Stable Isotope Labeling of *Arabidopsis thaliana* Cells and Quantitative Proteomics by Mass Spectrometry*

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Quantitative analysis of protein expression is an important tool for the examination of complex biological systems. Albeit its importance, quantitative proteomics is still a challenging task because of the high dynamic range of protein amounts in the cell and the variation in the physical properties of proteins. Stable isotope labeling by amino acids in cell culture (SILAC) has been successfully used in yeast and mammalian cells to measure relative protein abundance by mass spectrometry. Here we show for the first time that proteins from *Arabidopsis thaliana* cell cultures can be selectively isotope-labeled *in vivo* by growing cells in the presence of a single stable isotope-labeled amino acid. Among the tested amino acids ([13C6]-leucine, [13C6]arginine, and [6H4]lysine), [13C6]arginine proved to be the most suitable. Incorporation of [13C6]arginine into the proteome was homogeneous and reached efficiencies of about 80%. [13C6]Arginine-labeled *A. thaliana* suspension cells were used to study the regulation of glutathione S-transferase expression in response to abiotic stress caused by salicylic acid and to identify proteins that bind specifically to phosphorylated 14-3-3 binding motifs on synthesized bait peptides in affinity purification experiments. In conclusion, the combination of stable isotope labeling of plant cells and mass spectrometry is a powerful technology that can be applied to study complex biological processes that involve changes in protein expression such as cellular responses to various kinds of stress or activation of cell signaling. *Molecular & Cellular Proteomics* 4:1697–1709, 2005.

Protein expression in cells is highly dynamic, and the abundance and activity of specific proteins can vary greatly during development, growth, and differentiation as well as in response to biotic and abiotic stress. Therefore, it is of great biological interest to be able to quantitatively compare the subproteomes of different developmental stages or to quantitatively examine the activation of signaling pathways. The combination of stable isotope labeling, advanced multidimensional chromatography, and mass spectrometry enables the identification and quantitation of many hundred proteins in a single comparative experiment. In addition, mass spectrometry can be used to identify and quantitate changes in post-translational modifications, which often play important roles in protein function and regulation (1, 2). Thus, quantitative proteomics can contribute to our understanding of complex biological signal transduction processes.

Most quantitative proteomic strategies rely on the incorporation of stable isotopes into proteins or peptides, which are then compared with an unlabeled control sample by mass spectrometry (3). Relative protein abundance can be calculated from the intensities of normal versus isotope-labeled forms of the same tryptic peptides in the same mass spectrum. Isotope labeling of protein samples can be achieved either by chemical modification of proteins and tryptic peptides (4–7) or *in vivo* by metabolic labeling.

The first *in vivo* labeling studies used for protein quantitation by mass spectrometry relied on growth of cells in 15N-enriched medium (8, 9). This method has also been applied to label plants with 15N for analysis of proteins by NMR (10). However, the mass shift introduced by the incorporated 15N is different for each tryptic peptide depending on the length and amino acid composition. This complicates the subsequent analysis of the mass spectra as pairs of labeled and unlabeled peptides cannot easily be recognized and automatically quantified. Therefore, a different technique has been developed, termed stable isotope labeling of amino acids in cell culture (SILAC)† (11, 12), which relies on the growth of cells in a medium containing a single stable isotope-labeled amino acid that is incorporated into the proteome.

SILAC allows direct comparison of changes in protein expression between two closely related samples (11, 13). In contrast to chemical modifications of proteins or tryptic peptides, SILAC metabolically introduces a mass difference into a given proteome that directly allows distinguishing the SILAC proteome from the control proteome in the same combined analysis. This technology has successfully been applied to

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† The abbreviations used are: SILAC, stable isotope labeling by amino acids in cell culture; SA, salicylic acid; GST, glutathione S-transferase.
study the formation of signal-dependent protein complexes (14), to study modification-dependent protein-protein interactions (15), and to display the dynamics of signal-dependent phosphorylation events (16–18).

SILAC has been developed using mammalian cells in which full incorporation can easily be achieved using the essential amino acids as the metabolic label. Recently a double auxotrophic yeast strain was successfully metabolically labeled with lysine and arginine to study differential phosphorylation in the yeast pheromone pathway (17). So far, the SILAC technology seemed inapplicable to plant cells as they are autotrophic cells that can synthesize all amino acids from inorganic nitrogen. Thus, in plant biology, quantitative proteomic approaches so far mainly relied on chemical modification of proteins (e.g. ICAT) to study membrane lipid rafts (19) or used a two-dimensional gel approach with quantitation via spot intensity (20). Direct quantitation of intensities within the same mass spectrum, as it is done with SILAC, has the advantage of significantly reducing variation of chemical tagging and of giving better statistics on the quantitation due to the large set of uniformly labeled peptides. Here we describe the successful stable isotope labeling of plant suspension cell cultures. The SILAC plant cells were subsequently used to study (i) the induction of glutathione S-transferases in response to salicylic acid treatment and (ii) to identify specific interaction partners of peptides containing phosphoserine sequence motifs.

Salicylic acid (SA) is an important signaling molecule for the induction of systemic acquired resistance in plants (for a review, see Ref. 21). Plants respond to pathogenic attacks by bacteria, virus, or fungi with local necrosis, lignification, and the expression of pathogenesis-related proteins. These proteins function in protecting the plant against the invaders by inducing degradation of the pathogenic proteins or inducing apoptotic responses. Systemic acquired resistance primes the plant against further attack even in organs that were previously uninfected, and salicylic acid is the major signaling molecule inducing this response. In addition, SA is involved in the expression of genes induced by abiotic stresses such as ozone and salt (22, 23). One class of proteins with SA-dependent up-regulation in response to stress is GSTs.

