Proteins of Diverse Function and Subcellular Location Are Lysine Acetylated in Arabidopsis[1][W][OA]

Iris Finkemeier[2], Miriam Laxa, Laurent Miguet[3], Andrew J. M. Howden[4], and Lee J. Sweetlove*

Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom

Acetylation of the ε-amino group of lysine (Lys) is a reversible posttranslational modification recently discovered to be widespread, occurring on proteins outside the nucleus, in most subcellular locations in mammalian cells. Almost nothing is known about this modification in plants beyond the well-studied acetylation of histone proteins in the nucleus. Here, we report that Lys acetylation in plants also occurs on organellar and cytosolic proteins. We identified 91 Lys-acetylated sites on 74 proteins of diverse functional classes. Furthermore, our study suggests that Lys acetylation may be an important posttranslational modification in the chloroplast, since four Calvin cycle enzymes were acetylated. The plastid-encoded large subunit of Rubisco stands out because of the large number of acetylated sites occurring at important Lys residues that are involved in Rubisco tertiary structure formation and catalytic function. Using the human recombinant deacetylase sirtuin 3, it was demonstrated that Lys deacetylation significantly affects Rubisco activity as well as the activities of other central metabolic enzymes, such as the Calvin cycle enzyme phosphoglycerate kinase, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, and the tricarboxylic acid cycle enzyme malate dehydrogenase. Our results demonstrate that Lys acetylation also occurs on proteins outside the nucleus in Arabidopsis (*Arabidopsis thaliana*) and that Lys acetylation could be important in the regulation of key metabolic enzymes.

Acetylation of Lys side chains is a reversible and highly regulated posttranslational modification of both prokaryotic and eukaryotic proteins. Lys acetylation was first discovered for histones (Gershey et al., 1968), where it acts to regulate chromatin structure and gene expression (Lusser et al., 2001; Kurdistani and Grunstein, 2003; Martin and Zhang, 2005). However, in addition to its occurrence on nuclear proteins, Lys acetylation has recently emerged as a widespread posttranslational modification, occurring in a large number of proteins of diverse biological function (Choudhary et al., 2009). Lys acetylation is catalyzed by specific protein acetyltransferases and reversed by deacetylases. Enzymes of central carbon metabolism are a particular target, with most enzymes of glycolysis and the tricarboxylic acid (TCA) cycle being acetylated in *Salmonella enterica* and their acetylation status regulating flux through these pathways (Wang et al., 2010). The role of Lys acetylation in the regulation of central metabolism appears to be conserved in higher eukaryotes, with widespread acetylation of enzymes occurring in human liver (Zhao et al., 2010). As well as regulating enzymes directly, Lys acetylation links metabolic status to specific patterns of gene expression through histone modification (Wellen et al., 2009).

In plants, Lys acetylation has to date been exclusively studied in the context of chromatin. A number of histone acetyltransferases have been identified in plants, classified as A or B type depending on their nuclear or cytosolic localization, respectively (Chen and Tian, 2007). There are also many histone deacetylases, with Arabidopsis (*Arabidopsis thaliana*) containing 18 members of a putative histone deacetylase family (Pandey et al., 2002). Histone acetylation has been implicated in the regulation of gene expression associated with a number of developmental transitions (Tian and Chen, 2001; Tian et al., 2005) as well as being involved in the response to different environmental cues, including light (Chua et al., 2003) and low temperature (Sheldon et al., 2006). Histone Lys acetylation state is also thought to be important in integrating stress hormone signals (Chen and Tian, 2007).

The Lys acetylation status of nonhistone proteins is thought to be regulated by the sirtuin (SIRT) family of NAD^+-dependent deacetylases (Blander and Guarente, 2004), which, in humans, includes nucleus-, cytosol-,

[1] This work was supported by a Junior Research Fellowship (Christ Church College, University of Oxford) and by a Feodor Lynen Research Fellowship (Alexander von Humboldt Foundation) to I.F., by the Biotechnology and Biological Sciences Research Council (United Kingdom), and by the Deutsche Forschungsgemeinschaft (Emmy Noether Programme, grant no. FI 1655/1–1).

[2] Present address: Department of Biology, Ludwig-Maximilians-University Munich, Grosshaderner Strasse 2, 82152 Planegg-Martinsried, Germany.

[3] Present address: Laboratoire de Spectrométrie de Masse Bio-Organique, Ecole de Chimie, Polymères et Matériaux, Bât. R5–0 25, Rue Becquerel, 67087 Strasbourg, France.

[4] Present address: Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK.

* Corresponding author; e-mail lee.sweetlove@plants.ox.ac.uk.

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www.plantphysiol.org/cgi/doi/10.1104/pp.110.171595

and mitochondria-localized members (Schwer et al., 2002; North et al., 2003). Two SIRT homologs are present in the Arabidopsis genome, but it is currently not clear the extent to which Lys acetylation of nonhistone proteins occurs in plants. The aim of this study, therefore, was to characterize the extent of Lys acetylation in the Arabidopsis proteome and to undertake an initial examination of its functional significance beyond chromatin modification.

RESULTS AND DISCUSSION

To gain an initial overview of the extent of Lys acetylation on plant cell proteins, western-blot analysis was done using a commercially available antibody against acetyl-Lys residues that was used in previous studies to detect Lys-acetylated proteins in human cell lines and bacteria (Kim et al., 2006; Choudhary et al., 2009; Wang et al., 2010). Multiple proteins were immunoreactive in Arabidopsis cell culture extracts and leaf tissue (Fig. 1A, lanes 2 and 4). A competition assay with acetylated bovine serum albumin confirmed the specificity of the antibody for Lys-acetylated sites in Arabidopsis (Fig. 1A, lane 3). Several proteins of molecular mass higher than histones (10–30 kD) were detected, suggesting that it is not just histones that are acetylated in Arabidopsis. Furthermore, Lys-acetylated proteins could be detected by immunoprecipitation of proteins isolated from Arabidopsis whole leaf extract as well as from organellar fractions such as mitochondria and chloroplasts (Fig. 1B). The strongest immunoreactive bands were detected around the 55-kD H chain of the anti-acetyl-Lys antibody at an exposure time of 2 min. Longer exposure times resulted in completely blackened lanes, suggesting that there were proteins of all sizes pulled down by immunoprecipitation in the different fractions.

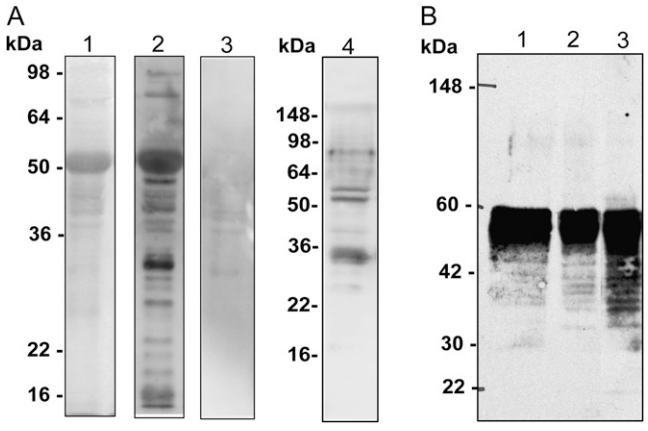

Figure 1. Detection of Lys-acetylated proteins in Arabidopsis using anti-acetyl-Lys antibody. A, Western-blot analysis of acetyl-Lys-containing proteins from Arabidopsis leaves (lanes 1–3) and heterotrophic cell cultures (lane 4). Lane 1, Ponceau S stain of 50 μg of Arabidopsis total leaf protein transferred to a nitrocellulose membrane; lane 2, anti-acetyl-Lys antibody (1:1,250) on leaf proteins; lane 3, competition assay with 1 mg of acetylated bovine serum albumin and anti-acetyl-Lys antibody (1:1,250) on leaf proteins; lane 4, anti-acetyl-Lys antibody (1:1,250) on cell culture proteins. B, Western-blot analysis of immunoprecipitated Lys-acetylated proteins from leaves (lane 1) and isolated organelles (mitochondria [lane 2] and chloroplast [lane 3]) using anti-acetyl-Lys antibody (1:1,250).

