A non-enzymatic acetylation of lysine residues adversely affects the Rubisco activase protein stability

Li-Li Yang^{1,4#}, Hui Hong^{2,3,4#}, Xiang Gao⁴, Jemaa Essemine⁴, Xin Fang⁴, Zhan Shu⁴, Guljannat Ablat⁴, Meng Wu⁵, Hua-Ling Mi⁶, Xiao-Ya Chen⁴, Mingnan Qu^{4,6*}, Gen-Yun Chen^{6*}

¹Peking University Shenzhen Hospital, Guangdong, China, 518036

²Department of Thoracic Surgery, Fudan University Shanghai Cancer Center, Shanghai, 200032, China

³Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, 200032, China

⁴State Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Shanghai, 200032, China

⁵Core Facility of molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai, 200032, China

⁶Laboratory of Photosynthesis and Environmental Biology, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Shanghai, 200032, China

These authors contributed equally to this article.

*Correspondence: Gen-Yun Chen (chenggy@sibs.ac.cn); Mingnan Qu (qmn@sippe.ac.cn)

Numbers of Figures: 5

Numbers of Tables: 0

Numbers of Supplementary Figures: 7

Numbers of Supplementary Tables: 4

Total words: 7501

29 **Abstract**

30 The post-translational modifications of non-histone (PTMs) proteins functions are
 31 crucial for the plant adaption to the changing environment. The Rubisco activase
 32 (RCA) plays a key role in the CO₂ fixation through the Rubisco activation process.
 33 We reported that the RCA from tobacco leaf could be acetylated at several lysine
 34 residues including K126 and K164. The acetylation level changes under different light
 35 conditions (night and day) as well as under heat stress (45 °C). We further showed
 36 that the RCA can be non-enzymatically acetylated *in vitro*, especially by the
 37 acetyl-CoA (Ac-CoA) through direct interaction between them. Our results of the *in*
 38 *vitro* assay with deuterium labeled Ac-CoA (D²-Ac-CoA) show that the two
 39 conserved RCA lysine residues (K126 and K164) were acetylated by Ac-CoA,
 40 entraining a dramatic decline in its ATPase activity and a slight effect on the Rubisco
 41 activation process. Furthermore, we revealed that the higher RCA acetylation level
 42 induced its faster degradation in the chloroplast, which was not a direct consequence
 43 of ubiquitination. Eventually, our findings unraveled a new prominent role for the
 44 protein acetylation in modulating the RCA stability, which could certainly regulate
 45 the carbon assimilation efficiency towards a different energy status of the plants.

46 **Key Words:** ATPase activity, Non-enzymatic protein acetylation, Post-translational
 47 modifications, Rubisco, RCA, Stability, Ubiquitination.

48

49 Introduction

50 Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a key photosynthetic
51 CO₂-fixing enzyme, catalyzes the first reaction of the photosynthetic CO₂
52 assimilation, a rate-limiting but notoriously inefficient, step in photosynthesis
53 (Spreitzer and Salvucci, 2002). It forms mostly the inactivated complex with its
54 substrate RuBP and other sugar phosphate inhibitors. The enzyme Rubisco activase
55 (RCA) facilitates the Rubisco activity by the removal of sugar phosphate inhibitors,
56 e.g., RuBP, to maintain a dynamic balance between its deactivation and activation
57 duality (Portis, 2003).

58 The RCA belongs to the AAA⁺ (an ATPase associated with various cellular
59 activities) family, which is characterized by its prominent ATP hydrolysis activity
60 (Robinson and Portis, 1989). In the presence of Mg²⁺-ATP, a closed-ring hexamers
61 was observed, whereas Mg²⁺-ADP produced amorphous particles (Kuriata *et al.*,
62 2014). *Nicotiana tabacum* (hereinafter, *N. tabacum*) RCA comprises an N-terminal (N
63 domain) with 67-residues important and crucial for the Rubisco activation process,
64 followed by an AAA⁺ module and a flexible C-terminal extension (Esau *et al.*, 1996).
65 The core AAA⁺ module consists of a nucleotide-binding α/β sub-domain (from helix
66 H₀ to H₅) and α helical sub-domain (i-helix H₆-H₁₀) (Stotz *et al.*, 2011; Hasse *et al.*,
67 2015; Flecken *et al.*, 2020).

