Protein Abundance Changes and Ubiquitylation Targets Identified after Inhibition of the Proteasome with Syringolin A*

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As proteins are the main effectors inside cells, their levels need to be tightly regulated. This is partly achieved by specific protein degradation via the Ubiquitin-26S proteasome system (UPS). In plants, an exceptionally high number of proteins are involved in Ubiquitin-26S proteasome system-mediated protein degradation and it is known to regulate most, if not all, important cellular processes. Here, we investigated the response to the inhibition of the proteasome at the protein level treating leaves with the specific inhibitor Syringolin A (SylA) in a daytime specific manner and found 109 accumulated and 140 decreased proteins. The patterns of protein level changes indicate that the accumulating proteins cause proteotoxic stress that triggers various responses. Comparing protein level changes in SylA treated with those in a transgenic line over-expressing a mutated ubiquitin unable to form polyubiquitylated proteins produced little overlap pointing to different response pathways. To distinguish between direct and indirect targets of the UPS we also enriched and identified ubiquitylated proteins after inhibition of the proteasome, revealing a total of 1791 ubiquitylated proteins in leaves and roots and 1209 that were uniquely identified in our study. The comparison of the ubiquitylated proteins with those changing in abundance after SylA-mediated inhibition of the proteasome confirmed the complexity of the response and revealed that some proteins are regulated both at transcriptional and post-transcriptional level. For the ubiquitylated proteins that accumulate in the cytoplasm but are targeted to the plastid or the mitochondrion, we often found peptides in their target sequences, demonstrating that the UPS is involved in controlling organelar protein levels. Attempts to identify the sites of ubiquitylation revealed that the specific properties of this post-translational modification can lead to incorrect peptide spectrum assignments in complex peptide mixtures in which only a small fraction of peptides is expected to carry the ubiquitin footprint. This was confirmed with measurements of synthetically produced peptides and calculating the similarities between the different spectra. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.036269, 1523–1536, 2014.

The dynamics of protein abundance and activity that drive biological processes is regulated at different levels, including transcription, translation, post-translational modification and degradation. However, research in various organisms has also revealed that protein dynamics is often partly decoupled from RNA expression levels (e.g. recent data in (1–6)). In Arabidopsis, especially diurnal transcript level fluctuations were not matched by corresponding changes in the detected proteome (7). In addition to the protein synthesis rate, protein abundance levels are also determined by the rate of protein degradation. For example, in human cells the combined contribution of sequence features related to translation and protein degradation was estimated to explain two thirds of protein abundance variation (4). Protein degradation adjusts protein levels in response to internal or external stimuli, but also in steady-state conditions the protein pool is dynamic and most cellular proteins are continuously synthesized and degraded (8).

Controlled and selective protein degradation is mainly achieved through the action of the ubiquitin-26S proteasome system (UPS). As targeted protein degradation by the UPS allows for fast down-regulation of protein levels, it has evolved as an important biological control mechanism especially targeting central regulators of hormone signaling, chromatin structure, transcription, biotic response pathways, morphogenesis, and circadian rhythm (9–12). The 26S proteasome is a 2.5 MDa ATP-dependent protease complex composed of a 20S core protease (CP) and two 19S regulatory particles (RP), each of which contains a lid and a base subunit. Degradation through the UPS begins with the ligation of one or

* The abbreviations used are: UPS, Ubiquitin-26S proteasome system; DEX, Dexamethasone; ED, End of the day; EN, End of the night; RP, 19S Regulatory Particle; SylA, Syringolin A; UBA, Ubiquitin-binding domain.
more ubiquitin proteins to specific target proteins through E1, E2, and E3 enzymes. Polyubiquitination with K48-linkages targets proteins for degradation to the 26S proteasome. After recognition of the ubiquitylated target protein by the proteasome, the polyubiquitin chain is cleaved off and released, whereas the target protein is threaded into the CP and cleaved into small peptides. In Arabidopsis, it is estimated that about 6% of all encoded proteins are involved in the UPS (10). This large number of around 1600 proteins comprises the ubiquitin proteins, the subunits of the proteasome complex, the E1, E2, and E3 ligases, and the deubiquitylating enzymes. Especially the E3 ligases constitute a very large group of around 1400 different proteins that define substrate specificity and attachment of ubiquitin moieties. The specificities of most E3 ligases is still unknown, but based on their large number it can be expected that in plants many proteins are targets for ubiquitylation.

In the ubiquitylation reaction the highly conserved 76 amino acid long ubiquitin is covalently attached to the target protein by an isopeptide bond between the ε-amino group of lysine in the target protein and the C-terminus of ubiquitin. The attachment of a single ubiquitin to one (monoubiquitylation) or several lysines of a protein (multiubiquitylation) has been associated with endocytosis of plasma membrane proteins, sorting of proteins to the vacuole, DNA repair, and transcriptional regulation (13–16). However, ubiquitin itself contains several lysines to which additional ubiquitin moieties can be attached, resulting in polyubiquitylation. The best known polyubiquitin chains are branched at K48 or K63, but branching at K6, K11, K27, K29, or K33 has also been reported. Although polyubiquitylation at K48 or K11 is in general associated with protein degradation through the UPS, K63-linked polyubiquitylation and other nonclassical linkage types have other physiological roles in nonproteolytic signaling such as stress and DNA damage response and protein endocytosis (17–21).

The numerous roles of the UPS in cellular control have triggered interest in the biological functions of target proteins and their sites of ubiquitylation. The identification of ubiquitylated proteins was mainly achieved using ubiquitin binding domain (UBAs) affinity chromatography and mass spectrometry (22–26). Here we report a similar approach for the identification of ubiquitylated Arabidopsis proteins, but using a different experimental method, which allowed us to detect proteins that until now have been refractory to identification. We also found that identification of ubiquitin footprints produces potentially erroneous results, which we subsequently confirmed by mass spectrometry of synthetically produced ubiquitylated peptides. Syringolin A is a potent specific inhibitor of the proteasome, which also allowed us to investigate changes in protein abundance after interfering with the UPS. This approach was complemented using a mutant overexpressing ubiquitin that cannot form K48-linked polyubiquitin chains. Previously we had reported that in Arabidopsis unexpectedly few proteins showed diurnal abundance changes, whereas more than 50% of their transcripts changed significantly (7). We therefore performed the experiment in a day-time specific manner to investigate the extent to which the UPS regulates diurnal protein levels.

