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Research Article

Rubisco lysine acetylation occurs at very low stoichiometry in mature Arabidopsis leaves: implications for regulation of enzyme function

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Multiple studies have shown ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39; Rubisco) to be subject to Lys-acetylation at various residues; however, opposing reports exist about the biological significance of these post-translational modifications. One aspect of the Lys-acetylation that has not been addressed in plants generally, or with Rubisco specifically, is the stoichiometry at which these Lys-acetylation events occur. As a method to ascertain which Lys-acetylation sites on Arabidopsis Rubisco might be of regulatory importance to its catalytic function in the Calvin–Benson cycle, we purified Rubisco from leaves in both the day and night-time and performed independent mass spectrometry based methods to determine the stoichiometry of Rubisco Lys-acetylation events. The results indicate that Rubisco is acetylated at most Lys residues, but each acetylation event occurs at very low stoichiometry. Furthermore, *in vitro* treatments that increased the extent of Lys-acetylation on purified Rubisco had no effect on Rubisco maximal activity. Therefore, we are unable to confirm that Lys-acetylation at low stoichiometries can be a regulatory mechanism controlling Rubisco maximal activity. The results highlight the need for further use of stoichiometry measurements when determining the biological significance of reversible PTMs like acetylation.

Introduction

Cells rely on a variety of protein post-translational modifications (PTMs) to regulate biological processes including metabolic activities [1]. Our understanding of the role of non-histone Lys N^{ε}-acetylation (Lys-acetylation) as a regulatory PTM within metabolism began with the discovery that many enzymes of the central metabolic pathways in animals and bacteria are Lys-acetylated in a manner that depends on nutritional status, affects enzyme activities and correlates with the direction of carbon flux [2–5]. Lysine acetylation has since been shown to occur on thousands of plant proteins involved in all manner of biological processes, including metabolism [6–10]. In rice and Arabidopsis, reversible histone acetylation controls plant metabolism epigenetically and acetylation also regulates the activity of transcription factors [11,12]. In Arabidopsis, the loss of the mitochondrial deacetylase SRT2 has been shown to increase protein acetylation levels in mitochondria and to affect aspects of mitochondrial transport and metabolism [13]. The loss of the putative chloroplast acetyl-transferase NSI causes a defect in photosynthetic state transitions and photosystem complex formation [14]. However, despite these discoveries, the overall role of Lys-acetylation in plants as a reversible mechanism of metabolic control remains very unclear [15]. Specifically, it remains unclear whether plant metabolic enzyme activities are directly controlled by reversible acetylation. Differences in the maximal enzyme activities of Rubisco, malate dehydrogenase and phosphoglycerate kinase from crude

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leaf extracts have been attributed changes in enzyme acetylation status [6]; however, to our knowledge, no purified plant enzyme has been characterised in terms of the kinetic effects of specific lysine acetylation events.

Protein phosphorylation represents one of the best-studied reversible PTMs within plant metabolism. Its occurrence on specific Ser, Thr and Tyr residues of target enzymes is controlled by the combined action of protein kinases and protein phosphatases, where it acts as a molecular switch to control the activity of most cellular processes [16,17]. Due to its reversible nature and similarly widespread occurrence, acetylation has been described to potentially operate in a manner analogous to phosphorylation in plants [15]. However, there exist important distinctions between acetylation and phosphorylation in plants that indicate that they are not operationally similar. Firstly, although acetyl-transferases exist that can catalyse attachment of Lys acetyl groups, Lys-acetylation also occurs spontaneously (i.e. non-enzymatically) under conditions of elevated pH and elevated acetyl-CoA levels, conditions found in the mitochondria and chloroplast [7,18,19]. Non-enzymatic lysine acetylation is facilitated, for example, by acetylated cysteine thiol groups, which more easily undergo spontaneous acetylation and can subsequently transfer the acetyl group from thiols to lysine amino groups [19]. A second difference is that, although thousands of Lys-acetylation sites have been identified, the gene families encoding acetyl-transferases are not expanded like those encoding protein kinases: in Arabidopsis, there are approximately 16 Lys acetyl-transferases vs 1085 kinases [15]. At face value, it appears unlikely that so many Lys-acetylation events could be regulated in a complex manner by the action of such a limited set of acetyl-transferases. Widespread targeting of Lys-acetyl-transferases by protein binding partners to specific Lys residues of enzymes, as occurs with histones, has not currently been established [5]. Given current evidence, spontaneous acetylation may explain the existence of most non-histone acetylated proteins, especially in mitochondria where no acetyl-transferase has yet been confirmed in plants [20]. A third major difference between protein phosphorylation and acetylation is stoichiometry (i.e. the proportion of protein molecules where a given target residue is occupied by that PTM). From non-plant studies, it has been shown that the stoichiometry of acetylation is particularly low: median 0.02% in humans [21]; median 0.04% in *Escherichia coli* [22], median <1% in *E. coli* [23] and median <0.02% in yeast [24]. The observed distributions of lysine acetylation stoichiometries are clearly much lower than those observed with phosphorylation, which has median stoichiometry of ∼30% in yeast [25,26]. In plants, assessments of lysine acetylation stoichiometries have not been reported to our knowledge.