68 The RCA activity responds to different environmental stimuli, such as light and
69 temperature (Chen *et al.*, 2010) and physiological conditions such as source-sink
70 relationship and balance, cellular ionic homeostasis, pH, ADP/ATP ratio (Portis,
71 2003; Osorio *et al.*, 2014). The RCA thermal stability varies among species, and
72 directly correlates with its photosynthesis thermotolerance threshold (Salvucci and
73 Crafts-Brandner, 2004). Thus, the enhanced RCA thermal stability has been shown to
74 improve the photosynthesis and plant growth under moderate heat stress (Kurek *et al.*,
75 2007). Moreover, the response of RCA to the oxidoreductive process constitutes an
76 appropriate way to fine-tuning the RCA activity and the Rubisco activation state to
77 the prevailing light intensity. Hence, two Cys-residues in the C-terminal of the large
78 RCA α isoform can reversibly form or break the disulfide bond via thioredoxin-*f* in

vivo (Zhang and Portis, 1999). The oxidized RCA, rather than its reduced form, was much more sensitive to the inhibition by the adenosine diphosphate, ADP (Carmo-Silva and Salvucci, 2013). Therefore, the RCA activity was found to be highly sensitive to the ADP/ATP ratios in the stromal chloroplast (Carmo-Silva and Salvucci, 2013).

Some previous reports suggested also that the variation in the RCA content contributes to some extent to the Rubisco activation speed during the changes in the light intensity but not to the overall final activated status of Rubisco (Fukayama *et al.*, 2012; Yamori *et al.*, 2012). The RCA is a thermo-labile protein, as it dissociates, denatures and aggregates under even a moderate heat stress, which could partially be attributed to the disrupted interactions between the RCA monomers and oligomers (Feller *et al.*, 1998; Salvucci *et al.*, 2001). Therefore, the RCA thermal stability varies among species and directly contributes to the photosynthetic efficiency under heat stress (Salvucci and Crafts-Brandner, 2004; Kurek *et al.*, 2007); However, the molecular mechanism underlying the RCA content adjustment and enzymatic regulation under different physiological and environmental conditions remains unclear.

A range of post-translational modifications (PTMs) has been attributed to the plant stress responses (Grabsztunowicz *et al.*, 2017; Zhou *et al.*, 2017; O'Leary *et al.*, 2020). RCA PTMs have been previously well elucidated, such as acetylation (Finkemeier *et al.*, 2011; Wu *et al.*, 2011) and phosphorylation (Kim *et al.*, 2019). Compared to the phosphorylation process, the proteins involved in the photosynthesis typically seems much easier to be modulated by reversible acetylation during the photosynthetic responses to high temperatures (Li *et al.*, 2020). This is due to the fact that Lys-acetylation typically represents a dynamic and reversible PTM occurring mainly on either the α -amino group at the N-terminal or the ϵ -amino group on the side chain of the Lys-residues (Mischerikow and Heck, 2011).

In this study, we revealed the presence of a dynamic regulation in the RCA acetylation level in tobacco leaf during both day and night times. We further assessed the changes in the RCA acetylation level induced by different metabolites and

109 acetyltransferase together with inhibitors. We investigated as well the crucial
110 relationship of RCA acetylation with its stability, which is required for the higher
111 plants adaption to the changing environmental conditions such as temperature and
112 circadian rhythm.

113 **Materials and methods**

114 *Plant growth*

115 Tobacco (*N. tabacum*) plants were grown in phytotron under a photoperiod of
116 12/12h light/dark at 25 ($\pm 1^\circ\text{C}$), light intensity of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a relative
117 humidity (RH) of 65~70%. The Arabidopsis mutant, *sp2* was provided by Prof. Qihua
118 Ling in CAS Center for Excellence in Molecular Plant Sciences. The Arabidopsis
119 were grown in growth chamber at light intensity of $120 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperature
120 23/22 $^\circ\text{C}$ day/night and RH of 70%.

121 *Total proteins extraction from tobacco leaves*

122 The total soluble proteins were rapidly extracted according to the method as
123 previously described (Chen *et al.*, 2010) with minor modifications. Two histone
124 deacetylase inhibitors (HDACi), 5 mM Nicotinamide (NAM) and 0.2 mM trichostatin
125 A (TSA), were added to the extraction buffer. Firstly, the leaf tissue was grounded
126 using pestle and mortar, then homogenate was transferred into Eppendorf tube, and
127 homogenized in 1:10 protein extraction buffer. The samples were then centrifuged at
128 14,000 rpm for 10 min at 4 $^\circ\text{C}$. The supernatant obtained via the bicinchoninic acid
129 (BCA) method through chloroplast isolation from tobacco leaves was carried out as
130 described elsewhere (Kubis *et al.*, 2008).

131 *Extraction and purification of RCA and Rubisco*

132 Tobacco fresh leaves were detached in the morning, then immediately frozen in
133 liquid nitrogen. Thereafter, the RCA was purified from the frozen leaves as reported
134 previously (Chen *et al.*, 2010). The obtained RCA was supplemented with 0.4 mM
135 ATP, and immediately preserved in liquid nitrogen. Rubisco was purified following
136 the protocol as documented previously (Gao *et al.*, 2016). Afterwards, the purified

137 Rubisco was re-suspended in 10% glycerol and preserved at -80 °C, till use. The
138 protein concentration was determined using Bradford protein assay reagent (Bio-Rad,
139 Hercules) followed the manufacturer's instructions.

