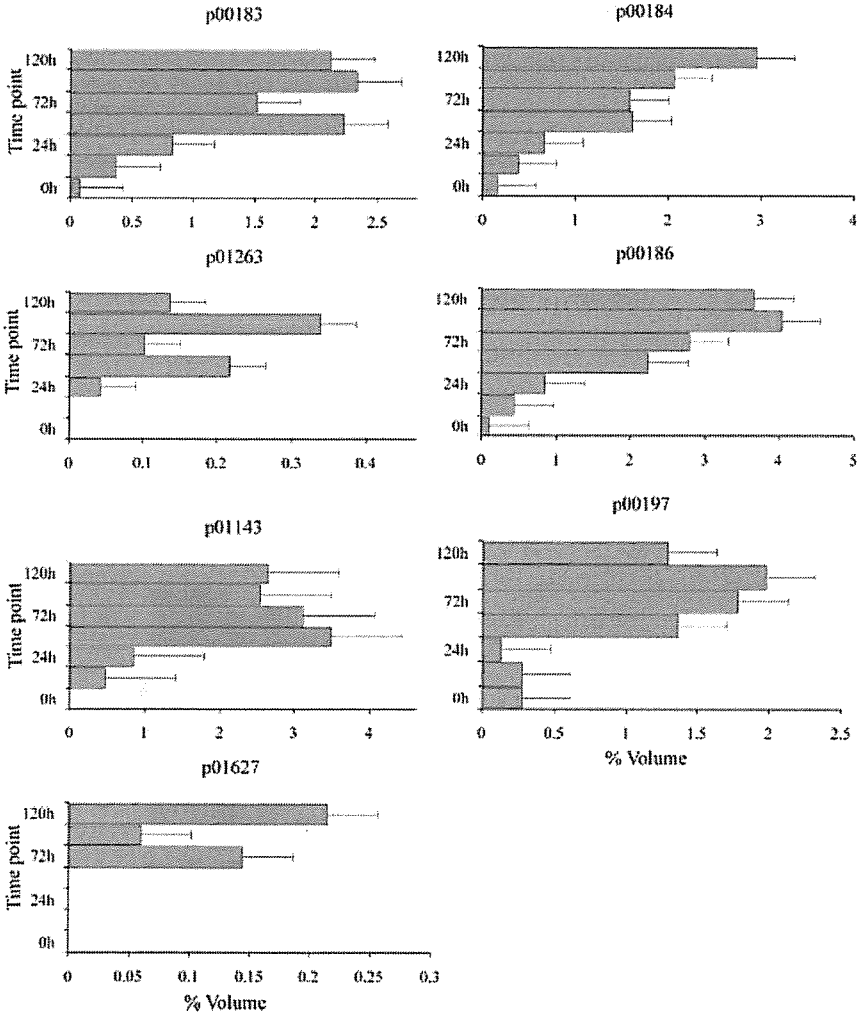


Figure 2. The abundance of the proteins identified as PR10-like. The figure shows the changes in protein abundance over the 0–120 h culture period of *M. truncatula* protoplasts. The abundance of the proteins is represented by the percentage volume of the spot. The error bars show the SDs of protein abundance (in % volume) from gels of six biological repeats.

folding. This group of proteins was present during all the analysed time points but appeared to increase in accumulation between 96 and 120 h of protoplast culture.

4.1 Protein synthesis and folding

During both the early and late phase a broad range of proteins involved in protein synthesis and folding showed high accumulation. During the intermediate phase these proteins were down-regulated. This result suggests that the synthesis of new proteins is an important part of the early and late phase of protoplast proliferation.

4.2 Energy metabolism

Most of the identified proteins play a role in energy metabolism. This can be related back to the origin of the protoplasts [3]. Mesophyll cells are highly specialized in photosynthesis, resulting in high accumulation of proteins that play a role in energy metabolism in these cells. This makes it more likely that proteins involved in energy metabolism are identified compared to other functional classes.

Proteins involved in energy metabolism were considered to play an important role in protoplast proliferation. The majority of proteins involved in energy metabolism were down-regulated, up to ten-fold. This may be a result of the cellular reorganization. However, it can also be that because protoplasts were cultured on glucose they do not have to use photosynthesis to obtain their energy. A previous proteomic study on embryogenesis in *M. truncatula*, which considered later developmental stages than those studied here [29] also found significant down-regulation in abundance of photosynthetic enzymes to be correlated with embryogenesis.