To identify novel Lys-acetylated proteins in Arabidopsis, protein extracts were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS; from at least three biological replicates). To reduce the complexity of the protein mixture and thereby maximize the number of Lys-acetylated proteins that could be identified, two different fractionation strategies were used (Fig. 2). First, proteins extracted from a heterotrophic Arabidopsis cell suspension culture (May and Leaver, 1993) were fractionated by size-exclusion chromatography, yielding 10 fractions that were then digested with trypsin prior to MS analysis (LTQ Orbitrap). Second, proteins extracted from leaf tissue or from isolated organelles (mitochondria or chloroplasts) were digested with trypsin and then enriched for Lys-acetylated proteins by immunopurification prior to MS analysis (LTQ Velos). The purity of the organellar fractions was confirmed by MS; more than 50% of the identified proteins were previously confirmed to reside in the respective organelle (Heazlewood et al., 2005) and were identified as most abundant in the organellar fractions by spectral counting (Supplemental Table S1).

To identify Lys-acetylated proteins, MS/MS spectra were searched for the modification $K_{170.11}$, as acetylation of the Lys residue results in a mass gain of 42.0105 D due to the substitution of a hydrogen atom with an acetyl group (CH_3CO-). All spectra containing this modification were manually inspected to ensure diagnostic b- or y-ion series (Fig. 2B; Supplemental Fig. S1). Furthermore, although Lys-acetylated peptides can be distinguished from Lys-trimethylated peptides in spectra from the LTQ Orbitrap mass spectrometer (the two modifications differing in mass by only 0.0363 D), we did not obtain sufficient mass accuracy with the LTQ Velos mass spectrometer (which has a dual cell linear ion trap) to make this distinction. Therefore, LTQ Velos MS/MS spectra were searched for the neutral loss ion "MH^+-59," that can be used as a unique marker for trimethylation (Zhang et al., 2004). Peptides for which this neutral loss ion was observed were removed from the analysis. However, it should be noted that failure to detect the neutral loss ion does not rule out its presence at levels below the detection limit, and the possibility remains that some false negatives of Lys-trimethylated peptides could remain.

All spectra were analyzed using the central proteomics facilities pipeline (CPFP) as described in "Materials and Methods" (Trudgian et al., 2010). Only peptides from complex mixtures passing a 1% false discovery rate were searched for internal Lys-acetylated sites and selected for manual inspection. In total, 91 Lys-acetylated sites and only seven trimethylated sites were identified (Supplemental Table S2). The 91

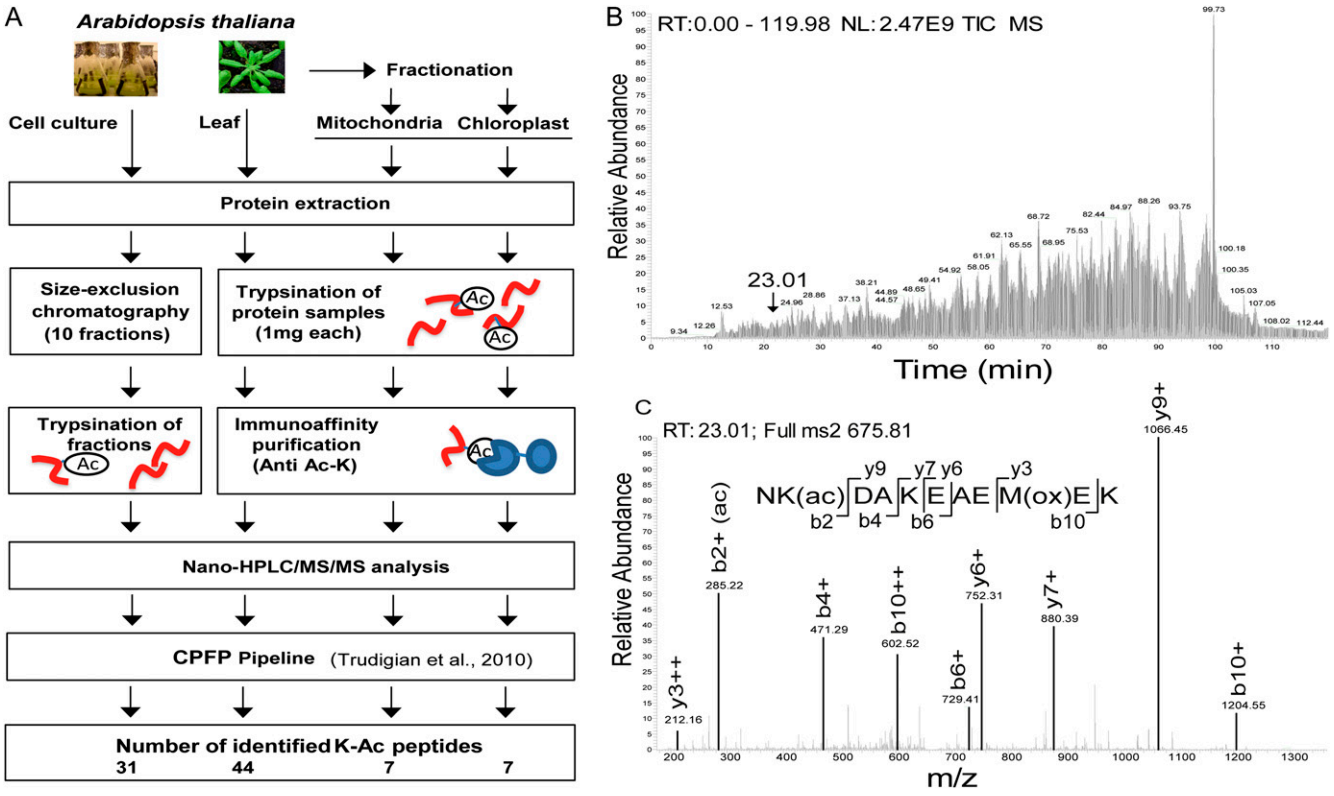

Figure 2. Gel-free LC-MS/MS experimental strategy for the analysis of Arabidopsis Lys-acetylated peptides. A, Scheme of the experimental procedure. B, Example LC-MS chromatogram. The precursor ion detected in the MS spectrum at the retention time (RT) 23.01 min was further fragmented by MS/MS as shown in C. C, Fragmentation spectrum of an acetylated peptide unique to formin homology 2-domain containing protein (At5g07650).