140 *Expression and purification of the recombinant RCA protein*

141 The amino acids substitution for the different RCA sites was carried out on tobacco
142 DNA, and the primers sequences used were listed in Supplementary Table S1. For
143 RCA mRNA and protein sequences, please referred to supporting datasets 1 and 2,
144 respectively. The expression and purification of the recombinant RCA was performed
145 as described earlier (Zhang and Portis, 1999). The site-directed mutagenesis was also
146 achieved through introducing the desired mutation by PCR (Vazyme), followed by the
147 DNA sequencing for confirmation, then cloned into PET28a vector (Novagen). All
148 the constructs were transferred into *E. coli* strain BL21 (DE3) cells, grown at 37 °C
149 for an attenuation at 0.6 optical density (OD) of 600 nm wavelength in Luria-Bertani
150 (LB) medium, followed by induction step with 0.1 mM isopropyl
151 beta-D-thiogalactoside (Sigma) overnight at 20 °C. The cells were then disrupted
152 under French press (Constant systems limited) in a lysis phosphate buffered saline
153 (PBS, pH 7.3), thereafter injected into a fast protein liquid chromatography (FPLC)
154 system (Pharmacia) for purification. Eventually, the purified protein was subsequently
155 stored as described above, till next use.

156 *Western blot analysis*

157 The standard western-blot procedures were followed in our study; however, for the
158 acetylation approach, the western-blot was performed as reported previously (Wang *et*
159 *al.*, 2010). The blots were developed with the enhanced chemi-luminescent (ECL)
160 detection system (Thermo Fisher Scientific) and signals were detected and processed
161 using a luminescent image analyzer (LAS-400 mini, GE Healthcare). The levels of
162 RCA protein, lysine acetylation (AcK) and lysine ubiquitin were detected by western
163 blots with anti-RCA (Orizymes), anti-AcK (PTM-105, Jingjie PTM Biolab) and
164 anti-ubiquitin antibodies (MM-0029-P, MEDIMABS), respectively.

165 *The RCA ATPase activity assay*

166 The RCA ATPase assay was measured by Quanti Chrom TM ATPase/GTase assay
167 kit (BioAssay Systems, DATG-200).

168 *Acetylation assay of RCA with Ac-CoA, ACP and Ac-AMP*

169 The purified RCA was added to a mixture containing 100 mM Tris-HCl (pH 8.0 at
170 25 °C), 60 mM NaCl, 0.5 mM Ac-CoA , 10 mM ACP or 10 mM Ac-AMP, 1 mM
171 NAM, 1 mM Sodium butyrate, 0.1 mM TSA , 2 mM MgCl₂, 1 mM adenosine
172 triphosphate (ATP). Therefore, 20 µL of the purified RCA (0.5 µg. µl⁻¹) was added to
173 30 µL assay. The reaction mixture was incubated for 30 min at 25 °C before
174 processing.

175 *Mass spectrum analysis and data processing*

176 The total protein from tobacco leaf was separated in 12% SDS-PAGE and stained
177 with Coomassie brilliant blue (CBB). Thereafter, the protein bands on the gel were
178 sliced, then the gel slices were minced and destained, subsequently the proteins were
179 reduced with dithiothreitol (DTT, Sigma), alkylated with iodo-acetamide (Sigma) and
180 afterwards digested overnight at 37 °C with trypsin (Sigma; in-gel tryptic digestion
181 kit, Thermo Fisher Scientific), desalted with C₁₈ Ziptips (Millipore). The resultant
182 peptides were examined by liquid chromatography mass spectrometry (LC-MS). The
183 obtained peptides were identified by LC/MS analysis through Orbitrap and MS/MS.

184 The MS results were initially investigated through the National Center for the
185 Biotechnology Information (NCBI) database with the aid of the Maxquant software
186 (version 1.4.1.2) as described earlier (Cox *et al.*, 2011). The seeking for the acetylated
187 peptides was carried out against the *N. tabacum* uniprot proteins database
188 (www.uniprot.org). The overall data were processed by Mascot Software (Matrix
189 Science). Accordingly, the peptides were identified by monitoring the maximum false
190 discovery rate (FDR) to be less than 0.01 (< 1%), stricter criteria for the acetylated
191 sites identification which requires a minimum Andromeda peptide score of 60%. An
192 Andromeda is a peptide search engine based on probabilistic scoring. It performs as

193 well as Mascot, can handle data with arbitrarily high fragment mass accuracy, is able
194 to assign and score complex patterns of the post-translational modifications.

195 The mass recalibration was performed using high confidence identifications based
196 on an initial “first search” using a 20 parts per million (ppm) mass tolerance for parent
197 ion masses and 20 ppm (HCD) or 0.5 Dalton (CID) for fragment ions. Spectra were
198 subsequently sought with a mass tolerance of 6 ppm for parent ions and 20 ppm
199 (HCD) or 0.5 Dalton (CID) for fragment ions. Thus, with the Thermo-Fisher Fusion
200 Orbitrap mass spectrometer, we can perform both CID (collision-induced
201 dissociation) and HCD (higher energy C trap dissociation) collision-induced
202 dissociation studies. In our current investigation, we considered and presented
203 uniquely the acetylated sites and/or peptide with a score more than 40%.