Our ability to catalogue the existence of acetylation and other PTMs by mass spectrometry screening greatly exceeds our ability to characterise the effect of PTMs on protein function [27]. In this study, we quantify the Lys-acetylation stoichiometry of the key plant enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (E.C. 4.1.1.39; Rubisco) as a means to assess the potential regulatory significance of individual acetylation events. Rubisco is a plant enzyme of crucial importance during photosynthesis, and regulation of its activity contributes to plant photosynthetic performance [28,29]. Rubisco activity is known to be controlled by several mechanisms including Rubisco activase activity [30–33]. Control of Rubisco activity by Lys-acetylation also been reported: two studies have described Arabidopsis Rubisco to be inhibited by Lys-acetylation [6,34], while a third study reported Rubisco maximal activity to be increased by greater Lys-acetylation arising from the absence of a major plastidial deacetylase, hda14 [35]. Furthermore, it was reported that RBL Lys-acetylation was greater at night when Rubisco is less active [34]. The specific Lys residues that may be responsible for the post-translational regulation of Rubisco activity have been suggested [6,34]. To independently assess the regulatory Lys-acetylation of Rubisco, we purified Rubisco from leaves of *Arabidopsis thaliana* in the daytime and night-time and established Lys-acetylation stoichiometries by two independent methods. Our findings indicate that Rubisco is Lys-acetylated at very low stoichiometries that could not meaningfully influence Rubisco activity *in vivo*, and moderate increases in acetylation state did not significantly alter Rubisco maximal activity.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. accession Col-0 (N6000) were sown into a 3 : 1 : 1 mix of potting soil, perlite and vermiculite, supplemented with slow-release fertiliser and covered with a transparent plastic cover until established. Plants were grown in a controlled environment growth chamber maintaining a short-day photoperiod of 8 h light to 16 h dark (23 : 00–7 : 00 light), a photon flux of 120 μmol m^2 s^{-1}, a relative humidity of 75% and a day-night temperature cycle of 22–17°C. The soil was kept well moistened with regular

watering. Leaves from entire rosettes of 10-week-old plants were harvested at 8 h into the light period or at 4 h into the dark period and snap frozen in liquid nitrogen.

Enzyme purifications

Rubisco was purified from 30 g of Arabidopsis leaves according to the method of Carmo-Silva, Barta and Salvucci [36]. The addition of the de-acetylase inhibitors 1 mM butyrate and 2 mM nicotinamide was made to all buffers to prevent Rubisco deacetylation. Following ammonium sulfate precipitation and sucrose gradient centrifugation as described, anion exchange chromatography was performed with a mono-Q 10/10 column (GE Lifesciences) by applying a gradient of 0–500 mM KCl in 50 mM Tris pH 7.6, 20 mM $MgCl_2$, 20 mM $NaHCO_3$ and 0.2 mM EDTA at 3 ml min^{-1} over 60 min. Pooled Rubisco fractions were precipitated with 60% $(NH_4)_2SO_4$, then stored as a saturated $(NH_4)_2SO_4$ suspension at −80°C.

Detection of rubisco acetylation sites

For initial detection of Rubisco acetylation sites, purified Rubisco was separated on a 12% SDS–PAGE gel and the bands for RBL and RBS were excised. Gel pieces were washed with 50 mM $(NH_4)HCO_3$ for 10 min then washed three times with 100% acetonitrile for 15 min and allowed to dry. Gel pieces were then incubated in 10 mM DTT, 50 mM $(NH_4)HCO_3$ for 45 min at 56°C, followed by incubation in 55 mM iodoacetamide for 45 min at room temperature in the dark. Gel pieces were then washed once with 50 mM $(NH_4)HCO_3$, twice with 50% acetonitrile in 25 mM $(NH_4)HCO_3$ and once with 100% acetonitrile and allowed to dry. Gel pieces were then digested with 80 ng of trypsin overnight. The liquid was then collected and the tryptic peptides were extracted twice with 50% (v/v) acetonitrile, 2% (v/v) formic acid for 20 min. The pooled liquid was then dried down in a vacuum concentrator. Samples were then resuspended in 5% (v/v) acetonitrile, 0.1% (v/v) formic acid and subject to solid-phase extraction using C18 MacroSpin Columns (The Nest Group, Inc.) according to the manufacturer's instructions.

Orbitrap MS/MS was performed according to [37]. Samples were analysed by LC–MS/MS on a Thermo Orbitrap Fusion mass spectrometer using data-dependent acquisition. The analysis consisted of direct injection onto a self-packed 150 mm × 75 μm Dr Maisch Reprosil-Pur 120 C18-AQ 1.9 μm column. Water/acetonitrile gradients with 0.1% formic acid were formed by an Ultimate U3000 nano pump running at 250 nl min^{-1} from 2% to 27% acetonitrile over 30 min.

Parallel MS/MS data analyses were performed using both MaxQuant (v1.6.7.0) [38] and Mascot (Matrix Science) software. For MaxQuant analysis, default parameters were used for the Andromeda database search except for the addition of Lys-acetylation as a variable modification, a peptide spectrum match false discovery rate of 0.05, up to 3 missed cleavages and the use of peptide matching between runs with a time window of 1 min. For Mascot analysis, raw data files were converted to .mgf files and MS/MS spectra were matched against the TAIR10 peptide database with the following parameters: Lys-acetylation and Met-oxidation were variable modifications; Cys-carbamidomethylation set as a fixed modification; digestion was by trypsin with up to 3 missed cleavages; MS peptide tolerance was 5 ppm, MS/MS tolerance was 0.6 Da. The significance threshold for peptide identification was set at $P < 0.05$.