EXPERIMENTAL PROCEDURES

Purification of Syringolin A— Cultures of Pseudomonas syringae pv. syringae B301D-R transformed with the plasmid pOEC for overproduction of SyIA (27) were grown in SRMAF medium for 10 days at 28 °C. Isolation of SyIA was performed as previously described (28–30). In short, 1 liter of conditioned SRMAF medium was filtered (0.45 μm pore size), supplemented with 100 ml Amberlite XAD-16 resin (Fluka) equilibrated with distilled water and shaken for 2 h. The XAD-16 resin was removed and first washed twice with 500 ml distilled water for 10 min and then twice with 20% methanol in water for 1 h. Bound substances were eluted by addition of 100 ml 70% isopropanol and shaking for 15 min. The last step was repeated four times. The combined eluted fractions were evaporated to 10% of the original volume using a Rotavapor-R evaporator (Büchi) at 50 °C. After centrifugation and filtration (0.22 μm pore size), 5 ml aliquots were supplemented with trifluoroacetic acid to a final concentration of 0.06% and isocratically separated by reverse-phase-HPLC on a Nucleosil 100 7 C18 250/20 column (Macherey-Nagel) with 20% (v/v) acetonitrile and 0.06% (v/v) trifluoroacetic acid in water using a flow rate of 10 ml/min. The peak containing SyIA eluted at 40 min (peak 1 in Wäspi et al., (30)), and the fraction was collected and lyophilized. Lyophilized eluate from five runs was dissolved in a total of 5 ml 100 mM NaOH and chromatographed using a Superdex 30 HiLoad 16/60 gel filtration column (GE Healthcare) and an Äkta FPLC system (GE Healthcare) at a flow rate of 1 ml/min using distilled water as the running solvent. Absorption was monitored at 254 nm. The major peak eluting between 50 and 75 min was collected and lyophilized, the later eluting minor peak was discarded. Lyophilized SyIA was weighed and stored under dry conditions in the dark. Based on HPLC and mass spectrometry, purity is estimated to be >99%. SyIA solutions with defined concentrations were prepared by dissolving weighted amounts of SyIA in water.

Plant Growth and Inhibition of the Proteasome— The plant material used in this study was either from Arabidopsis thaliana ecotype Col-0 (wild type) or the ubR48 mutant in which four transgenic ubiquitin proteins mutated at their K48 position can be expressed under the control of a dexamethasone (DEX)-inducible 35S promoter (31). Seeds were stratified for 2 days in darkness at 4 °C and after germination, the plants were grown in short day conditions with 8 h light and 16 h darkness. The proteasome was inhibited by spraying leaves of 55 day old plants with 10 μM Syringolin A in 0.02% (v/v) Tween20. As a mock control, leaves were sprayed with 0.02% (v/v) Tween20 only. For the experiments with total leaf extracts, only leaf 6 was treated. For the end of day (ED) samples, the time point of treatment was 30–60 min before the light went on and leaves were harvested before the light went off after 8 h of incubation. For the midnight (MN) and end of night (EN) samples, the time point of treatment was 30–60 min before the light went off and leaves were harvested either after 8 h (MN) or 16 h (EN) of incubation. For each condition, three to four leaves were harvested for both treatment and control. Fifty-five day old ubR48 mutant plants were treated with 0.6 mM DEX in 2% DMSO, or with 2% DMSO only as mock control. The treatment was carried out the same way as described for the wild-type plants, but only ED and EN samples were harvested. For the affinity enrichment of ubiquitylated proteins, the whole rosette was treated. For the affinity enrichment experiment using roots, plants were grown on vertical plates containing Murashige and Skoog medium with 0.8% agarose for 17 days. Roots were sprayed with SyIA solution 30–60 min before
the light came on and harvested 8 h later before the light went off (similar to ED samples). After harvesting all leaf and root material was directly frozen in liquid nitrogen and stored at −80 °C until further processing.

**Sample Preparation for Total Leaf Extracts**—Frozen plant material was ground with a pre-cooled mortar and pestle. Proteins were extracted by incubating the tissue powder with SDS buffer (4% SDS, 40 mM Tris-base, 5 mM MgCl₂, and 2× protease inhibitor mix (Roche)) at room temperature (RT) for 20 min. Nonsoluble material was pelleted by centrifugation at RT for 10 min at 16,200 × g. The supernatant was subsequently cleared by ultracentrifugation at RT for 45 min at 100,000 × g. Protein concentrations in the supernatants of the ultracentrifugation step were determined with the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). For each sample, 250 μg of protein were subjected to SDS-PAGE on 10% SDS gels. After electrophoretic separation of the proteins, the gels were stained with Coomassie brilliant blue R250 and each lane was cut into five sections. Each section was then further diced into small pieces, which were completely destained in 50% methanol, 50 mM ammonium bicarbonate pH 8 at 37 °C and washed three times with 50% acetonitrile for 15 min each and subsequently vacuum-dried. Proteins were reduced with 10 mM DTT, 5 mM ammonium bicarbonate pH 8 at 50 °C for 45 min followed by carbamidomethylation with 50 mM iodoacetamide, 50 mM ammonium bicarbonate in the dark for 1 h. Afterward the gel pieces were washed three times with 50% acetonitrile for 15 min each and vacuum-dried. The proteins were then digested in-gel with trypsin solution (4.8 μg/ml trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate for 16–19 h at 30 °C. After digestion, the peptides were extracted three times with 50% acetonitrile, 5% formic acid. Acetonitrile was vaporized before the peptides were desalted using Sep-Pak reverse-phase cartridges (Waters, Milford, MA). The solvent was vaporized and the purified peptides stored at −80 °C. Before subjecting to mass spectrometric measurements, each sample was dissolved in 3% acetonitrile, 0.1% trifluoroacetic acid.

**Affinity Enrichment Experiment**—Frozen plant material was ground with a mortar and pestle. To 0.75 g fresh weight plant material, 3 ml of cold native protein extraction buffer was added (20 mM HEPES pH 7.5, 0.1 mM EDTA, 0.1 mM KCl, 10% glycerol, 0.02% Tween20, 0.5 mM DTT, and 2× protease inhibitor mix (Roche)). Proteins were extracted on ice for 20 min and the extracts were cleared after centrifugation at 4 °C for 15 min at 16,200 × g. The supernatant was collected and protein concentration was determined using the Bradford assay. To the pellet, 1.5 ml of urea buffer was added at RT (8 M urea, 20 mM Tris-base, 5 mM MgCl₂, and 2× protease inhibitor mix (Roche)). Proteins were extracted at RT for another 20 min, followed by centrifugation at RT for 10 min at 16,200 × g. The supernatant was collected in dialysis bags and the urea concentration was reduced in a two-step dialysis to about 10 mM urea with a first dialysis for 40 min against 500 ml of 80 mM urea, 20 mM Tris base, 5 mM MgCl₂, and 0.02% Tween20, and a second dialysis for 1 h against 1 liter of 10 mM urea, 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 0.02% Tween20. Afterward, the extract was collected and the protein concentration was determined using the Bradford assay. If not stated otherwise, the following steps were carried out at 4 °C. First, 500 μg protein in a total volume of 800 μl was precleared with 100 μl washed Sepharose CL4B (Sigma-Aldrich) under constant agitation for 2 h. Afterward the Sepharose was pelleted and half of the extract was added to 50 μl washed p62-derived UBA domain agarose conjugate (Enzo Life Sciences), whereas the other half was added to 50 μl Sepharose CL4B as a control. After incubation for 2 h under agitation the beads were pelleted, the supernatant was removed and the beads were washed three times with 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 0.02% Tween20 for 5 min, 10 min, and 20 min. Proteins were eluted with 25 μl 0.25 M Tris-HCl pH 6.8, 50% glycerol, 8% SDS, 0.05% bromphenol blue, and 0.5 mM DTT at 80 °C for 10 min and subjected directly to SDS-PAGE on 10% gels. After electrophoretic separation of the proteins, each lane was cut into five sections. In-gel tryptic digestion and purification of the peptides was performed as described above.