204 *Surface plasmon resonance analysis*

205 The kinetic studies of the interaction between the Rubisco activase and the
206 substrate Ac-CoA were performed by the surface plasmon resonance (SPR) method
207 using a Biacore T100 instrument (GE Healthcare) at 25 °C as described (Graciet *et al.*,
208 2004). Thus, the purified RCA protein was immobilized on a carboxy-methylated
209 dextran sensor chip (CM5) chip (GE Healthcare) using an amine coupling method kit
210 (GE Healthcare). A blank immobilization was performed in following the same
211 method and was taken as the reference surface. The RCA was dialyzed against PBS
212 buffer and used as a coupling analyte to the surface of CM5 in the binding assay, then
213 injected over the flow cells at a flow rate of 30 $\mu\text{l min}^{-1}$ for 60 s. The association of
214 the Ac-CoA was monitored for a time period of 120 s, followed by waiting for 180 s,
215 subsequently the disassociation was monitored by the PBS buffer for 420 s. Finally,
216 the regeneration of the surface was performed with a short pulse of 50 mM NaOH.
217 The interaction between RCA and the Ac-CoA was detected and displayed as a
218 sensogram by plotting resonance (RU) units against time. All datasets were analyzed
219 with Biacore evaluation software.

220 *The RCA degradation assay in vitro*

221 The intact tobacco chloroplast was equally sampled in 24-well plate in 1.5 ml PBS
222 buffer, treated with different inhibitors or acetyl donors for 0, 3, 6 h, then the
223 chloroplast was harvested and detected by western-blot analysis with anti-RCA,
224 anti-AcK and anti-ubiquitin antibodies raised against RCA, AcK and ubiquitin,
225 respectively.

226 *The Rubisco activation assays*

227 The RCA acetylation by 0.5 mM Ac-CoA was performed as described in detail
228 above. The RCA activity was also assayed as reported earlier (Portis, 2003). Briefly,
229 the purified Rubisco from tobacco leaves was inactivated by desalting on a HiTrap
230 Desalting columns in CO₂-free buffer containing 20 mM Tricine pH 8.0, 150 mM
231 NaCl and 0.2 mM EDTA, followed by the addition of 1 mM RuBP to form the
232 Rubisco-RuBP complex. The activation process was triggered in a buffer containing
233 100 mM Tricine pH 8.0, 5 mM NaH¹⁴CO₃, 5 mM MgCl₂, 50 mM DTT, 1 mM ATP, 3
234 mM creatine phosphate, 14 U ml⁻¹ creatine phosphokinase. Therefore, the Rubisco
235 activation was quantified by comparing the initial rate of activation during the first 6
236 min of the reaction.

237 *Statistical analysis*

238 A two-tailed Student's *t*-test was performed for the entire statistical analyses. The
239 data are expressed as the mean of at least three independent experiments ($\pm$ SD). The
240 differences between samples were assessed at a *P*-value < 0.05(*), *P* < 0.01(**), *P* <
241 0.001 (***) which mean significant, very significant and strongly significant,
242 respectively.

243

244 **Results and discussion**

245 *Temperature and diurnal changes affect acetylation levels of RCA*

246 To compare the RCA acetylation level in tobacco leaves between dark and light
247 conditions, we performed the western-blot analysis with anti-acetylated lysine residue
248 (AcK). The protein acetylation level at ~42 KD, the same molecular weight as RCA,
249 appears oscillating between the day and night times. Thus, the acetylation level was

the lowest at noon compared to the other daytime points (Fig. 1A). Meanwhile, the RCA protein amount evaluated by western-blot was also oscillating during the day (Fig. 1A). Notably, the amount of total RCA was the lowest early morning at dawn before the light turning on, and the highest at noon. Furthermore, the acetylation registered at the molecular weight of RCA (42 KD) increased in heat-treated tobacco leaves for 30 min to 45 °C (Fig. 1B), while the RCA amount decreased obviously. In addition, the RCA acetylation level was gradually increased following the increase in the RCA protein amount, especially in stroma-based assays. This suggests that the RCA acetylation level is a temperature- and light-dependent process.

Acetylated lysine residues of RCA is related to ATPase activity

Afterwards, a subsequent analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) has identified four acetylated lysine residues, K126, K164, K313 and K360 in tobacco leaves (Fig. 1C-E; Supplementary Table S2). Among them, K126 and K164 are conserved AA residues, which are located at the nucleotide-binding α/β sub-domain (residue 68-252), whereas K313 and K360 residues are situated on the α -helical subdomain and the flexible C-terminal extension, respectively (Stotz *et al.*, 2011). The K313 residue in *Arabidopsis* RCA has been reported as one of the thermo-stable related sites (Kurek *et al.*, 2007). The functions of the other three lysine residues (K126, K164 and K360) had not yet been reported elsewhere. Our results regarding the ATPase activity assay show that the substitutions of these four lysine residues (K126R, K164R, K313R and K360R) significantly decreased the RCA ATPase activity ($P < 0.05$; Fig. 1F-H).