Quantification of Lys-acetylation stoichiometry using heavy-labelled acetic anhydride

Chemical acetylation of Rubisco was performed according to the method of [39]. Rubisco samples (100 μg) were resuspended in 100 μl of 8 M urea, 200 mM triethylammonium bicarbonate followed by the addition of 2 μl of 1 M dithiothreitol (DTT) and incubation at 37°C for 30 min. Twenty-two microlitres of 200 mM IAA was then added, followed by incubation at room temperature for 1 h. Three rounds of chemical acetylation were then performed by adding 6 μl of acetic anhydride-d_6 (10.57 M; Sigma) and incubating at 4°C for 20 min, followed by neutralisation to approximately pH 7.5 with 7.5 M NaOH. Ten microlitres of 50% hydroxylamine solution was then added to revert O-acetylation side reactions. Samples were then digested with 1 μg of trypsin overnight at 37°C. Samples were then subject to solid-phase extraction using C18 MacroSpin Column (The Nest Group, Inc.) according to the manufacturer's instructions, eluted in 70% acetonitrile, 0.1% formic acid and dried down in a vacuum desiccator.

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Orbitrap MS/MS analysis of Rubisco samples was then performed as above. MaxQuant analysis of MS/MS data was performed as above except that Lys-acetylation-D_3 (C_2D_3O) was added as a variable modification and missed cleavages were set at 5.

Lys-acetylation stoichiometry measurements using synthetic peptides

Purified Rubisco samples were resuspended in 50 mM $(NH_4)HCO_3$ pH 8.0. Each sample was adjusted to 1% (v/v) SDS and 10 mM DTT followed by incubation at 56°C for 45 min. In total 15 mM iodoacetamide was then added and samples were incubated in the dark for 45 min. Samples were then diluted 10 fold with 50 mM$(NH_4)HCO_3$ pH 8.0 followed by digestion overnight with trypsin at a 50 : 1 protein : trypsin ratio. Samples were subject to solid-phase extraction on a C18 HPLC column (Waters) column and 10 μg aliquots were stored at −80.

Synthetic peptides were purchased from JPT as SpikeTides TQL, which are absolutely quantified and contain a heavy-labelled Arg or Lys (U-13C, U 15N) at the C-terminus followed by a quantification tag that is removed by digestion. Peptides were dissolved in 50 mM $(NH_4)HCO_3$ pH8.0 and 1.5 mM DTT to a final concentration of 20 μM. Peptides were then pooled and digested with trypsin at a molar ratio of 50 : 1 (peptide : trypsin). Peptides containing an internal Lys (see Table 2) were pooled and digested with Arg-C (sequencing grade, Promega) at a molar ratio of 50 : 1 (peptide : Arg-C). The digested peptide mixes were then dried down and resuspended in 5% (v/v) acetonitrile and 0.1% (v/v) formic acid to various concentrations. Thirty microlitres of each dilution of peptide mixtures was then used to dissolve 10 μg dried aliquots of pre-digested Rubisco samples (see above) in order to generate a titration curve of synthetic peptides within a representative sample matrix. These samples were used to create standard curves of peptide abundances using multiple-reaction monitoring (MRM) with LC-QQQ MS/MS.

For MRM samples were loaded onto an Agilent Advance Bio Peptide Map column (2.1 × 250 mm, 2.7 μm particle size, P.N. 651750-902), which was heated to 60°C. Peptides were analysed according to [40] on an Agilent 6495 Triple Quadrupole MS. Transitions used for multiple-reaction monitoring are given in Supplementary Table S2.

In vitro acetylation, deacetylation and immunoblotting

For *in vitro* Rubisco acetylation treatments, 2 μg of purified Rubisco samples were incubated in 3 mM acetyl-CoA and 50 mM $(NH_4)HCO_3$ pH 8.0 for 3 h at 37°C. For deacetylation treatments, 2 μg of purified Rubisco samples were incubated with 10 mU of human Sirt3 (Cayman Chemical) for 3 h at 37°C following the manufacturer's instructions. SDS–PAGE was conducted using stain-free AnyKd gels (Bio-Rad). Blots were probed with anti-acetyl-Lys antibodies (Immunechem) following the manufacturer's instructions.

For *in vitro* peptide deacetylation assays, 50 pmol of synthetic peptides, digested as above, were incubated in 50 μg of crude night-time leaf extract, as per the Rubisco purification protocol, in the presence or absence of deacetylation inhibitors (1 mM butyrate and 2 mM nicotinamide) for 2 h at room temperature. Separate synthetic peptide reactions contained 50 pmol of synthetic peptide and 10 mU of human Sirt3 incubated in deacetylation buffer (50 mM Tris pH 8.0, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 6 mM NAD^+, 125 mM NaCl and 1.5 mM KCl) for 2 h at 37°C. Peptides were then extracted according to the differential solubilisation method of Kawashima et al. 2009 [41]. Briefly, 25 μl peptide reactions were diluted in 50 μl of 7 M urea, 2 M thiourea and 20 mM DTT and then dropped into 1800 μl of cold acetone and incubated on ice for 2 h. Following centrifugation the peptides were extracted in 200 μl of 70% (v/v) acetonitrile and 0.1% (v/v) formic acid. The samples were then dried down and resuspended in in 5% (v/v) acetonitrile and 0.1% (v/v) formic acid and analysed by LC-QQQ MS/MS, as above, alongside the creation of matching standard curves of synthetic peptides.

