Proteins of diverse function and subcellular location are lysine-acetylated in Arabidopsis

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Journal area: Biochemical processes and macromolecular structures

Running title: Lysine acetylation in Arabidopsis

Key words: lysine acetylation, Arabidopsis, post-translational regulation of metabolic enzymes, Rubisco
Abstract

Acetylation of the ε-amino group of lysine is a reversible post-translational modification recently discovered to be widespread, occurring on proteins outside the nucleus, in most sub-cellular 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 lysine acetylation in plants also occurs on organellar and cytosolic proteins. We identified 91 lysine-acetylated sites on 74 proteins of diverse functional classes. Furthermore, our study suggests that lysine acetylation maybe an important post-translational 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 lysine residues that are involved in Rubisco tertiary structure formation and catalytic function. Using the human recombinant deacetylase, sirtuin 3 (SIRT3) it was demonstrated that lysine 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 TCA cycle enzyme, malate dehydrogenase. Our results demonstrate that lysine acetylation also occurs on proteins outside the nucleus in Arabidopsis and that lysine acetylation could be important in the regulation of key metabolic enzymes.
Introduction

Acetylation of lysine side chains (Kac) is a reversible and highly regulated post-translational modification of both prokaryotic and eukaryotic proteins. Lysine 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, lysine acetylation has recently emerged as a widespread post-translational modification, occurring in a large number of proteins of diverse biological function (Choudhary et al., 2009). Lysine acetylation is catalysed by specific protein acetyltransferases and reversed by deacetylases. Enzymes of central carbon metabolism are a particular target, with most enzymes of glycolysis and the TCA cycle being acetylated in *Salmonella enterica* and their acetylation status regulating flux through these pathways (Wang et al., 2010). The role of lysine 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, lysine acetylation links metabolic status to specific patterns of gene expression through histone modification (Wellen et al., 2009).

In plants, lysine 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 localisations, respectively (Chen and Tian, 2007). There are also many histone deacetylases, with Arabidopsis 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 lysine acetylation state is also thought to be important in integrating stress hormone signals (Chen and Tian, 2007).

The lysine acetylation status of non-histone proteins is thought to be regulated by the SIRT family of NAD⁺-dependent deacetylases (Blander and Guarente, 2004), which, in humans, includes nucleus-, cytosol- and mitochondria-localised members (Schwer et al., 2002; North et al., 2003). Two SIRT homologues are present in the Arabidopsis genome but it is currently not clear the extent to which lysine acetylation of non-histone proteins occurs in plants. The aim of this study was therefore to characterize the extent of lysine 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 lysine acetylation on plant cell proteins, a Western-blot analysis was done using a commercially available antibody against acetyl-lysine residues that was used in previous studies to detect lysine-acetylated proteins in human cell lines and bacteria (Kim et al., 2006; Choudhary et al., 2009; Wang et al., 2010). Multiple proteins were immuno-reactive in Arabidopsis cell culture extracts and leaf tissue (Fig. 1A, lane 2 and 4). A competition assay with acetylated BSA confirmed the specificity of the antibody for lysine-acetylated sites in Arabidopsis (Fig. 1A, lane 3). Several proteins of molecular mass higher than histones (10 to 30 kDa) were detected, suggesting that it is not just histones that are acetylated in Arabidopsis. Furthermore, lysine-acetylated proteins could be detected by immunoprecipitation of proteins isolated from Arabidopsis whole leaf extract, as well from organellar fractions such as mitochondria and chloroplasts (Fig. 1B). The strongest immuno-reactive bands were detected around the 55 kDa heavy chain of the anti-acetyllysine 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.

To identify novel lysine-acetylated proteins in Arabidopsis, protein extracts were analysed by LC-MS/MS (from at least three biological replicates). To reduce the complexity of the protein mixture and thereby maximise the number of lysine-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 mass spectrometry analysis (LTQ Orbitrap). Second, proteins extracted from leaf tissue or from isolated organelles (mitochondria or chloroplasts) were digested with trypsin and then enriched for lysine-acetylated proteins by immuno-puriﬁcation prior to mass spectrometry analysis (LTQ Velos). The purity of the organellar fractions was conﬁrmed by mass spectrometry, i.e. 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 (Suppl. Tab.1).

To identify lysine-acetylated proteins, MS/MS spectra were searched for the modiﬁcation K₁₇₀,₁₁ as acetylation of the lysine residue results in a mass gain of 42.0105 Da due to the substitution of a hydrogen atom with an acetyl group (CH₃CO⁻). All spectra containing this modiﬁcation were manually inspected to ensure diagnostic b- or y-ion series (see Fig. 2B and Suppl. Fig.1). Furthermore, although lysine-acetylated peptides can be distinguished from lysine-trimethylated peptides in spectra from the LTQ Orbitrap mass spectrometer (the two modiﬁcations differing in mass by only by 0.0363Da), 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
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 lysine-trimethylated peptides could remain.