Besides, the K111 of the *Arabidopsis* RCA, one of the P-loop region sites of the Walk-A domain, has earlier been reported to be acetylated (Finkemeier *et al.*, 2011). Interestingly, we did not identify acetylation modification of RCA K111 in tobacco leaves, which might be related to low acetylation levels. Consistent with the previous results (Shen *et al.*, 1991), the K111R substitution in the tobacco RCA has also led to a lower ATPase activity (Supplementary Fig. S1). Taken together, the RCA

279 acetylation seems to be tightly related and/or dependent to the hydrolysis of its ATP
280 activity as documented previously (Wu *et al.*, 2011).

281

282 *RCA can be acetylated by Ac-CoA in absence of GCN5 and Pat*

283 The acetylation of the metabolic enzymes was obtained to be regulated by the
284 histone acetyltransferase and deacetylase (Wang *et al.*, 2010; Lima *et al.*, 2011).
285 Accordingly, several photosynthesis-related proteins were also found to be
286 acetylated (Jiang *et al.*, 2018; Li *et al.*, 2020), as the RCA is a chloroplastic protein
287 (Portis, 2003); however, no canonical acetyltransferase was reported in *Arabidopsis*
288 chloroplast proteome (Peltier *et al.*, 2006). In this regard, we first attempted to
289 acetylate the RCA *in vivo* using two canonical acetyltransferases reported for animal,
290 the general control non-derepressible 5 (GCN5) and the protein acetyl-transferase
291 (Pat); however, the RCA acetylation level was almost the same after incubation with
292 or without any canonical acetyltransferase (GCN5 or Pat) in the assay (Supplementary
293 Fig. S2A-B). Consistently, the acetylation level of RCA proteins expressed in *Pat*
294 deficient *E. coli mutant (pat)* was also nearly similar to that of the wild type
295 (Supplementary Fig. S3). Therefore, we speculated that the RCA acetylation could be
296 orchestrated in a concerted manner by a different biological pathway.

297 Enormous evidences showed that proteins can be acetylated by small molecular
298 metabolites. In mitochondria, the protein acetylation could be facilitated by the
299 alkaline pH and the high concentrations of Ac-CoA and succinyl-CoA (Wagner and
300 Payne, 2013). Besides, the acetyl-phosphate (ACP) can globally and chemically
301 acetylate the lysine residues of protein in *E. coli* (Weinert *et al.*, 2013). The Ac-AMP
302 could also non-enzymatically acetylate the proteins residues (Ramponi *et al.*, 1975).
303 Our previous study showed that the Rubisco can be directly acetylated by a
304 plant-derived metabolite, 7-acetoxy-4-methylcoumarin (AMC) (Gao *et al.*, 2016).

305 To investigate the eventual RCA acetylation by these metabolites candidates, we
306 examined the RCA acetylation level following *in vitro* incubation with Ac-CoA,
307 Ac-AMP, AMC and ACP, separately, without acetyltransferases furnished in the
308 assay. Notably, the western-blot analysis shows that all the four metabolites

309 candidates mentioned above could merely acetylate RCA (Fig. 2A-C; Supplementary
310 Fig. S2C). The RCA acetylation level enhances with increasing the concentration of
311 the four metabolites, especially for the Ac-AMP (Fig. 2A-C; Supplementary Fig.
312 S2C). Interestingly, the increased RCA acetylation levels due to the Ac-AMP effects
313 also promote the Rubisco acetylation level (Supplementary Fig. S4A).

314 It is worthy to mention that the RCA could be acetylated by just 0.004 mM
315 Ac-CoA, much less than its prevailing physiological concentration (0.01-0.02 mM),
316 by up to 5 times in spinach chloroplasts (Post-Beittenmiller *et al.*, 1992). Except the
317 Ac-CoA, the three other metabolites candidates (Ac-AMP, ACP and AMC) are not
318 natural or common metabolites for the higher plants. Therefore, these results suggest
319 that the RCA acetylation seems to be mediated non-enzymatically by Ac-CoA *in vivo*,
320 a major abundant metabolite in the chloroplast.

321 Furthermore, Ac-CoA and Ac-AMP could dramatically decrease the RCA ATPase
322 activity, but slight decrease the Rubisco activation (Fig. 2E-F; Supplementary Fig.
323 S4B), which remains similar as for the K313 mutation with slight changes in the
324 Rubisco activation process (Kurek *et al.*, 2007). This might be attributable to the fact
325 that the conserved RCA lysine sites in the C-terminal flexible extension and the
326 N-terminal were not modified by the Ac-CoA. These two sub-domains are very
327 important for the Rubisco activation and the truncation of these two sub-domains
328 would lead to a loss in the Rubisco activation properties or peculiarities (Esau *et al.*,
329 1996).