GSTs constitute a family of ubiquitously expressed proteins found in aerobic organisms from bacteria to plants and human. They catalyze the conjugation of glutathione to electrophilic substrates and have been associated with a number of different functions including protection of cells against oxidative damage, detoxification of organic compounds and natural toxins, and the intracellular transport of anthocyanin (24, 25). Arabidopsis thaliana has 53 GST genes, which fall into different subclasses. Recently it has been reported that SA induces the expression of GST mRNA, which leads to up-regulation also at the protein level (26). GST protein abundance in SA-treated cells has been compared with control cells by Western blotting and densitometry of stained two-dimensional gel spots with subsequent identification of proteins by mass spectrometry. Here we used SILAC-labeled A. thaliana suspension cells to accurately quantify the changes in abundance of GST isoforms upon treatment with SA.

In cell signaling, phosphorylation events are key processes in the regulation of enzyme activities, and a large number of phosphopeptides have been identified from Arabidopsis membranes in a previous large scale study (27). In this study, we used a recently developed method for unbiased proteomic screening for modification-dependent peptide-protein interactions (15) to demonstrate the use of SILAC plant cells for the identification of phosphorylation-dependent interactions. This screen has been successfully carried out using phosphotyrosine motifs of mammalian receptor kinases (28), but it remained unclear whether the affinity of protein-protein interactions depending on phosphoserine motifs would be sufficient for detection. Therefore, here we applied this peptide-protein interaction screen to nitrate reductase and sucrose-phosphate synthase, two enzymes known to be regulated by serine phosphorylation and dephosphorylation. The inactive, phosphorylated forms of these enzymes are stabilized during the night period by interaction with 14-3-3-binding proteins (29–31). Thus, to validate the use of SILAC plant cells in unbiased phosphorylation-dependent interaction screening, the 14-3-3 protein binding motifs of the enzymes nitrate reductase and sucrose-phosphate synthase were used as bait peptides in comparative pull-down experiments to identify proteins specifically interacting with the phosphorylated bait peptide.

In this study, we describe the first successful metabolic labeling of plant cell cultures with specific amino acids. We believe that SILAC of plant cells and subsequent proteome analysis by mass spectrometry are powerful tools that enable studies of the dynamics of post-translational modifications, protein-protein interactions, and protein expression in a single experiment. Especially in plant cells, many key regulatory processes involving modifications and specific protein expression changes are still uncharacterized. In addition, SILAC can be applied to comparatively characterize protein expression in response to stress treatments or to the comparison of wild types and mutants.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

A. thaliana suspension cell cultures (ecotype Landsberg) were used in the SILAC labeling experiments. Cells were grown in flasks rotating on a shaker with 120 rpm at 20 °C under constant light from a 60-watt light bulb. Standard Murashige and Skoog growth medium was supplemented with 100 μg ml⁻¹ myo-inositol, 100 μg ml⁻¹ nicotinic acid, 1 μg ml⁻¹ pyridoxine HCl, 10 μg ml⁻¹ thiamine HCl, 0.5 μg ml⁻¹ 1-naphthaleneacetic acid, 0.05 μg ml⁻¹ benzylaminopurine, and the pH value was adjusted to pH 5.7 with KOH.

**SILAC Labeling**

For SILAC labeling, the Murashige and Skoog medium was supplemented with either l-3,3,3-(3H)leucine (Leu-d₃), l-[¹⁵N]arginine
(\[^{13}\text{C}_6\text{Arg}\]) or L-2,2,4,4-[^{2}\text{H}]lysine (Lys-d\(_4\)) at various concentrations. Leu-d\(_3\) was added at 105 μg ml\(^{-1}\) (800 μM), Lys-d\(_2\) was added at 73 μg ml\(^{-1}\) (333 μM), and \[^{13}\text{C}_6\text{Arg}\] was added at 80–320 μg ml\(^{-1}\) (380–1500 μM). For the initial tests, cell cultures were grown for the time periods indicated in the legends of Figs. 1 and 2. Culture medium was replaced after 4 days to ensure a high level of labeled stable isotope amino acids in the medium. Samples were taken for analysis of incorporated stable isotope label at different time intervals.

For the affinity purification of GSTs after SA treatment and the protein interaction screens, 2 × 10\(^{10}\) ml of Murashige and Skoog medium containing either 160 μg ml\(^{-1}\) \[^{13}\text{C}_6\text{Arg}\] or unmodified arginine were inoculated from a stationary A. \textit{thaliana} culture and grown for 4 days. 100 ml of fresh medium with 160 μg ml\(^{-1}\) \[^{13}\text{C}_6\text{Arg}\] or unmodified arginine, respectively, were added to each culture, and cells were grown for an additional 2 days. 150 ml of control and SILAC-labeled cell cultures were directly used for modification-dependent protein interaction screens. For SA treatment and GST affinity purification, 50 ml of SILAC-labeled cell cultures received 1 mM SA from a stock solution in ethanol, whereas the corresponding amount of ethanol was added to control cells. After 24 h, cells were harvested by centrifugation and frozen at −80 °C.