**Tandem Affinity Enrichment Experiment**—The method for the tandem affinity experiment described above was applied with the following modifications. First, 1 mg leaf protein in a total volume of 2 ml was used for the affinity enrichment with UBA-conjugated beads and instead of an elution with SDS buffer, proteins were eluted by incubation with 2 mM NaCl, 25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 0.02% Tween20 for 10 min at 80 °C. The NaCl concentration was subsequently reduced by dialysis against the buffer without NaCl for 20 min. Half of the eluate was then applied to 25 μl α-Ubiquitin conjugated agarose (Ub (P4D1), Santa Cruz Biotechnology, Santa Cruz, CA), and the other half was used as control.

**Western Blot Analysis**—Proteins were extracted with SDS buffer as described above omitting the ultracentrifugation step. For the Western blot 40 μg protein were subjected to SDS-PAGE on 10% gels and afterward blotted onto nitrocellulose membranes. The blots were probed with α-HSP90-1 (1:1000 dilution, Agrisera, Sweden), α-Ubiquitin, K48-specific (1:1000, Millipore), and α-POR (1:1000, Agrisera) antibodies and α-rabbit (1:5000, BioRad, Hercules, CA) as secondary antibody. As loading controls α-Tubulin (1:2000, Sigma) antibody with α-mouse (1:5000, Roche) secondary antibody and α-DPE2 (1:1000, Agrisera) antibody with α-rabbit (1:5000, Millipore) secondary antibody were used.

**Mass Spectrometry Measurements**—Mass spectrometry measurements were performed on an LTQ Orbitrap XL MS (Thermo Fisher) coupled to a NanoLC-AS1 (Eksigent) using electrospray ionization. For LC separation, a capillary column packed with 8 cm C18 beads with a diameter of 3 μm and a pore size of 100 Å was used. Peptides were loaded on the column with a flow rate of 500 nl/min for 16 min and eluted by an increasing acetonitrile gradient from 3% acetonitrile to 50% acetonitrile for 60 min with a flow rate of 200 nl/min. Full scans were obtained using the FT Orbitrap at a range of 300–2000 mass/charge followed by MS/MS scans of the five highest parent ions. Dynamic exclusion was enabled at a duration of 120 s.

**Interpretation of MS/MS Spectra**—The acquired raw spectra were transformed to mgf data format and searched against the TAIR10 database (32, download on January 17th, 2011) with concatenated decoy database and supplemented with common contaminants (71,032 entries) using the Mascot algorithm (version 2.3.02) (Mascot Science). The search parameters used were: mass = monoisotopic, requirement for tryptic ends, two missed cleavages allowed, precursor ion tolerance = ± 10 ppm, fragment ion tolerance = ± 0.8 Da, variable modifications of methionine (M, PSID-MOD name: oxidation, mono Δ = 15.995) and static modifications of cysteine (C, PSI-MOD name: iodoacetamide derivatized residue, mono Δ = 57.0215). Peptide spectrum assignments with ionscore > 30 and expect value < 0.15, except those of known contaminants, were filtered for ambiguity. Peptides matching to several proteins were excluded from further analyses. This does not apply to different splice variants of the same protein or to different loci sharing exactly the same amino acid sequence. All remaining spectrum assignments were inserted into the pep2pro database (33–34). The false discovery rate (FDR) was calculated by dividing the number of reverse hits by the number of true hits times 100%. The total number of spectra and the FDR per experiment are shown in supplemental Table S1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (35) with the data set identifiers PXD000521, PXD000565 - PXD000568. The data are also publicly available in the pep2pro database at www.pep2pro.ethz.ch from where all informa-
tion on peptide and protein identifications can be retrieved and spectra can be viewed.

Statistical Analyses—Proteins were quantified by normalized spectral counting according to Baerenfaller et al. (36) and Bischof et al. (37) by calculating the expected contribution of each individual protein to the samples total peptide pool correcting the values using a normalization factor that balances for the theoretical number of tryptic peptides per protein and sample depth according to the formula:

\[ nSPC_k = \frac{\text{Spectra}_k \times (\text{TPP}_k \times \text{MS})^{-1}}{\text{MP}} \]

where \( nSPC_k \) is the normalized spectral count for protein K, Spectra\(_k \) is the number of spectra assigned to protein K, TPP\(_k \) is the number of theoretical tryptic peptides of protein K, MS is the total number of measured spectra in the data set, and MP is the total number of theoretical tryptic peptides of the identified proteins in the data set.

In the experiments using full extracts of either wild type or the ubR48 mutant, only proteins were included in the quantification that were detected with a minimum of 10 spectra within an experiment. Proteins were considered changing in abundance between two different treatments, when the fold-change of the average relative abundance over three biological replicates was more than 1.5 and the corresponding \( p \) value in a paired \( t \) test was smaller than 0.05. The proteins that were identified in at least two of the three biological replicates in one treatment and not at all in the other treatment were also considered as changing in abundance. In the affinity enrichment experiments, we required a minimum of 10 different spectra to call a protein identified, except for the tandem experiment, where we required only five spectra. With these data an index was calculated, where each protein identification in the UBA-domain affinity enrichment counts +1 and each identification in the Sepharose control counts −1 toward the final index. As an example, an index of 2 either indicates that the protein was identified in two of the three UBA affinity enrichment replicates and never in the Sepharose control, or that it was identified in all three UBA affinity enrichment replicates and only once in the Sepharose control. For accepting a protein to be ubiquitylated we applied the cut-off criteria for leaves and roots separately that the protein had a minimum index of 2 and was either not detected at all in the Sepharose background control or was at least fivefold enriched. For those proteins that were identified in the single and the tandem affinity enrichment experiment and also fulfilled the above mentioned cut-off criteria, we correlated the number of spectra for each protein in the individual experiments.

Comparison of the Identified Ubiquitylated Proteins in this Study with those Reported in Literature—For the comparison of the identified ubiquitylated proteins we downloaded the lists of ubiquitylated proteins reported in Maor et al., (22), Manzano et al., (23), Igawa et al., (24), Saracco et al., (25), and Kim et al., (26).

Search for Ubiquitylation Target Sites in the Data Set Reported by Maor et al., (22)—The raw mass spectrometry data described in Maor et al., (22) were converted into mZXML file format and the data were searched with SEQUEST/Peptide Prophet against the TAIR7 decay database with supplemented contaminants (fgcz_3702_TAIR7_20070425decay.fasta). The search parameters were similar to those used by Maor et al., (22) and were: mass = average, precursor ion tolerance = ±2 Da, enzyme = trypsin, allowed missed cleavages = 3, variable modifications of methionine, and lysine (+114.1026 Da) to account for the diglycine tag, and static modifications of cysteine. Spectrum assignments to unambiguous peptides with a probability > 0.1 were inserted into the pep2pro database.