330 *The RCA acetylation by Ac-CoA is required for their interaction*

331 As mentioned above, the RCA could be acetylated by the Ac-CoA at a lower
332 concentration than its physiological concentration in the chloroplast. Therefore, the
333 non-enzymatic acetylation method by the Ac-CoA may constitute an important and
334 pioneering biochemical approach for the RCA acetylation *in vivo*. We hence
335 examined the possible interaction between RCA and the Ac-CoA through the SPR.
336 The steady-state affinity data shows that the RU increases when the Ac-CoA
337 concentration increase (Fig. 2G-H), which is consistent with the results of acetylation
338 assay (Fig. 2D). The calculated dissociation equilibrium constant (K_D) was 0.36 mM;

339 however, when the Ac-CoA was replaced by its analogue malonyl-CoA, the
340 interaction signal was disappeared (Supplementary Fig. S5A-B). The SPR data
341 indicates the existence of a specific interaction between the Ac-CoA and RCA,
342 suggesting the possibility of RCA direct acetylation by Ac-CoA *in vivo*.

343 *RuBP inhibits the acetylation levels of RCA by Ac-CoA*

344 Previous studies suggested that the content of Ac-CoA was maintained at a stable
345 level during the day time, indicating that the difference in the RCA acetylation level
346 in dark and under light is not ascribable to the changes in the Ac-CoA concentration
347 *in vivo* (Tumaney *et al.*, 2004; Chen *et al.*, 2017). It is actually known that the RuBP
348 has a competitive binding ability with the RCA (Jensen, 2000). In this context, we
349 have previously reported that the Rubisco acetylation could be inhibited by RuBP, a
350 Rubisco substrate with higher expression under light than under dark conditions (Gao
351 *et al.*, 2016). Thus, we expect that this competitiveness binding capacity between them
352 (RuBP and RCA) could inhibit the acetylation process by the Ac-CoA, thus leading to
353 a decrease in the RCA acetylation level. We then examined the RuBP effect on the
354 RCA acetylation process by the Ac-CoA, and confirmed its inhibitory effects on RCA
355 acetylation levels. (Fig. 2D).

356 *Validation of acetylated lysine residues based on stable isotope labeling mass* 357 *spectrometry*

358 To further validate the RCA lysine residues non-enzymatic acetylation by the
359 Ac-CoA, we synthesized a deuterium labeled Ac-CoA (D²-Ac-CoA), where three
360 hydrogen atoms of the acetyl group were replaced by the deuterium (D). Based on
361 LC-MS/MS analysis, 6 acetylated RCA lysine residues were characterized from the
362 acetylation assays *in vitro* by the D²-Ac-CoA. These are K17, K34, K72, K116, K126
363 and K164 (Fig. 3A-B; Supplementary Table S3), which are located at the N-terminal
364 sub-domain (Supplementary Fig. S6). Strikingly, the number of the identified
365 acetylated lysine residues *in vitro* was more than expected for the native RCA, which
366 might be owing to the high Ac-CoA concentration or the different biomolecular
367 structure of the RCA between *in vivo* and *in vitro*. This supports the evidence as
368 observed in previous studies that the RCA polymerization could be altered when ATP

369 and RuBP are present in the assay (Mueller-Cajar *et al.*, 2011). This suggests the
370 presence of some other unknown inhibitors in addition to RuBP that could potentially
371 be also involved in the reversible RCA acetylation in the chloroplast.

372 *The deacetylase inhibitor promotes the RCA acetylation level and decreases its*
373 *stability*

374 To gain more insights in this subject, we used three known histone deacetylase
375 inhibitors (HDACi), i.e., Nicotinamide (NAM), Trichostatin A (TSA) and sodium
376 butyrate (NaB). Notably, the NAM selectively inhibits the Sirtuin (SIRT) HDACi
377 family, the TSA is a multiple HDACi family inhibitor and the sodium butyrate (NaB)
378 is a histone deacetylase inhibitor. As predicted, the western blots analysis shows that
379 the RCA acetylation level increased in the treated chloroplast with the deacetylase
380 inhibitors if compared with that of the control sample (Fig. 4A), while, interestingly,
381 the RCA content decreased significantly ($P < 0.05$, Fig. 4B). In this regards, it has
382 been earlier revealed that the acetylation features could affect the proteins stability
383 and turnover (Mateo *et al.*, 2009). We thus speculate that the acetylation process
384 might be somehow related to the RCA stability.