**Analysis of SILAC Labeling Efficiency**

To determine the labeling efficiency of the different amino acids and the optimal \[^{13}\text{C}_6\text{Arg}\] concentration (see Figs. 1 and 2), \textit{Arabidopsis} cells were harvested by centrifugation and resuspended in lysis buffer containing 8 mM urea, 100 mM H\(_4\)CO\(_3\), 1% SDS, 25 mM DTT, and protease inhibitors (Complete tablet, Roche Applied Science). Cells were broken mechanically and by multiple freeze-thaw cycles, and proteins were separated by one-dimensional SDS gel electrophoresis. Gel slices from the same molecular weight range were in-solution digested with 1 μg ml\(^{-1}\) \[^{13}\text{C}_6\text{Arg}\] (as described previously (17)) and analyzed by LC-MS/MS.

**Affinity Purification of GSTs**

Frozen cell pellets were resuspended in 10 ml of lysis buffer (137.93 mM NaCl, 8.06 mM Na\(_2\)HPO\(_4\), 2.67 mM KCl, 1.47 mM KH\(_2\)PO\(_4\), 0.5% Nonidet P-40, protease inhibitor mixture (Complete tablet, Roche Applied Science). The cells were broken by two freeze-thaw cycles and by multiple strokes with a tight fitting glass pistil in a homogenizer. Cell extracts were cleared by centrifugation for 15 min at 20,000 \(×\) g at 4 °C, and protein concentrations were determined with a Bradford assay. 20 mg of protein extracts from SILAC-labeled, SA-treated cells and control cells were mixed and rotated with 300 μl of equilibrated glutathione-Sepharose beads (Amersham Biosciences) for 18 h at 4 °C. The beads were washed with 10 ml of lysis buffer, twice with 10 ml of lysis buffer containing 0.05% Nonidet P-40, and once with 10 ml of lysis buffer without detergent and protease inhibitors. The beads were transferred into a spin column and washed with 800 μl of H\(_2\)O, and bound proteins were eluted twice with 200 μl of elution buffer (50 mM Tris, pH 8, 10 mM reduced glutathione). beads were incubated with elution buffer for 80 and 15 min, respectively. Both eluates were pooled and concentrated to a volume of 10 μl.

**Two-dimensional Gel Electrophoresis**

An IPG strip (11 cm, pH 4–7, Bio-Rad) was passively rehydrated overnight with 190 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.5% Triton X-100, 0.5% Ampholine 3.5–10, bromphenol blue) to which the affinity-purified GST protein sample had been added. Isoelectric focusing was performed on a Multiphor instrument (Amersham Biosciences) for 2 h each at 100, 200, 400, 600, 1000, and 2000 V followed by 12 h at 3500 V. To reduce and alkylate proteins, the IPG strip was incubated twice for 15 min with equilibration buffer (6 mM urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8, bromphenol blue) containing first 10 μg ml\(^{-1}\) DTT and subsequently 40 μg ml\(^{-1}\) iodoacetamide. Gel electrophoresis in the second dimension was performed with Criterion 12% Tri-HCl gels (Bio-Rad) according to the manufacturer’s instructions. Protein spots were visualized by silver staining and excised from the gel, and peptides were generated by digestion with trypsin. The supernatants of the in-gel digestions were desalted with reversed phase microcolumns and used for analysis by LC-MS.

**Modification-dependent Protein Interaction Screens**

Cell cultures were collected by centrifugation and resuspended in ice-cold homogenization buffer (250 mM sucrose, 100 mM HEPES/KOH, pH 7.5, 10 mM EDTA, 1% Nonidet P-40 with addition of protease inhibitor mixture (Complete tablet, Roche Applied Science), 50 mM sodium pyrophosphate, 25 mM sodium fluoride, and 1 mM sodium molybdate as phosphatase inhibitors. The cells were then broken up by multiple strokes in a Dounce homogenizer and by freeze-thaw cycles. The yield of protein concentration was 2–3 mg ml\(^{-1}\).

Desthiobiotinylated peptides were synthesized on a solid-phase peptide synthesizer using amide resin (Intavis) as described previously (15). Peptides were synthesized as pairs in an “active” (i.e. phosphorylated) and “control” (non-phosphorylated) form. For affinity pull-downs, 10 nmol of immobilized peptide were added to an average of 2 mg of cell extract. Equal amounts of protein from SILAC-encoded and control cells were incubated with the respective immobilized peptides at 4 °C for 4 h. After six rounds of washing with lysis buffer, beads of pull-down pairs with control and “phosphorylated” were combined, and bound proteins were eluted using 20 μM biotin. Eluted proteins were precipitated and subsequently digested with trypsin for LC-MS/MS.

**LC-MS/MS Analysis and Quantitation**

Salicylic Acid Stimulation and GST Analysis—Nano-HPLC-MS/MS analysis of GSTs was performed on an LC Packings system (Ultimate; Switchos2; Famos; LC Packings, Amsterdam, The Netherlands) coupled to a Q-TOF Ultima mass spectrometer (Micromass UK Ltd., Manchester, UK). The mass spectrometer was operated in the positive ion mode, and parent ions were selected for fragmentation by data-dependent analysis (three most abundant ions in each cycle): 1-s MS (m/z 350–1500) and maximum 2-s MS/MS (m/z 50–2000, continuum mode), 30-s dynamic exclusion. A charge state recognition algorithm was used to determine optimal collision energy for low energy CID MS/MS of peptide ions. The LC-MS/MS data were searched by VEMS version 3.0 (yas.sdu.dk) (32) against a database of non-redundant GST proteins from A. \textit{thaliana} generated from the National Center for Biotechnology Information (NCBI) database. For protein quantitation, individual LC-MS runs were combined, and integrated peak intensity ratios of SILAC pairs from unique arginine-containing peptides were determined. Values were corrected by VEMS for the incorporation efficiency of 75% \[^{13}\text{C}_6\text{Arg}\].