Mass Spectrometry Measurements of Synthetic Peptides and Relative Intensity Analysis—In the initial searches for the ubiquitin footprint, the Mascot searches were performed with a change in the following parameters: allowed missed cleavage sites = 3 and variable modification of lysine (+114.1026 Da) to account for the diglycine tag. For the identified ubiquitylated peptides synthetic peptides with identical amino acid sequences and containing the diglycine tag were produced (JPT Berlin). The synthetic peptides were measured as described above using 400 fmol per peptide either in a mixture of only synthetic peptides, or spiked into affinity enrichment protein mixtures with the same concentration. The resulting spectra were searched with Mascot using the same parameters. Only peptide spectrum assignments to doubly charged peptides with ionscore > 30 and expect value < 0.015 were considered in the correlation analyses with the exceptions detailed in supplemental Table S2, for which there were no peptide identifications fulfilling these criteria. In all corresponding spectra the intensities of the b- and y-ions were recorded and for each fragment ion the relative intensity was calculated as percentage of the highest intensity. To obtain a quantitative measure for the similarity of two spectra the dot product between the relative intensity values in these spectra was calculated according to Shedd et al. (38). For this, the relative intensities of the peptide fragment ions that were identified in both spectra were assembled into a vector for each spectrum, if at least four different fragments were in common between the two spectra. Each vector was then normalized to its magnitude and the dot product was calculated between the two normalized intensity vectors. The dot product mathematically describes the similarity between two vectors and gives 0 for complete orthogonality and 1 for identical vectors.

GO Categorization—GO categorization was performed using the Ontology software (http://compbio.charite.de/ontologizer) in combination with the Arabidopsis annotation file considering aspect Biological Process. Annotations with GO evidence codes IEA (Inferred from Electronic Annotation) or RCA (Inferred from Reviewed Computational Analysis) were excluded from analyses. As background lists the organ-specific protein maps for leaves or roots of the pep2pro TAIR10 was data set were used (33). Over-representation was assessed with the Topology-eliminated method and the \( p \) values were corrected for multiple testing using the Bonferroni method. GO categories with a \( p \) value < 0.05 were considered significant.

RT-qPCR—RNA was extracted from leaves using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions with subsequent DNase I treatment. For RT-qPCR 495 ng total RNA was reverse-transcribed using an oligo(dT) primer and reverse transcriptase (SuperScript II, Invitrogen). In the RT-qPCR reactions for HSP90 (AT5G52640), HSP70 (AT3G12580), ASN1 (AT3G74370), P0RB (AT4G27440), AT3G19010, and PIFI (AT3G15840) 10 ng RNA were reverse-transcribed using an oligo(dT) primer and reverse transcriptase (SuperScript II, Invitrogen). In the RT-qPCR reactions for HSP90 (AT5G52640), HSP70 (AT3G12580), ASN1 (AT3G74370), P0RB (AT4G27440), AT3G19010, and PIFI (AT3G15840) 10 ng RNA were used, and 20 ng for AtNBr1 (AT4G24690), ATG5 (AT5G17290), ATG12A (AT1G54210), ATG12B (AT3G13970), GDH2 (AT5G07440), CAO (AT2G47450), and ATG8A (AT4G21980). The TaqMan system (Roche) was used to perform RT-qPCR reactions with primers designed using Universal Probes (Roche) according to manufacturer’s instructions (supplemental Table S3). Measurements were performed on a LightCycler 480 System (Roche). Calculation of the relative mRNA abundance was performed using the equation

\[ \frac{\text{Efficiency}_{\text{average Ct(SylA)}}}{\text{Efficiency}_{\text{average Ct(mockcontrol)}}} \]

The efficiency of each primer with the particular cDNA was calculated using the LinRegPCR 12 program.

Microarray Transcript Data—We downloaded the microarray transcript data of Arabidopsis plants treated with SylA for 1 h and 12 h (40) from the ArrayExpress database and normalized the data using RMA (41) in R (42) using the Affy package. Significant changes were assessed with a two-sided Welch test followed by correction with the Benjamini-Hochberg method. Transcripts with a \( p \) value < 0.05 and a fold-change > log2(1.5) were considered to be regulated.
RESULTS

Syringolin A Inhibits the Arabidopsis Proteasome—For the identification of proteins that are regulated by the UPS between the end of the day (ED) and the end of the night (EN) we used Syringolin A (SylA). SylA is produced by Pseudomonas syringae pv. syringae and is a potent and specific inhibitor of the UPS that irreversibly inhibits all catalytic subunits of the proteasome (43). SylA does not affect any other cellular proteases, in contrast to proteasome inhibitors such as MG132, which also inhibits papain-like cysteine proteases (44), or leupeptin that also acts on serine and cysteine proteases (45). Using an irreversible inhibitor was important for our experiments because of the expected accumulation of ubiquitylated proteins, which excluded the use of reversible proteasome inhibitors such as Bortezomib (45). Also, SylA inhibits the proteasome function in Arabidopsis (43), which had not been demonstrated for the other selective proteasome inhibitor epoxomicin. To test whether spraying leaves with SylA is indeed a suitable method for inhibiting the UPS we used a marker line expressing a CyclinB1;1:GUS fusion protein (46). CyclinB1;1 accumulates at the late G2/M phase of the cell cycle and at the end of the M phase it is degraded by the proteasome (46–47). Inhibition of the proteasome can therefore be monitored using GUS staining in dividing cells (43, 48). In expanding leaves, most cells have ceased cell division and grow by expansion except for meristemoid and procambial cells, the stem cells that continue to produce cells of the stomatal cell lineage in the epidermis and the vascular tissue in the mesophyll cell layer, respectively (47, 49–50). After spraying leaves with SylA we observed significantly more GUS staining in vascular tissue and in dispersed epidermal cells compared with control leaves after 9 h and 18 h of treatment (supplemental Fig. S1), demonstrating that SylA can penetrate all leaf tissues. As an additional control we monitored the accumulation of polyubiquitylated proteins, which are detectable at a low level in untreated leaves and significantly increased after addition of SylA (Fig. 1). Together, SylA effectively inhibits the proteasome in leaf cells.

Proteins Change in Abundance after Proteasome Inhibition—For the identification of proteins that change their abundance after inhibition of the proteasome we adopted the workflow shown in Fig. 2. In total, we detected 4043 proteins in all the samples and 2526 with at least 10 spectra (supplemental Table S4). Proteins that changed in abundance were considered only if they were identified with at least 10 spectra and the fold-change between SylA and mock treated samples at a time point (ED, MN, or EN) was >1.5 with a p value <0.05, or the protein was identified in at least two biological replicates of one condition but not the other. Based on these criteria, we identified 109 proteins that accumulated, 140 proteins that decreased and six proteins that accumulated or decreased in abundance depending on the sampling times after treatment with SylA (Table I, supplemental Table S5).

There was little overlap between the sampling time points, most probably because of the stringent selection criteria we applied. However, the trend is the same for 68% of the proteins that are significantly changing at EN or MN and 39% at ED or EN, suggesting daytime-specific differences in the response to inhibition of the proteasome.

