Studont of Early Leaf Senescence in *Arabidopsis thaliana* by Quantitative Proteomics Using Reciprocal $^{14}$N/$^{15}$N Labeling and Difference Gel Electrophoresis*\[S\]

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Leaf senescence represents the final stage of leaf development and is associated with fundamental changes on the level of the proteome. For the quantitative analysis of changes in protein abundance related to early leaf senescence, we designed an elaborate double and reverse labeling strategy simultaneously employing fluorescent two-dimensional DIGE as well as metabolic $^{15}$N labeling followed by MS. Reciprocal $^{14}$N/$^{15}$N labeling of entire *Arabidopsis thaliana* plants showed that full incorporation of $^{15}$N into the proteins of the plant did not cause any adverse effects on development and protein expression. A direct comparison of DIGE and $^{15}$N labeling combined with MS showed that results obtained by both quantification methods correlated well for proteins showing low to moderate regulation factors. Nano HPLC/ESI-MS/MS analysis of 21 protein spots that consistently exhibited abundance differences in nine biological replicates based on both DIGE and MS resulted in the identification of 13 distinct proteins and protein subunits that showed significant regulation in *Arabidopsis* mutant plants displaying advanced leaf senescence. Ribulose 1,5-bisphosphate carboxylase/oxygenase large and three of its four small subunits were found to be down-regulated, which reflects the degradation of the photosynthetic machinery during leaf senescence. Among the proteins showing higher abundance in mutant plants were several members of the glutathione S-transferase family class phi and quinone reductase. Up-regulation of these proteins fits well for proteins showing low to moderate regulation factors. The abbreviations used are: At, *Arabidopsis thaliana*; cpr5, constitutive expressor of pathogenesis-related genes 5; CyDyes, cyanine dyes; Fl, fluorescent intensity; GST, glutathione S-transferase; MLP, major latex protein; RP, reversed-phase; RuBiCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; wt, wild-type.

1 The abbreviations used are: At, *Arabidopsis thaliana*; cpr5, constitutive expressor of pathogenesis-related genes 5; CyDyes, cyanine dyes; Fl, fluorescent intensity; GST, glutathione S-transferase; MLP, major latex protein; RP, reversed-phase; RuBiCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; wt, wild-type.
same fundamental restrictions as traditional two-dimensional PAGE. It exhibits a strong bias against hydrophobic proteins such as membrane proteins and proteins with an extreme isoelectric point and/or molecular weight, for example. In addition, accurate relative protein quantification is impaired when two or more protein species are present in the same spot (7).

Stable isotope labeling of proteins or peptides combined with MS analysis represents an alternative strategy for accurate, relative quantification of proteins on a global scale. In this approach, proteins or peptides of two different samples are differentially labeled with stable isotopes, combined in equal ratio, and then jointly processed for subsequent MS analysis. Relative quantification of proteins is based on the comparison of signal intensities or peak areas of isotope-coded peptide pairs extracted from the respective mass spectra. Stable isotopes can be introduced either chemically into proteins/peptides via derivatization of distinct functional groups of amino acids or metabolically during protein biosynthesis. Metabolic labeling strategies are based on the in vivo incorporation of stable isotopes during growth of organisms. Nutrients or amino acids in a defined medium are replaced by their isotopically labeled (\(^{15}\)N, \(^{13}\)C, or \(^2\)H) counterparts eventually resulting in uniform labeling of proteins during the processes of cell growth and protein turnover (1). As a consequence, differentially labeled cells or organisms can be combined directly after harvesting. This minimizes experimental variations due to separate sample handling and thus facilitates relative protein quantification of high accuracy. Since metabolic labeling is best applicable to biological systems that can be maintained under controlled conditions, it has been predominantly applied to unicellular organisms such as bacteria (8) and yeast (9) as well as cell culture systems (10). However, the feasibility to label multicellular organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster*, and a rat metabolically was shown as well (11, 12). Recently, in an approach reported by Kolkman et al. (23), we designed a double and reverse labeling strategy employing both two-dimensional DIGE and metabolic \(^{15}\)N labeling for relative quantification of plant proteins. *A. thaliana* plants used in this work were grown on solid medium in a growth chamber simulating growth conditions as natural as possible. Reverse \(^{14}\)N/\(^{15}\)N labeling experiments showed that quantitative incorporation of \(^{15}\)N-isotopes (95%) had no adverse effects on plant development. The concomitant use of DIGE as alternative quantification technique provided us with a control to assess the consistency of the results obtained by both methods. Overall, we performed nine independent experiments, which led to the reliable identification of 13 different proteins or protein subunits with significant changes in abundance in the *old1–1* mutant plant. Ribulose 1,5-bisphosphate carboxylase/oxygenase large as well as three of its four small subunits showed decreased abundance in mutant plants. Proteins found to be up-regulated were, among others, several isoforms of the glutathione S-transferase (GST) family class phi and quinone reductase. These proteins are generally known to be involved in the detoxification of reactive oxygen species (24, 25).

**EXPERIMENTAL PROCEDURES**

**Growth and Metabolic Labeling of Plants Using \(^{14}\)N/\(^{15}\)N Isotopes—** *A. thaliana* accession Landsberg erecta wt and *old1–1* mutant plants were grown under standard conditions (22 °C, 60–70% relative humidity, and 16 h of light). For metabolic labeling with \(^{15}\)N, seeds were sown onto 19-cm Petri dishes containing stone wool (Grodan BV, Roermond, The Netherlands) wetted with \(^{15}\)N-medium consisting of 0.75 mM MgSO\(_4\), 3 mM K\(^{15}\)NO\(_3\), 0.5 mM KH\(_2\)PO\(_4\), 1.5 mM CaCl\(_2\), and 0.11 mM \(^{15}\)NH\(_4\)\(^{15}\)NO\(_3\) supplemented with Hoagland's micronutrients (26). \(^{15}\)N-labeled salts were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA) and were >98% enriched in \(^{15}\)N. The Petri dishes were kept at 4 °C for 3 to 7 days and were then transferred to a growth chamber. During growth of the plants, the Petri dishes were kept humid with distilled water; medium was replenished every other week. After harvesting the seeds after 7–10 weeks of growth, they were sterilized with 1% bleach in 96% ethanol, washed twice with 96% ethanol, and dried. The sterilized seeds were then sown onto 15-cm Petri dishes containing medium solidified with 0.6% micro agar (Duchefa, Haarlem, The Netherlands). They were stratified at 4 °C for 3 to 7 days and subsequently transferred to the growth chamber. After 16 days of growth, the plants were frozen in liquid nitrogen, scraped off the dishes and stored at –80 °C prior to protein extraction. At this age, both *A. thaliana* wt and *old1–1* mutant plants did not show visible symptoms of leaf senescence under the growth conditions applied in this work. \(^{14}\)N-labeled *A. thaliana* plants were grown in parallel under the same conditions in the presence of salts containing \(^{14}\)N- and \(^{15}\)N-isotopes at their natural abundance.