Lys-acetylated sites belonged to 74 unique proteins of various functional categories (Fig. 3A; Supplemental Table S3). The majority of the Lys-acetylated proteins were identified in the fractions immunoenriched for protein-acetylated Lys residues, with most proteins belonging to the functional categories "photosynthesis" (Calvin-Benson cycle and light reactions) and "protein" (protein synthesis, degradation, and post-translational modification). In protein extracts from cell cultures, 31 Lys-acetylated peptides were uniquely identified (Supplemental Table S2). These peptides belonged to proteins of various functional categories, including "cell organization," "protein targeting," "signaling," and "metabolism" (Supplemental Table S3). Interestingly, more than half of these 31 proteins were acetylated on a Lys very close to the N-terminal end of the protein within the first 30 amino acids. A study by Zybailov et al. (2008) identified 47 N-terminal acetylation sites on nucleus-encoded proteins in Arabidopsis plastid preparations, but it did not investigate internal Lys-acetylated sites. We can exclude N-terminal acetylation on these 31 proteins, as we only selected peptides that confirmed the location of the Lys acetylation site by indicative b- and y-ion series.

To further investigate Lys acetylation sites, the amino acids surrounding the observed acetylated Lys residues were analyzed using the WebLogo tool (Crooks et al., 2004). Only the two flanking amino acids at either side of the acetylated Lys were analyzed, as several Lys-acetylated sites occurred close to the N terminus of proteins or were adjacent to the next Lys-acetylated site separated by only two amino acids. Only a very weak motif could be detected, with the most common amino acids Gly, Ala, Leu, and Arg surrounding the acetylated Lys residues (Fig. 3B), in comparison with Tyr, Phe, Glu, and Gly in human acetylated proteins (5). Only Gly at −1 is common to Lys-acetylated peptides from other organisms and has been found mainly in acetylation sites of nucleus-located proteins (Yang, 2004; Choudhary et al., 2009). However, it is interesting that 21 of the 91 identified Lys residues were conserved in the respective human homolog protein (Supplemental Text S1).

Lys Acetylation of Nuclear Proteins

As was to be expected, several histone proteins were found to be Lys acetylated, validating the ability of the proteomics strategy to correctly identify Lys-acetylated proteins. Four Lys-acetylated sites of histones H3 and H4 (At1g09200 and At1g07660) were identified that were previously shown to be Lys acetylated in Arabi-

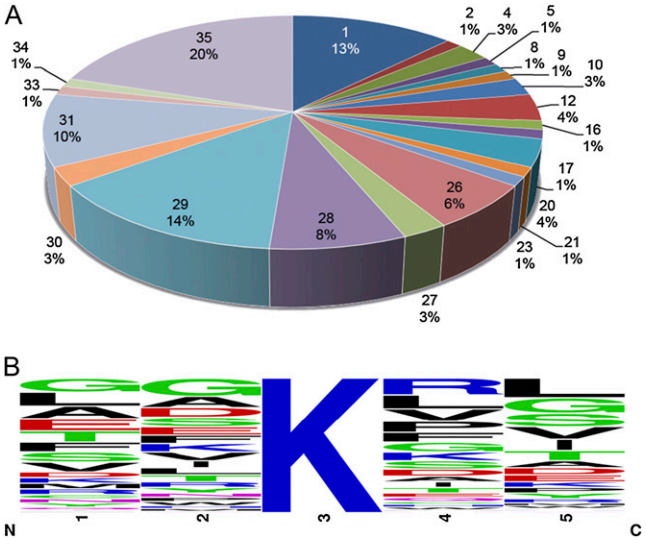

Figure 3. Overview of Lys-acetylated proteins in Arabidopsis. A, Pie chart of functional categories of Lys-acetylated proteins. The numbers represent the following categories: 1, photosynthesis; 2, major carbohydrate metabolism; 4, glycolysis; 5, fermentation; 8, TCA cycle; 9, mitochondrial respiratory chain; 10, cell wall; 12, nitrogen metabolism; 16, secondary metabolism; 17, hormone metabolism; 20, stress; 21, redox regulation; 23, nucleotide metabolism; 26, miscellaneous; 27, RNA regulation of transcription; 28, DNA synthesis; 29, protein synthesis, degradation, targeting, and posttranslational modification; 30, signaling; 31, cell organization and cell cycle; 33, development; 34, transport; 35, not assigned unknown proteins (PageMan analysis; Usadel et al., 2006). B, Sequence plot of the Lys-acetylated amino acid motif showing two amino acids on either side of the acetylated Lys residue. The sequence plot was generated with the WebLogo tool (Crooks et al., 2004) using 88 identified Lys-acetylated sites from 74 Arabidopsis proteins.

dopsis (Zhang et al., 2007). In addition to histones, three other Lys-acetylated nuclear proteins were found. These were NUCLEOSOME ASSEMBLY PROTEIN1 (At4g26110), a zinc finger protein transcription factor (At2g24500) and multidomain cyclophilin 59 (At1g53720), a multidomain cyclophilin with an RNA recognition motif, suggesting that Lys acetylation may regulate other aspects of DNA structure and gene expression in Arabidopsis. For several transcription factors from nonplant systems, it was shown that Lys acetylation adjacent to the DNA-binding site can result in a stimulation of DNA binding, while Lys acetylation in the DNA-binding domain disrupts DNA binding and transcription (Kouzarides, 2000; Yang and Seto, 2008).

Plastid Proteins Are Lys Acetylated

Unique to plants, proteins involved in photosynthesis represented a significant proportion of the total number of observed Lys-acetylated proteins. In total, nine individual photosynthetic proteins were Lys acetylated (approximately 12% of the total; Fig. 3A). These included both proteins of the photosynthetic electron transport chain, such as subunit H of the PSI reaction center (At1g52230), chlorophyll *a*/*b*-binding protein 3 (At1g29910), and the β-subunit of the chloroplast ATP synthase (AtCg00480), and the Calvin-Benson cycle (Rubisco, both large [AtCg00490] and small [At1g67090] subunits), Rubisco activase (At2g39730), aldolase (At2g21330), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; At1g12900), and phosphoglycerate kinase (At1g56190; Table I).

Lys Acetylation of Enzymes of Central Metabolism

In contrast to both bacteria (Wang et al., 2010) and animals (Zhao et al., 2010), where all the enzymes of glycolysis and the TCA cycle were Lys acetylated, we recovered relatively few enzymes of these pathways. This most likely reflects the fact that a lower depth of coverage of the Lys-acetylated proteome was achieved in this study, rather than a lack of Lys acetylation of these enzymes. In glycolysis, a putatively cytosolic aldolase (At3g52930) was Lys acetylated, and linked to the TCA cycle, a putatively cytosolic isoform of NAD^+-dependent malate dehydrogenase (MDH; At1g04410) was Lys acetylated. Only one Lys-acetylated protein of the mitochondrial respiratory chain was found: cytochrome *c* (At1g22840). In addition, the fermentative enzyme pyruvate decarboxylase (At5g17380) was Lys acetylated. Several other proteins associated with central carbon or nitrogen metabolism were Lys acetylated. These include the chloroplast ATP/ADP exchanger (AAC1; At3g08580), Gln synthase (At3g17820), and two enzymes of secondary metabolism (a cytochrome P450 [At5g45340] and a cinnamyl alcohol dehydrogenase [At5g19440] involved in phenylpropanoid metabolism). This suggests that sectors of both primary carbon and nitrogen metabolism as well as aspects of secondary metabolism could be regulated by Lys acetylation. The occurrence of Lys acetylation on the plastid ATP/ADP translocator, ATP synthase, and on the LHCB antenna protein demonstrates that Lys acetylation is not limited to soluble proteins but can also occur on integral membrane proteins.