Rubisco activity measurements

Rubisco samples were desalted into buffer consisting of 20 mM Tricine pH 8, 20 mM $NaHCO_3$, 10 mM $MgCl_2$ using Sephadex G50 resin. The activity of Rubisco was then determined by the NADH-coupled enzymatic assay as outlined by Sharwood et al. [42]. Ten micrograms of purified Rubisco, determined by Bradford reagent (Bio-Rad), was added to a final reaction solution volume of 200 μl. The reaction solution consisted of 20 mM Tricine pH 8, 20 mM $NaHCO_3$, 10 mM $MgCl_2$, 4 mM Ribulose-1,5-bisphosphate (99% pure), 1 mM ATP, 5 mM phosphocreatine, 0.8 mM NADH, 25 U of 3-phosphoglycerate kinase, 55 U of triose-phosphate isomerase, 20 U glycerophosphate dehydrogenase, 0.1 mg carbonic anhydrase, 25 U of creatine phosphokinase and 25 U of glyceraldehyde-3-phosphate dehydrogenase. All reagents were purchased from Sigma (Merck). Reaction solutions were placed in Coster 96-well flat-bottom polystyrene plates (Corning) heated to 25°C and,

immediately following the addition of Rubisco, absorbance at a wavelength of 340 nm was measured on an Infinite M200 Pro plate reader (Tecan) every 15 s over a 2-min period. A linear regression fit to the first 60 s of absorbance readings provided the rates of Ribulose-1,5-bisphosphate carboxylation per mg of Rubisco added factoring in a NADH extinction coefficient of 6.2 mM and oxidation of 4 NADH molecules per Ribulose-1,5-bisphosphate molecule carboxylated.

Data accessibility

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [43] partner repository with the dataset identifier PXD019268.

Results

There are opposing reports on the regulatory effects of lysine acetylation on Rubisco. To address this uncertainty we purified native Rubisco to apparent homogeneity from two samples of both daytime and night-time Arabidopsis leaves. A total protein stained SDS–PAGE gel of purified Rubisco showed dominant Rubisco large subunit (RBL) and Rubisco small subunit (RBS) bands (Supplementary Figure S1). To maintain the *in vivo* Lys-acetylation status of Rubisco, deacetylation inhibitors that were used previously in another report [34] were present throughout the purifications. We subjected the trypsin digested Rubisco samples to MS/MS analysis to identify acetylation sites without the need for prior enrichment of acetyl-Lys containing peptides. Data analysis with MaxQuant and Mascot demonstrated that 10 out of 24 RBL Lys residues could be detected in an acetylated state in one or more samples (Figure 1, Supplementary Table S1, Supplementary Data S1). For RBS, no significant Lys-acetylated peptide matches were detected (Supplementary Data S1). The positions of the identified RBL acetyl-Lys residues were then compared with those previously detected in other studies within Arabidopsis (Figure 1B). The comparison revealed a highly variable degree of detection among the 18 RBL acetyl-Lys residues that have been identified overall. Only one RBL acetylation site, Lys^{252}, was consistently detected in all studies.

In general, the MS signal strength of acetylated peptides was quite low compared with non-acetylated versions of the same peptides (Supplementary Table S1). However, direct comparison of signal intensities from acetylated vs non-acetylated versions of the same peptide to determine acetyl-Lys stoichiometry is inaccurate because acetylation interferes with trypsin digestion and because peptides display differing ionisation

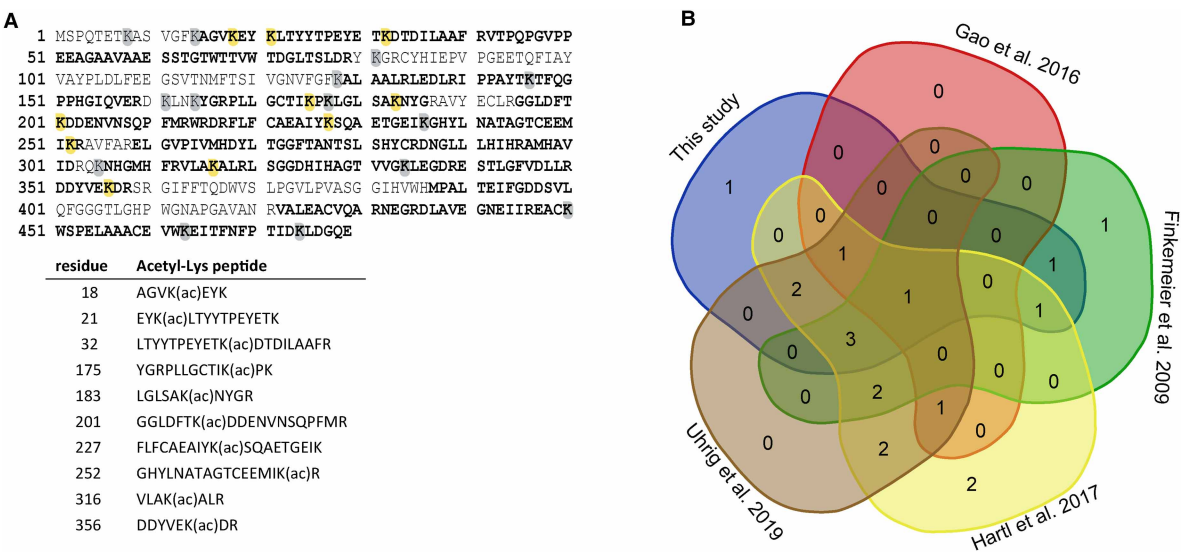

Figure 1. Acetylated Lys residues detected from Arabidopsis Rubisco large subunit.

