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Flower proteome: changes in protein spectrum during the advanced stages of rose petal development

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Abstract Flowering is a unique and highly programmed process, but hardly anything is known about the developmentally regulated proteome changes in petals. Here, we employed proteomic technologies to study petal development in rose (*Rosa hybrida*). Using two-dimensional polyacrylamide gel electrophoresis, we generated stage-specific (closed bud, mature flower and flower at anthesis) petal protein maps with ca. 1,000 unique protein spots. Expression analyses of all resolved protein spots revealed that almost 30% of them were stage-specific, with ca. 90 protein spots for each stage. Most of the proteins exhibited differential expression during petal development, whereas only ca. 6% were constitutively expressed. Eighty-two of the resolved proteins were identified by mass spectrometry and annotated. Classification of the annotated proteins into functional groups revealed energy, cell rescue, unknown function

(including novel sequences) and metabolism to be the largest classes, together comprising ca. 90% of all identified proteins. Interestingly, a large number of stress-related proteins were identified in developing petals. Analyses of the expression patterns of annotated proteins and their corresponding RNAs confirmed the importance of proteome characterization.

Keywords Flower development · Petals · Proteome · Rose

Abbreviations 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis · EST: Expression sequence tag · IEF: Isoelectric focusing · ESI-MS/MS: Electrospray ionization mass spectrometry · MALDI-TOF: Matrix-assisted laser-desorption time-of-flight · CID: Collision-induced dissociation

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Introduction

The flower's basic architecture is rather simple, despite its almost unlimited variability in showy traits, such as color, fragrance, shape, etc. The constant development of processes generating and fine-tuning these traits has strongly contributed to the evolutionary success of flowering plants. Nevertheless, in many plant systems, petals are the main source of this enormous variability.

Flower development is a uniquely programmed and tightly regulated process (Rolland-Lagan et al. 2003). Two discrete growth phases are distinguishable in petal development: one involves cell division, the other cell expansion (Martin and Gerats 1993; Yu et al. 2004). Very little is known about the genetic machinery involved in corolla-shape determination in general and phase transition in particular: in *Gerbera*, *GEG* has been proposed to be involved in the former (Kotilainen et al. 1999) and in *Arabidopsis*, *NAP* has been shown to function in the transition from active cell division to cell expansion

(Sablowski and Meyerowitz 1998). The major increase in petal size occurs during the later stages of flower development, when mitotic activity has basically ceased (Martin and Gerats 1993; Yu et al. 2004). During the cell-expansion phase, various metabolic pathways are activated to produce unique secondary metabolites, e.g. flavonoids, carotenoids and numerous scent compounds (Ben-Meir et al. 2002; Dudareva et al. 2004). With very few exceptions—involving genomic technologies—pathways which are active in petals have been studied via reductionist approaches that are based on identifying and characterizing a single gene/product, mainly in model plants (Vainstein et al. 2001; Dudareva et al. 2004).

For centuries, rose (*Rosa hybrida*) has been the most important crop in the floriculture industry (Krusmann 1981). With an annual value of ca. \$10 billion, it is used for both cut flowers and garden plants (VBN 1995; Zuker et al. 1998). Its economic importance also stems from the use of its petals as a source of natural fragrances, flavorings and colorings. As such, the physiology and biochemistry of rose flowers have been studied for years. Nevertheless, only a few molecular studies have been conducted and even in those, research was focused on a single protein/gene. We and others (Channeliere et al. 2002; Guterman et al. 2002) recently used genomics approaches to perform a large-scale study of rose petal functionality at the RNA level. We generated an annotated rose petal EST database of 2,500 unique genes, and rose DNA chips were prepared and used for analyses of the petal transcriptome during flower development in the cell-expansion phase (Guterman et al. 2002; Izhaki 2004). Based on these analyses, several genes involved in the formation of rose aroma were isolated and characterized (Guterman et al. 2002; Lavid et al. 2002; Shalit et al. 2003).

The advances made in high-throughput protein technologies have opened up new possibilities for studying proteomes on a large scale. Improvements made in mass spectrometry (MS) and in 2D gel electrophoresis techniques now allow the highly reproducible resolution of proteins/peptides. These techniques, in combination with the constantly expanding genomic and EST databases, enable the simultaneous characterization/analysis of the expression profiles of a large set of proteins. In the last few years, the first few steps have been made in realizing the potential of protein profiling for the dissection of specific processes in several plant systems (Peltier et al. 2000; Gallardo et al. 2001, 2003; Krufft et al. 2001; Peck et al. 2001; Bae et al. 2003; Watson et al. 2003; Taylor et al. 2004). Nevertheless, almost nothing is known about global proteome changes during flower development. Further, proof of the importance of proteome elucidation can be found in recent studies, which have revealed discrepancies in global gene expression when analyzed at the protein versus RNA level (Gygi et al. 1999; Watson et al. 2003; Johnson et al. 2004).

In the present study, a proteomics approach was used to analyze changes in gene expression during advanced stages of rose petal development. We generated stage-specific petal protein maps and characterized changes in

the levels of ca. 1,000 unique protein spots. About 10% of these proteins were identified, annotated and classified into functional groups. Analyses of the expression patterns of the annotated proteins and their corresponding RNAs confirmed the importance of the proteome characterization.

Materials and methods

Plant material

Rose plants (*R. hybrida* cv. Fragrant Cloud) were grown in pots filled with a mixture of peat and volcanic gravel (1:1 v/v) in a greenhouse under controlled temperature (28°C/20°C day/night) and a natural photoperiod. Flower development was divided into three stages. At stage 1, petals start to emerge from the sepal, and their color changes to red. Stage 4 is characterized by rapid petal elongation and further accumulation of pigments. At stage 6, flowers are fully open (Fig. 1).