However, between 0–24 h a group of proteins involved in photosynthesis showed an up-regulation of up to four-fold. A byproduct of the photosynthesis pathway is the production of

Table 3. Identified protein spots. Spot numbers are the same as shown in Fig. 1. The p*I* and MW were calculated from the 2-DE gel relative to molecular weight markers, with the MW in kDa. For each identified protein spot the *p*-value of time point and the *p*-value of interaction are given. The *p*-value of the time point is from the significant difference between the analysed time points. The *p*-level of interaction is from the significant difference between the interaction between the analysed time points and genotypes. Graphical representation of relative expression (%vol.) is given for each identified protein spot. The columns in the graphs are: A17 (grey) and 2HA (black) and from left to right 0–120 h. Category: The category of accumulation pattern, according to Table 2.

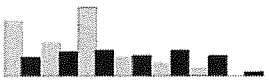
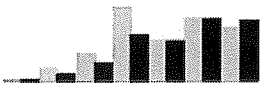
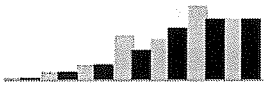
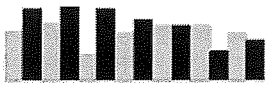
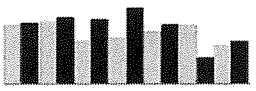
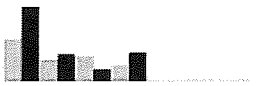
Spot no.	p*I*/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
Cell structure							
p00390	5.27/40.0	TC107326	Actin isoform B	<0.001	0.099		
p00393	5.37/40.0	TC107326	Actin isoform B	0.574	0.047		
p00469	5.15/47.2	AC135800_26.1	Tubulin alpha-1 chain	<0.001	0.849		5
Defence/stress							
p00183	5.13/19.3	TC106340	Pprg2 protein	<0.001	0.705		2
p00184	5.15/18.3	TC106314	Pprg2 protein	<0.001	0.614		5
p00186	4.98/18.2	TC106355	Pprg2 protein	<0.001	0.834		3
p00197	4.64/18.2	TC94217	Disease resistance response protein Pi49 (PR10)	<0.001	0.509		3
p01143	4.76/17.6	TC94217	Disease resistance response protein Pi49 (PR10)	<0.001	0.552		
p01263	5.14/19.0	TC106340	Pprg2 protein	<0.001	0.630		
p01627	4.36/19.4	P93333	PR10-1 protein	<0.001	0.303		3, 7
Energy metabolism							
p00030	4.59/62.1	TC100730	RuBisCO subunit binding-protein alpha subunit	0.711	0.301		
p00032	4.63/61.8	TC100730	RuBisCO subunit binding-protein alpha subunit	0.334	0.266		
p00058	4.91/50.7	TC109855	Ribulose-bisphosphate carboxylase activase large isoform	<0.001	0.807		

Table 3. Continued

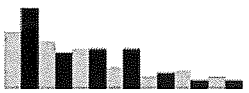
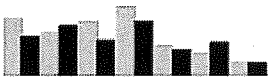
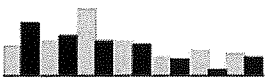
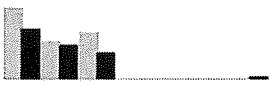
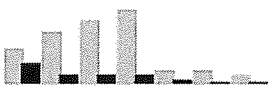
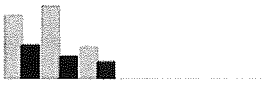
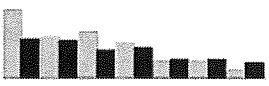
Spot no.	p*I*/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
p00066b	5.10/42.4	AW775333	RuBisCo activase	<0.001	0.135		4
p00124	4.80/30.5	TC94226	Oxygen-evolving enhancer protein 1	<0.001	0.797		
p00141	4.59/25.3	TC107375	Ribose-5-phosphate isomerase precursor	<0.001	0.696		4
p00200	4.47/18.0	TC100616	Glycine cleavage system H protein, mitochondrial precursor	0.269	0.074		
p00245	5.20/20.0	TC102788	ATP synthase beta subunit	<0.001	0.632		6
p00269	5.38/23.4	TC106626	Oxygen-evolving enhancer protein 2	<0.001	0.536		
p00301	5.08/30.5	TC94226	Oxygen-evolving enhancer protein 1	<0.001	0.831		4
p00302	5.13/30.8	TC94226	Oxygen-evolving enhancer protein 1, chloroplast precursor	<0.001	0.089		4
p00303	5.15/30.0	TC95201	Chloroplast inorganic pyrophosphatase	<0.001	0.005		3, 5
p00305	5.07/31.1	TC94226	Oxygen-evolving enhancer protein 1, chloroplast precursor	<0.001	0.357		4
p00306	5.11/30.9	TC94226	Oxygen-evolving enhancer protein 1, chloroplast precursor	0.002	0.806		
p00311	5.23/31.0	TC94226	Oxygen-evolving enhancer protein 1, chloroplast precursor	<0.001	0.391		
p00313	5.24/30.2	TC95125	Lactoylglutathione lyase	<0.001	0.563		4
p00315	5.33/30.2	TC94226	Oxygen-evolving enhancer protein 1, chloroplast precursor	<0.001	0.525		
p00345	5.29/35.4	TC97674	Quinone oxido-reductase-like protein	<0.001	0.526		4