385 Therefore, we further investigated the changes in RCA content in the chloroplast
386 treated with additional Ac-CoA and Ac-AMP. Consistently, the RCA content also
387 obviously decreased after incubation for 3 hour with AMP or Ac-CoA (Fig. 4C). To
388 prevent the acetylation impact of other proteins which might also directly or indirectly
389 affect the RCA stability in the chloroplast, we added the purified prokaryotic
390 recombinant RCA from *E. coli* (E-RCA), which exhibited much more acetylated
391 lysine residues and RCA acetylation level in the crude chloroplast extracts (Fig. 4D;
392 Supplementary Table S2; Supplementary Table S4). The western-blot analysis
393 demonstrates that the degradation of the recombinant RCA was faster than that of the
394 native one (RCA) in chloroplast, which indicates that the faster RCA degradation is
395 mainly attributed to the extent of RCA acetylation level. These results unravel that the
396 RCA acetylation might serve a sign for the RCA degradation *in vivo*.

397 Given that the RuBP could inhibit the RCA acetylation by the Ac-CoA, the low
398 RuBP level would result in a higher RCA acetylation level, and in turn leads to a

399 faster RCA degradation in the chloroplast (Figs. 2D and 4C). This statement was also
400 supported by the decreased RCA amount as well as by its (RCA) increased
401 acetylation level in tobacco leaves under a moderate heat stress (Fig. 1B). Actually, it
402 becomes well known that the RCA is a sensitive protein to high temperature (Salvucci
403 *et al.*, 2001). In line with this, our results indicate that the RCA degradation induced
404 following its acetylation may somehow contribute to its thermosensitivity *in vivo* to
405 high temperature.

406 *RCA degradation following its acetylation is not related to the ubiquitination*

407 In this regards, several reports suggested that the acetylation operation could
408 regulate the protein stability through its ubiquitination *in vivo* (Grice and Nathan,
409 2016). However, the ubiquitination process is not necessary involved in the protein
410 degradation in the chloroplast (Smalle and Vierstra, 2004; Moon *et al.*, 2004).
411 Consistently, our results showed also that the RCA protein level in the chloroplast
412 was not affected by the ubiquitination upon treatment with 25 μ M MG132, an
413 ubiquitin-proteasome inhibitor (Supplementary Fig. S7A-B). In the same trend, it has
414 been reported in a very recent study that the proteins ubiquitination may occur when
415 proteins are exported out of the chloroplast compartment using SP2 and CDC48, a
416 chloroplast-associated protein degradation, CHLORAD (Ling *et al.*, 2019); however,
417 no significant differences were observed in the RCA amount between the mutant *sp2*
418 and *Col* in Arabidopsis at night (Supplementary Fig. S8). This suggest that some other
419 degradation systems and special protease could regulates RCA degradation in
420 chloroplast as documented previously (Striebel *et al.*, 2009; Fukayama *et al.*, 2012).

421 Taken together, as shown in Fig. 5, the RCA can be acetylated *in vivo* in the
422 presence of Ac-CoA especially under high temperature. However, RCA acetylation
423 level could be inhibited under light as well as by RuBP. The RCA acetylation has led
424 to a considerable decrease in the ATPase activity and its protein amount. Therefore,
425 the degraded protein, as reflected by the substantial decrease in the RCA protein
426 amount, resulted eventually in a low Rubisco activation activity. Hereby, we deduce
427 that the ubiquitination does not constitute the sole reason standing behind the protein
428 degradation process. Nevertheless, to well understand the mechanism of the RCA

degradation further investigations are needed to better decipher the detailed and inter-related biochemical and molecular pathways orchestrating in a well concerted manner this sequential phenomenon (acetylation, ubiquitination and degradation).

Conclusion

In this study, we revealed that the RCA acetylation level is feasible to temperature and light stimulus, and it could be acetylated non-enzymatically by various metabolites including Ac-CoA through their direct interactions. The acetylated RCA affects its stability, and together with decrease its ATPase activity and Rubisco activation activity. Overall, the finding provides novel insights about the PTMs regulating the RCA function and a model by which the acetylation might react in an enzyme-independent manner in the plant chloroplast.

Conflict of interest statement

There authors have no conflicts of interest to declare.

Acknowledgments

We thank Professor Qihua Ling for providing *sp2* mutant seeds and professors Wenli Hu, Xiaoyan Xu, and Shimin Zhao for their kindness and patient guidance on the academic knowledge. We also thank all the colleagues Xinyu Liu, Zhaoxue Ma, Xunlin Han, Min Xu, Juan Chen, Zhiyong Xu and Zongzhu Li for generously supporting this study. This work was supported by grants from National Natural Science foundation of China, NSFC (31702019 to H. H., 81803139 to L.Y., 31700201 to M.Q.).

Author contributions

G.C., X.C., H.M., M.Q. planed and designed the research; M.Q., H.H., X.G., X.F., L.Y., Z.S., A.G., M.W. performed experiments; L.Y., M.Q., J.E., G.C. wrote the manuscript; G.C., M.Q., X.C. supervised the research; L.Y., H.H., contributed equally.