Protein extraction

Petals (1 g) were ground in liquid nitrogen and the resulting powder was extracted with 5 ml buffer containing 0.1 M Tris-HCl pH 8.0, 5% (w/v) sucrose, 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol and 2 mM PMSF. The extract was mixed with 25 ml of 0.1 M ammonium acetate in methanol, and left overnight at -20°C . The mixture was centrifuged at 12,000 *g* for 10 min and the pellet was washed three times with 10 ml ammonium acetate in methanol and once with 5 ml of 80% acetone. The pellet was then air-dried, solubilized in 0.5 ml rehydration solution containing 8 M urea, 2% (w/v) CHAPS and 0.5% (v/v) immobilized pH gradient (IPG) buffer pH 3–10 NL (Amersham Pharmacia Biotech, Uppsala, Sweden), and stored at -70°C until use.

Two-dimensional (2D) gel electrophoresis

Isoelectric focusing (IEF) was performed using an IPGphor instrument (Amersham Pharmacia Biotech) as



Fig. 1 Rose flowers of cv. Fragrant Cloud at different developmental stages

previously described (Gorg et al. 1998). Prior to electrophoresis, DTT, to a final concentration of 0.28% (w/v), and a few grains of bromophenol blue were added to the samples, which were brought to a final concentration of 0.7 mg protein in 250 μ l rehydration solution. Immobilized dry strips (13 cm, pH 3–10 NL, Amersham Pharmacia Biotech) were rehydrated with the protein sample for 6 h at 30 V, followed by 6 h at 60 V. Protein separation was performed at 500 V for 1 h, 2,000 V for 1 h and 8,000 V for 4 h (for a total of 34,500 V/h).

Following IEF, the gel strips were equilibrated in a buffer consisting of 1.5 M Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS, 1% DTT and bromophenol blue, twice for 15 min. The strips were placed on top of an SDS-polyacrylamide gel, with 5 and 15% stacking and resolving gels, respectively. Electrophoresis was performed in a standard Tris-Gly running buffer at 25 mA for 1 h, followed by 35 mA for 6 h. After electrophoresis, proteins were stained overnight with staining solution containing 0.1% (w/v) Coomassie Brilliant Blue R-250, 10% (v/v) acetic acid and 45% (v/v) methanol. At least three biological and two technical replicates were performed for each analyzed sample.

Gel analysis

The Coomassie Blue-stained gels were scanned with an ImageScanner (Amersham Pharmacia Biotech). Gel images were analyzed using the ImageMaster 2-D Elite (version 3.01) software (Amersham Pharmacia Biotech). After spot detection and background subtraction (mode: average on boundary), 2D gels were aligned, matched, and a quantitative determination of the spot volumes was performed (mode: total spot normalization). Specific spots were described as up-/down-regulated during flower development when their volumes were significantly different (at least a twofold difference in intensity with non-overlapping errors) in the analyzed gels. Average maps were created from five gels. For the analyses of developmental changes in protein levels three biological replicates (i.e. three different protein extractions) were used.

In-gel proteolysis and MS analysis

Stained protein spots in the gel (randomly selected) were excised with a razor blade; they were reduced in the gel with 10 mM DTT and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate. The gel pieces were treated with 50% acetonitrile in 10 mM ammonium bicarbonate to remove the stain from the proteins followed by drying. The dried gel pieces were rehydrated with 10 mM ammonium bicarbonate containing about 1 μ g trypsin per sample. The gel pieces were incubated overnight at 37°C and the resulting peptides were recovered with 60% acetonitrile and 0.1% (v/v) trifluoroacetate.

The peptides were deposited on a metal target as co-crystals with α -cyano-4-hydroxycinnamic acid. The MS analysis was performed using matrix-assisted laser-desorption time-of-flight (MALDI-TOF) (2E, Micromass, UK), in the positive ion mode. The MS data were compared to simulated proteolysis of the *Arabidopsis* proteins and all plant and rose EST data in “genpept”, using Masslynx software (Micromass). Some of the samples were analyzed by liquid chromatography-MS. The tryptic peptides were resolved by reverse-phase chromatography on a 1 \times 150 mm Vydac C-18 column. The peptides were separated on a linear gradient of 4 to 65% acetonitrile in 0.025% trifluoroacetate A, at 1% per minute and a flow rate of 40 μ l/min. The liquid from the column was electrosprayed into an ion-trap MS (LCQ, Finnigan, San Jose, CA, USA). MS was performed in the positive-ion mode using a repetitive full MS scan followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The MS data was compared to simulated proteolysis and CID of the proteins in “genpept” using Sequest software (J. Eng and J. Yates, University of Washington and Finnigan, San Jose, CA, USA). Sequence tags were used to screen NR (the non-redundant proteins database), all plant ESTs (EST_pln database), and the *Arabidopsis* genomic sequence databases as previously described (Guterman et al. 2002).

RNA gel blot analysis

Total RNA (10 μ g) was fractionated in a 1% agarose gel containing formaldehyde and blotted onto Hybond N⁺ membranes (Amersham Pharmacia Biotech). The blots were hybridized in 0.263 M Na₂HPO₄, 7% SDS, 1 mM EDTA, and 1% (w/v) BSA at 60°C with ³²P-labeled cDNA probes (Rediprime; Amersham Pharmacia Biotech). The membranes were washed twice in 2X SSC and 0.1% SDS at 60°C for 20 min each and exposed to X-ray film (Fuji, Tokyo, Japan) with two intensifying screens at -70°C.

Collection and analysis of volatiles

Individual intact rose flowers, still attached to the plants, were enclosed in a 1-l glass container with the appropriate openings, and headspace was trapped for 24 h at 25°C, using a method modified from Raguso and Pichersky (1995), utilizing a Porapak Q 80/100 (Waters Corp., Milford, MA) polydivinylbenzene filter. Volatiles were eluted utilizing 10 ml HPLC-grade hexane containing 100 μ g ethylmyristate as an internal standard, and evaporated to 0.5 ml. A 1- μ l aliquot of each sample was analyzed by GC-MS (Guterman et al. 2002). The volatile compounds were quantified by integrating the total mass ions detected and comparing to the internal standard (Lavid et al. 2002; Shalit et al. 2003).