Lys Acetylation of Structural Proteins Involved in Plant Development

Several Lys-acetylated proteins involved in cytoskeleton organization were found. Among them are a protein involved in microtubule organization (At2g35630; MOR1), an actin-depolymerizing factor (At3g4600; ADF2), and a microtubule motor armadillo repeat-containing kinesin-related protein (At3g54870; ARK1/MRH2) involved in modulating microtubule depolymerization during root hair tip growth (Yoo et al., 2008). Furthermore, CAP1 (At4g34490), a member of the cyclase-associated protein family that acts as a fundamental facilitator of actin dynamics over a wide range of plant tissues (Deeks et al., 2007), was Lys acetylated. In HeLa cells, Lys acetylation is an important regulator of cytoskeleton dynamics, and many structural proteins such as actin and tubulin as well as regulators of the cytoskeleton dynamics were found to

Table I. *Plastid-localized Lys-acetylated proteins*

Immunoenriched Lys-acetylated peptides from Arabidopsis leaves and chloroplasts were analyzed with the LTQ Velos/LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific; Supplemental Table S2). Spectra files were processed using the CPFP as described in "Materials and Methods." Fragment ion spectra were manually inspected for indicative b- and y-ion series (Supplemental Fig. S1). i-probability shows confidence score of Peptide Prophet (CPFP; Trudgian et al., 2010).

Description	Protein	Peptide	Lys (K) Side Chain Site	i-probability
Fru-bisP aldolase	At2g21330	K.YTGEGESEEAKEGMFVK(ac)GYTY.-	K395	1
GAPA-2 (GAPDH)	At1g12900	K.LNGIALRVPTPNVSVVDLVVQVSK(ac)K.T	K314	0.95
PGK1	At3g12780	K.GVTPK(ac)FSLAPLVPR.L	K146	1
PGK1	At3g12780	K.IGVIESLLEK(ac)CDILLLGGGMIFTFYK.A	K288	1
PGK1	At3g12780	R.LSELLGIEVTK(ac)ADDCIGPEVESLVASLPEGGVLLLENVR.F	K169	1
RCA (Rubisco activase)	At2g39730	K.NFLTLPNIKVPLILGIWGGK(ac)GQGK.S	K167	1
Large subunit of Rubisco	AtCg00490	K.ASVGFK(ac)AGVK.E	K14	0.98
Large subunit of Rubisco	AtCg00491	K.AGVK(ac)EYK.L	K18	0.99
Large subunit of Rubisco	AtCg00490	R.IPPAYTK(ac)TFQGPPHGIQVER.D	K146	1
Large subunit of Rubisco	AtCg00490	K.YGRPLLGCTIK(ac)PK.L	K175	1
Large subunit of Rubisco	AtCg00490	K.AGVKEYK(ac)LTYYTPEYETK.D	K21	0.98
Large subunit of Rubisco	AtCg00490	K.GHYLNATAGTCEEMIK(ac)R.A	K252	1
Large subunit of Rubisco	AtCg00490	R.VLAK(ac)ALR.L	K316	0.95
Large subunit of Rubisco	AtCg00490	R.DDYVEK(ac)DR.S	K356	0.99
Large subunit of Rubisco	AtCg00490	K.WSPELAAACEVWK(ac)EITFNFPTIDKLDGQE.-	K463	0.85
Rubisco small subunit 1A (RBCS-1A)	At1g67090	R.YWTMWK(ac)LPLFGCTDSAQVLK.E	K126	0.96
ATP synthase F1 sector subunit β	AtCg00480	K.LSIFETGIK(ac)VVDLLAPYRR.G	K154	1
PSI reaction center, putative/PSI-H	At1g52230	K.FFETFAAPFTK(ac)R.G	K99	1
CAB3 (chlorophyll *a*/*b*-binding protein 3)	At1g29910	R.NGVK(ac)FGEAVWFK.A	K125	0.74
GSH2	At5g27380	K.MESQK(ac)PIFDLEKLDDEFVQK.L	K66	1
AAC1 (ADP/ATP carrier 1)	At3g08580	R.MMMTSGEAVK(ac)YK.S	K325	1
GS2 (Gln synthetase 2)	At5g35630	K.WPLGWPVGAFPGPQGPYYCGVGADK(ac)IWGR.D	K223	1
Catalytic/coenzyme-binding chloroplast precursor	At2g37660	R.TGQIVYK(ac)K.L	K94	0.62

be Lys acetylated (Kim et al., 2006; Choudhary et al., 2009). Lys acetylation of tubulin also occurs in primitive eukaryotes such as *Tetrahymena* and *Chlamydomonas* and has an impact on cell motility (Westermann and Weber, 2003). It is possible, therefore, that Lys acetylation is also an important regulator of microfilaments in plants. The other main structural component of the plant cell, the cell wall, also had associated proteins that were Lys acetylated. Two proteins involved in cell wall synthesis, β-galactosidase 8 (At2g28470) and UDP-Xyl synthase (At3g46440), were found to be Lys acetylated.

Lys Acetylation of Proteins Involved in Cell Signaling and Plant Stress Responses

Several proteins linked to cellular signaling were Lys acetylated. These included a protein involved in hormone responses, the ethylene receptor 2 (At3g23150; ETR2). ETR2 is a two-component His kinase involved in ethylene perception (Plett et al., 2009). Lys acetylation of ETR2 occurs at Lys-507, which resides between the His kinase and the signal receiver domain, potentially having an impact on the interaction of these two domains. A phosphatidylinositol 3- and 4-kinase (At5g09350) was also Lys acetylated. Interestingly, Lys acetylation also occurred in the pentatricopeptide repeat (PPR) domain of the mitochondrial PPR40 protein (At3g16890). PPR40 is a potential signaling link between mitochondrial electron transport and transcriptional regulation of stress and hormonal responses in the nucleus (Zsigmond et al., 2008). As well as glutathione synthetase (At5g27380; GSH2), three other proteins involved in abiotic stress responses were Lys acetylated: a DNAJ heat shock domain-containing protein (At3g06340), a dehydrin (At1g76180; ERD14), and a late embryogenesis abundant-like protein (At4g02380.2; LEA5/SAG21). A mitochondrial calcium-binding protein (At3g59820) and an F-box protein (At2g36090) involved in protein turnover were Lys acetylated. Several F-box proteins and two major proteasome-bound deubiquitinases were also found to be Lys acetylated in human cell lines (Choudhary et al., 2009). Regulation of protein turnover by Lys acetylation is a distinct possibility, as Lys is the ubiquitinated residue and acetylation of the Lys would prevent ubiquitination and thus protein degradation (Yang and Seto, 2008).

Lys Acetylation of Proteins Involved in Protein Synthesis and Targeting

Seven Lys-acetylated proteins were identified that play a role in different steps of protein biosynthesis and targeting. Of proteins involved in protein biosynthesis,

these included a lysyl-tRNA synthetase (At3g11710) that catalyzes the attachment of Lys to its cognate RNA molecule, the translation factor EF1-α (At1g07920), which was also found to be Lys acetylated in HeLa cells (Choudhary et al., 2009), as well as the 60S ribosomal proteins L35a (At1g41880) and L5-1 (At3g25520). Two proteins were Lys acetylated that are involved in protein targeting via the endomembrane system. VSP35 (At3g51310) is part of a retromer protein complex involved in endosome-to-lysosome protein transport (Oliviusson et al., 2006), and a sec34-like family protein (At1g73430) of unknown function that contains a sec34 domain important for tethering vesicles to the Golgi.