Peptide Pull-downs—Eluted proteins from peptide pull-downs were in-solution digested with 1 μg of trypsin after reduction in 1 μg of DTT, alkylation with 5 μg of iodoacetamide, and dilution of the sample with 4 volumes of 50 mM NH\(_4\)HCO\(_3\). Tryptic peptide mixtures were then desalted on STAGE tips (33) and loaded onto reversed phase analytical columns for liquid chromatography (34). Peptides were eluted from the analytical column by a multistep linear gradient.
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Running from 5 to 30% acetonitrile in 90 min and sprayed directly into the orifice of a QSTAR-Pulsar quadrupole time-of-flight hybrid mass spectrometer (PE-Sciex). Proteins were identified by MS/MS by information-dependent acquisition of fragmentation spectra of multiply charged peptides that were then searched against a non-redundant version of the NCBI database using the Mascot algorithm (35) with search parameters as described previously (36). For quantitation and spectra validation, the open source software MSQuant (msquant-sourceforge.net) was used. Ratios were calculated from the average of all quantified peptides of a single protein. A protein was defined as a significant interaction partner if the average ratio of that protein was significantly (p < 0.05) higher than the average variation of all quantified proteins.

RESULTS

Within the last few years, stable isotope labeling has been used to quantify protein concentrations and relative expression levels by mass spectrometry. One of these approaches, termed SILAC, uses the replacement of specific amino acids by stable isotope-labeled counterparts in the cell culture medium. Cells grown under such conditions will incorporate the isotopic marker into their proteins, leading to an increase in their protein masses accordingly. Thus, in a mixture of tryptic peptides from protein extracts of isotopically labeled and unlabeled control proteins, the mass difference introduced by the labeled amino acid allows relative quantitation of intensities of the two forms in the same mass spectrum. Relative abundance levels then can be deduced from the intensity ratios of the labeled and unlabeled tryptic peptides.

SILAC of Plant Cells—In contrast to mammalian cells, plant cells can synthesize all amino acids and therefore might not incorporate the exogenously supplied labeled amino acids efficiently. On the other hand, it has been described that most of the amino acid biosynthesis pathways are feedback-regulated and that high concentrations of the respective amino acids lead to a down-regulation of their synthesis (37). Therefore, in a first experiment the incorporation efficiencies of different amino acids were tested. Leucine was chosen as it is the most abundant amino acid in proteins. Arginine and lysine were chosen as they occur as terminal amino acids in peptides after digestion with trypsin. Tryptic peptides have inherent properties that make them well suited for sequencing by tandem mass spectrometry.

A. thaliana suspension cells were grown in the presence of either [13C6]Arg, [2H3]leucine (Leu-d3), or [2H4]lysine (Lys-d4) for up to 8 days, and proteins were subsequently analyzed by gel electrophoresis and LC-MS/MS. Ratios of tryptic peptides from ribulose-bisphosphate carboxylase large chain precursor were compared between the differently labeled cell cultures (Fig. 1A). For cell cultures labeled with [13C6]Arg or Leu-d3, the amino acid incorporation increased over time up to about 70% after 8 days. In the Lys-d4-labeled cell culture, there was no significant increase in labeling efficiency after 4 days, indicating that there is an equilibrium between synthesis of lysine and use of Lys-d4 from the medium (Fig. 1A). The overall labeling efficiency of all (n = 17 for [13C6]Arg, 16 for Leu-d3, and 14 for Lys-d4) proteins that could be quantified from the one excised gel band of each sample showed only little variation, indicating that the isotopically distinct amino acids are incorporated specifically and uniformly into proteins (Fig. 1B and supplemental information).

Among the three tested amino acids, [13C6]Arg and Lys-d4 are especially suitable for subsequent mass spectrometric analysis because trypsin cleaves C-terminally of arginine and lysine, leading to a high efficiency in the detection and sequencing of labeled tryptic peptides. Thus, [13C6]Arg was used for further experiments due to its better incorporation efficiency.

For the mass spectrometric analysis of differential protein expression using SILAC, close to complete incorporation of labeled amino acids into proteins is necessary. Therefore, it was tested whether higher concentrations of [13C6]Arg could shift the equilibrium between synthesis of unmodified arginine and use of [13C6]Arg supplied through the growth medium toward complete labeling of proteins. Arabidopsis suspension cells were grown in the presence of 80, 160, or 320 µg ml⁻¹ [13C6]Arg (380, 760, and 1500 µM, respectively) for up to 7 days. Cell morphology and growth did not change detectably at higher arginine concentrations. Stable isotope labeling of proteins was assessed by gel electrophoresis and LC-MS/MS (Fig. 2). Increasing the [13C6]Arg concentration from 80 to 160 µg ml⁻¹ led to a higher proportion of isotope incorporation, which is exemplified by a peptide from Hsc70 (Fig. 2A). The maximal labeling efficiency reached about 80% after 5 days in culture. Further increase in arginine concentration to 320 µg ml⁻¹ or growth of cells for a total of 7 days did not significantly change this ratio.