All spectra were analysed 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 (FDR) were searched for internal lysine-acetylated sites and selected for manual inspection. In total, 91 lysine-acetylated sites and only 7 trimethylated sites were identified (Supplemental Table S2). The 91 lysine-acetylated sites belonged to 74 unique proteins of various functional categories (Fig. 3A, Supplemental Table S3). The majority of the lysine-acetylated proteins were identified in the fractions immuno-enriched for protein acetylated lysines with most proteins belonging to the functional categories ‘photosynthesis’ (Calvin-Benson cycle and light reactions) and ‘protein’ (protein synthesis, degradation and posttranslational modification). In protein extracts from cell cultures, 31 lysine-acetylated peptides were uniquely identified (Supplemental Table S2). These peptides belonged to proteins of various functional categories including ‘cell organisation’, ‘protein targeting’, ‘signalling’ and ‘metabolism’ (Supplemental Table S3). Interestingly, more than half of these 31 proteins were acetylated on a lysine 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 nuclear-encoded proteins in Arabidopsis plastid preparations, but did not investigate internal lysine-acetylated sites. We can exclude N-terminal acetylation on these 31 proteins as we only selected peptides that confirmed the location of the lysine acetylation site by indicative b- and y-ion series.

To further investigate lysine acetylation sites, the amino acids surrounding the observed acetylated lysines were analysed using the WebLogo tool (Crooks et al., 2004). Only the
two flanking amino acids at either side of the acetylated-lysine were analysed, as several lysine-acetylated sites occurred close to the N-terminus of proteins or were adjacent to the next lysine-acetylated site separated only by two amino acids. Only a very weak motif could be detected with the most common amino acids glycine, alanine, leucine and arginine surrounding the acetylated-lysine residues (Fig. 3B) in comparison to tyrosine, phenylalanine, glutamic acid and glycine in human acetylated proteins (5). Only glycine at -1 is common to lysine-acetylated peptides from other organisms and has been found mainly in acetylation sites of nuclear-located proteins (Yang, 2004; Choudhary et al., 2009). However, it is interesting to note that 21 of the 91 identified lysine-residues were conserved in the respective human homologue protein (Supplemental Text S1).

Lysine acetylation of nuclear proteins

As was to be expected, several histone proteins were found to be lysine-acetylated, validating the ability of the proteomics strategy to correctly identify lysine-acetylated proteins. Four lysine-acetylated sites of histones H3 and H4 (At1g09200, At1g07660) were identified that were previously shown to be lysine-acetylated in Arabidopsis (Zhang et al., 2007). In addition to histones, three other lysine-acetylated nuclear proteins were found. These were NAP1, a nucleosome assembly protein (At4g26110), C2H2 zinc finger protein FZF transcription factor (At2g24500), and Cyp59 (At1g53720), a multidomain cyclophilin with RNA recognition motif, suggesting that lysine acetylation may regulate other aspects of DNA structure and gene expression in Arabidopsis. For several transcription factors from non-plant systems it was shown that lysine acetylation adjacent to the DNA-binding site can result in a stimulation of DNA binding, while lysine acetylation in the DNA-binding domain disrupts DNA binding and transcription (Kouzarides, 2000; Yang and Seto, 2008)

Plastid proteins are lysine-acetylated
Unique to plants, proteins involved in photosynthesis represented a significant proportion of the total number of observed lysine-acetylated proteins. In total, 9 individual photosynthetic proteins were lysine-acetylated (~12 % of the total; Fig. 3A). These included both proteins of the photosynthetic electron transport chain (subunit H of the photosystem I reaction centre (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 subunits (At1g67090)), Rubisco activase (At2g39730), aldolase (At2g21330), glyceraldehyde 3-P dehydrogenase (At1g12900) and phosphoglycerate kinase (At1g56190) (Table 1).

**Lysine 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 lysine-acetylated, we recovered relatively few enzymes of these pathways. This most likely reflects the fact that a lower depth of coverage of the lysine-acetylated proteome was achieved in this study, rather than a lack of lysine acetylation of these enzymes. In glycolysis, a putatively cytosolic aldolase (At3g52930) was lysine-acetylated, and linked to the TCA cycle a putatively cytosolic isoform of NAD+-dependent malate dehydrogenase (At1g04410) was lysine-acetylated. Only one lysine-acetylated protein of the mitochondrial respiratory chain was found: cytochrome c (At1g22840). In addition, the fermentative enzyme pyruvate decarboxylase (At5g17380) was lysine-acetylated. Several other proteins associated with central carbon or nitrogen metabolism were lysine-acetylated. These include the chloroplast ATP/ADP exchanger (AAC1, At3g08580), glutamine 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 lysine acetylation. The occurrence
of lysine acetylation on the plastid ATP/ADP translocator, ATP-Synthase and on the LHCB antenna protein demonstrates that lysine acetylation is not limited to soluble proteins but can also occur on integral membrane proteins.