Lys Acetylation Regulates Enzyme Functions

Lys acetylation of the ε-amino group of the Lys side chain results in neutralization of the positive charge and impairs the formation of hydrogen bonds, which is important for the biological function of Lys in many proteins and enzymes. Thus, Lys acetylation could affect diverse protein functions such as enzyme activity and protein-protein and protein-nucleic acid interactions, depending on the position and function of the Lys residue for the overall protein function. To gain an insight into the position of the identified acetylated Lys residue within the protein molecule, we generated three-dimensional structures of six selected Arabidopsis enzymes of central metabolism using published structural data of homologous proteins from other species that share a high sequence similarity with the respective Arabidopsis protein (Supplemental Fig. S2).

Phosphoglycerate kinase and Rubisco stand out in terms of the extent of Lys acetylation. Rubisco was the most abundantly Lys acetylated, with nine different Lys acetylation sites (Lys-14, -18, -21, -146, -175, -252, -316, -356, and -463) identified in the chloroplast-encoded large subunit (Supplemental Fig. S2; Supplemental Text S1). We confirmed the Lys-acetylated sites of Rubisco with the LTQ Orbitrap (Supplemental Fig. S2; Supplemental Text S1). Only for the peptides containing Lys-14 and Lys-463, we did not obtain a fragmentation spectrum with the LTQ Orbitrap but found the peptides in the LC scan at the correct retention time, ion charge, and with a δ mass of less than 10 ppm.

Lys-175 is a catalytically active Lys residue that accepts protons after enolization of ribulose-1,5-bisphosphate and protonates the acicarboxylate in the last step of the reaction (Cleland et al., 1998). Thus, Lys acetylation of Lys-175 would very likely interfere with Rubisco activity, which was also observed after site-directed mutations of this residue in *Rhodospirillum rubrum* (Knight et al., 1990). Furthermore, Lys acetylation occurred on Lys-252 and Lys-356, which are known to be important for ionic interactions at the dimer-dimer interface between two Rubisco large subunits, and on Lys-146, which normally functions in interdimer salt links with Glu-110 buried deep in the crevice between two dimers (Knight et al., 1990). Lys acetylation also occurred on Lys-316, which is strictly conserved in all known Rubisco sequences and forms a hydrogen bond to Leu-138 important for domain-domain interactions between the C and N termini in the hydrophobic core of the large subunit (Knight et al., 1990). Several posttranslational modifications have already been identified to occur on the Rubisco large subunit, mainly at the stage of protein assembly, such as carbamylation of the active

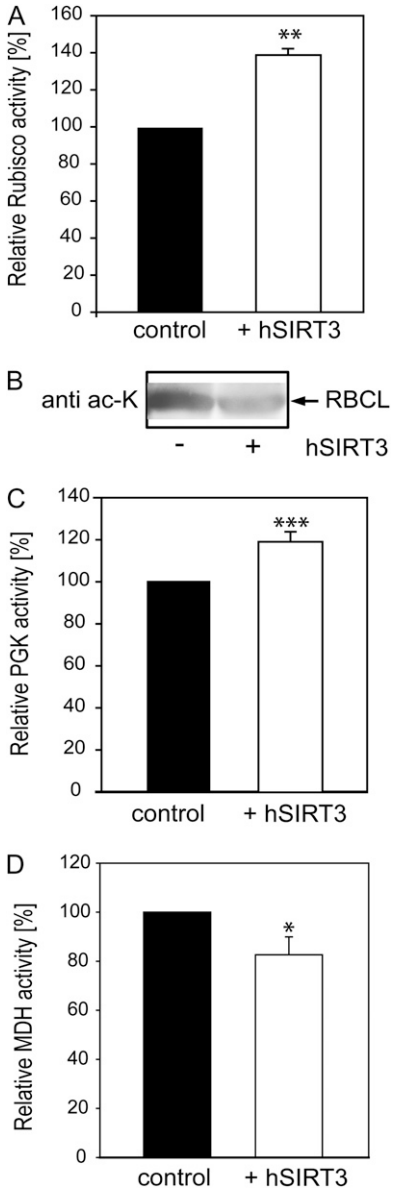

Figure 4. Lys acetylation affects enzyme activities of Rubisco (A and B), phosphoglycerate kinase (C), and NAD^+-dependent MDH (D) in Arabidopsis leaf extracts. Enzyme activities were measured after incubation with (+) or without (−) hSIRT3 deacetylase enzyme for 3 h at 37°C ($n = 5$, ±SD for MDH and PGK; $n = 3$, ±SD for Rubisco). Asterisks indicate significant differences (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) from control treatment without deacetylase (one-tailed paired t test). B shows a western-blot analysis of Lys-acetylated Rubisco large subunit (RBCL) in Arabidopsis leaf extract treated with or without hSIRT3 enzyme.

Table II. *hSIRT3 deacetylates Rubisco at several Lys residues*

Rubisco peptides were analyzed by MS on an LTQ Orbitrap XL system, and peak areas of the precursor ions (mass tolerance ≤ 10 ppm) were manually quantified from two biological replicates treated and untreated with hSIRT3 using the peak detection function of the Xcalibur program (Thermo Fisher Scientific). Retention times of the precursor ions are indicated in parentheses after the respective peak area. Ratio = peak area (+hSIRT3)/peak area (−hSIRT3); $n = 2$. n.d., Not detected.

Lys (K) Side Chain Site	Peptide	Precursor Mass	z	Peak Area Replicate (1)		Peak Area Replicate (2)		Ratio (1)	Ratio (2)
				−hSIRT3	+hSIRT3	−hSIRT3	+hSIRT3		
K14	K.ASVGFK(ac)AGVK.E	503.2893	2	2,024,658 (38.68)	1,743,313 (39.43)	4,323,830 (44.89)	1,994,744 (45.89)	0.86	0.46
K18	K.AGVK(ac)EYK.L	418.7293	2	6,042 (18.87)	n.d.	181,431 (21.57)	78,107 (22.07)	0.00	0.43
K21	K.EYK(ac)LTYYTPEYETK.D	935.4443	2	540,204 (55.79)	n.d.	166,379 (61.00)	92,189 (61.34)	0.00	0.55
K146	R.IPPAYTK(ac)TFQGPPHGI QVER.D	760.0713	3	105,885 (48.06)	97,448 (47.75)	n.d.	n.d.	0.92	n.d.
K175	K.YGRPLLGC(ca)TIKPK(ac) LGLSAK.N	705.4099	3	371,434 (44.7)	n.d.	n.d.	n.d.	0.00	n.d.
K252	K.GHYLN(da)ATAGTC(ca) EEMIK(ac)R.A	997.459	2	2,139,141 (22.98)	1,454,392 (23.57)	271,229 (30.3)	48,605 (30.52)	0.68	0.18
K252	K.GHYLNATAGTC(ca)EEMIK (ac)R.A	664.9806	3	147,742 (56.13)	n.d.	n.d.	n.d.	0.00	n.d.
K252	K.SQAETGEIKGHYLN(da)A (TAGTCca)EEMIK(ac)R.A	735.0985	4	6,310,874 (31.29)	3,437,305 (32.02)	1,862,689 (36.36)	1,457,631 (36.41)	0.54	0.78
K252	K.SQAETGEIKGHYLNATA GTC(ca)EEMIK(ac)R.A	979.4679	3	114,592 (55.33)	n.d.	71,688 (51.17)	29,682 (51.02)	0.00	0.41
K252	K.SQAETGEIKGHYLN(da) ATAGTC(ca)EEMIK(ac)R.A	979.7959	3	52,038 (40.01)	14,805 (40.43)	79,698 (44.37)	25,745 (43.79)	0.28	0.32
K316	R.VLAK(ac)ALR.L	406.7712	2	560,705 (22.32)	447,572 (22.91)	7,311,999 (25.42)	n.d	0.80	0.00
K356	R.DDYVEK(ac)DR.S	541.2435	2	931,502 (21.57)	8,349 (21.32)	n.d.	n.d.	0.01	n.d.
K356	R.ESTLGFVDLLRDDYVEK (ac)DR.S	771.3849	3	10,723,480 (51.75)	3,560,598 (52.44)	1,457,839 (52.42)	244,713 (53.04)	0.33	0.17
K356	R.ESTLGFVDLLRDDYVEK (ac)DR.S	1,156.5739	2	3,375,184 (87.55)	n.d.	1,095,145 (91.52)	286,040 (90.31)	0.00	0.26