**Preparation of Protein Extracts—** Protein extracts were prepared according to Giavalisco et al. (27). In brief, frozen *A. thaliana* plants of an entire Petri dish (~500 mg) were supplied with 0.125 parts (w/w) of solution 1 [one tablet of Complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) dissolved in 2 ml of 100 mM KCl, 20% (v/v) glycerol, and 50 mM Tris, pH 7.1] and 0.05 parts (w/w) of solution 2 [1 mM pepstatin A (Serva, Heidelberg, Germany) and 1.4 \(\mu\)M PMSF (AppliChem, Darmstadt, Germany) dissolved in ethanol]. Plants were ground to a fine powder in a mortar placed in liquid nitrogen. After centrifugation of the homogenate (30 min at 226,000 g and 4 °C), the supernatant containing the soluble proteins was collected and either
directly applied to two-dimensional PAGE or stored at −80 °C. Protein concentration was determined by amino acid analysis. After acidic hydrolysis of samples, the concentrations of free amino acids were analyzed by precolumn derivatization with 6-aminoquinoliny-N-hydroxysuccinimidyl carbamate using a Waters AccQFluor reagent kit (Waters Corporation, Milford, MA) (28, 29). Derivatives of amino acids were separated by reversed-phase (RP-) HPLC using a Waters AccQTag column (25 cm × 2 mm inner diameter) on a Waters Alliance 2695 Separation Module. After fluorescence detection on a Waters 2475 Multi Fluorescence Detector, the concentrations of the separated amino acids were determined.

**Labeling of Proteins Using CyDyes**—Proteins were labeled with CyDyes according to the manufacturer’s protocol (Amersham Biosciences/GE Healthcare). Before labeling, 54 mg of urea per 50 μl of protein sample (corresponding to −130 μg of protein) were added (27); the pH was adjusted to 8.5 using 1 M Tris-base. Stock solutions of cyanine dyes (1 nmol/μl) were diluted with anhydrous dimethyl formamide (dimethylformamide, pro analysis; Sigma, St. Louis, MO) to 400 pmol/μl. 50 μg of protein were mixed with 400 pmol of cyanine dye (Cy2, Cy3, or Cy5; Amersham Biosciences/GE Healthcare), briefly centrifuged and incubated on ice in the dark for 30 min. The labeling reaction was stopped by addition of 1 μl of 10 mM 1-lysine (Sigma) and incubation on ice for 10 min. In half of the experiments, proteins from wt and mutant plants were labeled with Cy3 and Cy5, respectively. In the other half of the experiments, fluorescent labeling was reversed. An internal standard generated by pooling equal amounts of proteins from each sample was labeled with Cy2. Differentially labeled samples were immediately combined in a 1:1:1 ratio and then subjected to protein separation by two-dimensional PAGE.

**Separation of Differentially 14N/15N- and CyDye-labeled Proteins by Two-Dimensional PAGE**—Proteins were separated via two-dimensional PAGE following the protocol of Klose (30). To allow for relative quantitative analysis of proteins based on both fluorescent and metabolomic labeling, extra 125 μg of protein per A. thaliana phenotype were added, resulting in a total of 400 μg protein per gel. 5 μl of 1.4 M DTT (BioRad, Munich, Germany) and 10 μl of the ampholyte mixture Servalyte 2–4 (Serva) were added per 100 μl of protein sample. IEF was performed according to Klose and Kobalz (31) in an IEF chamber produced in-house using tube gels (20 cm × 1.5 mm) that contained carrier ampholytes. Following IEF, the tube gels were ejected and incubated in equilibration buffer [125 mM Tris, 40% (w/v) glycerol, 3% (w/v) SDS, 65 mM DTT, pH 6.8] for 15 min. Gels were then washed three times with SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.2% SDS), placed on top of polyacrylamide gels (20 cm × 30 cm × 1.5 mm; 15.0% total acrylamide, 1.3% bisacrylamide), and fixed with 1.0% (w/v) agarose containing 0.01% (w/v) bromphenol blue (Riedel de-Haen, Seelze, Germany). SDS-PAGE was performed at 15 °C using a Desaphor VA 300 system (Desaga, Heidelberg, Germany) at a constant current flow of 75 mA for 15 min followed by a constant current flow of 200 mA for 6 to 7 hours.

**Image Acquisition, Analysis, and Visualization of Proteins Separated by Two-Dimensional DIGE**—After gel electrophoresis, two-dimensional DIGE gels were scanned using the Typhoon 9400 Imager (Amersham Biosciences/GE Healthcare). Excitation wavelengths and emission filters were chosen specifically for each of the fluorescent dyes according to the manufacturer’s user guide. Scans were acquired at a resolution of 100 μm. After cropping and filtering using the ImageQuant software (version 5.2, Amersham Biosciences/GE Healthcare), images were subjected to difference in-gel analysis and biological variation analysis using the DeCyder software, version 6.0 (Amersham Biosciences/GE Healthcare). Spot intensities were normalized based on the internal standard labeled with Cy2. Following the enhancement of the gel, proteins were visualized by colloidal Coomassie Brilliant Blue staining (32). ImageQuant 5.2 was used to convert two-dimensional DIGE images into the corresponding false color images.

**In-gel Digestion of Proteins**—Protein spots of interest were manually excised from the two-dimensional gel using a spot picker and transferred to glass mini-tubes. Gel spots were immediately destained by alternately incubating them with 20 μl of 10 mM ammonium hydrogen carbonate (NH₄HCO₃) and 20 μl of 5 mM NH₄HCO₃/50% ACN for 10 min each. This step was performed three times. Afterward, gel pieces were dried in vacuo and either stored at −80 °C or directly subjected to proteolytic digestion with trypsin (Promega, Mannheim, Germany) dissolved in 10 mM NH₄HCO₃ (pH 7.8) at a final concentration of 0.03 μl/μl. Protein spots were incubated overnight at 37 °C with 2 μl of trypsin solution and slight agitation. Proteolytic peptides were extracted twice with 10 μl of ACN and 5% formic acid mixed 1:1 (v/v); extracts were combined and ACN was removed in vacuo. For MS analysis, samples were acidified by addition of 5% formic acid to a final volume of 20 μl.