Table 3. Continued

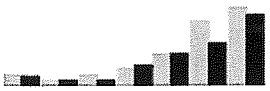
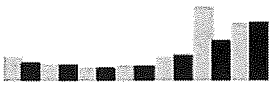
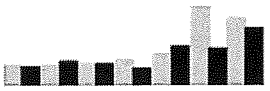
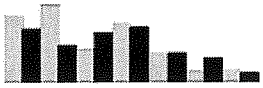
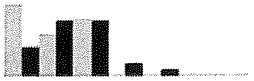
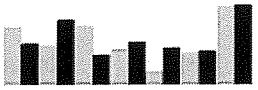
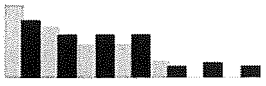
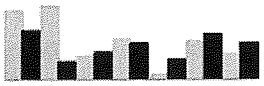
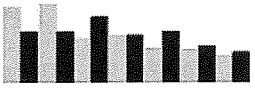
Spot no.	p*I*/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
p00376	5.37/43.0	TC94141	RuBisCo activase beta form precursor	<0.001	0.850		3, 5
p00378	5.16/36.9	TC107266	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	<0.001	0.781		5
p00382	5.22/38.1	TC107266	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	<0.001	0.233		5
p00384	5.27/37.3	TC107266	Sedoheptulose-1,7-bisphosphatase, chloroplast precursor	<0.001	0.836		4, 7
p00386	5.33/38.6	TC94141	RuBisCo activase beta form precursor	<0.001	0.297		5
p00398	5.41/39.2	TC94141	RuBisCo activase beta form precursor	<0.001	0.879		
p00399	5.43/38.4	TC94141	RuBisCo activase beta form precursor	<0.001	0.741		4
p00412	5.41/39.4	TC94027	Phosphoglycerate kinase, chloroplast precursor	<0.001	0.021		
p00419	5.43/46.6	TC102788	ATP synthase beta subunit	<0.001	0.171		5, 4
p00420	5.42/49.3	TC102788	ATP synthase beta subunit	0.022	0.563		
p00434	5.35/47.8	TC102788	ATP synthase beta subunit	0.003	0.317		5
p00435	5.35/50.6	ABE83482	AAA ATPase	<0.001	0.315		1, 8
p00446	5.26/50.7	TC93945	F1 ATPase	0.478	0.990		
p00451	5.19/44.2	AW775333	RuBisCo activase	<0.001	0.934		
p00453	5.10/42.5	Q9TKI7	ATP synthase subunit beta	0.032	0.738		
p00481	5.24/56.3	TC100508	RuBisCO subunit binding-protein beta subunit	0.123	0.576		