site residue Lys-201 and N-terminal N^{α}-acetylation. Posttranslational modifications were also reported to occur at the level of the holoenzyme, such as trimethylation of Lys-14 (Houtz et al., 2008). As we identified Lys-14 to be Lys acetylated, it apparently competes with trimethylation at this residue. Although the function of trimethylation at Lys-14 is not known, it is discussed to play a role in the regulation of the protein-protein interactions of Rubisco with other proteins such as chromodomain proteins that specifically recognize trimethylated residues (Houtz et al., 2008). However, the same can be suggested for Lys acetylation, as Lys acetylation functions as a binding motif for bromodomain-containing proteins. To test whether Lys acetylation has an impact on Rubisco enzyme activity, we incubated Arabidopsis leaf extracts with purified human SIRT3 (hSIRT3) enzyme. hSIRT3 is a Lys deacetylase that is known to have broad substrate specificity, deacetylating several mitochondrial key metabolic enzymes (Hirschey et al., 2009).

The functional importance of Lys acetylation of Rubisco was confirmed by the observation that treatment of leaf extracts with hSIRT3 deacetylase caused a 40% increase in maximum catalytic activity of Rubisco (Fig. 4A). Activities are presented as percentage values to the control, as the absolute activities were slightly variable between biological replicates (1–2 μmol min^{-1} mg^{-1} protein, which lies in the range of published activities of Rubisco from spinach [*Spinacia oleracea*; Siegel and Lane, 1975]), but consistently increased after deacetylase treatment. In addition to the nine Lys-acetylated residues detected in the large subunit of Rubisco, there was one site present in the small subunit as well as one site in the Rubisco activase enzyme, both of which could have an effect on overall Rubisco activity. Western-blot analysis with the deacetylase-treated and untreated leaf extract revealed that although hSIRT3 leads to an appreciable reduction in Rubisco Lys acetylation, residual Lys acetylation was still detected, suggesting that not all Rubisco Lys acetylation sites can be removed by hSIRT3 (Fig. 4B). In order to identify the Lys-acetylated sites affected by hSIRT3 treatment, we performed a MS analysis similar to those described by Schlicker et al. (2008) for investigation of hSIRT3 deacetylation activity on metabolic enzymes. Following their protocol, protein extracts treated and untreated with hSIRT3 were fractionated by SDS-PAGE, and equally loaded Rubisco bands were excised from the gel. Proteins were trypsin digested and subsequently analyzed by LC-MS/MS (LTQ Orbitrap). Seven of the previous nine acetylated peptides were detected in the trypsinated samples of the excised Rubisco bands (Table II). The peak areas of the precursor ions were manually quantified using the Xcalibur

program (Thermo Fisher Scientific). Interestingly, most of the detected peptides were deacetylated by more than 50%, while Lys-146, which is buried deep in the Rubisco structure, was only marginally deacetylated by hSIRT3 treatment (Table II). Acetylation of the active site Lys-175 was only detected in one replicate. Thus, it cannot be ruled out that the observed increase in Rubisco activity after deacetylase treatment is a cooperative effect of several Lys residues affecting the tertiary structure of Rubisco rather than deacetylation of the active site residue. It is not immediately obvious what the physiological significance of the regulation of Rubisco by Lys acetylation is. One possibility is that it contributes to the partial inactivation of the Rubisco pool in vivo (Salvucci and Crafts-Brandner, 2004) and controls rapid increases in activity to match higher photon flux rates.

For phosphoglycerate kinase 1 (PGK1), all three acetylated Lys residues are exposed at the protein surface (Supplemental Fig. S2). Lys acetylation of at least one of these sites seems to inhibit PGK function, as treatment of Arabidopsis extract with hSIRT3 deacetylase resulted in a significant 20% increase in PGK activity (Fig. 4C).

MDH is a tetrameric enzyme, and the Lys-acetylated residues are situated at the dimer-dimer interface between two subunits (Supplemental Fig. S2). In the thermophilic bacterium *Chloroflexus aurantiacus*, it was shown that two Lys residues located at the dimer-dimer interface are essential in the network of electrostatic interactions, which are important for the oligomeric integrity of the protein complex (Bjørk et al., 2004). Although the amino acid sequence is not highly conserved between Arabidopsis and *Chloroflexus*, it is likely that similar interactions are also important for oligomerization of the Arabidopsis enzyme. When we measured NAD^+-MDH activity after deacetylase treatment of Arabidopsis protein extract with hSIRT3 enzyme, we observed a significant decrease in enzyme activity of approximately 20% (Fig. 4D). This is consistent with results obtained with human MDH, where deacetylase treatment of purified MDH enzyme resulted in more than 70% decreased activity and in vivo inhibition of deacetylases in HEK293T cells resulted in 100% increased activity (Zhao et al., 2010).

In general, the effect of deacetylase treatment on enzyme activity for all three tested enzymes was below 50% change in activity and thus much less than observed for human enzymes (Zhao et al., 2010). This could be due to the fact that (1) only a small proportion of the respective enzyme is Lys acetylated in vivo in Arabidopsis, (2) the Lys-acetylated residues are not always accessible to the enzyme, (3) there are many substrates competing for deacetylation in whole leaf extracts, and (4) hSIRT3 has a slightly different specificity toward plant Lys acetylation sites, which seem to vary from their animal counterparts (Fig. 3B); thus, deacetylation by SIRT3 is not completely efficient.