Miscellaneous

Protein concentration was determined using a Lowry kit (BioRad). Anthocyanins were extracted from 1 g of fresh rose petals, ground in liquid nitrogen. The resulting powder was extracted with 20 ml of 1% HCl in methanol overnight at 20°C, and the level of anthocyanin was measured spectrophotometrically (A_{530}). For dry weight determination, petals were placed in an oven at 80°C for 3 days.

Results

Development of rose cv. Fragrant Cloud flowers was divided into six stages, as described previously (Guterman et al. 2002), from a small bud with petals still covered by sepals (stage 1) to a fully open flower at anthesis (stage 6). Flowers at the intermediate stage (stage 4) were characterized by fully pigmented petals that are just beginning to unfurl (Fig. 1). The transition from stage 1 to 4 is accompanied by a three- to fourfold increase in petal fresh and dry weight (Table 1). The protein content of petals (per flower) also increases approximately twofold during this transition. Upon further development of the flower to stage 6, the fresh weight of petals increases by another 100%, while dry weight and protein content increase by only ca. 40–50%, suggesting that flower growth from stage 4 to 6 is mainly due to cell expansion and water uptake. During flower development, rose petals accumulate anthocyanin pigments and emit volatile compounds. The levels of both increase dramatically during the transition of petals from stage 1 to 4 (Table 1).

To characterize the petal proteome, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out with proteins extracted from petals at the three developmental stages. Proteins were first resolved by means of IEF on strips with a nonlinear pH gradient in the range of 3.0 to 10.0 and then separated in the second dimension by SDS-PAGE and stained with Coomassie Blue. Representative gels (Fig. 2) show protein spots of different intensities at pI values from ca. 4.0 to 8.0, and molecular masses of ca. 14 to 100 kDa.

Average petal protein maps were created for each stage of flower development. Each average gel contained more than 600 protein spots (Table 2). A comparison of the maps revealed 978 different protein spots, of which 421 were present throughout development; the rest appeared in only one or two developmental stages. About

12% of the proteins accumulating at each of the three analyzed stages of flower development were stage-specific (Table 2).

To follow the accumulation patterns of proteins during petal development up to anthesis (stage 6), the stain intensities of the different protein spots were evaluated. Protein concentration in the samples, to be analyzed by 2D gels and representing different stages of flower development, was estimated by Lowry assays and 1D SDS-PAGE was run to confirm equal protein levels in the samples. In addition, to confirm equal loading on the 2D gels of the proteins from different developmental stages, we followed the level of glyceraldehyde-3-phosphate dehydrogenase (see further on), used in many studies (e.g. see Aubert et al. 2001) as a constitutively expressed reference. We also performed a western blot analysis of a low-molecular-weight heat-shock protein (LMW HSP) identified by 2D gel (spot 137, see further on): its pattern of expression during flower development was essentially identical to that revealed by the analysis of stain intensities on the 2D gel (data not shown).

Petal development from stage 1 to 6 was accompanied by constitutive expression (a less than twofold difference) of 64 proteins, representing 8 to 10% of the protein spots detectable at each developmental stage. During the early stages (1 to 4) of petal development (Table 3), the levels of 511 proteins, representing over half of all the different protein spots (899 proteins) detected at these stages, were downregulated (over twofold decrease in expression level). About a quarter of the proteins were upregulated (over twofold increase in expression level), and the expression level of ca. 21% of the proteins did not differ (less than a twofold change). Similar to the early stages of flower development, during the transition from stage 4 to 6, the largest group consisted of proteins that are downregulated, representing ca. 40% of all the different protein spots (886) detected at these stages. Essentially the same number of proteins were either upregulated or constitutively expressed in petals during this transition (each group making up ca. 30% of all the different spots identified at these stages).

In a parallel study, we generated a database containing ca. 3000 ESTs from rose petals at the intermediate stage 4 of development (Guterman et al. 2002; Izhaki 2004). This database was employed to further characterize the rose petal proteome. To annotate petal proteins, different protein spots were excised from gels of stage 4 petals and subjected to MS. Proteins identified by MALDI-TOF and/or electrospray ionization (ESI)-MS/MS analyses, a total of 82 spots (see supplemental data), were annotated

Table 1 Characteristics of rose (cv. Fragrant Cloud) petals at various stages of development

| Developmental stage | Fresh weight (g/flower) | Dry weight (g/flower) | Protein (mg/flower) | Anthocyanins (A_{530} /flower) | Volatiles (μ g/flower 24 h) |
|---------------------|-------------------------|-----------------------|---------------------|-----------------------------------|----------------------------------|
| 1 | 1.3 ± 0.1 | 0.2 ± 0.02 | 406.3 ± 27.4 | 1.4 ± 0.2 | 5.3 ± 0.4 |
| 4 | 4.4 ± 0.4 | 0.9 ± 0.10 | 838.8 ± 38.6 | 20.3 ± 1.3 | 168.1 ± 83.1 |
| 6 | 9.4 ± 0.3 | 1.3 ± 0.10 | 1151.5 ± 29.4 | 22.7 ± 2.4 | 42.0 ± 18.4 |