Another important factor for the successful establishment of SILAC in a new cell system is the uniform labeling of all proteins. Therefore, the same set of quantified proteins (n = 53) was examined for their isotope incorporation from cells grown for 5 days at different concentrations of [13C6]Arg. Several protein bands were excised from a gel and analyzed by LC-MS/MS (Fig. 2B). Quantitation of MS spectra confirmed that SILAC labeling was uniform for all tested proteins at all concentrations of [13C6]Arg, averaging around 71 ± 6.5% for 80 µg ml⁻¹, 80 ± 5.2% for 160 µg ml⁻¹, and 81 ± 6.9% for 320 µg ml⁻¹ [13C6]Arg (Fig. 2B). The quantitated proteins belonged to a variety of functional classes, including heat shock proteins, ribosomal proteins, and metabolic enzymes, and localize to different cellular subcompartments (see supplementary information for protein list). This indicates that incorporation of [13C6]Arg occurs homogeneously within the proteome. Thus, 160 µg ml⁻¹ of [13C6]Arg was sufficient for an average of 80% incorporation, and this concentration was used for further comparative experiments.

Application 1: Analysis of GST Expression in Response to SA—For experiments of quantitative comparison of two samples, a control cell culture is grown in unlabeled medium, and one is grown in the presence of [13C6]Arg. The isotope-labeled
FIG. 1. Stable isotope labeling of Arabidopsis suspension cells with different amino acids. A, incorporation rate of either Leu-\textsuperscript{d3}, \textsuperscript{13}C\textsubscript{6}\textsuperscript{Arg}, or Lys-\textsuperscript{d4} after 4 and 8 days in culture. Mass spectra are shown of peptides DTDILAAFR (m/z 511.19, unlabeled) and EITFNFTPIDKLDGQE (m/z 933.94, unlabeled) stemming from ribulose-bisphosphate carboxylase. B, average labeling efficiency (percentage of heavy form of the tryptic peptide) of identified proteins with Leu-\textsuperscript{d3}, \textsuperscript{13}C\textsubscript{6}\textsuperscript{Arg}, and Lys-\textsuperscript{d4}. 
cells are then treated differently from the control cells depending on the aim of the experiment (e.g. by stressing the cells, inducing signaling events, etc.). Equal amounts of protein extracts from both samples are mixed together and used for analysis by mass spectrometry. Changes in protein abundance in response to the differential treatment of the two cultures can be detected and quantified by intensity differences between isotope-labeled and unlabeled peaks of the SILAC peptide pairs. As an example for this type of quantitative analysis, we chose to study the expression of glutathione S-transferases upon treatment of cells with SA.

Plant GSTs constitute a large protein family with primary functions in detoxification and stress response. In A. thaliana, 53 genes encoding for GSTs have been found, some of which

**Fig. 2.** Labeling efficiency of *Arabidopsis* cells with different concentrations of $[^{13}C_6]$Arg. A, mass spectra of the peptide ATAGDTHLGGEDFDNR (m/z 838.38) of Hsc70.1p after labeling with different concentrations of $[^{13}C_6]$Arg for up to 7 days. B, average and S.D. of the labeling efficiency of more than 50 proteins after 5 days.
are regulated by various stress responses (20, 25, 26, 38, 39). For example, SA induced the expression of at least four distinct GSTs at the protein level (26) and led to transcriptional up-regulation of a number of additional GST genes (38). We expected that the SILAC approach would allow us to quantitate changes in protein expression with a higher level of confidence than in previous experiments, and in addition, new isoforms of SA-regulated GSTs might be found.

A. thaliana suspension cells labeled with [13C6]Arg were incubated for 24 h with 1 mM SA. Equal amounts of proteins extracts from [13C6]Arg-labeled, SA-treated and unlabeled, untreated control cells were mixed, and GSTs were isolated on immobilized glutathione. Purified GST proteins were separated by two-dimensional gel electrophoresis (Fig. 3). The majority of proteins were observed in the range between 20 and 30 kDa, which is in good agreement with the expected molecular masses of GSTs. Protein spots from this region of the gel were excised and digested with trypsin, and peptides were analyzed by nanoflow LC-MS. A total of 11 different GSTs were identified from 23 spots of the two-dimensional gel (Table I). Most GSTs were identified from at least two spots, supporting the notion that GSTs undergo extensive post-translational modification (26).

Three members of the GST family, GSTF8, GSTF10, and GSTU19, could be detected in more than eight spots each, indicating a large degree of molecular heterogeneity. In addition, GSTF8 and GSTU19 were in many cases detected as the proteins with the highest score in a particular gel spot (Table I, marked in bold numbers), indicating that they are highly expressed in the Arabidopsis suspension cells. It has to be noted that the sensitive mass spectrometry analysis used allows the detection of peptides in the low femtomole range. This sensitivity would be sufficient to detect traces of proteins, which because of their high amounts were not completely resolved during isoelectric focusing. The identified GSTs belonged to the Phi and Tau subfamilies in accordance with earlier findings that Zeta, Theta, and dehydroascorbate reductase class GSTs do not bind to glutathione-Sepharose (40).