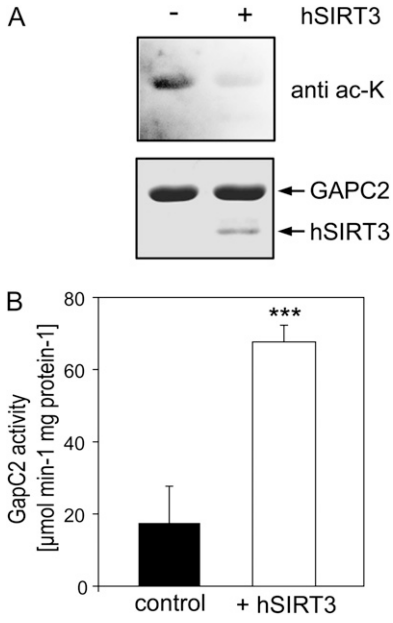

Figure 5. Effect of Lys acetylation on enzyme activity of recombinant GapC2 protein. A, Western-blot analysis of Lys-acetylated GapC2 protein with and without deacetylase treatment (top panel) and Coomassie blue-stained gel (bottom panel). B, Enzyme activity of GapC2 after treatment with and without hSIRT3 deacetylase ($n = 4$, ±SD). Asterisks indicate significant differences (*** $P < 0.001$, t test) from control treatment without deacetylase.

For the two glycolytic enzymes GAPDH and aldolase, we detected only one Lys-acetylated Lys residue each. Interestingly, in both enzymes, the Lys residues are very exposed on the protein surface, protruding like needles (Supplemental Fig. S2). Lys acetylation of these residues removes a surface charge, and one can assume that this has an impact on protein-protein interactions. For animal aldolase, it is known that several Lys residues located within the groove of the protein visible in the structure are important for binding to F-actin and thus for colocalization of glycolytic enzymes within the cell (Forlemu et al., 2007). Unfortunately, we were not able to test the effect of deacetylation on aldolase activity, as aldolase activity in total leaf extracts was too low to reliably quantify.

For plastidic GAPDH, it is known that it forms protein-protein interactions with other glycolytic enzymes or Calvin cycle enzymes in the chloroplast (Graciet et al., 2004). The acetylated Lys residue Lys-314 is conserved in plants and animal GAPDH sequences (Supplemental Text S1). As a recombinant GAPC-2 (At1g13440) protein was available in our laboratory, we tested whether the enzyme is Lys acetylated after purification from *Escherichia coli*. The recombinant GAPC-2 protein was Lys acetylated to a great extent in *E. coli*, and we were able to almost completely remove the Lys-acetyl groups by treatment with hSIRT3 enzyme (Fig. 5A). When we analyzed the recombinant GAPC-2 protein by MS (LTQ Orbitrap),

we detected four Lys-acetylated sites at Lys-130, -216, -220, and -255 (Supplemental Table S2). Interestingly, Lys-255 is homologous to the acetylated site Lys-314 in GAPA-2 and also homologous to the Lys-acetylated site Lys-203 from the GAPDH protein of *E. coli* (Zhang et al., 2009), indicating a conservation of Lys acetylation sites and a conservation of the acetyltransferase specificity between *E. coli* and a yet unidentified Arabidopsis plastidial acetyltransferase. Furthermore, the Lys-acetylated residue Lys-130 is homologous to Lys-128 from *Chlamydomonas* GAPDH (Lys-189 in GAPA-2), and it was shown that Lys-128 is essential for complex formation with the Calvin cycle enzyme phosphoribulokinase (Graciet et al., 2004). For the cytosolic GAPC-2 protein, Lys acetylation of Lys-130 could influence protein-protein interactions of GAPC-2 with other glycolytic enzymes (Graham et al., 2007). All four Lys-acetylated sites are predicted to be located at the outer surface of the GAPC-2 protein in a GAPC-2 structural model (Supplemental Fig. S2). Removal of the acetylation sites resulted in a more than 3-fold increase in enzyme activity (Fig. 5B), suggesting that Lys acetylation could also regulate GAPDH enzyme activity in Arabidopsis. However, no significant changes in total NADH- and NADPH-dependent GAPDH activity could be measured in Arabidopsis leaf extract after treatment with SIRT3. This could be due to the fact that not all GAPDH isoforms are Lys acetylated in Arabidopsis or that only a minority of GAPDH protein is acetylated under the tested conditions.

CONCLUSION

While several recent papers demonstrate that Lys acetylation regulates the activity of enzymes of central metabolic pathways (Schwer et al., 2006; Ahn et al., 2008; Nakagawa et al., 2009; Yu et al., 2009; Wang et al., 2010; Zhao et al., 2010), this is, to our knowledge, the first report that nonnuclear proteins are Lys acetylated in plants (see also Wu et al., 2011). Unique to this study is the observation that the activities of Calvin cycle enzymes can be regulated by Lys acetylation. Calvin cycle enzymes are already strongly regulated through a redox-dependent activation by thioredoxins in the light (Buchanan and Balmer, 2005) as well as other mechanisms, such as dynamic recruitment of enzymes into multiprotein complexes (Howard et al., 2008). Lys acetylation could provide an additional layer of regulation that might integrate metabolic cues such as energy or carbon status through the availability of acetyl-CoA. For Rubisco, this opens up a potentially novel strategy to engineer its activity. Significantly, Wu et al. (2011). have undertaken a similar approach to ours and also found many of the same acetylated photosynthetic proteins as reported here. Interestingly, they found that the Lys-acetylated form of LHCB is more abundant in the mobile antennae than in the PSII-bound antennae. Together, these data indicate that Lys acetylation is more widespread in Arabidopsis than previously thought and most likely is an important regulatory mechanism for in vivo enzyme function and for the regulation of photosynthesis.

MATERIALS AND METHODS

Cell Cultures and Plant Growth

Cell suspension cultures of Arabidopsis (*Arabidopsis thaliana* ecotype Landsberg *erecta*) were maintained as described previously (Williams et al., 2008). Arabidopsis plants (ecotype Columbia 0) were grown on compost supplemented with vermiculite at 22°C with a photoperiod of 14 h and a light intensity of 80 μE m^{-2} s^{-1}.

Organelle Isolation

Mitochondria were isolated from 50 g (fresh weight) of 10-d-old Arabidopsis seedlings (Day et al., 1985). Chloroplasts were isolated from 10 g of 4-week-old Arabidopsis leaves (Lamkemeyer et al., 2006). Nuclei were isolated from 10 g of 4-week-old Arabidopsis leaves (van Blokland et al., 1997). Organellar fractions were stored at −80°C until further use.

Protein Extraction

Proteins from Arabidopsis cell cultures were extracted from freeze-dried cells in a 6 M urea, 2 M thiourea, and 10 mM HEPES buffer (pH 8). The cell debris was pelleted by centrifugation. The protein-containing supernatant was filtered (0.22 μm) and fractionated into 10 fractions on a size-exclusion column (Agilent Zorbax GF-250) at a flow rate of 0.5 mL min^{-1} using extraction buffer as the mobile phase. Proteins from organellar fractions were isolated by acetone precipitation. Proteins from total leaves were isolated by phenol extraction (Isaacson et al., 2006).

In-Solution Digestion of Proteins

The precipitated proteins were reduced with dithiothreitol (DTT), alkylated with iodacetamide, and digested using modified sequencing-grade trypsin (Sigma-Aldrich) as described (Choudhary et al., 2009).