Values represent means of four replicates ± SE

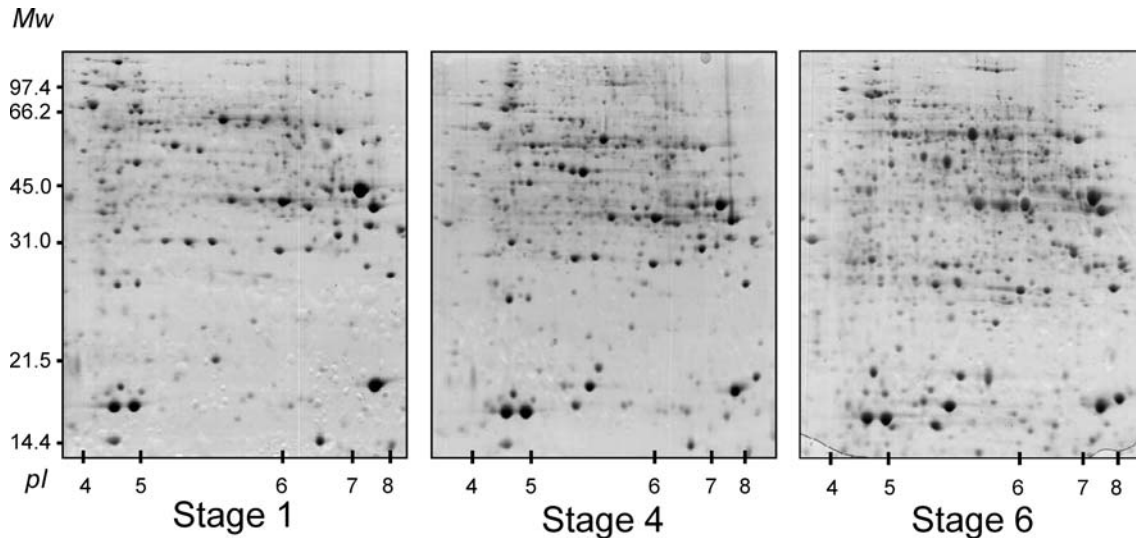


Fig. 2 Reference maps of rose petal proteins at different developmental stages. Proteins were extracted from petals at three different developmental stages and equal amounts were separated by 2D-PAGE. Representative gels for each stage, stained with Coomassie Blue, are presented

on a master gel (Fig. 3). Since the sequence information in rose is mainly from an EST database (i.e. partial sequence only), almost all theoretical MW and pI values for identified spots are based on genomic databases of plants other than rose. This may explain the relative lack of fit observed in several cases between the experimental and theoretical values, e.g. spots#98, 123.

Among the annotated proteins, 11 groups of spots were characterized, each consisting of two to four proteins with different molecular masses and/or pIs but apparently encoded by the same gene (Table 4). Proteins in these groups may have arisen from post-translational modifications, alternative splicing, allelic variation, etc.

The identified proteins were classified into functional groups according to the Munich Information Center for Protein Sequences (MIPS, <http://mips.gsf.de>). The largest group contained proteins (26% of total annotated) with a putative function in energy (Table 4).

Table 2 Distribution of rose petal protein spots during flower development

| Developmental stage | Protein spots (no.) | Stage-specific | |
|---------------------|---------------------|----------------|------------|
| | | No. | % of total |
| 1 | 735 | 92 | 12.5 |
| 4 | 766 | 94 | 12.3 |
| 6 | 611 | 79 | 12.9 |
| 1,4 | | 181 | |
| 4,6 | | 70 | |
| 1,6 | | 41 | |
| 1,4,6 | | 421 | |

Spots revealed by 2D-PAGE (five gels) of proteins extracted from petals at three different developmental stages, presented in Fig. 2, were counted. These gel images were overlaid and spots present in only one stage were characterized as stage-specific proteins. The number of protein spots present in two (stages 1 and 4; stages 4 and 6; stages 1 and 6) and three (stages 1, 4 and 6) developmental stages is also shown

Proteins classified into cell-rescue categories and those with unknown function represented the next largest groups, with ca. 18% of total identified proteins each. Similarly, proteins with putative function in metabolism represented ca. 17% of the total identified proteins. A much smaller number of identified proteins belonged to the other functional categories.

Two proteins (#78, 102) could be identified only in the rose EST database (BQ104638 and BQ104241, respectively). These ESTs have no homologues in other databases, as revealed by BLAST analysis against NR (non-redundant proteins database), all plant ESTs (EST_pln) and the Arabidopsis genomic sequences. Five additional proteins from the no-hit category could have been identified in the database based on tryptic peptide sequences of ESI-MS/MS. Nevertheless, since their theoretical MWs differed greatly (by over 20%) from the experimental values, these proteins were classified in the no-hit category.

Table 3 Expression profiling of petal proteins during rose flower development

| Expression pattern | Developmental stages | | | |
|------------------------|----------------------|------|-------------|------|
| | From 1 to 4 | | From 4 to 6 | |
| | No. | % | No. | % |
| Downregulated proteins | 511 | 56.8 | 356 | 40.2 |
| Upregulated proteins | 197 | 21.9 | 248 | 28.0 |
| Constitutive proteins | 191 | 21.3 | 282 | 31.8 |

Proteins extracted from petals at three different developmental stages were separated by 2D-PAGE and average spot intensities were compared. Proteins whose level increased or decreased more than twofold during flower development, as revealed by the intensity of Coomassie Blue stain, were considered upregulated or downregulated, respectively: 899 and 886 different protein spots were identified in stages 1 to 4 and 4 to 6, respectively. All spots with an at least twofold difference in intensity have non-overlapping errors