The proteins REGULATORY PARTICLE NON-ATPASE 12A (RPN12a, AT1G64520), CALCIUM-BINDING EF HAND FAMILY PROTEIN (TCH3, AT2G41100), GLUTAMINE-DEPENDENT ASPARAGINE SYNTHASE 1 (ASN1, AT3G47340), and HEAT SHOCK PROTEIN 70 (HSP70, AT3G12580) that accumulated after SylA treatment were responsible for significant over-representation of response to absence of light and response to light intensity as two of the five categories in the gene ontology (GO) category Biological Process with a p value < 0.01, suggesting that the proteasome is indeed involved in diurnal protein level regulation. The 12 GO categories that were over-represented in the set of proteins that decreased after SylA treatment included tRNA aminoacylation for protein translation, tRNA aminoacylation and amino acid activation, suggesting that SylA treatment negatively affects the translation machinery and thus attenuates de novo protein synthesis. To validate the above results we analyzed the
HSP90 and POR proteins in SylA treated samples by Western blot (Fig. 3). We confirmed that HSP90 accumulated after SylA treatment and also detected signals in the higher molecular weight range indicating the accumulation of polyubiquitylated HSP90 (Fig. 3A). The increase of unmodified HSP90 after SylA treatment suggests de novo protein synthesis, but could also be because of the activity of deubiquitylating enzymes (DUBs) that remove the polyubiquitin tag from proteins not degraded by the proteasome. We also confirmed the decrease of the POR protein after SylA treatment (Fig. 3B). Unexpectedly, more proteins decreased than increased after SylA treatment. Possible reasons for the protein level decrease after inhibition of the proteasome include: (1) down-regulation of transcript levels; (2) reduced translation; (3) degradation by other proteases; or (4) protein degradation by autophagy. To obtain a better understanding of the reasons for the observed protein abundance changes we performed the following analyses.

**Processes Determining Protein Levels**—In addition to comparing the protein abundance between SylA treated and mock treated samples we also assessed the differences to the respective T0 control, which represents the sample at the time point of SylA treatment (Fig. 2). This allowed us to distinguish the scenarios shown in Fig. 4 and to propose possible explanations for the observed protein abundance level changes. For proteins in scenario A, their increase in SylA treated samples suggest a function of the UPS in the homeostasis of these proteins (seven proteins, Table II). This category com-

**TABLE I**

Proteins that are changing in abundance after inhibition of the proteasome with SylA in at least two samples. Proteins that are higher in SylA treatment are indicated in blue, those that are higher in control in orange, and those that are higher in control in one and higher in SylA in another sampling time in green. The numbers in the columns ED, MN and EN indicate: 1: p value < 0.05 and fold-change SylA/control > 1.5; 2: identified in at least two biological replicates of SylA treatment and not at all in control; -1: p value < 0.05 and fold-change control/SylA > 1.5; -2: identified in at least two biological replicates of control and not at all in SylA treatment.

| Proteins| AGI| ED| MN| EN| Description |
|---------|----|----|----|----|-------------|
| O-methyltransferase family protein, IGMT4 | AT1G21130 | 0 | 2 | 2 | |
| ATP binding cassette subfamily B4, ABCB4 | AT2G47000 | 2 | 0 | 0 | |
| Fes1A | AT3G0350 | 2 | 2 | 0 | |
| ubiquitin family protein, DNA-damage inducible 1, DDI1 | AT3G13235 | 2 | 0 | 1 | |
| glutamine-dependent asparagine synthase 1, ASN1 | AT3G47340 | 2 | 0 | 2 | |
| ATPase, AAA-type, CDC48 protein, ATC48B | AT3G53230 | 0 | 2 | 2 | |
| AAA-type ATPase family protein | AT4G02480 | 0 | 1 | 2 | |
| Translation initiation factor 3 protein | AT4G30690 | 1 | 2 | 0 | |
| Heat shock protein 70 (Hsp 70) family protein | AT5G02490 | 1 | 0 | 1 | |
| glutamate dehydrogenase 2, GDH2 | AT5G07440 | 2 | 0 | 2 | |
| Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein | AT5G24650 | 2 | 0 | 2 | |
| AMP-dependent synthetase and ligase family protein | AT2G17650 | -2 | 0 | 2 | |
| unknown protein | AT3G53470 | 2 | -1 | 0 | |
| myo-inositol monophosphatase like 2, IMPL2, HISN7 | AT4G39120 | 1 | 0 | -1 | |
| RP non-ATPase subunit 8A, RPN8A | AT5G05780 | 0 | -1 | 1 | |
| histone B2 | AT5G2880 | 2 | -2 | 0 | |
| nitrile specifier protein 5, NLS5 | AT5G48180 | 1 | -1 | 0 | |
| GroES-like zinc-binding dehydrogenase family protein | AT1G64710 | -2 | 0 | -2 | |
| cold regulated 15b, COR15B | AT2G42530 | -1 | -1 | 0 | |
| guanosine nucleotide diphosphate dissociation inhibitor 1 | AT2G44100 | -2 | -1 | 0 | |
| chloroplast signal recognition particle component (CAO) | AT2G47450 | -1 | 0 | -1 | |
| Protein of unknown function (DUF1997) | AT5G04440 | -2 | 0 | -2 | |
| phosphoglycerate/bisphosphoglycerate mutase family protein | AT5G22620 | 0 | -1 | -1 | |
| tryptophan synthase beta-subunit 1 | AT5G54810 | -1 | -2 | 0 | |
| US small nuclear ribonucleoprotein helicase | AT5G61140 | -1 | -1 | 0 | |
Inhibition of the Proteasome Might Target Proteins to Degradation by Autophagy—Autophagy degrades large cellular constituents up to whole organelles, protein complexes, protein aggregates, and soluble proteins (54–55). In human cells, activation of autophagy also decreases proteasome levels (56). In Arabidopsis, NEXT TO BRCA1 GENE 1 (NBR1, AT4G24690) targets ubiquitylated protein aggregates to the autophagy pathway (56). NBR1 belongs to the subgroup of ATG8-interacting motif containing proteins that recruits specific cargo to autophagy (57). We investigated whether inhibition of the proteasome caused changes in transcript levels of several autophagy-related genes using RT-qPCR because we did not obtain quantitative data for the encoded proteins in our high-throughput approach. We found that the transcript levels of both NBR1 and AUTOPHAGY 8A (ATG8A, AT4G21980) were indeed significantly increased in samples collected after treatment with SylA, whereas those of ATG5 (AT5G17290), ATG12A (AT1G54210), and ATG12B (AT3G13970) were not significantly changed (supplemental Fig. S2). The increased transcript levels of NBR1 and ATG8A indicate that the accumulating polyubiquitylated proteins are likely targeted to degradation by autophagy.

Inhibition of the Proteasome has a Differential Effect on the Transcriptome—For the proteins that changed their levels after SylA treatment we first investigated if they had corresponding changes in their transcript levels using the transcriptome data reported by Michel et al., (40) from Arabidopsis plants that were treated with SylA or Tween only and sampled after 8 and 12 h. Under these experimental conditions there was no significant change at the transcript level for 52 of the proteins that we found increased after SylA treatment, whereas others had either increased or decreased transcript levels (supplemental Table S5). We then specifically tested the transcript levels for HSP70 (AT3G12580), HSP90 (AT5G52640), AT3G19010, ASN1 (AT3G47340), and GLUTAMATE DEHYDROGENASE 2 (GDH2, AT5G07440) using RT-qPCR on the mRNA from the same ED and EN samples as used in the proteomics experiment. For HSP70, HSP90, and GDH2 the transcript levels were significantly up-regulated providing a plausible explanation for the protein accumulation after inhibition of the proteasome, whereas those for AT3G19010 and ASN1 are not significantly changing in both transcript data sets (supplemental Fig. S3, supplemental Table S5). For 37 proteins that are decreasing in abundance the transcript level also decreased, for 12 it increased and for 85 it was not changing significantly (supplemental Table S5). We were specifically interested in the transcript levels for
the plastid-localized SIGNAL RECOGNITION PARTICLE 43 (CAO, AT2G47450), POST-ILLUMINATION CHLOROPHYLL FLUORESCENCE INCREASE (PIFI, AT3G15840), and PROTOCHLOROPHYLIDE OXIDOREDUCTASE B (PORB, AT4G27440) proteins (supplemental Fig. S3, supplemental Table S5). RT-qPCR of CAO and PORB mRNAs revealed a significant decrease also at transcript level, whereas the PIFI mRNA did not change significantly. Together, the decrease of at least some proteins after proteasome inhibition can be explained by their down-regulation at the transcript level and likely reduced \textit{de novo} protein synthesis.