Table 3. Continued

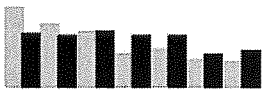
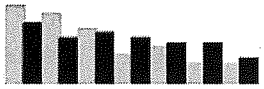
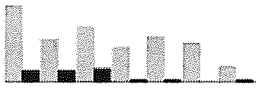
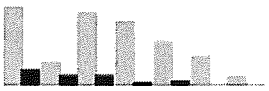
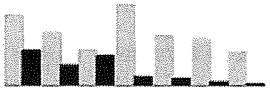
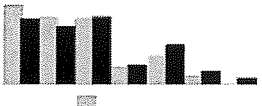
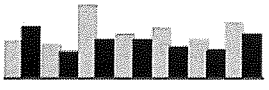
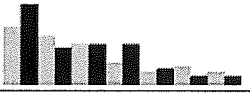
Spot no.	p*I*/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
p00484	5.30/56.5	TC100508	RuBisCO subunit binding-protein beta subunit	0.007	0.435		
p00485	5.36/56.4	TC100508	RuBisCO subunit binding-protein beta subunit	<0.001	0.425		
p00668	6.10/50.6	ABE86890	Ribulose bisphosphate carboxylase, large chain	<0.001	0.287		4, 6
p00669	6.15/50.5	ABE93511	Ribulose bisphosphate carboxylase, large chain	<0.001	0.693		4, 6
p00670	6.19/50.5	ABE86890	Ribulose bisphosphate carboxylase, large chain	<0.001	0.149		1, 4
p00671	6.24/50.1	ABE93511	Ribulose bisphosphate carboxylase, large chain	<0.001	0.448		6
p00701	6.14/39.4	TC94407	12-oxophytodienoic acid 10, 11-reductase	<0.001	0.990		3
p00741	5.87/35.7	TC100316	Plastidic aldolase	<0.001	0.024		1, 5
p00746	5.68/36.3	TC100316	Plastidic aldolase	<0.001	0.295		1, 7
p00783	6.07/25.3	TC94553	FQR1 (flavodoxin-like quinone reductase 1)	<0.001	0.864		3, 4
p00806	6.03/17.1	TC106571	Ribulose bisphosphate carboxylase small chain	<0.001	0.371		4, 6
p00821	6.42/17.1	TC106570	Ribulose bisphosphate carboxylase small chain	<0.001	0.319		
p00827	6.60/17.2	TC93917	Ribulose 1,5-bisphosphate carboxylase small subunit precursor	<0.001	0.946		6
p00893	6.34/34.9	TC106473	Malate dehydrogenase precursor	0.396	0.630		
p01067	5.32/27.5	TC95719	Oxidoreductase	<0.001	0.538		
Primary metabolism							
p00066a	5.10/42.4	TC107437	Magnesium-chelatase subunit chlI	<0.001	0.135		4

Table 3. Continued

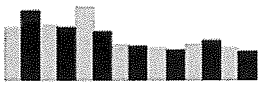
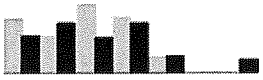
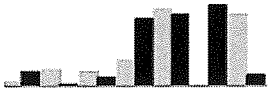
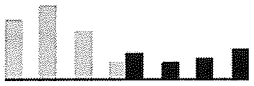
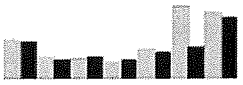
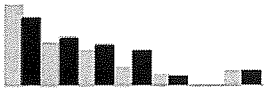
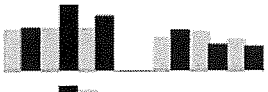
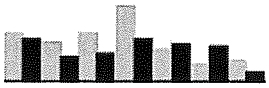
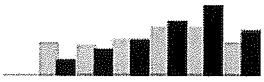
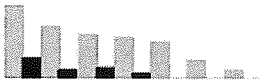
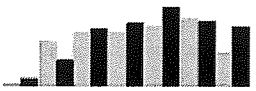
Spot no.	pI/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
p00287	5.39/27.8	TC94819	Putative 3-beta hydroxysteroid dehydrogenase/ isomerase protein	0.004	0.699		4
p00388	5.25/39.9	TC106913	Glutamine synthetase	<0.001	0.255		
p00392	5.34/39.8	TC106913	Glutamine synthetase	<0.001	0.014		6
p00452	5.19/39.9	TC106913	Glutamine synthetase	<0.001	0.615		7
p00755	5.88/33.0	TC100271	Cysteine synthase	<0.001	0.002		3
Protein synthesis and folding							
p00011	4.79/67.5	ABE79560	Heat shock protein Hsp70	0.124	0.783		
p00012	4.83/67.0	ABE79560	Heat shock protein Hsp70	0.008	0.366		
p00013	4.87/66.6	ABE79560	Heat shock protein Hsp70	<0.001	0.238		
p00015	4.95/66.4	ABE79560	Heat shock protein Hsp70	<0.001	0.071		
p00040	5.12/56.4	TC106707	Protein disulphide-isomerase precursor (PDI)	<0.001	0.347		5
p00041	5.05/66.6	TC106707	Protein disulphide-isomerase precursor (PDI)	<0.001	0.621		5
p00064	5.00/39.4	TC94943	30S ribosomal protein S1, chloroplast precursor (CS1)	<0.001	0.705		4, 7
p00065	5.08/39.3	TC94943	30S ribosomal protein S1, chloroplast precursor (CS1)	<0.001	0.830		4
p00072	4.81/35.5	TC107662	RNA-binding region RNP-1	<0.001	0.418		4
p00156	4.94/24.1	TC93962	2-Cys peroxiredoxin	<0.001	0.752		1
p00192	4.73/19.1	TC106480	50S ribosomal protein L12-1, chloroplast precursor (CL12-A)	<0.001	0.163		1, 4