**Mass Spectrometric Analysis and Protein Identification**—Tryptic digests were analyzed by nano HPLC/ESI-MS/MS using a Dionex LC Packings system (Dionex LC Packings, Idstein, Germany) coupled to a QSTAR XL instrument (Applied Biosystems, Foster City, CA). Peptide mixtures were separated by online RP capillary HPLC as previously described by Schaefer et al. (33). The mass spectrometer was equipped with a nanoelectrospray ion source (SCIEX, Toronto, Ontario, Canada) and distal coated SilicaTips (FS360–20–10–D; New Objective, Woburn, MA). For external calibration of the QSTAR XL instrument in the enhanced product ion mode, reserpine (m/z 609.280; Agilent Technologies, Santa Clara, CA) and two of its fragments with m/z 174.100 and 195.065 were used. To guarantee high reproducibility of MS scans, calibration was routinely performed before and after the analysis of biological samples.

The general mass spectrometric parameters were set as follows: ion spray voltage (IS), 1800–2000 V; curtain gas (CUR), 10–14; gas 1, 0; declustering potential (DP), 50 V; focusing potential (FP), 220 V; declustering potential 2 (DP2), 15 V. In the Analyst QS 1.1 software (Applied Biosystems), the so-called “Information Dependent Acquisition” (IDA) method was chosen, which consists of a survey MS scan (m/z 400–1200) followed by sequential isolation and fragmentation of the three most intense peaks (enhanced product ion scans, m/z 100–200). To obtain most informative peptide fragmentation spectra, only multiply charged peptide ions of interest were isolated in the quadrupole Q1 (set to low resolution) and subjected to collision-induced dissociation using nitrogen as collision gas. Furthermore, previously fragmented ions were dynamically excluded for the following 16 s. Depending on the mass-to-charge ratio of isolated peptides, collision energies for the fragmentation in the collision cell q2 were dynamically adjusted by the software (rolling collision energy).

Peaklists of MS/MS spectra were generated using the software Analyst QS 1.1 with default parameter settings. For peptide and protein identification, uninterpreted peptide ESI-MS/MS spectra were correlated with the A. thaliana EBI protein sequence database (ipi.ARA4.v3.17.fasta) containing 34,559 protein entries using SEQUEST (34, 35) (TurboSEQUEST v.27) after converting and exporting the raw data into the DTA format using the software tool wiff2dta (36). Species restriction to A. thaliana is justified by the fact that exclusively proteins from A. thaliana were analyzed. Database searches were performed with tryptic specificity allowing two missed cleavages and a mass tolerance of 0.2 Da for parent and fragment ions. Oxidation of methionine residues and formation of propionamide at cysteine residues were considered as variable modifications; no fixed modifications were included. Cut-off scores for accepting individual MS/MS spectra of doubly and triply charged peptides were 2.0 for the cross-correlation factor (Xcorr) and 0.1 for the delta normalized cross-correlation factor (Scan). Protein identification was based on at least two
distinct peptides. If a protein appeared under different names and accession numbers, the entry with the highest sequence coverage was selected. In the case that different protein isoforms were listed by SEQUEST, these entries were inspected manually and the presence of each protein isoform was confirmed by the identification of at least two unique peptides.

**Relative Protein Quantification**—For relative quantification of proteins showing differences in abundance based on fluorescence labeling and two-dimensional DIGE, the software DeCyder 6.0 was used. Criteria for significant protein regulation were as follows: Protein spots needed to 1) be present and analyzed in at least three gels per set of experiments and 2) show an average factor of ≥ 1.5 in both sets as well as 3) a Student’s t-test value of \( p < 0.05 \). For calculations of protein abundance ratios based on two-dimensional DIGE experiments, fluorescence intensities (“normalized spot volumes”) for each channel of the different CyDyes were manually exported out of DeCyder and used for further processing. The fluorescence intensity of a \(^{15}\text{N}\)-labeled protein was divided by the fluorescence intensity of the corresponding \(^{14}\text{N}\)-labeled protein. Individual ratios obtained for proteins in reciprocally labeled sets of experiments were averaged and arithmetic mean values as well as the corresponding standard deviation were calculated for each protein per set. For relative protein quantification based on metabolic \(^{14}\text{N}/^{15}\text{N}\) labeling, the in-house developed software Peakardt was employed. For the export of peaklists from Analyst QS 1.1 for use in Peakardt, the threshold for “PeakFinding” in MS spectra and total ion chromatograms was set to 1.0%. Total ion chromatograms acquired in LC-MS/MS runs were divided into distinct time segments of 1 min; MS spectra within these intervals were summed up and used for further *in silico* processing. Abundance ratios of \(^{14}\text{N}\)- (light) and \(^{15}\text{N}\)-coded (heavy) peptides were calculated by dividing peak areas of light peptides by those of heavy peptides using the “FindPairs” algorithm of Peakardt. For relative quantification, the monoisotopic peak and generally two \(^{13}\text{C}\)-isotope peaks of both light and heavy peptides as well as the first \(^{14}\text{N}\)-satellite peak of the heavy peptide were taken into account. In case that peptide signal intensities were low, only one \(^{13}\text{C}\)-isotope peak was considered. To improve the confidence of relative peptide quantification, an *in silico* digestion of identified proteins with trypsin was performed via Peakardt. Masses of theoretically determined \(^{14}\text{N}/^{15}\text{N}\)-labeled peptides were then searched and matched with peptide masses present in the experimentally acquired mass spectra. Only peptide pairs that did not overlap with other peptides in their \(m/z\)-range were considered for further calculation of relative protein abundances. In addition, peptide pairs exceeding the 2.5-fold of the arithmetic mean determined by “FindPairs” were considered as outliers and removed. The determination of protein abundance ratios was based on at least two adequate peptide pairs. If not stated otherwise, a protein was considered as significantly regulated based on metabolic labeling and MS analysis when it 1) could be identified and quantified reliably in at least three gels per set of experiments and 2) showed an average regulation factor of ≥ 1.5 as well as 3) a Student’s \( t \)-test value of \( p < 0.05 \). The arithmetic mean of all peptides detected for each protein in all biological replicates per set, the deviation (in %), biological and technical variance, \( p \) value as well as the 95% confidence interval were calculated using the “ExperiAna” module of Peakardt.