\textbf{Regulation of the Proteasome Components—} According to KEGG (58) and TAIR (32), the proteasome is composed of a total of 56 different proteins (supplemental Table S6). It has been reported that transcripts encoding proteasome subunits were up-regulated after SylA treatment (40). Mapping the transcript data to the protein identifiers we found that this was the case for 52 of the 53 genes encoding proteasome subunit proteins represented on the microarray (supplemental Table S6). This prevalent increase at the transcript level is in contrast to the protein data, because of the 32 proteasome subunit proteins that were identified with at least 10 spectra only five significantly changed after SylA treatment (supplemental Table S6). One of the accumulating proteins is RPN12A that physically interacts with the U-box E3 ligases PUB22 and PUB23, and its ubiquitylation leads to partial dissociation from the RP (59). It is possible that proteasome complexes irreversibly inhibited by SylA are targeted to degradation by autophagy and that accelerated protein turnover prevents accumulation of the proteasome subunit proteins despite their increased transcript levels.

\textbf{Different Methods of Interfering with the UPS Result in Different Sets of Accumulating Proteins—} We investigated if interference with targeted UPS protein degradation was comparable to SylA-mediated inhibition of the proteasome using a conditional \textit{Arabidopsis} ubiquitin mutant line that over-expresses ubiquitin with a K48R substitution, which cannot form K48-linked polyubiquitylation chains (31). However, we found that the overlap was small between the accumulating proteins in both experiments (supplemental Table S8). Furthermore, the identity of the accumulating proteins suggests that a different response is triggered by over-expression of the ubiquitin K48R mutant and inhibition of the proteasome with SylA.

\textbf{Identification of New Direct Targets of the UPS—} The above results demonstrated that interfering with UPS-mediated protein degradation considerably affects protein homeostasis in \textit{Arabidopsis} and has additional indirect effects that are triggered by the accumulation of polyubiquitylated proteins generated by the proteasome.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|l|c|}
\hline
\textbf{Category} & \textbf{AGI} & \textbf{Description} & \textbf{Daytime} \\
\hline
A & AT1G21130 & O-methyltransferase family protein, IGMT4 & EN \\
A & AT3G44340 & Clone eighty-four & EN \\
A & AT4G19006 & Proteasome component (PCI) domain protein, RPN6 & EN \\
A & AT5G26240 & Heat shock protein 90.1 & EN \\
A & AT5G02490 & Heat shock protein 70 (Hsp 70) family protein & ED, EN \\
A & AT5G25440 & Bacterial sec-independent translocation protein mttA/HCF106 & EN \\
A & AT5G60160 & Zn-dependent exopeptidases superfamily protein & EN \\
B & AT5G24650 & Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein & EN \\
C & AT1G32900 & UDP-Glycosyltransferase superfamily protein & EN \\
C & AT5G24400 & NagB/RpiA/CoA transferase-like superfamily protein & ED \\
C & AT5G47860 & Protein of unknown function (DUF1350) & ED \\
D & AT1G64550 & General control non-repressible 3 & EN \\
D & AT2G2170 & Lipase/lipoxygenase, PLAT/LH2 family protein & EN \\
D & AT2G25840 & Nucleotidyl transferase superfamily protein & ED \\
D & AT2G31810 & ACT domain-containing small subunit of acetolactate synthase protein & EN \\
D & AT2G35860 & FASCIIN-like arabinogalactan protein 16 precursor & EN \\
D & AT2G45060 & Uncharacterised conserved protein UCP022280 & EN \\
D & AT2G47450 & Chloroplast signal recognition particle component, CAO & EN \\
D & AT3G02770 & Ribonuclease E inhibitor RraA/Dimethylmenaquinone methyltransferase & EN \\
D & AT3G15840 & Post-illumination chlorophyll fluorescence increase, PIFI & EN \\
D & AT4G0183 & Polyketide cyclase / dehydrase and lipid transport protein & EN \\
D & AT4G16390 & Pentatricopeptide (PPR) repeat-containing protein & EN \\
D & AT4G27440 & Protochlorophyllide oxidoreductase B (PORB) & ED \\
D & AT4G31500 & cytochrome P450 family 83 subfamily B polypeptide 1 & EN \\
D & AT4G33220 & Pectin methylesterase 44 & EN \\
D & AT5G04440 & Protein of unknown function (DUF1997) & EN \\
D & AT5G13720 & Uncharacterized protein family (UPF0114) & EN \\
D & AT5G14220 & Flavin containing amine oxidoreductase family & EN \\
D & AT5G20650 & Copper transporter 5 & EN \\
\hline
\end{tabular}
\caption{Proteins belonging to the different categories as illustrated in Fig. 5. The last column indicates, whether the change was observed during the day (ED) or during the night (EN).}
\end{table}
erating a proteotoxic stress. In order to find the direct targets of the proteasome we therefore aimed at identifying ubiquitylated proteins directly by affinity chromatography using the p62 ubiquitin-binding domain (UBA) (23, 60). Using UBAs from various proteins, either alone or in combination, together with non-denaturing buffer conditions have already identified 1556 different Arabidopsis proteins (22–26). These approaches have used a non-denaturing buffer system for the extraction of proteins to avoid interference in the subsequent binding assays. As these native conditions cannot solubilize membranes, ubiquitylated integral membrane proteins or proteins attached to membranes will be excluded from detection. This will for example affect substrates of endoplasmic reticulum-associated degradation (ERAD), as these proteins get ubiquitylated while being translocated through the ER membrane and will not reach a soluble state before degradation (61). To capture these proteins as well, we employed a serial extraction method with native and denaturing buffers in combination with UBA affinity chromatography. Using this experimental approach and applying cut-off criteria that also included an index calculation, we were able to identify 1080 ubiquitylated proteins in leaves and 1083 in roots with a total of 1791 distinct proteins (supplemental Table S9).

To verify the validity of the experimental approach we performed a tandem affinity enrichment experiment in which the UBA affinity purification was followed by an immunoprecipitation using an anti-ubiquitin antibody. We considered the proteins identified in the tandem affinity experiment as bona fide ubiquitylated proteins. We observed a positive correlation in the number of spectra with which the proteins were identified in the single and the tandem affinity enrichment experiment, but not between the Sepharose control and the tandem affinity enrichment experiment (supplemental Fig. S4). This indicates that also bona fide ubiquitylated proteins can be identified with few spectra in the Sepharose control and that the criteria applied here will still allow for their identification.