**Lysine acetylation of structural proteins involved in plant development**

Several lysine-acetylated proteins involved in cytoskeleton organisation were found. Among them 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 lysine-acetylated. In HeLa cells, lysine 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 be lysine-acetylated (Kim et al., 2006; Choudhary et al., 2009). Lysine 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 therefore possible that lysine 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 lysine-acetylated. Two proteins involved in cell wall synthesis, β-galactosidase 8 (At2g28470) and UDP-Xylose synthase (At3g46440) were found to be lysine-acetylated.

**Lysine acetylation of proteins involved in cell signalling and plant stress responses**

Several proteins linked to cellular signalling were lysine-acetylated. These included a protein involved in hormone responses, the ethylene receptor 2 (At3g23150, ETR2).
is a two-component histidine kinase involved in ethylene perception (Plett et al., 2009). Lysine acetylation of ETR2 occurs at K507 which resides between the histidine 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 lysine-acetylated. Interestingly, lysine acetylation also occurred in the pentatricopeptide repeat (PPR) domain of the mitochondrial PPR40 protein (At3g16890). PPR40 is a potential signalling 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 lysine-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 lysine-acetylated. Several F-box proteins and two major proteasome-bound de-ubiquitinases were also found to be lysine-acetylated in human cell lines (Choudhary et al., 2009). Regulation of protein turnover by lysine acetylation is a distinct possibility as lysine is the ubiquitinated residue and acetylation of the lysine would prevent ubiquitination and thus protein degradation (Yang and Seto, 2008).

**Lysine acetylation of proteins involved in protein synthesis and targeting**

Seven lysine-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 catalyses the attachment of lysine to its cognate RNA molecule, the translation factor EF 1-alpha (At1g07920) which was also found lysine-acetylated in Hela cells (Choudhary et al., 2009), as well as the 60S ribosomal proteins.
L35a (At1g41880) and L5-1 (At3g25520). Two proteins were lysine-acetylated which 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.

**Lysine acetylation regulates enzyme functions**

Lysine acetylation of the ε-amino group of the lysine side chain results in a neutralization of the positive charge and impairs the formation of hydrogen bonds, which is important for the biological function of lysine in many proteins and enzymes. Thus lysine acetylation could affect diverse protein functions such as enzyme activity, protein-protein and protein-nucleic acid interactions, depending on the position and function of the lysine residue for the overall protein function. To gain an insight into the position of the identified acetylated lysine 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 lysine acetylation. Rubisco was the most abundantly lysine-acetylated with nine different lysine acetylation sites (K14, K18, K21, K146, K175, K252, K316, K356, K463) identified in the chloroplast-encoded large subunit (LSU) (Supplemental Fig. S2, Supplemental Text S1). We confirmed the lysine-acetylated sites of Rubisco with the LTQ Orbitrap (Supplemental Fig. S2, Supplemental Text S1). Only for the peptides containing K14 and K463, respectively, we did not obtain a fragmentation spectrum with the LTQ Orbitrap but found the peptides in the LC chromatogram at the correct retention time, ion charge and with a delta mass of less than 10 ppm.
K175 is a catalytically-active lysine residue that accepts protons after enolization of ribulose-1, 5-bisphosphate and protonates the aci-carboxylate in the last step of the reaction (Cleland et al., 1998). Thus, lysine acetylation of K175 would very likely interfere with Rubisco activity which was also observed after site-directed mutations of this residue in *R. rubrum* (Knight et al., 1990). Furthermore, lysine acetylation occurred on K252 and K356 which are known to be important for ionic interactions at the dimer-dimer interface between two Rubisco LSU subunits, and on K146 which normally functions in inter dimer salt links with E110 buried deep in the crevice between two dimers (Knight et al., 1990). Lysine acetylation also occurred on K316 which is strictly conserved in all known Rubisco sequences and forms a hydrogen bond to L138 important for domain-domain interactions between the C- and N-terminus in the hydrophobic core of the large subunit (Knight et al., 1990). Several posttranslational modifications have already been identified to occur on the Rubisco LSU subunit mainly at the stage of protein assembly, such as carbamylation of the active site residue K201 and N-terminal N\(^\alpha\)-acetylation. Posttranslational modifications were also reported to occur at the level of the holoenzyme such as trimethylation of K14 (Houtz et al., 2008). As we identified K14 to be lysine acetylated it apparently competes with trimethylation at this residue. Although the function of trimethylation at K14 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 recognise trimethylated residues (Houtz et al., 2008). However, the same can be suggested for lysine acetylation as lysine acetylation functions as a binding motif for bromodomain-containing proteins. To test, whether lysine acetylation has an impact on Rubisco enzyme activity we incubated Arabidopsis leaf extracts with purified human sirtuin 3 (hSIRT3) enzyme. hSIRT3 is a lysine deacetylase that is known to have broad substrate specificity, deacetylating several mitochondrial key metabolic enzymes (Hirschey et al., 2009). The functional importance of lysine 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 (from 1 - 2 µmol/min mg protein which lies in the range of published activities of Rubisco from spinach (e.g. Siegel and Lane, 1975)), but consistently increased after deacetylase treatment. In addition to the 9 lysine-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 lysine acetylation, residual lysine acetylation was still detected suggesting that not all Rubisco lysine acetylation sites can be removed by hSIRT3 (Fig. 4B). In order to identify the lysine-acetylated sites affected by hSIRT3 treatment, we performed a mass spectrometry 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 analysed by LC-MS/MS (LTQ Orbitrap). Seven of the previously nine acetylated peptides were detected in the trypsinated samples of the excised Rubisco bands (Tab. 2). The peak areas of the precursor ions were manually quantified using the Xcalibur program (Thermo Fisher Scientific, UK). Interestingly, most of the detected peptides were deacetylated by more than 50 %, while K146 which is buried deep in the Rubisco structure was only marginally deacetylated by hSIRT3 treatment (Tab. 2). Acetylation of the active site K175 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 lysine 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 regulation of Rubisco by lysine acetylation is. One possibility
is that it contributes to the partial inactivation of the Rubisco pool \textit{in vivo} (Salvucci et al.,
2004)) and controls rapid increases in activity to match higher photon flux rates.