Table 3. Continued

Spot no.	pI/MW	Accession no.	Protein identity	*p*-Level time point	*p*-Level interaction	Relative %vol.	Category
p00238	5.46/19.7	TC93939	Glycine-rich RNA binding protein	<0.001	0.786		
p00282	5.59/25.6	TC101147	20 kDa chaperonin, chloroplast precursor (Protein Cpn21)	<0.001	0.168		6
p00370	5.09/38.3	TC104263	Peptidyl-prolyl *cis-trans* isomerase CYP37, chloroplast precursor	<0.001	0.159		7
p00790	5.78/24.9	TC107500	Chaperonin 21 precursor	<0.001	0.505		6
p01113	4.56/20.3	TC95578	Peroxiredoxin-like protein	0.218	0.556		1
Secondary metabolism							
p00289	5.29/25.3	TC100522	Chalcone–flavonone isomerase 1 (Chalcone isomerase 1)	<0.001	0.959		
p00752	5.72/32.4	TC100786	Isoflavone reductase homolog 1	<0.001	0.685		3
p01186	5.32/27.3	TC107427	Putative CCoAOMT	<0.001	0.734		2, 3
Transcription							
p00082	4.70/38.4	TC101331	Poly(A) polymerase	0.001	0.556		1
p00133	4.66/28.8	TC99849	RNA-binding protein precursor	<0.001	0.951		1
p00321	5.43/30.1	ABE85149	NA-binding region RNP-1	<0.001	0.143		4, 6
Unclassified							
p00448	5.22/43.1	TC103229	Unknown protein	<0.001	0.055		
Unclear classification							
p00761	5.80/28.3	TC106966	Embryo-abundant protein EMB	<0.001	0.790		

reactive oxygen species (ROS) [30]. Under normal conditions, the production of ROS is well controlled, however, stress situations such as drought, heat or light stress may cause an over production of ROS by the chloroplasts [30, 31]. The culture of protoplasts takes place under continuous low light conditions. Therefore, it could be that these light conditions may stress the protoplasts, resulting in an overproduction of ROS by the chloroplasts and as a result the activation of genes involved in the stress response.

4.3 PR10-like proteins

Several PR10 proteins were highly up-regulated, up to fivefold, at 48 h and were the most abundant proteins on the gel, they were, therefore, more closely analysed. PR10-like proteins and related ABA-responsive proteins were also found to be the most abundant proteins in *M. truncatula* embryonic cultures [21, 29], cell suspension cultures [32] and roots [33]. Interestingly, neither this nor a previous study comparing A17 with 2HA during later stages of embryogenesis [29] found any significant differences in expression of PR10-like proteins between the two genotypes. In both the cases, changes were observed in their expression over time.

Typical PR10-like proteins are acidic cytosolic proteins with a molecular weight ranging between 16 and 20 kDa. PR10-like proteins contain two binding sites, a highly conserved glycine rich loop and a hydrophobic cavity. The glycine rich loop shows similarity with the P-loop of nucleotide binding proteins and has been shown to bind zeatin (cytokinin) and similar molecules. The hydrophobic cavity has the ability to bind hydrophobic molecules such as fatty acids, brassinosteroids, steroids, and flavonoids [34–37]. It is possible that during the phases of protoplast proliferation for which the PR10-like proteins were considered to be of importance, there is a change in the need for such hydrophobic molecules. Because the glycine rich loop is highly conserved along the different members of the PR10-like family [34], the hydrophobic cavity is likely to specify the function of the different PR10-like proteins. Hence, the diversity of PR10-like proteins and their specific regulation suggest that there is a diverse range of hydrophobic molecules involved at the different stages of protoplast proliferation.