**Statistical Analyses**—In the reverse labeling experiments described in this work, a protein with a distinct abundance ratio in one experiment (referred to as Set 1) is expected to have the reciprocal ratio in the experiment with reversed labeling (referred to as Set 2). Consequently, multiplication of both ratios is expected to result in a factor of 1. For the calculation of the standard deviation (S.D.) for this kind of analysis, the “propagation of error model” (37) was applied using the formula \( \Delta X = \sqrt{(\sigma_A/A)^2 + (\sigma_B/B)^2} \times X \), with \( X = A \times B \), and \( A \) and \( B \) being the abundance ratio of a distinct spot or protein in Set 1 and Set 2; \( \sigma_A \) and \( \sigma_B \) denote the respective S.D.: \( \Delta X \) is the value “propagation of error” to be calculated as shown in Equation 1.

\[
\Delta X = \sqrt{(\sigma_A/A)^2 + (\sigma_B/B)^2} \times X \quad \text{(Eq.1)}
\]

The same formula was used to determine the “propagation of error” when the average abundance ratios of proteins based on \(^{15}\text{N}\) labeling were divided by the corresponding values obtained in DIGE experiments.

To detect outliers in data sets obtained in double and reverse labeling experiments, box plots were utilized (38, 39). They were plotted using the software R, version 2.5.1. With a box plot, the distribution characteristics of continuous data can be visualized. As with a histogram, accumulation points, distribution width, skew, and outliers can be described. The horizontal, bold line within the “box” represents the median and the small rectangle shows the arithmetic mean of the data set. The box itself contains the middle half of the data from first quartile to third quartile (interquartile distance). The vertical lines ending in horizontal lines above and below the box are called “whiskers.” The horizontal lines lie on the last data point inside the 1.5-fold of the interquartile distance spanning from the box. Data points outside the whiskers are interpreted as “outliers” and drawn as filled circles.

**RESULTS**

**\(^{15}\text{N}/^{15}\text{N}\) Labeling of Plants Grown on Solid Medium**—So far, comparative proteomics studies on *A. thaliana* employing metabolic labeling have been performed using cultured suspension cells or plants grown in liquid cultures (13–18). However, in order to acquire biologically meaningful information on complex events related to, among others, plant development, metabolism, or responses to environmental influences, one should aim at growing plants under conditions that reflect their natural habitat as close as possible.

A major aim of the work presented here was to achieve complete metabolic labeling (i.e. ≥ 95% incorporation of stable isotopes) of entire *A. thaliana* plants grown on solid medium using \(^{15}\text{N}\). For this purpose, *A. thaliana* wt and *old1–1* mutant plants exhibiting advanced leaf senescence (20–22) were grown under standard conditions in a growth chamber as described in “Experimental Procedures.” \(^{15}\text{N}\) labeling of plants was performed by substituting KNO\(_3\) and NH\(_4\)NO\(_3\) in the medium with the respective \(^{15}\text{N}\)-containing salts (>98% enriched in \(^{15}\text{N}\)). To ensure efficient metabolic labeling, seeds of first-generation plants were harvested and sown once again onto solid, \(^{15}\text{N}\)-containing growth medium. Control plants (\(^{14}\text{N}\)-labeled) were subjected to the same procedure. To study early leaf senescence, second generation plants were harvested after 16 days of growth. At this stage, incorporation of \(^{15}\text{N}\)-isotopes into the proteins from both wt and mutant plants reached 95% (data not shown) as estimated using the software IsoPro 3.0.

**Linearity of Relative Protein Quantification Based on \(^{14}\text{N}/^{15}\text{N}\) Labeling**—To evaluate the linearity of relative protein quantification based on \(^{14}\text{N}/^{15}\text{N}\) labeling and MS, protein extracts from differentially labeled wt plants were mixed in distinct concentration ratios ranging from 1:5 (\(^{14}\text{N}/^{15}\text{N}\)) to 5:1.
ing two-dimensional PAGE and visualization by colloidal Coomassie Blue, 18 corresponding protein spots covering a wide range of molecular weight and pl were excised from each gel and subjected to nano HPLC/ESI-MS/MS analysis. Since we observed co-elution of 14N/15N-labeled peptides from the RP column (data not shown), differences in the chromatographic behavior of peptide pairs did not need to be considered. MS-based relative protein quantification in the entire study was performed by calculating peak ratios of 14N/15N-labeled peptides based on peak areas using the in-house developed software Peakardt (see “Experimental Procedures”). Protein abundance ratios were calculated based on 4.5 peptide pairs on average. The average protein concentration ratios were in good accordance with the theoretical ratios ranging from 1:5 to 5:1 as reflected by the correlation coefficient of 0.9965 (supplemental Fig. 1). Relative errors between theoretical and experimental values were in the range of 7% for the 1:1 and 21% for the 1:5 ratios.

**Design of Double and Reverse Labeling Strategy**—For assessment whether metabolic labeling is applicable to address biological questions in plant proteomics, a double and reverse labeling strategy concomitantly employing DIGE and 14N/15N labeling was designed. Reverse labeling experiments were carried out to reveal potential inconsistencies in protein quantification caused by the labeling technique employed. Moreover, reverse 14N/15N labeling allows the detection of possible adverse effects on an organism’s development and protein expression that may occur during the process of differential labeling, including heavy isotope effects (11, 16). An additional benefit of reverse 15N labeling is the improved ability to detect and quantify strongly regulated proteins by MS (40). Double labeling provides a reciprocal control for both strategies and allows to evaluate the consistency of the results obtained by the two different techniques for protein quantification (23).