For phosphoglycerate kinase (PGK1) all three acetylated-lysine residues are exposed at
the protein surface (Supplemental Fig. S2). Lysine 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).

Malate dehydrogenase is a tetrameric enzyme and the lysine-acetylated residues are
situated at the dimer-dimer interface between two subunits (Supplemental Fig. S2). In the
thermophilic bacterium \textit{Chloroflexus aurantiacus} it was shown that two lysine 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 (Bjork 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 \textit{in vivo} inhibition of deacetylases in HEK293T
cells resulted in a 100% increased activity (Zhao et al., 2010).

In general, the effects of deacetylase treatment on enzyme activity for all three tested
enzymes were 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 (i) only a small proportion of
the respective enzyme is lysine-acetylated \textit{in vivo} in Arabidopsis, (ii) the lysine-acetylated
residues are not always accessible to SIRT 3 enzyme, (iii) that there are many substrates competing for deacetylation in whole leaf extracts and (iv) that the human SIRT3 has a slightly different specificity towards plant lysine acetylation sites which seem to vary from their animal counterparts (Fig. 3B) and thus deacetylation by SIRT3 is not completely efficient.

For the two glycolytic enzymes GAPDH and aldolase we detected only one lysine-acetylated lysine residue each. Interestingly, in both enzymes the lysine residues are very exposed on the protein surface protruding like needles (Supplemental Fig. S2). Lysine 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 lysine residues located within the groove of the protein visible in the structure are important for binding to F-actin and thus for co-localisation 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 lysine residue K314 is conserved in plants and animal GAPDH sequences (Text S1). As a recombinant GACP-2 (At1g13440) protein was available in our laboratory we tested whether the enzyme is lysine-acetylated after purification from *E. coli*. The recombinant GACP-2 protein was lysine-acetylated to a great extent in *E. coli* and we were able to almost completely remove the lysine-acetyl groups by treatment with hSIRT3 enzyme (Fig. 5A). When we analysed the recombinant GACP-2 protein by mass spectrometry (LTQ Orbitrap) we detected four lysine-acetylated sites at K130, K216, K220...
and K255, respectively (Suppl. Tab. 2). Interestingly, K255 is homologous to the acetylated site K314 in GAPA-2 and also homologous to the lysine-acetylated site K203 from GAPDH protein of *E. coli* (Zhang et al. 2009) indicating a conservation of lysine acetylation sites and a conservation of the acetyltransferase specificity between *E. coli* and a yet unidentified Arabidopsis plastidial acetyltransferase. Furthermore, the lysine-acetylated residue K130 is homologous to K128 from Chlamydomonas GAPDH (K189 in GAPA-2) and it was shown that K128 is essential for complex formation with the Calvin cycle enzyme phosphoribulokinase (Graciet et al., 2004). For the cytosolic GAPC-2 protein lysine acetylation of K130 could influence protein-protein interactions of GAPC-2 with other glycolytic enzymes (Graham et al., 2007). All four lysine-acetylated sites are predicted to be located at the outer surface of the GAPC-2 protein in a GAPC-2 structural model (Suppl. Fig. S2). Removal of the acetylation sites resulted in a more than 3-fold increase in enzyme activity (Fig. 5B), suggesting that lysine 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 lysine-acetylated in Arabidopsis or that only a minority of GAPDH protein is acetylated under the tested condition.