Although to date no clear function of PR10-like proteins has been identified, research points to a function as binding or transport proteins in intracellular signalling [35–37], in which PR10-like proteins function as a cytoplasmic 'taxi' for hydrophobic molecules. Overexpression of a pea PR10 protein was reported to cause elevated cytokinin and decreased ABA levels in *Brassica napus*, suggesting that changes in expression of PR10 could be relevant for phytohormone regulated developmental processes [38] It should be noted that some PR10-like proteins have been reported to have low RNase activity [35, 38]. It is possible, however, that when cytokinin is bound it may prevent RNAse activity of the PR10 proteins.

The up-regulation of PR10 and PR10-like proteins during protoplast culture might be an effect of a wound or stress response from the protoplast isolation. The fact that their expression did not differ between the embryogenic and nonembryogenic line suggests that PR10-like proteins might be involved in a general stress response encountered during protoplast regeneration rather than embryogenesis itself. However, their strong accumulation after 48 h suggests an additional or alternative developmental role during protoplast proliferation which requires further investigation.

4.4 Flavonoid metabolism

Isoflavone reductase (p00752) and chalcone isomerase (p00289) were up-regulated, up to 5 and 30 fold, respectively, during the intermediate and late phase of protoplast proliferation respectively. Furthermore, chalcone isomerase showed a 0.8-fold down-regulation during the initiation of the cell division (96–120 h). Isoflavone reductase and chalcone isomerase are both involved in the flavonoid biosynthesis pathway. Flavonoids are wide spread in the plant kingdom and have functions that range from pigments in flowers to protection of the plant from stress such as UV-radiation in the epidermis to signal molecules in plant–plant and plant–microbe interactions [39, 40]. The accumulation patterns of isoflavone reductase and chalcone isomerase suggest an involvement of flavonoids during the period of 72–120 h of protoplast proliferation, possibly in stress response to the culturing conditions and the cell wall disruption. PR10-like proteins have been found to bind flavonoids [35], and the concomitant changes in flavonoid pathway enzymes and PR10 expression could indicate the regulation of flavonoids by both synthesis and intracellular homeostasis.

5 Concluding remarks

The large number of protein spots together with the accumulation patterns of some of the identified protein spots suggests that cellular reorganization takes place as a result of protoplast isolation. The majority of the identified proteins classified as defence or stress response proteins are the PR10-like proteins. The accumulation profiles of the PR10-like proteins do not support the idea that they are only involved in the wound response activated by protoplast isolation. Alternatively, the accumulation profile of PR10-like proteins suggests that they are involved in later developmental processes, possible through their role in binding plant hormones and other regulatory molecules.

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6 References

[1] Hartmann, H. T., Kester, D. E., Davies, J. F. T., Geneve, R. L., in: Hartmann, H. T., Kester, D. E., Davies, J. F. T., Geneve, R. L. (Eds.), *Plant Propagation: Principles and Practice*, Prentice Hall, New Jersey, 1997, pp. 549–589.

[2] Tao, W., Verbelen, J.-P., *Plant Sci.* 1996, *116*, 107–115.

[3] Sheen, J., *Plant Physiol.* 2001, *127*, 1466–1475.

[4] Sheahan, M. B., Rose, R. J., McCurdy, D. W., *Plant J.* 2004, *37*, 379–390.

[5] Pilet, P. E., in: Pilet, P. E. (Ed.), *The Physiological Properties of Plant Protoplasts*, Springer, Heidelberg, 1985, pp. 1–5.

[6] Eriksson, T. R., in: Fowke, L. C., Constable, F. (Eds.), *Plant Protoplasts*, CRC Press, Florida, 1985, pp. 1–20.

[7] Meyer, Y., Aspart, L., Chartier, Y., *Plant Physiol.* 1984, *75*, 1034–1039.

[8] Meyer, Y., Aspart, L., Chartier, Y., *Plant Physiol.* 1984, *75*, 1027–1033.

[9] Meyer, Y., Cooke, R., *Planta* 1979, *147*, 181–185.

[10] Carle, S. A., Bates, G. W., Shannon, T. A., *J. Plant Growth Reg.* 1998, *17*, 221–230.