In this work, two sets of experiments were carried out (Fig. 1). In the first set, referred to as Set 1, wt plants were grown in a medium containing 14N while oldf1–1 mutant plants were labeled with 15N. In the second set (Set 2), labeling was reversed. To take into account biological variations and to gain statistically significant data on differences in protein abundance, five (Set 1) and four (Set 2) independent experiments were performed. After harvesting the plants and protein extraction, proteins were differentially labeled with CyDyes as follows: In three of five replicates in Set 1, proteins derived from wt plants were labeled with Cy5 while proteins from mutant plants were labeled with Cy3. In the remaining two replicates, fluorescent labeling was reversed. The same labeling strategy was applied to the experiments of Set 2, with two replicates each. This color switch is routinely used to eliminate effects of a possible preferential affinity of different fluorescent dyes to proteins of one of the samples analyzed (41).
However, no such effects were observed in this study (data not shown). Differentially labeled protein samples and an aliquot of the corresponding internal standard were combined in a 1:1:1 ratio and subjected to two-dimensional PAGE. Following image analysis using the software DeCyder, selected protein spots were analyzed by nano HPLC/ESI-MS/MS on a QTOF instrument. Fig. 2 shows the overall distribution of protein spots on a representative two-dimensional DIGE gel poststained with colloidal Coomassie. Proteins derived from wt plants were labeled with Cy3 and mixed with Cy5-labeled proteins from old1–1 mutant plants as well as an internal standard labeled with Cy2 in equal ratios. Proteins were separated by two-dimensional PAGE and subsequently visualized with colloidal Coomassie Brilliant Blue. Numbers indicate regulated (arrow only) and nonregulated spots (arrows and *) as determined by DIGE that were further subjected to MS analysis for both protein identification and quantification. Squares highlight areas with protein spots exhibiting major differences in protein abundance. A–D, corresponding images of Cy3-labeled wt proteins (A and C) and Cy5-labeled old1–1 mutant proteins (B and D). Images were converted from fluorescence into false color images using the software ImageQuant 5.2.

However, no such effects were observed in this study (data not shown). Differentially labeled protein samples and an aliquot of the corresponding internal standard were combined in a 1:1:1 ratio and subjected to two-dimensional PAGE. Following image analysis using the software DeCyder, selected protein spots were analyzed by nano HPLC/ESI-MS/MS on a QTOF instrument. Fig. 2 shows the overall distribution of protein spots on a representative two-dimensional DIGE gel poststained with colloidal Coomassie Brilliant Blue. Numbers indicate both regulated (marked with an arrow only) and nonregulated spots (marked with arrow and asterisk) reported in this work (supplemental Tables S1 and S2).

15N Labeling Did Not Cause Adverse Effects on Plants—To address the question whether 15N labeling of A. thaliana plants resulted in artificial changes in protein abundance, 21 protein spots that did not show significant regulation based on DIGE in both Set 1 and Set 2 were analyzed by MS. A total of 20 different proteins were identified. In three spots, two distinct proteins were present (supplemental Table S2). Following MS-based protein identification and relative quantification, protein abundance ratios calculated for each protein in Set 1 were multiplied with the ratio of the corresponding protein in Set 2 (14N-labeled old1–1 versus 15N-labeled wt). This analysis was based on protein spots that did not show differences in fluorescent intensities. Numbers correspond to spot numbers in Fig. 2; abbreviations of protein names correspond to the UniProtKB/Swiss-Prot database. More detailed information about proteins, including full protein name, IPI accession number, peptide abundance ratios, and sequence coverage, are provided in supplemental Tables S2, S5, and S6. B, box plot based on the mean values of the data illustrated in A. *Deviation expressed as root mean squared error.

Fig. 3. Evaluation of metabolic labeling of A. thaliana plants with respect to possible artificial effects of 15N-incorporation on protein abundance. A, following MS-based relative protein quantification, the abundance ratios calculated for proteins in Set 1 (14N-labeled wt versus 15N-labeled old1–1) were multiplied with the ratios of the corresponding protein in Set 2 (14N-labeled old1–1 versus 15N-labeled wt). This analysis was based on protein spots that did not show differences in fluorescent intensities. Numbers correspond to spot numbers in Fig. 2; abbreviations of protein names correspond to the UniProtKB/Swiss-Prot database. More detailed information about proteins, including full protein name, IPI accession number, peptide abundance ratios, and sequence coverage, are provided in supplemental Tables S2, S5, and S6. B, box plot based on the mean values of the data illustrated in A. *Deviation expressed as root mean squared error.
Proteins showed significant differences in abundance in at least three independent replicates in Set 1 and Set 2 based on DIGE and $^{14}\text{N}/^{15}\text{N}$-labeling combined with MS. Protein names, IPI accession numbers, abbreviated protein names according to the UniProtKB/Swiss-Prot database, spot numbers corresponding to numbers in Figure 2, average abundance ratios for each protein as well as the corresponding regulation factors are provided. For the GST isoforms, the terms according to the current nomenclature for plant GSTs (42, 43) are indicated as well. RuBiSCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; CV, coefficient of variation; S.D., standard deviation.

**TABLE I**

Proteins found to be significantly regulated in *A. thaliana* old1–1 mutant plants