**Conclusion**

While several recent papers provide examples demonstrate that lysine 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 the first report that non-nuclear proteins are lysine-acetylated in plants. Unique to this study is the observation that the activities of Calvin cycle enzymes can be regulated by
lysine 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 multi-protein complexes (Howard et al. 2008)). Lysine acetylation could provide an additional layer of regulation that might integrate metabolic cues such as energy or carbon status through the availability of acetyl-Co A. For Rubisco this opens up a potentially novel strategy to engineer its activity. Significantly, Wu and colleagues have undertaken a similar approach to ours and also found many of the same acetylated photosynthetic proteins as reported here (Wu et al., 2010). Interestingly, they found that the lysine-acetylated form of LHCB is more abundant in the mobile antennae than in the photosystem II bound antennae. Together, these data indicate that lysine acetylation is more widespread in Arabidopsis than previously thought and most likely an important regulatory mechanism for in vivo enzyme function and for regulation of photosynthesis.

Materials and Methods

Cell Cultures and Plant Growth

Cell suspension cultures of Arabidopsis thaliana ecotype Landsberg erecta were maintained as described previously (Williams et al., 2008). Arabidopsis plants (ecotype Colombia 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⁻²s⁻¹.

Organelle isolation

Mitochondria were isolated from 50 g fresh weight of 10-day-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). Organelar 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, 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 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, alkylated with iodacetamide and digested using modified sequencing grade trypsin (Sigma-Aldrich, UK) as described (Choudhary et al., 2009).

Enrichment of lysine-acetylated peptides and mass spectrometric analysis

Lysine-acetylated peptides were enriched by immunoaffinity chromatography with polyclonal acetylated-lysine antibody (Cell Signaling Technology, Inc.), eluted with acidified water (0.1 % TFA) and purified for mass spectrometry (Choudhary et al., 2009).

Mass spectrometry data were acquired on an LTQ Velos linear ion trap (Thermo Fisher Scientific, Hemel Hempstead, UK) and on an LTQ orbitrap XL mass spectrometer (Thermo Scientific, Hemel Hempstead, UK) fitted with a nanospray source (Proxeon, Thermo Scientific) coupled to a nano HPLC system (LTQ Orbitrap: U3000 Dionex, Camberley, UK; LTQ Velos: Easy-nLC, Proxeon, Thermo Scientific). Samples were loaded onto a 5 cm long, 100 micron inner diameter, picotip column (New Objective, Massachusetts, USA) packed with C18 material (ProntoSIL C18 phase; 120 angstrom pore, 3 micron bead C18, Bischoff Chromatography, Leonberg, Germany). The HPLC was run in a direct injection configuration (buffer A: 5 % acetonitrile, 0.1 % formic acid and buffer B: 95 % acetonitrile, 0.1 % formic acid). Samples were loaded onto the column at a flow rate of 700 nL/min, and...
resolved using a 75-120 minute gradient at a flow rate of 300 nL/min. The Orbitrap was run in a data dependent acquisition mode in which the Orbitrap resolution was set at 60,000 and the top 5 multiply-charged species were selected for MS/MS. In the LTQ Velos the twenty most intense multiply-charged ions were sequentially isolated and fragmented in the linear ion trap by collision induced dissociation (CID). Charge state +1 ions were rejected. The ion selection threshold was set to 500 counts for MS², the activation Q was set to 0.25 and the activation time to 30 ms. Raw spectra files consisting out of full-scan MS and ion trap MS² 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 pipeline, 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 Da in MS/MS mode allowing up to 3 missed cleavage sites. LTQ Velos spectra were searched with a mass tolerance of 2.5 Da in MS mode and 0.5 Da in MS/MS mode, as the LTQ Velos sometimes selects the non-monoisotopic peak for fragmentation. Cysteine carbamidomethylation was searched as a fixed modification, whereas oxidized methionine and acetylation of lysine was searched as variable modification. Peptides identified at a 1% false discovery rate in the empirical/target decoy dataset (Peptide Prophet) and which contained an internal acetylated lysine 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 (i-prob) 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-prob value using Bayesian statistics (Keller et al., 2002). The closer the i-prob value is to 1 the more likely the peptide hit is to be correct. Quantitation of precursor ion peak areas were performed by using the manual peak area assignment tool in the Xcalibur software (Thermo Fisher
Scientific, UK). Precursor ion masses were selected (4 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 protein extraction buffer (100 mM Hepes-KOH pH 7.5, 5% glycerol, 15 mM EGTA, 5 mM EDTA, 0.5% w/v PVP-40, 1% TritonX-100, 3 mM DTT, 10 µM Leupetin, 1 mM PMSF, 2 µg/ml apicidin). For Immunoprecipitation (IP), proteins extracts were pre-cleared using 30 µl of Protein A Sepharose 4B (Invitrogen Ltd, Life Technologies, UK). Cleared extracts were incubated with 10 µl acetylated-lysine antibody for 2 h and were subsequently incubated with 30 µl Protein A Sepharose at 4°C overnight. Immunoprecipitates were washed three times with IP-buffer. Proteins were eluted by boiling sepharose in 5x gel loading buffer for 5 min. For Western-blot analysis proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane and probed using acetylated-lysine antibody in a 1:1,000 dilution (ImmuneChem Pharmaceuticals Inc., Canada). Secondary anti-HRP 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 protein extract with 2 µg of human recombinant SIRT3 protein (Cayman Chemical Company, USA) for 3h 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, 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 leave tissue in 500 µl ice-cold extraction buffer (100 mM bicine pH 7.8, 5 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 5 mM DTT, 1.5% (w/v) PVP-40) containing protease inhibitor (Complete Protease Inhibitor Cocktail, Roche...
Diagnostics Ltd., UK). The homogenate was clarified by a 10 s spin at 2000g. 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 RT. The assay consisted of 100 mM Tris-HCl (pH 8.1), 10 mM MgCl$_2$, 1 mM EDTA, 10 mM NaHCO$_3$, and 1.4 mM ribulose-1,5-bisphosphate and 1 µCi NaH$^{14}$CO$_3$. The reaction was stopped after precisely 30s by the addition of 2M HCl to evaporate unincooperated acid-labile $^{14}$CO$_2$ and dried to completeness at 60°C over-night. Incorporated radiolabel was determined by liquid scintillation counting.