(**A**) Sequence of Arabidopsis RBL with a list of detected Lys-acetylation sites. Yellow highlighted Lys residues were detected to be acetylated. Grey highlighted residues were not detected to be acetylated. Boldface indicates protein sequence coverage detected by MS/MS. The list of Lys-acetylation sites contains the highest scoring peptide for each acetylation site. (**B**) Venn Diagram depicting the overlap of RBL acetylation sites detected among acetylomics studies.

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efficiencies during MS analysis [26]. Therefore, to quantify the stoichiometry of Lys-acetylation events on Rubisco we applied two independent approaches. The first approach used the method of chemically acetylating all free Lys residues with heavy-labelled acetic anhydride, then quantifying the ratio of exogenous (heavy) to endogenous (light) versions of the same acetyl-Lys containing peptides, which should have similar ionisation efficiencies [39,44]. The purified daytime and night-time Rubisco samples were chemically acetylated, trypsin digested and subjected to LC-Orbitrap MS/MS. Resulting peptide matches included 16 of the RBL's 24 Lys residues and five RBS Lys residues (Table 1, Supplemental Data S2). These residues were detected predominantly as heavy-labelled acetyl-Lys with a small minority being detected as unmodified Lys. One RBS peptide bearing an endogenous (light) acetyl-Lys at position 92 was detected in one sample from night-time leaves. The stoichiometry of this acetylation event in this sample was 0.26% (Table 1). In contrast, other Rubisco peptides containing endogenous acetyl-Lys fell below the detection limit of this experiment. Note that Lys-acetylation protects most Lys residues from cleavage by trypsin, thus generating longer peptides that may be detected with diminished sensitivity. Nevertheless, these results further indicate that all RBL and RBS acetyl-Lys residues within these samples are of low stoichiometry.

As a second independent method to quantify the stoichiometry of Lys-acetylation within RBL, we designed four sets of heavy-labelled synthetic peptides corresponding to four of the Arabidopsis RBL Lys-acetylation sites: Lys residues 175, 201, 252 and 334. Based on their location within the Rubisco enzyme structure, these residues were all previously described as plausible sites of Rubisco regulation by Lys-acetylation [6,34]. Three different synthetic peptides were used to capture the stoichiometry of a specific Lys residue, as shown in Table 2. The first peptide was acetylated at the target lysine and contained no missed cleavages (with acetyl-Lys not considered a cleavage site). The two other peptides were non-Lys-acetylated, with either no missed cleavages or one missed cleavage at the Lys-acetylation site (except where that Lys was followed by Pro). Increasing amounts of the heavy-labelled synthetic peptides were spiked into purified Rubisco samples from daytime and night-time leaves. These samples were analysed by multiple-reaction monitoring (MRM) to generate standard curves of signal intensity to the absolute amount for each peptide (Supplementary Figure S2). The transitions used for MRM are shown in Supplementary Table S2. Product ion signals corresponding to endogenous versions of the peptides were then interpolated (Table 2). In each case, while the non-acetylated peptide was readily detected, the endogenous acetylated peptide was below the detection limit of the LC-QQQ MS/MS. Nevertheless, the analysis provided an estimate of each peptide's detection limit, allowing the upper limit of Lys-acetylation stoichiometry to be calculated as the maximal amount of Lys-acetylated peptide (e.g. less than 0.1 fmol) divided by the sum of non-acetylated peptides. Given that this upper-limit estimation is directly dependent on the signal intensity of non-acetylated peptides, samples with the highest signal intensities (Day 1 and Night 1) represent more accurate estimates of stoichiometry. In these samples, the acetylation stoichiometry for each of the four Lys residues examined was less than 1% (Table 2).

To ensure that these Lys residues of Rubisco are not actively deacetylated in whole-plant extracts, despite the presence of deacetylase inhibitors, we incubated the Lys-acetylated synthetic Rubisco peptides in crude leaf extracts to monitor any Lys-deacetylation activity (Figure 2). Standard curves for the synthetic peptides were also created for these conditions (Supplementary Figure S3). The ratios of acetylated to non-acetylated versions of the four synthetic Rubisco peptides were unaltered following extended 2 h incubations in crude leaf extracts in the presence or absence of deacetylation inhibitors. In contrast, incubations of the purified peptides with the recombinant human deacetylase Sirt3 (hSirt3) resulted in substantial reductions in acetylation ratios in three of the four peptide pairs. This indicates that the deacetylation of these diverse sites by plant enzymes or chemical processes in whole tissue extracts under the conditions used here is unlikely. However, we cannot exclude the possibility that Lys-deacetylation activity remained undetected for some other Rubisco sites, potentially influencing their Lys-acetylation stoichiometry determinations.

To assess the effect of acetylation on Rubisco activity, we incubated purified Rubisco samples in acetyl-CoA, which leads to non-enzymatic lysine acetylation without denaturing the enzyme. Analysis of Rubisco samples with anti-acetyl-lysine antibodies showed a consistent increase in ~50% in the signal from acetyl-CoA incubated samples compared with controls (Figure 3A,B). In contrast, incubation of samples in hSirt3, which has previously been used to deacetylate enzymes in plants [6], did not decrease the signal from anti-acetyl-Lys antibodies. This suggested that the anti-acetyl-Lys signal from RBL may be partly non-specific (i.e. not entirely related to acetyl-Lys residues) or that hSirt3 cannot effectively deacetylate Rubisco. Acetic anhydride treated samples were used as a positive control, representing almost complete lysine acetylation. The relative signal