| Protein (IPI accession) | Abbreviated protein name | Spot | Set | Average abundance ratio (± S.D.) | Regulation factor (± CV in %) | Average abundance ratio (± S.D.) | Regulation factor (± CV in %) |
|-------------------------|--------------------------|------|-----|-----------------------------------|-------------------------------|-----------------------------------|-------------------------------|
| RuBiSCO large subunit (IPI00535114.1) | RBL | 10 | 1 | 2.28 (± 0.53) | −2.3 (± 23.2) | 1.69 (± 0.43) | −1.7 (± 25.4) |
| | | | | | | | | |
| RuBiSCO small subunit 1A (IPI00539020.1) | RBS1A | 74 | 1 | 1.73 (± 0.44) | −1.7 (± 25.4) | 1.61 (± 0.48) | −1.6 (± 29.8) |
| | | | | | | | | |
| RuBiSCO small subunit 1B (IPI00521186.1) | RBS1B | 75 | 2 | 1.59 (± 0.35) | −1.6 (± 22.0) | 1.54 (± 0.26) | −1.5 (± 16.9) |
| | | | | | | | | |
| RuBiSCO small subunit 2B (IPI00523477.1) | RBS2B | 73 | 2 | 1.75 (± 0.11) | −1.8 (± 6.3) | 1.75 (± 0.41) | −1.8 (± 23.4) |
| | | | | | | | | |
| Major latex protein-related (IPI00531983.1) | Q8SUR0 | 71 | 2 | 3.00 (± 0.43) | −3.0 (± 14.3) | 2.16 (± 0.57) | −2.2 (± 26.4) |
| | | | | | | | | |
| Serine hydroxymethyltransferase, mitochondrial precursor (IPI00525727.1) | GLYM | 14 | 1 | 0.62 (± 0.07) | 1.6 (± 11.3) | 0.61 (± 0.12) | 1.6 (± 19.7) |
| | | | | | | | | |
| Enolase (IPI00526310.1) | ENO | 20 | 1 | 0.66 (± 0.11) | 1.5 (± 16.7) | 0.63 (± 0.13) | 1.6 (± 20.6) |
| | | | | | | | | |
| Quinone reductase (IPI00529112.1) | Q9LSQ5 | 55 | 2 | 2.43 (± 0.67) | 2.4 (± 27.6) | 3.25 (± 1.11) | 3.3 (± 34.2) |
| | | | | | | | | |
| Glutathione S-transferase PM24, AtGSTF2 (IPI00535149.2) | GSTF4 | 56 | 2 | 1.29 (± 0.68) | 1.3 (± 52.7) | 1.54 (± 0.40) | 1.5 (± 26.0) |
| | | | | | | | | |
| | | | | | | | | |
| Glutathione S-transferase AtGSTF9 (IPI00538125.1) | Q805B2 | 58 | 1 | 16.64 (± 3.64) | 16.6 (± 21.9) | 7.51 (± 2.41) | 7.5 (± 32.1) |
| | | | | | | | | |
| Glutathione S-transferase AtGSTF11 | GST11 | 60 | 2 | 16.64 (± 3.64) | 16.6 (± 21.9) | 7.51 (± 2.41) | 7.5 (± 32.1) |
| | | | | | | | | |
| Glutathione S-transferase 1 AtGSTF6 (IPI00548409.1) | GSTF1 | 62 | 2 | 0.22 (± 0.05) | 4.5 (± 22.7) | 0.32 (± 0.11) | 3.1 (± 34.4) |
| | | | | | | | | |
| Glutathione S-transferase 6 AtGSTF8 (IPI00536062.1) | GSTF6 | 63 | 2 | 1.83 (± 0.68) | 1.8 (± 37.2) | 1.50 (± 0.31) | 1.5 (± 20.7) |

*This protein did not meet the criteria for significant regulation based on DIGE.*

ana wt and mutant plants in both sets of experiments reciprocally labeled with $^{14}\text{N}/^{15}\text{N}$. MS analyses of these spots (marked in Fig. 2) resulted in the identification of 12 distinct proteins or protein subunits. In one of the spots (number 58), two different isoforms belonging to the GST superfamily could reliably be identified. Table I lists all proteins found to be significantly up- or down-regulated in this study, including the GST isoform present in spot number 63. In addition, the table provides information on the average protein abundance ratios determined based on both peak areas of $^{14}\text{N}/^{15}\text{N}$-labeled peptides and fluorescence intensities as well as the corresponding regulation factors. For the GST isoforms, the de-
molecules according to the current nomenclature for plant GSTs (42, 43), which will be used in the following, are given as well. Squares in Fig. 2 highlight areas showing major differences in protein abundance as revealed by two-dimensional DIGE analysis. Insets show corresponding images of Cy3-labeled wild type proteins (Figs. 2A and C) and Cy5-labeled old1–1 mutant proteins (Figs. 2B and D). The protein underlying all four spots in Figs. 2A and B, identified as the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), was down-regulated in mutant plants. Further proteins with decreased abundance in old1–1 plants were RuBisCO small subunits 1A, 1B, and 2B as well as a protein with similarity to major latex protein. Densitometric intensities of spots marked in Figs. 2C and D indicate significant up-regulation of the respective proteins in old1–1 mutant compared with wild type plants. These proteins predominantly belonged to the family of GSTs. In addition to AtGSTF2, AtGSTF6, AtGSTF7, AtGSTF8 (identified in spot number 63), and AtGSTF9, the proteins serine hydroxymethyltransferase mitochondrial precursor, enolase as well as quinone reductase showed higher abundances in the old1–1 mutant.

Comparison of Protein Quantification via Two-dimensional DIGE and14N/15N Labeling Combined with MS—The consistency of the results obtained for reciprocally14N/15N-labeled proteins based on (I) fluorescence scanning and (II) peptide MS analysis was validated by multiplying the average abundance ratios of corresponding protein spots/proteins of Set 1 and Set 2. On average, the products of protein abundance ratios based on DIGE (Fig. 4A) were 1.09 (± 0.26) and 1.09 (± 0.30) based on14N/15N labeling (Fig. 4B), which indicates high consistency of the reverse labeling experiment. The averages of biological and technical variance for the MS-based data were consistent with those observed for the nonregulated proteins (80.8% and 19.2% in Set 1 and 76.7% and 23.3% in Set 2; for values of individual proteins, see supplemental Tables S3 and S4). The box plot for the data obtained using DIGE (Fig. 4C) indicates the presence of two outliers. These are spot number 58, corresponding to two members of the GST family, and spot number 65 (AtGSTF6) with values of 1.83 and 1.65, respectively. No outliers were detected for the data based on15N labeling (Fig. 4D).

To evaluate the comparability of relative protein quantification based on DIGE and14N/15N labeling, corresponding average protein abundance ratios were divided. This was performed for each spot in Set 1 and Set 2, except for spot number 58, as this spot contained two different proteins. For Set 1 (Fig. 5A), an average value of 0.99 (± 0.46) with individ-
Fig. 6. Example of relative protein quantification using DIGE and 14N/15N labeling combined with MS. A, three-dimensional views of the fluorescence intensities (FI) of spot number 58 in Set 1 (top) and Set 2 (bottom) extracted from the DeCyder software. For relative quantification, the FI of the 14N-labeled proteins (FI14N) was divided by the FI of the 15N-labeled proteins (FI15N). B and C, Representative sections of mass spectra showing tryptic 14N/15N-labeled peptides derived from AtGSTF9 (B; SQGPDLLGK) and AtGSTF2 (C; VNEWVAEITK) identified in spot number 58 for Set 1 (top) and Set 2 (bottom). Protein abundance ratios were determined by dividing the peak area (PA) of the 14N-labeled peptide by the PA of the 15N-labeled peptide. Δm/z, shift in m/z resulting from the incorporation of 15N into the peptides; wt, wild type.