Because Arabidopsis cells do not incorporate [13C6]arginine completely, it is necessary to calculate a correction factor to obtain correct expression ratios. Therefore, an aliquot of SILAC-encoded protein extract from cells that had not been treated with SA was digested with trypsin and analyzed by LC-MS. Quantitation of the identified peptides yielded an average incorporation of [13C6]Arg of 75%. This value was used to normalize the relative GST protein expression.

Changes in protein abundance of the same GST isoforms detected in different spots on the two-dimensional gel lay within the experimental variation, indicating that SA treatment did not influence the post-translational modifications of individual GSTs. Therefore, protein quantitation was performed on the combined peptide mass spectra from all protein spots. The ratio of labeled to unlabeled peak intensity was calculated for all detected unique arginine-containing peptides. Examples for mass spectra and tandem MS fragmentation spectra of peptides from GSTs with different changes in expression are shown in Fig. 3, B–D. It has to be noted that these spectra are taken directly from the MS data files and that peak intensities therefore are not corrected for the incomplete incorporation of [13C6]Arg. This is apparent for the spectra in Fig. 3C where the heavy and light peaks appear to have similar intensity. However, applying the correction factor, the ratio between heavy and light peak was calculated to be 1.94. For protein quantitation, the average ratio was calculated from all peptides for individual GSTs.

Three of the identified GSTs, GSTF8, GSTU24, and GSTU27 (Fig. 3D), showed evidence of a strong increase in response to SA (3.5-, 4.7-, and 5.6-fold, respectively) (Table I). GSTU7 and GSTU19 were moderately up-regulated (2.0- and 1.7-fold, respectively), whereas the other isoforms did not change significantly or were even slightly reduced (GSTF9 and GSTF10, both 0.6-fold). Protein amounts of GSTF8, GSTU19, and GSTU24 were also found to be induced by SA in the study of Sappl et al. (26), corroborating the effectiveness of our SILAC approach.

A previous study investigating the mRNA levels of a subset of GSTs in Arabidopsis plants found that GSTF10, GSTF9, and GSTU5 were among the most highly expressed GSTs and did not change significantly in response to various stress conditions (38). Our data confirm this observation because protein expression levels of these GSTs also were reduced or stayed constant after incubation with SA (0.6-, 0.6-, and 1.0-fold changes for GSTF9, GSTF10, and GSTU5, respectively).

Some members of the Arabidopsis GST family are very similar, thereby aggravating sequence-based identification by mass spectrometry. Examples are GSTF6 and GSTF7, which are 93% identical in their amino acid sequence and in addition contain only seven arginines. Therefore, a theoretical digest of the proteins with trypsin yields only two arginine-containing peptides that differentiate the two proteins (assuming standard cleavage behavior of trypsin and taking into account that leucine and isoleucine cannot be distinguished in our MS analysis). Nevertheless one unique arginine-containing peptide and a few more lysine-containing peptides were found for GSTF6 and GSTF7, demonstrating the ability of mass spectrometry to discriminate between close relatives. Protein quantitation in these cases relies only on one peptide. Likewise GSTF2 is 92% identical to GSTF3 on the protein level with only one unique arginine-containing peptide for each protein.

The results obtained here confirm previous findings that SA leads to an increase in protein abundance of a subset of GSTs although to a different extent. Individual GSTs possess different enzymatic affinities for a number of xenobiotic substrates (38) and are induced by a large number of different stress factors. However, there is relatively little known about the physiological roles of individual members of the GST family. It
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(A) Image showing a gel with numbered peaks.

(B) MS-spectrum of RAVVTLVEK, with fragmentation spectra of unlabeled and labeled peptides.

(C) MS-spectrum of VTEFVSELVR, with fragmentation spectra of unlabeled and labeled peptides.

(D) MS-spectrum of CLTRPA/SK, with fragmentation spectra of unlabeled and labeled peptides.

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Table I

| Name     | Gene accession number | Protein accession number | Identified in spot | Unique peptides | Ratio H/L | Deviation |
|----------|-----------------------|--------------------------|--------------------|----------------|-----------|-----------|
| GSTF2    | At4g02520             | gi:15235401              | 15, 17, 18, 19     | 1              | 1.4       | 2.69      |
| GSTF6    | At1g02930             | gi:15218640              | 14, 16             | 1              | 0.8       | NA        |
| GSTF7    | At1g02920             | gi:15218639              | 21, 23             | 1              | 1.4       | 3.01      |
| GSTF8    | At2g47730             | gi:30690772              | 10, 11, 12, 13, 14, 16, 19, 20, 22 | 5 | 3.5 | 4.72 |
| GSTF9    | At2g30860             | gi:15224581              | 7, 15, 19, 22, 23  | 5              | 0.6       | 4.77      |
| GSTF10   | At2g30870             | gi:15224582              | 7, 8, 9, 10, 11, 12, 13, 14, 15, 22 | 5 | 0.6 | 4.81 |
| GSTU5    | At2g29450             | gi:15227085              | 3, 6, 7           | 3              | 1.0       | 4.61      |
| GSTU7    | At2g29420             | gi:15227082              | 12, 22            | 2              | 2.0       | 3.76      |
| GSTU19   | At1g78380             | gi:18411929              | 3, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 | 8 | 1.7 | 3.88 |
| GSTU24   | At1g17170             | gi:15220040              | 2, 3, 4, 5, 6, 7, 8 | 2 | 4.7 | 2.66 |
| GSTU27   | At3g43800             | gi:15229782              | 5                 | 2              | 5.6       | 2.52      |