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Table 1. Analysis of exogenous (heavy) and endogenous (light) acetylated peptides following *in vitro* Rubisco acetylation with deuterated acetic anhydride

		Heavy acetylated peptide						Light acetylated peptide					
				Peptide MS intensity						Peptide MS intensity			
Lysine residue	Peptide sequence	Mass	z	Day 1	Day 2	Night 1	Night 2	Mass	z	Day 1	Day 2	Night 1	Night 2
RBL													
18;21	AGVK(Ac)EYK(Ac)LTYYTPEYETK	2272.14	2	0	0	7.5×10^6	0	2269.14		0	0	0	0
32	LTYYTPEYETK(Ac)DTDILAAFR	2454.20	2;3	1.3×10^6	0	1.2×10^8	8.5×10^7	2451.20		0	0	0	0
81	VTPQPGVPPEEAGAAVAAESSTGTWT TVWTDGLTSLDRYK(Ac)GR	4403.17	4	0	0	3.7×10^8	0	4400.17		0	0	0	0
146	IPPAYTK(Ac)TFQGPPHGIQVER	2280.21	2;3;4;5	5.9×10^8	1.9×10^9	7.3×10^9	4.6×10^{10}	2277.21		0	0	0	0
161;164	DK(Ac)LNK(Ac)YGR	1082.60	2;3	1.8×10^9	5.9×10^9	6.3×10^9	4.1×10^{10}	1079.60		0	0	0	0
175;177;183	PLLGCTIK(Ac)PK(Ac)LGLSAK(Ac)NYGR	2320.33	2;3;4	9.1×10^8	3.5×10^9	3.8×10^9	2.4×10^{10}	2317.33		0	0	0	0
201	GGLDFTK(Ac)DDENVNSQPFMR	2214.01	2;3;4;5	4.7×10^8	4.9×10^9	1.0×10^{10}	7.3×10^9	2211.01		0	0	0	0
252	GHYLNATAGTCEEMIK(Ac)R	1994.94	3	1.7×10^6	0	1.2×10^7	0	1991.94		0	0	0	0
305	QK(Ac)NHGMHFR	1198.59	2;3	3.8×10^9	7.7×10^8	1.8×10^8	3.1×10^{10}	1195.59		0	0	0	0
316	VLAK(Ac)ALR	814.55	2	4.7×10^9	1.0×10^7	4.2×10^{10}	8.4×10^{10}	811.55		0	0	0	0
334	LSGGDHIHAGTVVGK(Ac)LEGDR	2062.06	2;3;4	1.2×10^9	8.1×10^8	5.8×10^9	1.5×10^{10}	2059.06		0	0	0	0
356	DDYVEK(Ac)DR	1083.49	2;3	1.3×10^9	4.2×10^9	2.5×10^9	2.5×10^{10}	1080.49		0	0	0	0
RBS													
92	NK(Ac)WIPCVEFELEHGFVYR[1–4]	2367.16	2;3;4	1.2×10^9	6.0×10^7	1.3×10^{10}	4.8×10^{10}	2364.16	3	0	0	3.3×10^7	0
140;146;147	LPLFGCTDSAQVLK(D3_Ac)EVEECK (D3_Ac)K(D3_Ac)EYPNAFIR[1]	3575.80	3	0	0	1.3×10^7	0	3572.80		0	0	0	0
146;147	EVEECK(Ac)K(Ac)EYPGAFIR[1]	2000.98	2;3	2.9×10^5	5.0×10^6	5.5×10^7	6.2×10^7	1997.98		0	0	0	0
146;147	EVEECK(Ac)K(Ac)EYPNAFIR[2–4]	1943.96	2;3	0	0	2.5×10^7	4.2×10^7	1940.96		0	0	0	0
174	QVQCISFIAYK(Ac)PPSFTDA[1]	1987.00	2;3	5.4×10^7	8.7×10^8	2.1×10^9	6.2×10^8	1984.00		0	0	0	0
174	QVQCISFIAYK(Ac)PPSFTEA[2,3]	2130.05	2;3	1.3×10^8	4.6×10^8	5.5×10^8	9.6×10^8	2127.05		0	0	0	0
174	QVQCISFIAYK(Ac)PPSFTG[4]	2116.04	2;3	7.6×10^7	2.0×10^8	2.8×10^6	3.2×10^8	2113.04		0	0	0	0

[1]At1G67090;
[2]At5G38410;
[3]At5G38420;
[4]At5G38430.

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Table 2. Stoichiometry of select acetyl-Lys sites on RBL determined following the creation of standard curves with heavy-labelled synthetic peptides