PGK and GAPDH enzyme activities were measured by spectrophotometric assay (Giegé et al., 2003). NADH-dependent malate dehydrogenase activity was assayed in 100 mM potassium phosphate (pH7.4), 60 mM MgCl$_2$ in the direction of OAA reduction.

**Cloning and heterologous expression of GAPC2**

GAPC2 (At1g13440) was amplified from *Arabidopsis* cDNA with both NcoI and BglII 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, La Jolla, CA, U.S.A.). Proteins were purified under reducing conditions (Laxa et al., 2007).

**Acknowledgments**

We would like to thank Jennifer Ho, Gary Woffendin and Anthony Sullivan from Thermo Fisher Scientific (Hemel Hempstead, UK) for the LTQ Velos measurements in the Thermo Demo laboratory. We would like to thank Benjamin Thomas and David Trudgian from the Central Proteomics Facility (CPF, Oxford University) for the LTQ Orbitrap measurements and for help with the usage of the CPFP pipeline. Furthermore, we would like to 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.

I.F. was supported by a Junior Research Fellowship (Christ Church College, University of Oxford, UK) and by a Feodor Lynen Research Fellowship (Alexander von Humboldt foundation, Germany). This work was supported by the Biotechnology and Biological Sciences Research Council (UK) and by the Deutsche Forschungsgemeinschaft (Emmy Noether Programme, grant FI 1655/1-1).
References


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Table 1. Plastid-localized lysine-acetylated proteins. Immuno-enriched lysine-acetylated peptides from Arabidopsis leaves and chloroplasts were analyzed with the LTQ Velos / LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, UK) (see Suppl. Tab. 2). Spectra files were processed using the CPFP proteomics pipeline as described in material and methods. Fragment ion spectra were manually inspected for indicative b- and y-ion series (see Suppl. Fig1) and iprob = i probability (confidence score Peptide Prophet, CPFP, Trudgian et al., 2010).