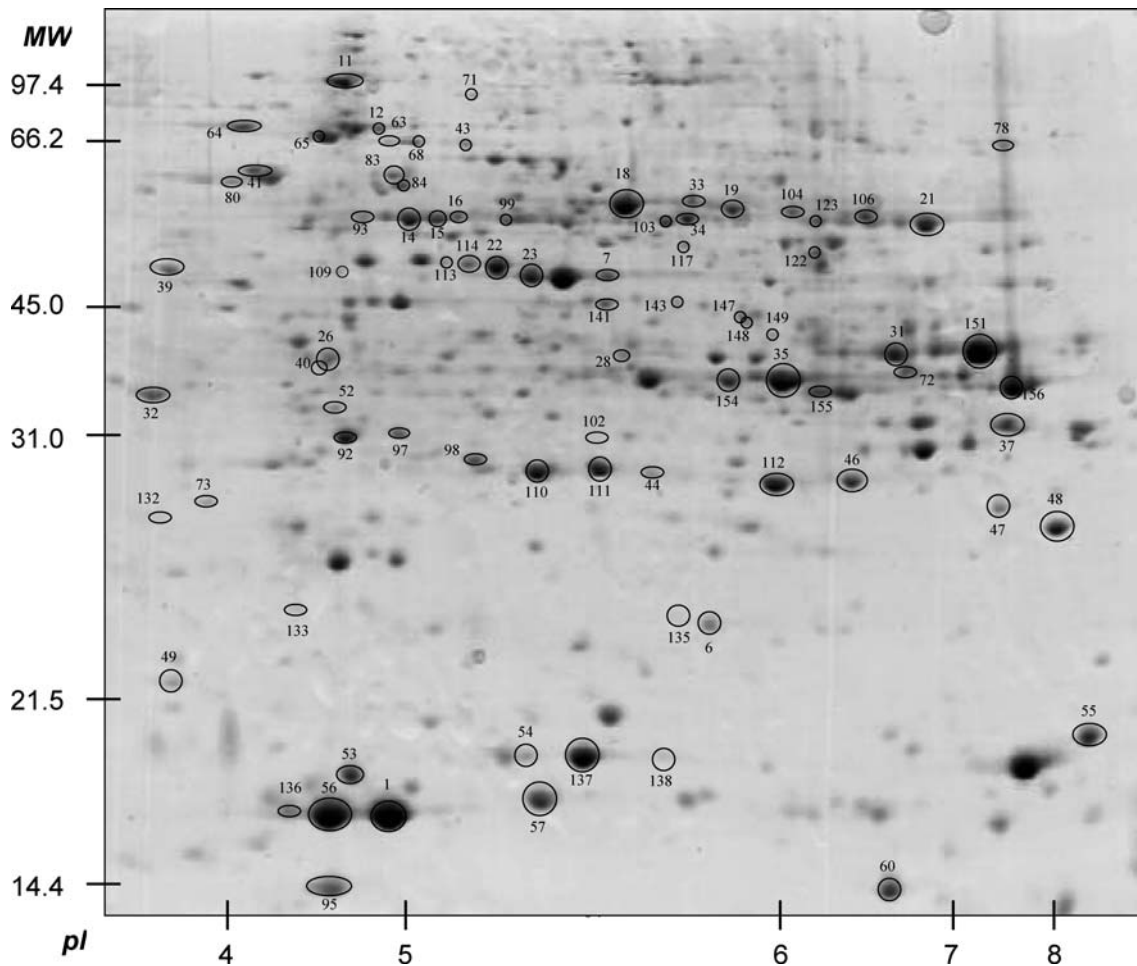


Fig. 3 Proteins identified by mass spectrometry. Identification numbers of circled proteins identified by mass spectrometry (see Table 4) are presented on the reference map of proteins extracted from stage-4 petals

Expression pattern at the RNA level was analyzed for several rose genes. Four genes, whose expression during flower development from stage 1 to 6 had been determined at the protein level, were studied (Fig. 4). Of the three proteins corresponding to BQ105069 (#1, 56, 136), only protein #136's expression corresponded to that of the RNA. Similarly, only a partial correlation was observed between the expression pattern of BQ106020 RNA and expression of the two corresponding proteins, #137 and 54. For BQ105169, expression at the RNA level was upregulated during flower development, while the level of the corresponding protein increased from stage 1 to 4 but then remained constitutive. The pattern of BQ104538 transcript accumulation also did not correspond to that of the encoded protein #123 during flower development. These results demonstrate discrepancies in gene expression at the RNA vs. protein levels. We also harnessed the available data from microarray experiments that followed transcript levels in petals of developing *cv.* Fragrant Cloud flowers (Guterman et al. 2002, Izhaki 2004). A comparison of the changes in expression (from stage 1 to 4) of genes coding for the identified proteins at the RNA and protein levels again revealed a lack of consistency in ca. 62% of the cases (Table 4).

Discussion

Advances in proteomics tools (Kruft et al. 2001; Bardel et al. 2002; Gallardo et al. 2002; Roberts 2002; Taylor et al. 2004) allowed us to assess rose petal development on a large scale at the protein level. 2D-PAGE was performed with total proteins extracted from petals at three different developmental stages. The sum of these three protein maps yielded ca. 2,000 protein spots and alignment of these maps revealed ca. 1,000 different protein spots. Almost 60% of these different proteins were not detectable at all three stages of flower development, with ca. 30% being stage-specific. These protein-expression dynamics suggest that numerous programs/pathways are activated/deactivated during the petal transition from stage 1 to 6, even though major changes, such as cell differentiation and division, have already ceased prior to stage 1 of flower development (Chmelnitsky et al. 2002). Advanced stages of rose petal growth (stage 1 to 6), as in many other plants, are due to cell expansion, which follows the cell-division phases. During the cell-expansion phase, the petals accumulate high levels of pigment (Weiss 2000). In parallel, high levels of volatiles are

Table 4 Functional classification and expression analyses of the identified rose petal proteins. Protein spots excised from Coomassie Blue-stained gels were subjected to digestion with trypsin and identified following mass spectrometry analysis (either ESI-MS/MS or MALDI-TOF, see supplemental data)