		Peptide amounts (fmol)			
Peptide	**Sequence**	**Day 1**	**Day 2**	**Night 1**	**Night 2**
Lys 175					
No Acetyl-K	YGRPLLGCTIKPK	32.14	15.17	24.22	2.36
No Acetyl-K missed cleavage	n/a				
Acetyl-K	YGRPLLGCTI(Kac)PK	n.d. <0.1	n.d. <0.1	n.d. <0.1	n.d. <0.1
Acetyl-K stoichiometry		<0.3%	<0.7%	<0.4%	<4.2%
Lys 201					
No Acetyl-K	GGLDFTK	3.55	0.97	1.02	1.19
No Acetyl-K missed cleavage	GGLDFTKDDENVNSQPFMR	32.98	3.64	20.85	6.74
Acetyl-K	GGLDFT(Kac)DDENVNSQPFMR	n.d. <0.1	n.d. <0.1	n.d. <0.1	n.d. <0.1
Acetyl-K stoichiometry		<0.3%	<2.2%	<0.5%	<1.3%
Lys 252					
No Acetyl-K	GHYLNATAGTCEEMIK	18.06	6.73	11.20	6.43
No Acetyl-K missed cleavage	GHYLNATAGTCEEMIKR	1.57	3.13	7.26	0.35
Acetyl-K	GHYLNATAGTCEEMI(Kac)R	n.d. <0.1	n.d. <0.1	n.d. <0.1	n.d. <0.1
Acetyl-K stoichiometry		<0.5%	<1.0%	<0.5%	<1.5%
Lys 334					
No Acetyl-K	LSGGDHIHAGTVVGK	7.92	0.95	8.02	1.63
No Acetyl-K missed cleavage	LSGGDHIHAGTVVGKLEGDR	n.d. <0.1	n.d. <0.1	n.d. <0.1	n.d. <0.1
Acetyl-K	LSGGDHIHAGTVVG(Kac)LEGDR	n.d. <0.01	n.d. <0.01	n.d. <0.01	n.d. <0.01
Acetyl-K stoichiometry		<0.1%	<1.1%	<0.1%	<0.6%

n.d. indicates that the signal was below the detection limit and therefore below the lowest detectable signal from the synthetic peptide standards as indicated.

from acetic anhydride treated samples was much stronger and outside the linear range of the signals from the other Rubisco samples, so they could not be quantitatively compared.

To assess the potential regulatory effect of altered acetylation status we subsequently performed Rubisco activity measurements with control and acetyl-CoA treated samples. The increased non-enzymatic Lys-acetylation of the acetyl-CoA treated samples did not alter the ribulose-1,5-bisphosphate carboxylation activity (Figure 3C). There was, however, greater activity for day than night samples, which is likely related to the presence of tightly bound Rubisco inhibitors during the night, as previously described [45,46], rather than an effect that could be attributed to Acetyl-Lys differences in Rubisco between the day and the night.

Discussion

This study shows that the Arabidopsis RBL and RBS contain multiple Lys-acetylation sites; however, independent methods indicated that these acetylation events occurred at a low stoichiometry in both the daytime and night-time leaf samples. First, using synthetic peptides to calibrate peptide abundance, four Lys-acetylation events at potential regulatory sites on RBL were demonstrated to have a stoichiometry below 1% in both the daytime and night-time (Table 2). Second, using *in vitro* chemical acetylation, pairings of exogenous and endogenous acetylated peptides could be directly compared for signal intensity (Table 1). However, despite detecting many exogenous acetyl-Lys containing peptides, only one endogenous acetylation event at Lys-92 of RBS in night-time leaves was detected, and at a very low stoichiometry (0.26% in one sample). All other Rubisco acetylation sites were below the detection limit of this method. Therefore, although precise stoichiometries for the RBL and RBS acetylation events could not be determined, the results indicated that their occurrence was relatively rare (Tables 1 and 2).

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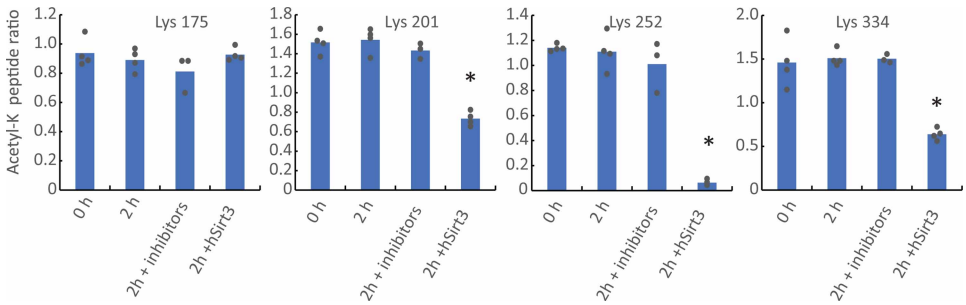

Figure 2. Peptide substrate assays for Lys-deacetylase activity in Arabidopsis crude leaf extracts.
The ratio of acetylated to non-acetylated versions of four synthetic peptides are quantified by MRM following incubations of the synthetic peptides in crude leaf extract for 2 h with or without deacetylation inhibitors. Separate peptide incubations were performed in the presence of hSirt3. Asterisks indicate significant differences compared with the corresponding 0 h controls (ANOVA and Holm-Sidak *post-hoc* test, $P < 0.05$, $n \geq 3$).

Low levels of RBL stoichiometry are consistent with other studies of protein acetylation levels in yeast and mammals that indicate most Lys-acetylation sites on proteins outside the nucleus are of very low stoichiometry [21,26]. Moreover, the inconsistency of acetylation site detection between published plant acetylomics studies (Figure 1B), the often spontaneous, non-catalysed nature of Lys-acetylation and the lack of an expanded set of acetyl-transferases in plant genomes, all favour a model where most proteins are acetylated in a non-regulated fashion and at low levels. This is clearly distinct from the paradigm of a tightly regulated PTM event controlling metabolism and instead more analogous to PTMs resulting from oxidative stress, e.g. carbamylation [21]. This does not mean that Lys-acetylation is unimportant, it has been demonstrated using genetic means that