[11] Pasternak, T. P., Prinsen, E., Ayaydin, F., Miskolczi, P. *et al.*, *Plant Physiol.* 2002, *129*, 1807–1819.

[12] Grafi, G., Avivi, Y., *Trends Biotechnol.* 2004, *22*, 388–389.

[13] Grafi, G., *Dev. Biol.* 2004, *268*, 1–6.

[14] Fehér, A., Pasternak, T. P., Dudits, D., *Plant Cell Tiss. Org. Cult.* 2003, *74*, 201–228.

[15] Zhao, J., Morozova, N., Williams, L., Libs, L. *et al.*, *J. Biol. Chem.* 2001, *276*, 22772–22778.

[16] Avivi, Y., Morad, V., Ben-Meir, H., Zhao, J. *et al.*, *Dev. Dynamics* 2004, *230*, 12–20.

[17] Rose, R. J., Nolan, K. E., *Plant Cell Rep.* 1995, *14*, 349–353.

[18] Nolan, K. E., Rose, R. J., Gorst, J. R., *Plant Cell Rep.* 1989, *8*, 278–281.

[19] Rose, R. J., Nolan, K. E., Bicego, L., *J. Plant Physiol.* 1999, *155*, 788–791.

[20] Thomas, M. R., Johnson, L. B., White, F. F., *Plant Sci.* 1990, *69*, 189–198.

[21] Imin, N., Jong, F. D., Mathesius, U., Noorden, G. v. *et al.*, *Proteomics* 2004, *4*, 1883–1896.

[22] Cannon, S. B., Crow, J. A., Heuer, M. L. *et al.*, *Plant Physiol.* 2005, *138*, 38–46.

[23] Reinders, J., Lewandrowski, U., Moebius, J. *et al.*, *Proteomics* 2004, *4*, 3686–3703.

[24] Bevan, M., Bancroft, I., Bent, E., Love, K. *et al.*, *Nature* 1998, *391*, 485–488.

[25] Franz, G., Blaschenk, W., in: Pilet, P.-E. (Ed.), *The Physiological Properties of Plant Protoplasts*, Springer-Verlag, Heidelberg, 1985, pp. 171–183.

[26] Grimmig, B., Kneusel, R. E., Junghanns, K. T., Matern, U., *Plant Biol.* 1999, *1*, 299–310.

[27] Arioli, T., Peng, L., Betzner, A. S. *et al.*, *Science* 1998, *279*, 717–720.

[28] Leon, J., Rojo, E., Sanchez-Serrano, J. J., *J. Exp. Bot.* 2001, *52*, 1–9.

[29] Imin, N., Nizamidin, M., Daniher, D. *et al.*, *Plant Physiol.* 2005, *137*, 1250–1260.

[30] Laloi, C., Apel, K., Danon, A., *Curr. Opin. Plant Biol.* 2004, *7*, 323–328.

[31] Couee, I., Sulmon, C., Gouesbet, G., El Amrani, A., *J. Exp. Bot.* 2006, *57*, 449–459.

[32] Lei, Z., Elmer, A. M., Watson, B. S. *et al.*, *Mol. Cell. Proteomics* 2005, *4*, 1812–1825.

[33] Mathesius, U., Kijzers, G., Natera, S. H. A. *et al.*, *Proteomics* 2001, *1*, 1424–1440.

[34] Biesiadka, J., Bujacz, G., Sikorski, M. M., Jaskolski, M., *J. Mol. Biol.* 2002, *319*, 1223–1234.

[35] Koistinen, K. M., Soininen, P., Venalainen, T. A., Hayrinen, J. *et al.*, *Phytochemistry* 2005, *66*, 2524–2533.

[36] Markovic-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E. *et al.*, *J. Mol. Biol.* 2003, *325*, 123–133.

[37] Mogensen, J. E., Wimmer, R., Larsen, J. N., Spangfort, M. D., Otzen, D. E., *J. Biol. Chem.* 2002, *277*, 23684–23692.

[38] Srivastava, S., Emery, R., Kurepin, L., Reid, D. *et al.*, *Plant Growth Reg.* 2006, *49*, 17–25.

[39] Taylor, L. P., Grotewold, E., *Curr. Opin. Plant Biol.* 2005, *8*, 317–323.

[40] Winkel-Shirley, B., *Curr. Opin. Plant Biol.* 2002, *5*, 218–223.