| Spot no. | Accession no. | Putative function/description | Exp. MW (kDa) | Exp. pI | Theo. MW (kDa) | Theo. pI | Expression protein RNA | | |
|--|---------------|--|---------------|---------|----------------|----------|------------------------|-----|-----|
| | | | | | | | 1-4 | 4-6 | 1-4 |
| Energy | | | | | | | | | |
| 14 | BQ105894 | Mitochondrial ATP synthase β -subunit | 55.7 | 5.0 | 60.3 | 6.0 | c | c | c |
| 15 | BQ105894 | Mitochondrial ATP synthase β -subunit | 55.7 | 5.1 | 60.3 | 6.0 | c | c | c |
| 16 | BQ105894 | Mitochondrial ATP synthase β -subunit | 56.2 | 5.2 | 60.3 | 6.0 | d | c | c |
| 93 | BQ105894 | Mitochondrial ATP synthase β -subunit | 55.7 | 4.7 | 60.3 | 6.0 | u | c | c |
| 48 | BQ105229 | ATP synthase subunit | 24.6 | 7.8 | 27.6 | 6.3 | c | c | c |
| 33 | AAB01582 | ATP synthase α -subunit | 59.0 | 5.7 | 55.3 | 6.5 | c | c | |
| 53 | BQ104437 | Mitochondrial F ₀ ATP synthase D chain | 18.7 | 4.7 | 19.6 | 5.1 | d | d | c |
| 18 | BQ104782 | Enolase | 58.4 | 5.5 | 47.9 | 5.6 | c | c | d |
| 103 | BQ104782 | Enolase | 55.3 | 5.6 | 47.9 | 5.6 | c | c | d |
| 117 | BQ104782 | Enolase | 50.52 | 5.6 | 47.9 | 5.6 | c | u | d |
| 19 | BQ105613 | Enolase | 57.5 | 5.8 | 47.9 | 5.6 | d | c | c |
| 151 | CF349790 | Cytosolic aldolase | 36.3 | 7.2 | 38.5 | 6.9 | d | u | c |
| 31 | CF349790 | Cytosolic aldolase | 36.3 | 6.5 | 38.5 | 6.9 | u | c | c |
| 143 | CF349790 | Cytosolic aldolase | 42.1 | 5.6 | 38.5 | 6.9 | d | c | c |
| 35 | BQ103935 | Malate dehydrogenase | 33.7 | 6.0 | 35.6 | 8.7 | c | c | d |
| 154 | BQ103935 | Malate dehydrogenase | 33.7 | 5.8 | 35.6 | 8.7 | c | c | d |
| 104 | BQ105334 | Glycine hydroxymethyltransferase | 59.3 | 6.0 | 57.4 | 8.1 | c | d | c |
| 148 | BQ104363 | Phosphoglycerate kinase | 40.6 | 5.9 | 42.1 | 5.5 | d | d | |
| 155 | CAB43638 | NAD(P)H oxidoreductase, isoflavone reductase | 31.2 | 6.1 | 34.1 | 6.3 | u | d | |
| 147 | AAC49964 | NAD ⁺ -dependent isocitrate dehydrogenase | 40.7 | 5.8 | 39.6 | 8.4 | d | d | |
| 149 | AAM61498 | NAD ⁺ -dependent isocitrate dehydrogenase subunit 1 | 39.9 | 6.0 | 39.6 | 8.4 | c | c | |
| Cell rescue, defense, cell death and ageing | | | | | | | | | |
| 54 | BQ106020 | LMW HSP | 18.8 | 5.3 | 17.4 | 6.2 | u | u | u |
| 137 | BQ106020 | LMW HSP | 18.8 | 5.4 | 17.4 | 6.2 | u | c | u |
| 57 | BQ105169 | LMW HSP | 18.8 | 5.4 | 17.5 | 5.6 | u | c | u |
| 55 | BQ105353 | LMW HSP | 19.1 | 8.0 | 18.2 | 5.4 | c | u | u |
| 64 | BQ104691 | HSP70 | 78.6 | 4.1 | 75.5 | 5.2 | c | c | |
| 65 | BQ104443 | HSP70 | 75.5 | 4.6 | 71.4 | 5.0 | d | u | c |
| 68 | CF349319 | HSP 70 | 74.0 | 5.0 | 71.2 | 5.3 | d | u | c |
| 12 | CF349462 | HSP70 | 77.9 | 4.8 | 70.9 | 5.4 | u | c | u |
| 110 | CF349827 | L-ascorbate peroxidase | 27.4 | 5.4 | 27.9 | 5.9 | c | c | c |
| 111 | CF349827 | L-ascorbate peroxidase | 27.5 | 5.5 | 27.3 | 5.7 | c | c | c |
| 135 | CF349736 | Superoxide dismutase | 23.7 | 5.6 | 25.4 | 8.5 | u | c | c |
| 6 | CF349736 | Superoxide dismutase | 23.6 | 5.7 | 26.3 | 6.7 | c | c | c |
| 32 | BQ105483 | Chromoplast carotenoid-associated protein (ChrC) | 32.6 | 3.6 | 35.2 | 5.2 | u | u | u |
| 122 | CF349678 | Catalase | 47.6 | 6.1 | 56.9 | 6.6 | c | c | c |
| 133 | CAA71503 | 2-Cys peroxiredoxin | 23.7 | 4.4 | 28.9 | 8.8 | d | c | |
| Metabolism | | | | | | | | | |
| 7 | BQ106376 | S-adenosylmethionine synthetase | 46.0 | 5.5 | 42.9 | 5.4 | c | c | u |
| 22 | BQ106376 | " | 47.0 | 5.3 | 43.3 | 5.7 | c | c | u |
| 23 | BQ106376 | S-adenosylmethionine synthetase | 45.7 | 5.3 | 43.1 | 5.5 | u | d | u |
| 114 | BQ106376 | S-adenosylmethionine synthetase | 47.7 | 5.2 | 42.9 | 5.7 | c | c | u |
| 112 | BQ106102 | Triosephosphate isomerase | 26.7 | 5.9 | 27.1 | 5.5 | c | c | c |
| 44 | BQ106102 | Triosephosphate isomerase | 27.4 | 5.6 | 27.1 | 5.5 | u | d | c |
| 98 | BQ106556 | Triosephosphate isomerase | 28.0 | 5.2 | 33.3 | 7.7 | d | c | c |
| 46 | BQ106636 | Triosephosphate isomerase | 26.9 | 6.3 | 27.1 | 5.5 | c | c | c |
| 156 | CF349690 | Glyceraldehyde-3-phosphate dehydrogenase | 33.3 | 7.4 | 39.1 | 6.4 | c | c | c |
| 39 | BQ106504 | Disulfide isomerase | 46.3 | 3.7 | 55.6 | 5.0 | c | c | c |
| 141 | BQ105675 | Dihydroflavonol reductase | 42.1 | 5.5 | 43.8 | 5.6 | c | c | c |
| 21 | BQ105334 | Hydroxymethyltransferase | 56.6 | 6.8 | 51.7 | 6.8 | c | c | c |
| 60 | BQ104381 | Nucleoside-diphosphate kinase | 17.5 | 6.5 | 16.3 | 7.9 | d | c | d |
| 34 | BQ104506 | UDP-glucose dehydrogenase | 55.3 | 5.6 | 53.2 | 5.7 | c | c | c |