<table>
<thead>
<tr>
<th>Description</th>
<th>Protein</th>
<th>Peptide</th>
<th>K(ac) site</th>
<th>iprob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>At2g13330</td>
<td>K.Y.G.E.E.S.E.E.A.N.E.G.M.Y.V.N(ac)G.Y.T.Y.</td>
<td>K395</td>
<td>1</td>
</tr>
<tr>
<td>GAPA2 (glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>At1g12900</td>
<td>K.L.N.G.I.A.L.R.V.P.T.P.N.V.S.V.D.L.V.G.O.V.S.N(ac)K.T</td>
<td>K314</td>
<td>0.95</td>
</tr>
<tr>
<td>PGK1 (PHOSPHOGLYCOLATE KINASE 1)</td>
<td>At3g127800</td>
<td>K.G.V.T.P.N(ac)F.S.L.A.P.L.V.P.R.L</td>
<td>K146</td>
<td>1</td>
</tr>
<tr>
<td>PGK1 (PHOSPHOGLYCOLATE KINASE 1)</td>
<td>At3g127800</td>
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</tr>
<tr>
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<td>At3g127800</td>
<td>R.L.E.L.L.G.E.V.T.N(ac)A.U.D.C.G.E.V.E.S.L.V.E.S.L.V.E.S.L.V.E.S.L.V.E.S.L.V.E.S.L</td>
<td>K169</td>
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<tr>
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<td>At1g03400</td>
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<td>0.96</td>
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<td>At1g03400</td>
<td>K.R.P.P.Y.T.N(ac)F.G.O.S.P.G.K.O.V.E.R.D</td>
<td>K146</td>
<td>1</td>
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<tr>
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<tr>
<td>ATP synthase F1 sector subunit beta</td>
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<tr>
<td>Photosystem reaction center, putative / PSI-H</td>
<td>At1g52230</td>
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<td>K89</td>
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<td>CAB3 (CHLOROPHYLL A/B BINDING PROTEIN 3)</td>
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<td>GDH2 (GLUTAMATE DEHYDROGENASE 2)</td>
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<td>K.M.E.S.O.K(ac)H.P.O.U.E.R.L.D.E.V.O.K.L</td>
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<tr>
<td>AACT (ADP/ATP CARRIER 1)</td>
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<td>R.M.M.M.S.Q.E.A.V.N(ac)Y.K.S</td>
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<td>1</td>
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<tr>
<td>Catalytic coenzyme binding, chloroplast precursor</td>
<td>At2g37060</td>
<td>R.T.O.C.G.N.Y(ac)K.L</td>
<td>K84</td>
<td>0.62</td>
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</table>
**Tab. 2: hSIRT3 deacetylates Rubisco at several lysine residues.** Rubisco peptides were analysed by mass spectrometry 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, UK). Retention times of the precursor ions are indicated in brackets behind the respective peak area, n.d. = not detected (n = 2; ratio = peak area (+hSIRT3) / peak area (-hSIRT3)).

<table>
<thead>
<tr>
<th>K(ac)</th>
<th>Peptide</th>
<th>Precursor mass</th>
<th>z</th>
<th>Peak Area Replicate (1)</th>
<th>Peak Area Replicate (2)</th>
<th>Ratio (1)</th>
<th>Ratio (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-hSIRT3</td>
<td>+hSIRT3</td>
<td>-hSIRT3</td>
<td>+hSIRT3</td>
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<tr>
<td>K14</td>
<td>K.ASVGF(ac)AGVK,E</td>
<td>503.2893</td>
<td>2</td>
<td>2024658 (38.68)</td>
<td>1743313 (39.43)</td>
<td>4323830 (44.89)</td>
<td>1994744 (45.89)</td>
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<tr>
<td>K18</td>
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<td>418.7293</td>
<td>2</td>
<td>6042 (18.87)</td>
<td>n.d.</td>
<td>181431 (21.57)</td>
<td>78107 (22.07)</td>
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<tr>
<td>K21</td>
<td>K.EYK(ac)LYYTPEYETK.D</td>
<td>935.4443</td>
<td>2</td>
<td>540204 (65.79)</td>
<td>n.d.</td>
<td>166379 (61.00)</td>
<td>92189 (61.34)</td>
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<tr>
<td>K148</td>
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<td>97448 (47.75)</td>
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<tr>
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<td>213941 (22.98)</td>
<td>145439 (25.57)</td>
<td>271229 (30.3)</td>
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<td>n.d.</td>
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<td>1095145 (91.52)</td>
<td>286040 (90.31)</td>
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</table>
**Figures**

**Fig. 1. Detection of lysine-acetylated proteins in Arabidopsis using anti-acetyl-lysine antibody.** (A) Western-blot analysis of acetyl-lysine-containing proteins from Arabidopsis leaves (1, 2, 3) and heterotrophic cell cultures (4). (1) Ponceau S-stain of 50 µg of Arabidopsis total leaf protein transferred to a nitrocellulose membrane, (2) anti-acetyllysine antibody (1:1250) on leaf proteins, (3) competition assay with 1 mg acetylated BSA and anti-acetyllysine antibody (1:1250) on leaf proteins, (4) anti-acetyllysine antibody (1:1250) on cell culture proteins. (B) Western-blot analysis of immuno-precipitated lysine-acetylated proteins from leaves (5) and isolated organelles ((6) mitochondria, (7) chloroplast) using anti-acetyl-lysine antibody (1:1250).