![Mass spectra](image)

Table 1: Comparison of relative quantification using DIGE and 14N/15N labeling combined with MS.

| Protein       | Set 1 | Set 2 |
|---------------|-------|-------|
| AtGSTF9       | 0.61  | 1.40  |
| AtGSTF2       | 3.18  | 1.70  |

DISCUSSION

To analyze changes in protein abundance occurring during early leaf senescence, we developed a comprehensive quantitative proteomics strategy comprising differential labeling of proteins from A. thaliana wt and old1–1 mutant plants using CyDyes and 14N/15N-isotopes followed by MS (Fig. 1). We used second-generation plants harvested after 16 days of growth. This time point was chosen because our focus was to identify alterations in protein abundance during early leaf senescence, i.e., the stage before visible symptoms of leaf senescence occur. In old1–1 mutants grown on solid medium, visible yellowing was not observed before day 17 (data not shown). We successfully demonstrated that full labeling with 15N, which has recently been reported for A. thaliana suspension cultures or plants grown in liquid culture (14–18), is equally feasible for entire A. thaliana plants grown on solid medium representing more natural growth conditions for this species. Following a labeling scheme spanning two generations, incorporation of 15N into the proteins of wt and old1–1 mutant plants amounted to 95%. Using distinct mixtures of protein extracts from 14N/15N-labeled wt plants, we evaluated...
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The simultaneous use of DIGE and 14N/15N labeling combined with MS provided us with two independent approaches, which allowed us not only to assess the applicability of metabolic 15N labeling for quantitative plant proteomics but also to directly compare the capacity of both methods. Reversed 14N/15N labeling enabled us to demonstrate that the labeling did not have any side effects on protein expression and development of *A. thaliana* plants. For this, we analyzed 21 protein spots showing no regulation based on DIGE and performed at least four independent experiments per set to gain statistically sound data. Multiplication of abundance ratios based on 14N/15N-labeled peptides calculated for proteins in Set 1 and Set 2 yielded an average value of 0.90 (± 0.27) (Fig. 3A), indicating high consistency between both data sets. Overall, MS-based quantification results confirmed the DIGE findings, i.e. that the proteins underlying the spots do not exhibit differences in abundance between wt and mutant plants. However, we could identify one protein, AtGSTF8, which consistently exhibited a regulation factor of approximately +2 in both sets that could not be detected by DIGE.

Our double and reverse labeling strategy resulted in the determination of 13 proteins and protein subunits that showed significant regulation in the *old1–1* mutant compared with *Arabidopsis* wt plants. All proteins showed consistent regulation in both sets based on either DIGE (Fig. 4A) or 14N/15N labeling and MS (Fig. 4B). In addition, average values of 1.09 (± 0.26) for DIGE and 1.09 (± 0.30) for quantitative MS across all proteins indicate a very good correlation between Set 1 and Set 2 for both quantification strategies. Statistical analyses did not reveal any outliers in the data set based on 14N/15N labeling (Fig. 4D), whereas two outliers were detected in the data set derived from DIGE (Fig. 4C). These were spots number 58 and 65 with average values of 1.83 and 1.65. Both spots exhibited diffuse spot borders, which may have introduced small errors leading to a poor correlation between both sets of experiments.

To assess the comparability of DIGE and 14N/15N labeling combined with MS for relative protein quantification, we divided the average abundance ratios of proteins determined by either method (note that only single protein-containing spots showing significant regulation were considered). The average values across all 20 protein spots were 0.99 (± 0.46) for Set 1 (Fig. 5A) and 1.40 (± 1.01) for Set 2 (Fig. 5B), indicating high consistency between both quantification methods. Statistical analyses revealed the presence of one outlier in Set 1, the protein quinone reductase in spot number 55, and two outliers in Set 2, namely AtGSTF7 in spot number 60 and AtGSTF2 in spot number 61. Both GST isoforms were consistently found to be highly regulated in all experiments (Table I). The average values determined for the individual proteins based on 14N/15N labeling, however, were 3- (AtGSTF2 in Set 2) to 8-fold (AtGSTF7 in Set 1) higher than those obtained by DIGE. This drift is more pronounced in Set 1, which is a reasonable explanation why these GSTs were classified as outliers in this data set. Interestingly, the tendency toward the determination of considerably higher regulation factors following MS-based quantification is true for all proteins in our study exhibiting regulation beyond the factor of ± 3 (Table I). Consequently, the correlation between DIGE and MS-based quantification is lower for proteins with regulation factors exceeding −3 and +3. Box plots visualize this observation by showing a skew to the lower quartile for Set 1 (Fig. 5C) and a skew to the upper quartile for Set 2 (Fig. 5D). Our results correspond to observations made by Kolkman et al. who evaluated the comparability of DIGE and 15N labeling combined with MS in a quantitative proteomic study of different yeast strains (23). They found a good correlation between both quantification techniques as long as the protein concentration ratios were within the range of −3 to +3, too. Beyond these margins, however, they also observed that MS provided higher values for protein concentration ratios. According to Huttlin et al. (16), MS-based quantification based on full metabolic labeling results in accurate values for regulation factors of ±12. To our knowledge, analogous data for DIGE experiments are not available. At present, it is therefore not possible to assess which quantification technique is more reliable and better suited for the determination of abundance ratios of proteins exhibiting rather extreme changes in abundance. Further studies are necessary to clarify this question. A promising approach to address this issue is the use of partial metabolic labeling. In a comparative study of full (> 98% 15N) versus partial metabolic labeling (5–6% 15N) of *A. thaliana* plants, Huttlin et al. demonstrated that the latter is superior for the analysis of proteins exhibiting large differences in protein abundance (16).

Fig. 6 demonstrates basic features of DIGE (Fig. 6A) and MS for relative protein quantification (Figs. 6B and C). While differences in protein abundance are typically immediately apparent following fluorescence image analysis and usually only regulated protein spots are subsequently identified by MS, stable isotope labeling combined with MS enables the global identification and quantification of proteins in the same experiment. Moreover, a general advantage of metabolic labeling and MS is the capability to quantify accurately different proteins comigrating during electrophoresis as illustrated for the proteins AtGSTF9 and AtGSTF2 both present in spot number 58 (Figs. 6B and C). MS-based quantification revealed that the two GST isoforms exhibited striking differences in abundance ratios; AtGSTF9 was ~2-fold up-regulated in *old1–1* mutant plants while AtGSTF2 exhibited a
regulation factor of roughly +20. Based on DIFE, however, only one value was obtained for both proteins, i.e. +1.7 in Set 1 and +3.1 in Set 2 (Table I).