Enrichment of Lys-Acetylated Peptides and MS Analysis

Lys-acetylated peptides were enriched by immunoaffinity chromatography with polyclonal acetylated Lys antibody (Cell Signaling Technology), eluted with acidified water (0.1% trifluoroacetic acid), and purified for MS (Choudhary et al., 2009). MS data were acquired on an LTQ Velos linear ion trap (Thermo Fisher Scientific) and on an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) fitted with a nanospray source (Proxeon; Thermo Fisher Scientific) coupled to a nano-HPLC system (LTQ Orbitrap, U3000 Dionex, LTQ Velos, Easy-nLC, Proxeon; Thermo Fisher Scientific). Samples were loaded onto a 5-cm-long, 100-μm i.d. picotip column (New Objective) packed with C18 material (ProntoSIL C18 phase, 120-Å pore, 3-μm bead C18; Bischoff Chromatography). The HPLC was run in a direct injection configuration (buffer A, 5% acetonitrile, 0.1% formic acid; buffer B, 95% acetonitrile, 0.1% formic acid). Samples were loaded onto the column at a flow rate of 700 nL min^{-1} and resolved using a 75- to 120-min gradient at a flow rate of 300 nL min^{-1}. The Orbitrap was run in a data-dependent acquisition mode in which the Orbitrap resolution was set at 60,000 and the top-five multiply charged species were selected for MS/MS. In the LTQ Velos, the 20 most intense multiply charged ions were sequentially isolated and fragmented in the linear ion trap by collision-induced dissociation. Charge state +1 ions were rejected. The ion selection threshold was set to 500 counts for MS/MS, the activation Q was set to 0.25, and the activation time was set to 30 ms. Raw spectra files consisting out of full-scan MS and ion-trap MS/MS spectra were converted to mzXML data formats and searched against an in-house-generated target/decoy database (generated from the Arabidopsis TAIR8 protein database) using the CPFP, which supports searches with Mascot (Matrix Science), X-Tandem! (native and k-score), and OMSSA (Trudgian et al., 2010). Spectra

were searched with a mass tolerance of 20 ppm in MS mode and 0.5 D in MS/MS mode, allowing up to three missed cleavage sites. LTQ Velos spectra were searched with a mass tolerance of 2.5 D in MS mode and 0.5 D in MS/MS mode, as the LTQ Velos sometimes selects the nonmonoisotopic peak for fragmentation. Cys carbamidomethylation was searched as a fixed modification, whereas oxidized Met and acetylation of Lys were searched as variable modifications. Peptides identified at a 1% false discovery rate in the empirical/target decoy data set (Peptide Prophet) and that contained an internal acetylated Lys residue were manually inspected for indicative b- and y-ion series and for the neutral loss ion MH^+-59 to exclude isobaric trimethylation (Zhang et al., 2004). i-probability values are given for each peptide. The i-probability value is calculated from the threshold scores used by different search engines such as the Mascot or X-Tandem! score and translated into a single i-probability value using Bayesian statistics (Keller et al., 2002). The closer the i-probability value is to 1, the more likely the peptide hit is to be correct. Quantitation of precursor ion peak areas was performed by using the manual peak area assignment tool in the Xcalibur software (Thermo Fisher Scientific). Precursor ion masses were selected (four-decimal mass precision) in MS mode with a mass tolerance of 10 ppm.

Immunoprecipitation and Western Blotting

Proteins were extracted from 100 mg fresh weight in 500 μL of protein extraction buffer (100 mM HEPES-KOH, pH 7.5, 5% glycerol,15 mM EGTA, 5 mM EDTA, 0.5% [w/v] polyvinylpyrrolidone-40, 1% Triton X-100, 3 mM DTT, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 2 μg mL^{-1} apicidin). For immunoprecipitation, protein extracts were precleared using 30 μL of Protein A-Sepharose 4B (Invitrogen Life Technologies). Cleared extracts were incubated with 10 μL of acetylated Lys antibody for 2 h and were subsequently incubated with 30 μL of Protein A-Sepharose at 4°C overnight. Immunoprecipitates were washed three times with immunoprecipitation buffer. Proteins were eluted by boiling Sepharose in 5× gel loading buffer for 5 min.

For western-blot analysis, proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed using acetylated Lys antibody in a 1:1,000 dilution (ImmuneChem Pharmaceuticals). Secondary anti-horseradish peroxidase antibody was used in a 1:10,000 dilution.

Enzymatic Assays

Prior to the enzymatic assays, protein extracts were subjected to deacetylase treatment by incubating approximately 10 μg of protein extract with 2 μg of recombinant hSIRT3 protein (Cayman Chemical) for 3 h at 37°C (Hirschey et al., 2009). As a control (−SIRT), SIRT3 protein buffer (50 mM sodium phosphate, pH 7.2, 100 mM sodium chloride, and 20% glycerol) was used instead of SIRT3 enzyme.

Rubisco activity assays were performed according to Kobza and Seemann (1989) with minor modifications. Proteins were extracted from 100 mg of leaf tissue in 500 μL of ice-cold extraction buffer (100 mM Bicine, pH 7.8, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, and 1.5% [w/v] polyvinylpyrrolidone-40) containing protease inhibitor (Complete Protease Inhibitor Cocktail; Roche Diagnostics). The homogenate was clarified by a 10-s spin at 2,000*g*. The activity of Rubisco was measured as the rate of incorporation of $^{14}CO_2$ into acid-stable products in a 30-s assay at room temperature. The assay consisted of 100 mM Tris-HCl (pH 8.1), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM $NaHCO_3$, 1.4 mM ribulose-1,5-bisphosphate, and 1 μCi of $NaH^{14}CO_3$. The reaction was stopped after precisely 30 s by the addition of 2 M HCl to evaporate unincorporated acid-labile $^{14}CO_2$ and dried to completeness at 60°C overnight. Incorporated radiolabel was determined by liquid scintillation counting. PGK and GAPDH enzyme activities were measured by spectrophotometric assay (Giegé et al., 2003). NADH-dependent MDH activity was assayed in 100 mM potassium phosphate (pH 7.4) and 60 mM $MgCl_2$ in the direction of oxaloacetate reduction.

Cloning and Heterologous Expression of GAPC2

GAPC2 (At1g13440) was amplified from Arabidopsis cDNA with both *Nco*I and *Bgl*II restriction sites for subsequent directed cloning into pQE60 (Qiagen). Heterologous expression of the C-terminally His-tagged proteins was performed in BL21(DE3)pLysS cells (Invitrogen). Proteins were purified under reducing conditions (Laxa et al., 2007).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. LC-MS/MS fragmentation spectra of all identified Lys-acetylated peptides.

Supplemental Figure S2. Acetylation sites located on the three-dimensional structures of aldolase, Rubisco, MDH, ATP synthase, phosphoglycerate kinase, and GAPDH.

Supplemental Table S1. Proteins identified in organellar fractions of chloroplasts and mitochondria.

Supplemental Table S2. List of all Arabidopsis Lys-acetylated peptides and proteins identified by LC-MS/MS.

Supplemental Table S3. Overrepresentation analysis of a functional class of Lys-acetylated proteins.

Supplemental Text S1. Protein sequences and alignments of all proteins found in this study.

ACKNOWLEDGMENTS

We thank Jennifer Ho, Gary Woffendin, and Anthony Sullivan from Thermo Fisher Scientific for the LTQ Velos measurements in the Thermo Demo laboratory. We thank Benjamin Thomas and David Trudgian from the Central Proteomics Facility at Oxford University for the LTQ Orbitrap measurements and for help with the usage of the CPFP. Furthermore, we thank Nick Kruger (Oxford University) for his advice on Rubisco activity measurements, Monika Kalde (Oxford University) for her advice on the pull downs, and Xia Wu and Steven C. Huber (University of Illinois) for providing the method for the western-blot analysis and for critical scientific discussions of the paper.

Received December 21, 2010; accepted February 4, 2011; published February 10, 2011.

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