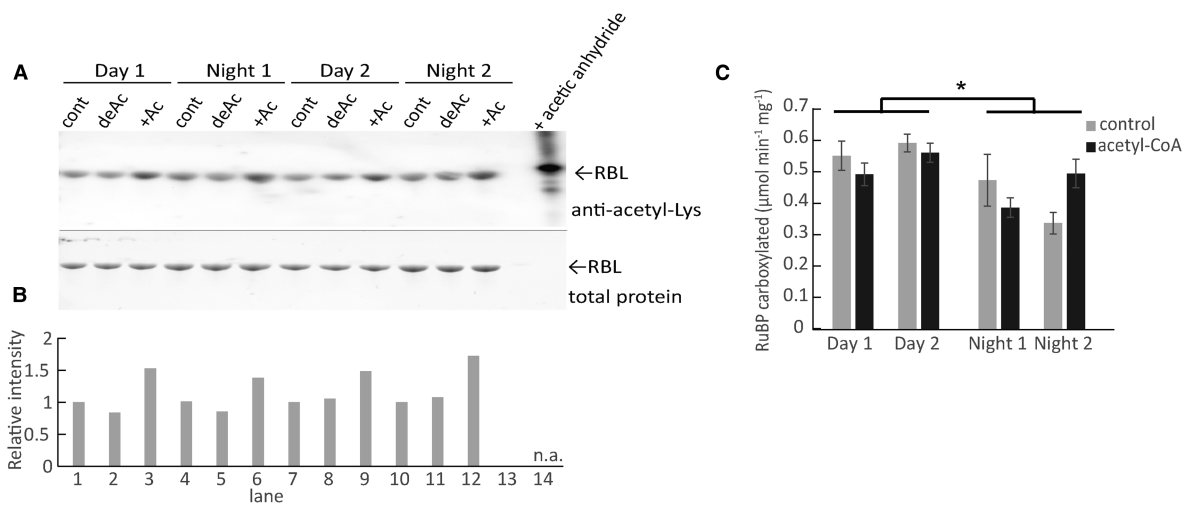

Figure 3. Rubisco maximal activity measurements following Lys-acetylation treatments. (**A**) Purified leaf Rubisco samples were subject to *in vitro* deacetylation (deAc) and acetylation treatments with acetyl-CoA (+Ac) or acetic anhydride followed by SDS–PAGE and western blotting with anti-acetyl-lysine antibodies. The lower panel shows the total protein of the stain-free gel prior to immunoblotting performed using UV fluorescence. 300 ng of protein was loaded per lane except for the lane containing acetic anhydride treated Rubisco which contains 3 ng. (**B**) The relative intensity of each band in (**A**) was measured as the ratio of western blot signal intensity to total protein signal intensity of the RBL band. The corresponding total RBL protein band in the +acetic anhydride sample was below the detection limit. (**C**) Acetyl-CoA and control-treated samples were assayed for Rubisco maximal activity. Analysis by two-way ANOVA indicated non-significant effect of the acetyl-CoA treatment but a significant day-night effect as indicated by the asterisk ($P < 0.05$, Holm-Sidak *post-hoc* test). Error bars represent standard error of the mean of three technical replicates.

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interfering with non-nuclear Lys-deacetylation activities has strong consequences for plant metabolism [13]. However, it remains to be determined which plant enzymes contain Lys residues that are acetylated at high enough stoichiometries to individually control metabolic activities.

As could be expected by their low stoichiometry, modest alteration of Lys-acetylation levels by incubation with acetyl-CoA did not change Rubisco maximal activity (Figure 3C). Furthermore, changes in the day and night activity of Rubisco did not correlate with significant changes in Lys-acetylation stoichiometries (Tables 1 and 2). Unlike a previous result [6], the incubation of Rubisco in hSirt3 did not reduce the immunoreactive anti-acetyl-Lys signal, despite hSirt3 being active against 3 out of 4 Lys-acetylated RBL peptide targets (Figure 2). In addition, we did not detect RBL acetylation at Lys-474, which was previously hypothesised to be involved in Rubisco activation under low light [35]. Therefore, overall, we were unable to confirm previous reports for a link between RBL Lys-acetylation and its function. However, it is possible that differences in experimental growth conditions between studies contributed to differences in RBL acetylation levels.

It is becoming widely acknowledged that many PTMs detected using advanced and highly sensitive MS techniques constitute biochemical noise of the system, not regulatory mechanisms [47,48]. Separating bona fide regulatory sites from biochemical noise now presents a major technological hurdle. In this regard, assessment of acetylation stoichiometries, as performed here, should be used as a means to rapidly assess the potential biological significance among Lys-acetylation sites before commencing the more laborious task of assessing PTM effects [26]. It is unlikely that a low stoichiometry PTM (e.g. <1%) such as those described here would meaningfully alter the catalytic activity of an enzyme pool [21]. Additionally, high stoichiometry PTM sites are more likely to be enzyme catalysed PTMs and therefore potentially linked to regulatory pathways. However, outside of metabolic enzymology, low stoichiometry PTMs may still be important within signal transduction pathways, which amplify by nature. For example, the moonlighting activity of rice GAPDH1 as a transcription factor, not a glyceraldehyde 3-phosphate dehydrogenase, is regulated by Lys-acetylation [11].

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contributions

B.M.O. and A.H.M. designed the research; B.M.O., R.F., O.D., E.S. and J.P., conducted the proteomics analyses; B.M.O and A.P.S. carried out the biochemical analyses; B.M.O. and A.H.M. wrote the article.

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Abbreviations

DTT, dithiothreitol; MRM, multiple-reaction monitoring; PTMs, post-translational modifications; RBL, Rubisco large subunit; RBS, Rubisco small subunit.

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