We reliably identified five distinct proteins or protein subunits that were significantly down-regulated in old1–1 mutant plants, RuBisCO large subunit, RuBisCO small subunits 1A, 1B, and 2B as well as a protein related to major latex protein. RuBisCO is the key enzyme of the photosynthetic CO₂ fixation and consists of eight large and eight small subunits. It catalyzes the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in carbon dioxide fixation, as well as the oxidative fragmentation of the pentose substrate. Reduction in RuBisCO levels has been associated with senescence and its degradation may be triggered by reactive oxygen species (reviewed in (45)), such as ozone. In agreement with this idea, our results consistently showed an ~2-fold decrease of all RuBisCO subunits at a developmental stage (16 days) before symptoms of leaf senescence are visible. Against the background of extensive changes in the metabolism of senescing leaves for the purposes of nutrient retrieval, reduction in RuBisCO levels is a necessary consequence. Indeed, it has been reported that the earliest structural hallmark of leaf senescence is the disintegration of chloroplasts, which is accompanied by, among others, the progressive loss of RuBisCO on the biochemical level (19). A further protein with a 2- to 3-fold lower abundance in the old1–1 mutant was a protein related to major latex protein (MLP). MLPs were first isolated from the latex of opium poppy and have since been found to be present in a number of other plants and tissues, including Arabidopsis (46, 47). The general function of this protein family is still unknown; follow-up studies are necessary to clarify whether MLPs are of relevance for early leaf senescence.

Most of the proteins found to be highly up-regulated in old1–1 mutant plants belong to the large family of glutathione S-transferases, which are generally known as detoxifying enzymes. Among these GSTs (AtGSTF2, AtGSTF6, AtGSTF7, AtGSTF8, AtGSTF9), AtGSTF7 showed particularly high regulation factors of up to +36 (± 15.1%) in Set 2 and +50 (± 50%) in Set 1 (calculated based on ¹⁵N/¹⁴N labeling and MS analysis; Table I). AtGSTF2, which was identified in four distinct spots, was ~10- to 20-fold up-regulated while the regulation factors of the other GSTs were in the range of +2 to +10. In plants, GSTs are mainly cytosolic and homodimeric or heterodimeric proteins that were shown to be involved in stress tolerance as well as in the detoxification of herbicides, organic pollutants, and natural toxins (48). Plant GSTs are currently classified into six distinct classes (49), two of which, phi and tau, are plant-specific (50). These are typically up-regulated in response to biotic stress including osmotic stress, extreme temperatures, and infection or treatments that evoke plant defense reactions (24). There are reports about increased expression of several GSTs during leaf senescence (51–53). Interestingly, the isoforms found to be up-regulated in the A. thaliana old1–1 mutant in our study are all assigned to the phi class. Among those, only AtGSTF8 has been linked to leaf senescence yet (51). In general, the expression of GSTs of class phi was reported to be predominantly induced by oxidative and pathogen stress (54). It is of interest to note that investigations of the transcriptome of a further A. thaliana mutant also exhibiting an early leaf senescence phenotype, constitutive expressor of pathogenesis-related genes 5 (cpr5), which is allelic to old1–1 (22), revealed an increased expression of the genes encoding for the two isoforms that were highly up-regulated in old1–1 plants in our study, AtGSTF7 (36- to 50-fold) and AtGSTF2 (10- to 20-fold) (55). In light of these concordant observations in old1–1 and cpr5 mutants, one may reason that the old1–1 plants undergo oxidative stress in the early stages of leaf senescence. This conclusion is supported by reports showing that in A. thaliana the expression of AtGSTF6, which was found to be 5- to 10-fold up-regulated in our study, is induced by ozone (56). Ozone is generally known to trigger the biosynthesis of ethylene, a plant hormone playing a key role in leaf senescence. In carnation, elevated levels of ethylene lead to membrane degradation, lipid peroxidation, and the induction of GST genes. The role of GSTs in the context of leaf senescence is probably the protection of cells against reactive oxygen species generated by lipid degradation during the process of leaf senescence (reviewed in (24)). As mentioned above, earliest structural changes during leaf senescence consist in the disintegration of the chloroplast (19), an event that involves membrane and lipid degradation. For this reason, the presence of GSTs even in early stages of leaf senescence may be fundamental for the plant in order to maintain the functionality of the cells for efficient mobilization of the nutrients in the senescing leaf.

Serine hydroxymethyltransferase, enolase, and quinone reductase showed significant up-regulation in the old1–1 mutant, too. To our knowledge, neither of these proteins has been associated with leaf senescence so far. As for the protein related to MLP, further studies are required to assess their significance for early leaf senescence. Interestingly, it has been hypothesized that the flavoprotein quinone reductase may have a role in the protection of plant cells from oxidative damage (25). Thus, the finding that quinone reductase is 2- to 4-fold up-regulated in the old1–1 mutant supports our assumption that these plants experience oxidative stress during early leaf senescence.

In conclusion, our comparative, quantitative proteomics study of A. thaliana wt and old1–1 mutant plants resulted in the determination of 13 distinct proteins significantly regulated in the early leaf senescence mutant. The differential regulation of some of these proteins, such as RuBisCO large and small subunits as well as some of the GST isoforms and quinone reductase, fits well into the context of early leaf senescence that had not been studied in Arabidopsis in such an extensive manner on the level of proteins before. However,
our list of proteins with different abundance in both A. thaliana phenotypes probably constitutes only a fraction of all proteins that are differentially regulated during this complex stage of leaf development. The detection of differences in rather low abundant proteins was most likely impeded by the presence of highly abundant proteins such as RuBisCO.

A promising approach for a more detailed study of proteins differentially expressed during early leaf senescence is the focused analysis of distinct sub-proteomes by employing organellar fractionation techniques, for instance. In addition, metabolic $^{15}$N/$^{14}$N labeling combined with MS allows for gel-free quantitative proteomics studies facilitating the study of membrane proteins (15). Since the amino acid sequence of the OLD1 protein presumably contains an N-terminal putative nuclear localization signal and a C-terminal domain with five transmembrane regions (22, 57, 58), a quantitative organellar or membrane proteomics approach or a combination of both provide promising means to gain deeper insight into the regulation and function of this protein in (early) leaf senescence. In general, we believe that metabolic $^{15}$N labeling of entire A. thaliana plants combined with MS for both accurate relative quantification and reliable identification of proteins provides a universal tool for comparative proteomics studies addressing a vast array of biological questions.

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