Protein spots of GSTs purified from a mixture of SILAC-encoded, SA-treated, and control cells were excised from a two-dimensional gel, proteolyzed with trypsin, and analyzed by LC-MS. Combined mass spectra from all spots were searched against a non-redundant database of A. thaliana GST proteins and subsequently quantitated with VEMS. Protein names are according to nomenclature by Wagner et al. (38); accession numbers are from GenBank™. Spot numbers are marked in bold for the top scoring protein. Number of unique peptides includes only arginine-containing peptides that have been used for quantitation. Modified forms of the same peptide have been counted only once. Note that the same peptide generally has been identified several times (in different spots or with modifications). A H/L (H, heavy, isotope-labeled peptide; L, light; unmodified peptide) ratio greater than 1 indicates increased protein amounts upon incubation of cells with SA. The deviation is given for all quantified peptides for a protein (including multiple identifications of the same peptide). NA, not applicable.

Application 2: Detection of Phosphorylation-dependent Protein-Protein Interactions—Modification-dependent protein-protein interactions between domains and characteristic peptide motifs are an important organizing principle of signal transduction pathways. For example, certain phosphotyrosine-containing peptide sequences mediate specific interactions with Src homology 2 or phosphotyrosine-binding domains (41–43). Consensus peptide sequences involved in such peptide motif interactions have been well studied using peptide libraries or phage display (44, 45). However, most of the methods for interaction screening, such as two-hybrid systems, lack specificity for modification-dependent interactions.

In plant cells, 14-3-3 proteins have been well studied as regulators of protein activity, and their binding to proteins is dependent on a specific motif around a phosphoserine residue (29, 46, 47). Therefore, the 14-3-3 binding motifs of nitrate reductase and sucrose-phosphate synthase were used as bait peptides in an interaction screen designed to identify specific interaction partners to phosphorylation motifs.

When phosphorylated and unphosphorylated bait peptides of the 14-3-3 binding site of serine 534 of nitrate reductase (PTLKRTASTPFMN) were used as bait in affinity pull-downs of intact proteins from plant cell lysates, 14-3-3 protein GF14 was identified via sequencing of three peptides (Table II). However, differentiation of 14-3-3 protein isoforms was not possible with this set of tryptic peptides. The peptide with C-terminal arginine showed high ratios of the peak intensities of the [13C6]Arg form versus the normal arginine-containing form, consistent with a specific interaction of 14-3-3 proteins with the serine-phosphorylated form of the bait peptide (Table II). The 14-3-3 protein was the only protein of 98 identified proteins that showed a ratio significantly exceeding the 3 s threshold of the average ratios (1.92 ± 0.95) of all identified proteins (Fig. 4A). In pull-down experiments in which phosphorylated and non-phosphorylated bait peptides with a mutated 14-3-3 binding site (PTLKAAASAFMN) were used, no 14-3-3-binding proteins were identified (Fig. 4B), and no tryptic peptides showed significantly different ratios from average (1.44 ± 0.3). Using phosphorylated and non-phosphorylated bait peptides bearing the 14-3-3 binding site of sucrose-phosphate synthase (DLLTRQVSAPGV), a 14-3-3 protein was again found as the only protein with a ratio significantly different from average (1.37 ± 0.41), indicating significant and specific interaction with the phosphorylated form of the bait peptide around phosphoserine 229 of sucrose-phosphate synthase (Fig. 4C). Thus, stable isotope labeling can efficiently
be used to determine specific versus nonspecific binding partners of peptide motifs.

**DISCUSSION**

In this study we successfully demonstrated that metabolic labeling by amino acids is possible for plant cells and that good results were achieved with \[^{13}\text{C}_6\]arginine, which we demonstrated in two application studies involving (i) a comparative study of protein expression and (ii) a modification-dependent interaction screen using affinity pull-downs.

**Labeling Efficiency between the Different Amino Acids with Respect to Their Synthesis Pathway**—Lysine biosynthesis in plants involves a branched pathway using aspartic acid as a precursor. Despite the importance of lysine in human and agricultural animal nutrition, the pathway of lysine synthesis has not been fully elucidated except for the first and last steps. The committing enzyme is dihydrodipicolinate synthase (DHDPDS) (37, 48). In plants, dihydrodipicolinate synthase activity is very sensitive to lysine inhibition (\(I_{50} = 10\text{–}50 \mu\text{M}\)), suggesting that lysine biosynthesis should have been fully inhibited at the concentration supplied to the medium in our experiments. However, supplement of high concentrations of lysine in the medium can be toxic due to inhibition of aspartate kinase. This enzyme catalyzes not only the first step in lysine biosynthesis but also in the synthesis of threonine and methionine. Thus, high extracellular amounts of lysine or threonine can cause deficiency in other amino acids, especially methionine. However, in our experiments, we did not observe any morphological changes and toxic effects on the cultured cells.

Likewise leucine biosynthesis in plants is largely unexplored, but the available evidence indicates that plants use the same pathway found in microorganisms (49). The precursor is pyruvate, and the committing enzyme is 2-isopropylmalate synthase. This enzyme is feedback-inhibited by micromolar concentrations of leucine. Thus, the concentration of leucine added to the medium in our experiments was sufficient to induce feedback inhibition, and possibly full incorporation could have been achieved with higher leucine concentrations. However, this has not been pursued here as arginine labeling proved to be the better strategy for mass spectrometric applications.