The identified ubiquitylated proteins included 1209 proteins unique to our study and 582 previously reported proteins (Fig. 5A) (22–26). Of the 1209 proteins, 510 were exclusively identified in roots and 467 in leaves, whereas 232 proteins were common to both organs. The advantage of the serial extraction was confirmed by the high number of proteins that were exclusively identified in the urea extracts. The large overlap between the proteins in the serial extracts unique to roots and leaves also suggests that they have organ-specific functions (Fig. 5B, 5C). Ubiquitylated proteins common to leaves and roots are over-represented for GO categories related to translation and response pathways. Soluble proteins exclusive to leaves are over-represented for translation-related processes, whereas proteins extracted with 8 M urea are found mostly in GO categories photosynthetic electron transport chain in photosystem I and chlorophyll biosynthetic process. Over-represented GO categories in root proteins included ubiquitin-dependent protein catabolic process.

Interestingly, according to the predictions in SUBAIII (62) most of the identified ubiquitylated proteins localized in only one compartment are targeted to plastids (39.6%) and mitochondria (8.1%), whereas only 26% reside in the cytosol. Because plastids and mitochondria do not contain a functional UPS, the precursors of these proteins are likely targeted to the UPS for degradation rather than entering the organelle. If the ubiquitylated plastid- and mitochondria-targeted proteins are the cytosolic precursors, we would expect to find peptides from their transit sequences. We therefore used the length of the transit sequences predicted by TransitP and ChloroP (63) and determined the most N-terminal localized peptide for these proteins (37). We detected a peptide located in the transit peptide sequence for 10.3% of the chloroplast- and 31.6% of the mitochondria-targeted proteins. Because not all transit sequences are expected to produce identifiable N-terminal peptides, these numbers are very high and confirm that many of the identified ubiquitylated proteins are indeed organelle protein precursors. Our data therefore confirm that the UPS regulates organellar protein levels (64–66) and demonstrate that the UPS control of organelle protein levels is likely more extensive than has been appreciated until to date.

We determined the overlap between the ubiquitylated proteins and proteins that change in abundance after SyIA inhibition of the proteasome and found that 77 of the 253 proteins from the SyIA dataset were also ubiquitylated (supplemental Table S5). Interestingly, the ubiquitylated proteins were found in both categories of increasing and decreasing proteins after SyIA treatment. For example, HSP90 and HCF106 that increased in abundance after SyIA treatment (category A in

**Fig. 5.** Overlap of reported ubiquitylated proteins in seedlings (Manzano et al., (23); Igawa et al., (24); Saracco et al., (25), and Kim et al., (26)) and cell culture (Maor et al., (22)) with the proteins from leaves and roots in this study (A) and with the proteins identified in the two serial native and urea extracts of leaves (B) and roots (C).
Table II) were ubiquitylated. However, CAO and PIFI (category D in Table II) were also ubiquitylated. Together, our results show that inhibition of the proteasome generates a differentiated response at the protein level such that UPS-targeted proteins do not necessarily accumulate after SyL treatment and that not all accumulating proteins are targets of the UPS.

Identification of Ubiquitylation Sites—We used our data from the affinity enrichment experiment to identify ubiquitylation sites. Proteins containing attached ubiquitin chains should produce tryptic peptides that contain the two C-terminal glycines of ubiquitin attached to lysine, termed ubiquitin footprint (supplemental Fig. S5). The delta mass of the ubiquitin footprint is 114.043 Da and its identification is usually considered to reveal the site of ubiquitylation. In addition to the mass shift, the peptide should also have a missed cleavage site because trypsin cannot cleave proteins at modified lysines (67). However, even reports of high-confidence ubiquitylated protein data sets revealed fewer than expected ubiquitin footprints given the high number of identified ubiquitylated proteins (26). When we included the ubiquitin footprint as a variable modification in our database-dependent searches and determined the local false discovery rate (lFDR) using the target decoy approach reported by Elias and Gygi (68) we found that 64 of the 96 peptide spectrum assignments to ubiquitylated peptides were against the decoy database. In addition, in 33 of the 96 peptides the modified lysine was the last amino acid of the peptide, although trypsin does not cleave proteins after the modified lysine. This indicates that the ubiquitin footprint assignments are problematic when analyzing complex peptide mixtures in which only a small fraction of peptides is expected to have the ubiquitin footprint.

In a previous study Maor et al., (22) reported 85 ubiquitylated peptides. We determined the lFDR for the ubiquitylation target sites in this dataset by searching the mass spectrometry data against the target decoy database using Sequest, requiring that peptides were doubly charged and had a minimum Xcorr of 2.5. Using these criteria we found that of the 970 peptide spectrum assignments to ubiquitylated peptides were against the decoy database. In addition, in 33 of the 96 peptides the modified lysine was the last amino acid of the peptide, although trypsin does not cleave proteins after the modified lysine. This indicates that the ubiquitin footprint assignments are problematic when analyzing complex peptide mixtures in which only a small fraction of peptides is expected to have the ubiquitin footprint.

Further evidence for low confidence in the assignment of ubiquitylation sites is the small overlap between identified modified and unmodified peptides, which was the case for a single peptide only in our data set. In addition, only 14.3% of the proteins identified with peptides containing the ubiquitin footprint were also identified based on other peptides from the same protein, although the affinity enrichment was on the protein level. In a phosphoproteomics data set that we had reported earlier (69), 11.6% of all phosphorylated peptides were also found in their nonphosphorylated form and 35.2% of the proteins identified with phosphorylated peptides were also identified based on nonphosphorylated peptides. Therefore we would expect a higher fraction of peptides identified with and without ubiquitin footprint. Furthermore, we would expect more proteins identified based on peptides containing the ubiquitin footprint and nonmodified peptides, if ubiquitylation assignments were correct.