Table 4 (Contd.)

| Spot no. | Accession no. | Putative function/description | Exp. MW (kDa) | Exp. pI | Theo. MW (kDa) | Theo. pI | Expression protein RNA | | |
|--|---------------|--------------------------------------|---------------|---------|----------------|----------|------------------------|-----|-----|
| | | | | | | | 1-4 | 4-6 | 1-4 |
| Protein destination | | | | | | | | | |
| 41 | BQ105563 | Chaperonin 60 β subunit | 63.8 | 4.1 | 62.0 | 5.2 | u | c | c |
| 83 | BQ105563 | Chaperonin 60 β subunit | 65.3 | 4.9 | 64.1 | 5.6 | d | u | c |
| 123 | BQ104538 | 26S protease regulatory subunit 7 | 55.3 | 6.1 | 47.7 | 6.0 | d | d | c |
| 47 | CF349909 | 20S proteasome α 4 subunit | 29.1 | 7.3 | 27.1 | 6.9 | d | d | c |
| 71 | CF349832 | ATP-dependent Clp protease | 91.1 | 5.2 | 99.5 | 8.8 | u | c | c |
| 72 | CAA05023 | Succinyl-CoA-ligase α subunit | 36.0 | 6.6 | 36.2 | 8.6 | c | c | |
| 99 | CF349560 | Leucyl aminopeptidase | 55.3 | 5.3 | 53.1 | 5.4 | u | c | c |
| Cell growth, cell division and DNA synthesis | | | | | | | | | |
| 11 | BQ104538 | CDC48 | 106.4 | 4.7 | 90.3 | 5.0 | d | c | c |
| Intracellular transport | | | | | | | | | |
| 52 | AAF01284 | NSF attachment | 32.0 | 4.5 | 32.8 | 5.2 | c | c | |
| Ionic homeostasis | | | | | | | | | |
| 43 | BQ104264 | V-ATPase catalytic subunit A | 74.0 | 5.2 | 68.6 | 5.3 | d | c | u |
| Unknown function | | | | | | | | | |
| 1 | BQ105069 | Unknown | 18.1 | 4.9 | 17.1 | 5.2 | c | c | u |
| 56 | BQ105069 | Unknown | 18.1 | 4.6 | 17.1 | 5.2 | c | c | u |
| 136 | BQ105069 | Unknown | 18.8 | 4.3 | 17.1 | 5.2 | u | d | u |
| 26 | AAC00619 | Unknown | 36.3 | 4.6 | 39.9 | 7.5 | c | c | c |
| 63 | CAB52464 | Unknown | 74.6 | 4.9 | 78.8 | 5.9 | d | u | |
| 73 | AAC24091 | Unknown | 27.7 | 3.9 | 18.9 | 10.0 | c | u | |
| 92 | CAB88540 | Unknown | 29.5 | 4.7 | 18.6 | 10.0 | c | c | |
| 97 | BQ104783 | Unknown | 28.4 | 4.9 | 27.1 | 5.1 | c | c | c |
| 113 | AAF99784 | Unknown | 47.6 | 5.1 | 46.1 | 9.5 | d | d | |
| 138 | BQ105155 | Unknown | 18.7 | 5.6 | 22.7 | 9.1 | u | c | c |
| 40 | BQ105025 | Unknown | 32.5 | 4.4 | 32.6 | 5.5 | d | c | c |
| 95 | CF349367 | Unknown | 14.4 | 4.6 | 16.6 | 4.9 | c | d | |
| 49 | BQ105680 | Unknown | 20.0 | 3.7 | 19.0 | 4.4 | c | c | c |
| 109 | BQ105117 | Unknown | 46.3 | 4.6 | 41.2 | 5.2 | d | c | c |
| 28 | CF349380 | Unknown | 36.3 | 5.5 | 41.6 | 5.7 | c | u | d |
| No hits found | | | | | | | | | |
| 102 | BQ104241 | No hits found | 32.0 | 5.5 | | | d | u | c |
| 78 | BQ104638 | No hits found | 73.3 | 7.3 | | | c | u | |
| 106 | CF349612 | No hits found | 59.3 | 6.4 | | | d | u | c |
| 84 | BQ104395 | No hits found | 62.0 | 4.9 | | | d | c | |
| 132 | BQ105170 | No hits found | 27.7 | 3.6 | | | c | c | |
| 37 | BQ104971 | No hits found | 30.2 | 7.4 | | | d | u | c |
| 80 | BQ106227 | No hits found | 42.7 | 5.6 | | | c | d | d |

Proteins were classified into functional groups according to the Munich Information Center for Protein Sequences (MIPS). Expression pattern of proteins during flower development from stage 1 to 4 and from stage 4 to 6, determined as in Table 3, is presented. Expression pattern of corresponding RNA during

flower development from stage 1 to 4 was determined by microarray (Guterman et al. 2002; Izhaki 2004). Protein spots corresponding to the same gene are clustered. Expression pattern: *c* constitutive, *u* upregulated, *d* downregulated. *Exp* experimental, *Theo* theoretical

emitted by the flowers (Dudareva et al. 2000). Hence, during flower development from stages 1 to 6, we expected to see mainly increases/decreases in protein levels, and not the drastic changes in protein composition found here, i.e. 30% of the proteins being stage-specific.