**Fig. 2. Gel-free LC-MS/MS experimental strategy for analysis of Arabidopsis lysine-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 23.01 min was further fragmented by MS/MS as shown in (C). (C) Fragmentation spectrum of an acetylated peptide unique to formin homology2-domain containing protein (At5g07650).

**Fig. 3. Overview of lysine-acetylated proteins in Arabidopsis.** (A) Pie chart of functional categories of lysine-acetylated proteins. The numbers represent the following categories: 1. photosynthesis, 2. major CHO metabolism, 4. glycolysis, 5. fermentation, 8. TCA cycle, 9. mitochondrial respiratory chain, 10. cell wall, 12. N-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 lysine-acetylated amino acid motif showing 2 amino acids on either sides of the acetylated lysine residue. The sequence plot was generated with the WebLogo tool (Crooks et al., 2004) using 88 identified lysine-acetylated sites from 74 Arabidopsis proteins.

**Fig. 4. Lysine-acetylation affects enzyme activities of (A, B) Rubisco, (C) phosphoglycerate kinase and (D) NAD+-dependent malate dehydrogenase in Arabidopsis leaf extracts.** Enzyme activities were measured after incubation with (+) or without (-) hSIRT3 deacetylase enzyme for 3h 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) to control treatment without deacetylase (one-tailed paired t-test). (B) Western-blot analysis of lysine-acetylated Rubisco large subunit (RBCL) in Arabidopsis leaf extract treated with (+) or without (-) hSIRT3 enzyme.
Fig. 5. Effect of lysine-acetylation on enzyme activity of recombinant GapC2 protein. (A) Western-blot analysis of lysine-acetylated GapC2 protein with and without deacetylase treatment (upper panel). Coomassie-stained gel (lower 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) to control treatment without deacetylase.

Supplemental Material

Supplemental Table S1: Proteins identified in organellar fractions of (A) chloroplasts and (B) mitochondria.

Supplemental Table S2: List of all Arabidopsis lysine-acetylated peptides and proteins identified by LC-MS/MS. (A) summary of all proteins, (B) peptides identified with the LTQ Orbitrap XL, (C) peptides identified with the LTQ Velos.

Supplemental Table S3. Lysine acetylation is found in proteins from diverse functional categories (Pagman statistical analysis of overrepresented functional categories (Usadel et al., 2006)).

Supplemental Text S1: Protein sequences and alignments of all proteins found in this study. (A) Alignment of Rubisco sequences from Arabidopsis thaliana, Brassica oleaeca, Chlamydomonas rheinhardtii and Synechococcus elongatus (B) Alignment of Arabidopsis GAPA-2 and GAPC-2 proteins. Conserved lysine residues are highlighted in black. Acetylated lysine residues are red. (C) Protein sequences and alignments of detected proteins. Lysine acetylated peptides are highlighted in yellow and acetylated lysine residues are highlighted in red.

Supplemental Fig. S1: LC-MS/MS fragmentation spectra of all identified lysine-acetylated peptides.

Supplemental Fig. S2. Acetylation sites located on the 3D structures of aldolase, Rubisco, malate dehydrogenase, ATP-Synthase, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. The 3D structures were modeled with Swiss-Pdb-Viewer 4.0 and modified with RasMol Version 2.6. Aldolase (At2g52930) was fitted to aldolase from rabbit muscle (NCBI entry 3DFQ), RUBISCO (large subunit – AtCg00490, small subunit – At1g67090) to RUBISCO from Synechococcus elongatus PC6301 (NCBI entry 1RBL), malate dehydrogenase (At1g53240) to the human type II malate dehydrogenase (NCBI entry 2DFD), glyceraldehyde 3-phosphate dehydrogenase (At1g12900/ GAPA-2; At1g13440/ GAPC-2) to Oryza sativa GAPDH (NCBI entry 3E6A) and phosphoglycerate kinase (At3g12780) to human phosphoglycerate kinase (NCBI entry 3C39). Given positions of acetylated lysine residues in phosphoglycerate kinase include the signal peptide amino acid sequence. Positions of acetylated lysine residues in plant chloroplastidic ATP-Synthase isoforms (AtCg00120 and AtCg00480) are illustrated in a 3D structure of the mitochondrial bovine ATP-Synthase (NCBI entry 2JDI). The corresponding lysine residue in the bovine amino acid sequence is K142 (K154) in the beta subunit. For a better overview lysine-acetylated residues in RUBISCO are only shown for one out of the eight subunits. The colour code is as follows:
red – acetylated residues, yellow – residues important for catalysis, black - homologous lysine residue in GAPDH proteins, dark grey – assigns one subunit in a complex.