Arginine is synthesized from glutamate via ornithine and citrulline. It can be converted to polyamines (putrescine, spermidine, and spermine), and also conversion to proline has been observed in mammalian cells. Our results indicate that arginine supplied from the external medium will readily and uniformly be incorporated into proteins and that the degree of incorporation is concentration-dependent.

**Labeling Efficiency of Different Proteins**—A prerequisite for using metabolic labeling for quantitation of protein abundance is that the incorporation occurs uniformly throughout all proteins. In the experiments carried out here, the highest average incorporation rate of \[^{13}\text{C}_6\]Arg was 80%. The average incorporation rate of an individual experiment was used to calculate a correction factor and to normalize the calculated ratios between labeled and unlabeled forms of the same tryptic peptides. This correction is very important if protein abundance is displayed as a ratio between labeled and unlabeled peak intensities as has been done in the study of GST protein abundance in response to SA treatment. In contrast, the peptide pull-down experiments aim at identification of those interaction partners that bind significantly more strongly to the bait peptide than to the unphosphorylated control peptide. In this case, the differences in \[^{13}\text{C}_6\]Arg to \[^{12}\text{C}_6\]Arg ratio of the tryptic peptides of specific interaction partners versus unspecific binding partners were compared. Thus, accurate normalization was not required.

**Quantitative Analysis of GST Abundance in Response to SA**—In this study, SILAC of Arabidopsis suspension cells was used to quantitatively examine the effect of SA treatment on the changes in abundance of GSTs. In a previous study using Western blotting and densitometry of Coomassie-stained two-dimensional gels to examine expression changes of GSTs in response to SA (26), it was shown that GSTF7 and GSTF8 were strongly (20-fold) induced by SA, and GSTF10, GSTU19, and GSTU24 were induced to a lesser degree (2-fold). However, because GSTU19 and GSTF10 co-migrated in the two-dimensional gel, it was not possible to determine conclusively whether the expression of GSTF10 was indeed increased. With the SILAC approach, relative expression levels can also be determined when proteins co-migrate in the
same gel spot as individual tryptic peptides are measured by mass spectrometry, and each peptide is quantitated separately (Fig. 3, B and C; both spectra are from spot 10). This allowed us to distinguish between expression levels of GSTF8, GSTF10, and GSTU19, which co-migrate to a large extent. The differences in SA-induced changes between the isoforms were found in several spots in which these enzymes co-migrated, demonstrating the robustness of quantitation with SILAC. We found that expression of GSTF10 decreased (0.6-fold), whereas GSTU19 increased moderately (1.7-fold).

Three GSTs, GSTF8, GSTU24, and GSTU27, were strongly up-regulated by SA (>3-fold) in our data set. Up-regulation of GSTU27 has to our knowledge not been reported before. Expression of GSTF8 and GSTU24 was also found to be induced by SA previously (26), although at different ratios (>20- and >2-fold, respectively), which could be a consequence of different experimental conditions or be due to the relatively low linear range of densitometry of Coomassie-stained proteins. SILAC combined with mass spectrometry enables highly accurate quantitation over a wide range of ratios of SILAC pairs and in addition combines sequence information with quantitative data (13). It allows for the unbiased quantitation of a high number of proteins in one analysis making it one of the prime methods for quantitative proteomics.

Because of the high sequence homology between some members of the Arabidopsis GST family, only a limited number of unique arginine-containing peptides exist for some proteins (e.g. only one peptide for GSTF2). Nevertheless all identified GSTs could be quantitated with at least one unique peptide, demonstrating the high sensitivity of our mass spectrometric analysis. Further improvements of SILAC in Arabidopsis cells could therefore include the double labeling of cells with $^{13}$C-Lys and $^{13}$C-Arg to enable the quantitation of all tryptic peptides. This would increase the number of unique peptides that can be used for quantitation and thereby increase the statistical confidence of the analysis.

SILAC in Combination with Proteomic Interaction Screens—
The peptide-protein interactions screen has been successfully applied previously to the characterization of the interactome of mammalian receptor kinases (15, 28). Here the same principle was applied to validate the proteomic peptide-protein interaction screen also for phosphoserine-based interactions in a plant system. The results are convincing in that SILAC labeling allowed discrimination of specific from unspecific binding partners in pull-down experiments also using plant cell cultures. Of 72–105 proteins identified in the pull-downs using the 14-3-3 binding site of nitrate reductase, sucrose-phosphate synthase, and a mutated form of the nitrate reductase binding site, only one protein significantly met the criteria set for significant interaction partners. Therefore, future applications in large scale interaction screening involving affinity pull-down screens can become a significant method for unbiased proteomic modification-dependent interaction screens also in plant science.

Conclusions—In this work, we show for the first time that specific in vivo labeling of plant cells is possible with single
Quantitative Proteomics of *Arabidopsis* by MS

among the tested amino acids, arginine proved to be the most suitable one as it is a favorable amino acid for mass spectrometric analysis of tryptic peptides, and a high rate of incorporation could be achieved. We believe that the SILAC technique will be very useful for comparative experiments that can be carried out using plant cell cultures; studies of stress responses, interaction screening, cell signaling events, and comparison of wild types and mutants are conceivable applications. The advantage of SILAC over other quantitative proteomic approaches (chemical modifications and imaging) lies in the efficient labeling of tryptic peptides by using arginine (or lysine) as a label and in the direct comparison of the two isotopic forms within the same mass spectrum. Thereby highly accurate and sensitive quantitations are possible.

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