During the transition from stage 1 to 4, the levels of ca. 55% of the proteins decreased. A more balanced number of up- and downregulated proteins characterized further development of the petals from stage 4 to 6. The initial massive downregulation of proteins may be indicative of the transition from one developmental program to another, i.e. from cell division to cell expansion. Indeed, protein #11, whose level was downregulated during the

petal transition from stage 1 to 4, was identified as a homologue of cell-division-cycle protein cdc 48 (Thoms 2002), lending support to this notion.

The transition from stage 1 to 4 is also accompanied by the accumulation of anthocyanin (Table 1; Ben-Meir et al. 2002) and the disintegration of chloroplast components (Vainstein and Sharon 1993). Indeed, the level of chloroplast Clp protease (protein #71) increases in petals during the transition from stage 1 to 4. On the other hand, the level of dihydroflavonol reductase (protein #141), the only representative of the anthocyanin biosynthetic pathway identified in this study, remains constant during pigment accumulation (stages 1 to 4).

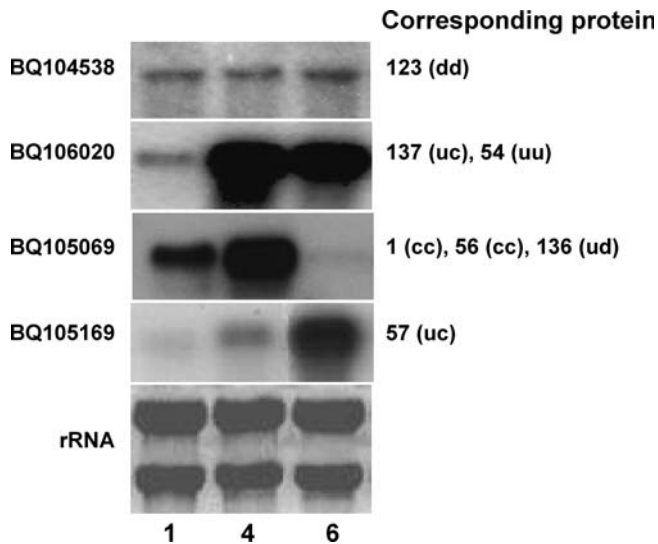


Fig. 4 Comparison of RNA expression pattern with that of its corresponding protein during rose flower development. Total RNA was extracted from petals at three developmental stages and northern blot analysis was performed with four different genes. rRNA is presented for comparison. Corresponding proteins (*spot numbers*) and their pattern of expression (*c*, constitutive, *u* upregulated, *d* downregulated) during petal development from stage 1 to 4 and from stage 4 to 6 are presented. First letter in parentheses indicates expression pattern from stage 1 to 4, and the second letter from stage 4 to 6

Classification of the annotated proteins into functional groups revealed energy, cell rescue, unknown function (including novel sequences) and metabolism to be the largest classes, together comprising ca. 90% of all identified proteins. Interestingly, a large number of stress-related proteins were identified in the developing petals, including low- and high-molecular-weight HSPs, peroxidase, superoxide dismutase, and catalase. This is not surprising as many stress-related genes are active in developing rose petals (Channeliere et al. 2002; Guterman et al. 2002). Another representative of this group was the homologue of a chromoplast carotenoid-associated protein (spot #32). It should be noted that Fragrant Cloud petals do not accumulate chromoplasts or carotenoids. However, these proteins have been shown to be involved not only in chromoplastogenesis during flower development, but also in protection against biotic and abiotic stresses (Langenkamper et al. 2001). The large proportion of stress-related proteins in developing petals is not unique to rose. In both *Arabidopsis* and *Antirrhinum*, stress-related genes represent a large proportion of the petal ESTs and are regulated by a class B floral homeotic gene (Zik and Irish 2003; Bey et al. 2004). The stress-related hormone jasmonic acid has also been shown to accumulate to high levels in developing flowers (Meyer et al. 1984). The accumulation of stress-related proteins in petals may be indicative of the requirement for protection of the cell during flower development against either intracellular stress (e.g. an oxidative environment) or external stresses. The flower in general, petals in particular, are extremely

sensitive tissues and even mild biotic or abiotic stress conditions can lead to organ death.

Despite the fact that secondary metabolism-related pathways are highly active in advanced stages of petal development (Francis and Allcock 1969; Suzuki et al. 2000), only one identified protein, namely dihydroflavonol reductase, could be directly related to secondary metabolism. This can be explained by the low level of relevant enzymes, membrane association, etc. It should also be noted that only a limited number of proteins were annotated: ca. 10% of the total identified by us and only ca. 1% of the putative total number of petal proteins.

We analyzed the expression pattern during flower development of four gene products at the RNA level. To a large extent, the pattern of expression of the corresponding proteins did not correlate with the respective transcript levels. Similar discrepancies were also revealed when the expression patterns of 65 proteins were evaluated on the basis of microarray analyses. This is not unexpected since in several cases (e.g. BQ105069) we show that the same gene corresponds to several different protein spots, which differ in their expression patterns. It is likely that at least some of these protein isoforms result from post-translational modifications. Furthermore, the generation of isoforms due to alternative splicing cannot be excluded (Jordan et al. 2002). We also cannot dismiss the idea that such groups of proteins result from recent gene duplication and/or allelic variation, especially since cv. Fragrant Cloud is tetraploid. In this case, the differences in expression at the transcript level between such closely related genes cannot be revealed by either RNA blot or cDNA array. To overcome this limitation, gene-specific probes must be used; however in our case, this was not an option since full-length sequences of the transcript/proteins are still unavailable.

In this work, the rose petal proteome was analyzed during flower development. The stage-specific protein maps generated here should be highly instrumental in producing detailed analyses of the late processes of flower development. In-depth characterization of gene expression at the protein level, and not only at the RNA level, is essential for understanding such complex biological processes.

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