Synthetic Peptides Reveal Incorrect Ubiquitin Footprint Assignments—It is possible that search algorithms cannot assign the ubiquitin footprint correctly because it introduces two additional amide bonds that fragment in collision induced dissociation (supplemental Fig. S5). The dissociation of the amide bonds will produce fragment ions with a mass loss of $-58.02$ or $-115.02$ Da, termed here uf1 and uf2, respectively. The detection of these fragment ions would be a good indication for the presence of the ubiquitin footprint, however these peaks are frequently outside of the detection window. In addition to the additional fragmentation sites produced by the dissociation of the amide bonds, the conversion of the ε-amino group of the lysine side chain into an amide group will likely influence the charge distribution in the peptides and might therefore change the fragmentation patterns. To distinguish between these possibilities we synthesized 21 of the identified ubiquitylated peptides with their diglycine tags and measured them either as a mixture of synthetic peptides only, or added them to one of the affinity-purified protein samples. To obtain a quantitative measure for the similarity between two spectra we calculated the dot product of the relative intensities of the fragment ions (38). These calculations revealed that different spectra for the same ubiquitylated peptide identified in different measurements of the affinity-purified protein samples were very similar with an average dot product of 0.9861. Also the correlations between technical replicate spectra for the synthetic peptides gave a high average dot product of 0.9966, whereas the average dot product between technical replicate spectra of different synthetic peptides and affinity-purified protein samples spiked with synthetic peptides was 0.9368. In contrast, the correlation between spectra of affinity-purified protein samples and synthetic peptides and between affinity-purified protein samples and spiked affinity-purified protein samples was lower with averages of 0.6503 and 0.7035, respectively (supplemental Table S10). The correlation frequencies in Fig. 6 illustrate that the correlation between spectra of affinity-purified protein samples and synthetic peptides was particularly low. Also the correlation between spectra of affinity-purified protein samples and spiked affinity-purified protein samples was low, indicating that the spiked affinity-purified samples only the synthetic peptides were identified, which were not identical to the ubiquitylated peptides originally identified in the affinity-purified protein samples. This is shown in detail for the peptides with sequence SAEGLGGLNKR (supplemental Table S11, supplemental Fig. S6) and AGVKGVEEPVK (supplemental Table S12, supplemental Fig. S7). One exception was the peptide with sequence TILYNKGGSR, which had a good correlation of 0.9852 between spectra from the affinity-purified protein sample and the spiked affinity-purified protein sample. However, this peptide belongs to a protein from the reverse database.
Proteasomes are cleared from the cell by autophagy. This molecular and cellular proteomics 13.6 1533. It is possible that protein aggregates and protein components that cannot be degraded by the 26S proteasome which serves as a recycling route for ubiquitylated molecular autophagy, protein synthesis and targets proteins to degradation by alternative protein degradation pathways such as autophagy, MET, PRO, and GLY PROTEIN 1 (CMPG1, AT1G66160), which is a protein quality control pathway for misfolded or incorrectly assembled proteins in the ER that are retranslocated to the cytosol for degradation by the proteasome. In plants CDC48 is required for this protein re-translocation (78). The Arabidopsis genome encodes three CDC48 proteins (CDC48A, AT3G09840; CDC48B, AT3G53230; CDC48C, and AT3G01610), and both CDC48A and CDC48B accumulated after SylA treatment. Expression of the ABC4 (AT2G47000) and AT5G24650 transport proteins was also induced after SylA treatment, suggesting that protein translocation is an important cellular response to accumulating protein levels.

SylA treatment also increased the deubiquitylating enzyme UBP6 (AT1G51710). In yeast UBP6 interacts with the base of the proteasome and together with the metalloprotease proteasome subunit Rpn11 contributes to deubiquitylation of UPS-targeted proteins (79). It is therefore likely that in Arabidopsis UBP6 also removes the polyubiquitin chain from polyubiquitylated proteins.

Among the proteins that accumulated after inhibition of the proteasome we were especially interested in the proteins targeted to the UPS for maintaining cellular protein homeostasis. We have shown that in Arabidopsis most quantified proteins do not follow the large fluctuations in their diurnal transcript levels (7). Such protein homeostasis could be achieved by tight regulation of protein synthesis and UPS-mediated protein degradation independent of mRNA levels during changing environmental conditions such as the transition from light to dark. This would allow for maintaining protein levels under non-stress conditions and, at the same time, for an increase in protein levels through decreased protein degradation in case of changing environmental conditions. As this type of regulation does not require de novo protein synthesis, a fast adaptation to changing environmental conditions can be achieved. This has for example been shown to be the case in early defense response, where the E3 ubiquitin ligase CYS, MET, PRO, and GLY PROTEIN 1 (CMPG1, AT1G66160), which itself is a target of the UPS, gets stabilized and accumulates (80). This also applies to the heat shock protein HSP70, as cytosolic HSP70 associates with FES1A (AT3G09350), which is induced by high temperatures and prevents HSP70 degradation (81). The level of FES1A was increased after SylA treatment (Table I), as were transcript levels for HSP70 and FES1A. In mice, both HSP70 and HSP90 interact with the ubiquitin ligase CHIP, which affects their stability (71). HSP90 also accumulated after SylA treatment at protein and transcript level and was ubiquitylated. Together, these results indicate that some proteins are regulated both at the level of transcription and protein degradation. IGMT4, a putative inositol glucosinolate O-methyltransferase also accumulated after SylA treatment, and its transcript level was increased.

**DISCUSSION**

Protein degradation by the UPS plays an important role in all important cellular processes. For example, in plants it was shown that defense against pathogens involves ubiquitylation of both positive and negative regulatory proteins, and also proteins that regulate responses to abiotic stress are targeted to the UPS for degradation (9–10, 21, 70–77). Interfering with the UPS revealed that only a subpopulation of the accumulating proteins can be directly attributed to UPS degradation. Our results suggest that inhibition of the proteasome reduced protein synthesis and targets proteins to degradation by alternative protein degradation pathways such as autophagy, which serves as a recycling route for ubiquitylated molecular components that cannot be degraded by the 26S proteasome (55). It is possible that protein aggregates and protein complexes such as ribosomes and non-functional (i.e. blocked) proteasomes are cleared from the cell by autophagy. This could explain why only few of the proteasome subunit proteins accumulate after SylA treatment, although all but one have increased transcript levels. Inhibition of the proteasome may also activate ER-associated protein degradation (ERAD), which is a protein quality control pathway for misfolded or incorrectly assembled proteins in the ER that are retranslocated to the cytosol for degradation by the proteasome. In plants CDC48 is required for this protein re-translocation (78).
increased after SylA treatment. As an O-methyltransferase IGMT4 could be involved in specific defense responses because its transcript level is induced after aphid feeding attacks, but not in response to microbial pathogens, caterpillars, or thrips (82). Thus, IGMT4 may be continuously synthesized and turned over during stress-free conditions and accumulates after aphid feeding attacks.

To assess whether we can detect diurnal regulation of protein degradation we investigated the proteins with opposing changes in accumulation at the end of the night and at the end of the day. The proteins that had accumulated to higher levels at the end of the day and that were reduced at the end of the night include NITRILE SPECIFIER PROTEIN 5 (NSP5 AT5G48180). NSP5 is a protein in the glucosinolate pathway that catalyzes the hydrolysis of aglucone into nitriles and therefore prevents the spontaneous rearrangement of aglucone to the reactive isothiocyanate (83). Both isothiocyanates and nitriles seem to play a role in the biotic defense response and glucosinolates have also been shown to form a link between secondary metabolism and the circadian clock (84). Given the roles of this protein in defense and circadian rhythm, its daytime-specific regulation seems plausible.

Our results demonstrate that peptide spectrum assignments with the ubiquitin footprint as variable modification result in a high occurrence of false positive ubiquitylation site assignments in complex peptide mixtures. As the affinity enrichment step using the UBA domain by definition targets the full-length ubiquitylated proteins, the majority of the resulting tryptic peptides are not expected to carry a diglycine tag. Although these unmodified peptides allow for the reliable identification of the affinity-enriched ubiquitylated proteins, they seem to interfere with the identification of the ubiquitylation sites. Indeed, when only peptides with attached diglycine tags were measured, as was the case for the synthetic peptides, the correct modified peptide sequences were identified. For correct ubiquitylation site assignment it therefore seems necessary to work with highly enriched peptides with attached diglycine tags. This can for example be achieved with the use of a diglycine-lysine specific antibody (85–86). In a refined workflow including this antibody, the identification and quantification of more than 20,000 distinct endogenous ubiquitylation sites has recently been reported in human cells (87). This demonstrates that the reliable determination of ubiquitylation sites is possible by the use of specialized workflows.

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[5] This article contains supplemental Figs. S1 to S7 and Tables S1 to S12.

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