Data and materials availability

458 All data is available in the manuscript or the supporting information.

459

460

Reference

- 462 **Carmo-Silva AE, Salvucci ME.** 2013. The regulatory properties of rubisco activase
463 differ among species and affect photosynthetic induction during light transitions.
464 *Plant Physiology* **161**, 1645 LP – 1655.
- 465 **Chen C, Li C, Wang Y, et al.** 2017. Cytosolic acetyl-CoA promotes histone
466 acetylation predominantly at H3K27 in Arabidopsis. *Nature Plants* **3**, 814–824.
- 467 **Chen J, Wang P, Mi H -l., Chen G-Y, Xu D-Q.** 2010. Reversible association of
468 ribulose-1, 5-bisphosphate carboxylase/oxygenase activase with the thylakoid
469 membrane depends upon the ATP level and pH in rice without heat stress. *Journal of*
470 *Experimental Botany* **61**, 2939–2950.
- 471 **Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen J V, Mann M.** 2011.
472 Andromeda: A peptide search engine integrated into the MaxQuant environment.
473 *Journal of Proteome Research* **10**, 1794–1805.
- 474 **Esau BD, Snyder GW, Portis ARJ.** 1996. Differential effects of N- and C-terminal
475 deletions on the two activities of rubisco activase. *Archives of biochemistry and*
476 *biophysics* **326**, 100–105.
- 477 **Feller U, Crafts-Brandner SJ, Salvucci ME.** 1998. Moderately high temperatures
478 inhibit ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase-mediated
479 activation of rubisco. *Plant Physiology* **116**, 539 LP – 546.
- 480 **Finkemeier I, Laxa M, Miguet L, Howden AJM, Sweetlove LJ.** 2011. Proteins of
481 diverse function and subcellular location are lysine acetylated in Arabidopsis. *Plant*
482 *Physiology* **155**, 1779 LP – 1790.
- 483 **Flecken M, Wang H, Popilka L, et al.** 2020. Article dual functions of a rubisco
484 activase in metabolic repair and recruitment to carboxysomes ll article dual functions
485 of a rubisco activase in metabolic repair and recruitment to carboxysomes. *Cell* **183**,
486 457-473.e20.
- 487 **Fukayama H, Ueguchi C, Nishikawa K, Katoh N, Ishikawa C, Masumoto C,**
488 **Hatanaka T, Misoo S.** 2012. Overexpression of rubisco activase decreases the
489 photosynthetic co2 assimilation rate by reducing rubisco content in rice leaves. *Plant*
490 *and Cell Physiology* **53**, 976–986.
- 491 **Gao X, Hong H, Li WC, Yang L, Huang J, Xiao YL, Chen XY, Chen GY.** 2016.
492 Downregulation of rubisco activity by non-enzymatic acetylation of RbcL. *Molecular*
493 *Plant* **9**, 1018–1027.
- 494 **Grabsztunowicz M, Koskela MM, Mulo P.** 2017. Post-translational modifications
495 in regulation of chloroplast function: recent advances. *Frontiers in plant science* **8**,
496 240.
- 497 **Graciet E, Lebreton S, Gontero B.** 2004. Emergence of new regulatory mechanisms
498 in the Benson-Calvin pathway via protein-protein interactions: A
499 glyceraldehyde-3-phosphate dehydrogenase/CP12/ phosphoribulokinase complex.
500 *Journal of Experimental Botany* **55**, 1245–1254.
- 501 **Grice GL, Nathan JA.** 2016. The recognition of ubiquitinated proteins by the
502 proteasome. *Cellular and molecular life sciences*□: *CMLS* **73**, 3497–3506.

503 **Hasse D, Larsson AM, Andersson I.** 2015. Structure of *Arabidopsis thaliana*
504 Rubisco activase. *Acta crystallographica. Section D, Biological crystallography* **71**,
505 800–808.

506 **Jensen RG.** 2000. Activation of Rubisco regulates photosynthesis at high temperature
507 and CO₂. *Proceedings of the National Academy of Sciences* **97**, 12937 LP – 12938.

508 **Jiang J, Gai Z, Wang Y, Fan K, Sun L, Wang H, Ding Z.** 2018. Comprehensive
509 proteome analyses of lysine acetylation in tea leaves by sensing nitrogen nutrition.
510 *BMC Genomics* **19**, 840.

511 **Kim SY, Harvey CM, Giese J, Lassowskat I, Singh V, Cavanagh AP, Spalding**
512 **MH, Finkemeier I, Ort DR, Huber SC.** 2019. In vivo evidence for a regulatory role
513 of phosphorylation of *Arabidopsis* Rubisco activase at the Thr78 site. *Proceedings of*
514 *the National Academy of Sciences* **116**, 18723 LP – 18731.

515 **Kubis SE, Lilley KS, Jarvis P.** 2008. Isolation and preparation of chloroplasts from
516 *Arabidopsis thaliana* plants. *Methods in molecular biology* (Clifton, N.J.) **425**,
517 171–186.

518 **Kurek I, Chang TK, Bertain SM, Madrigal A, Liu L, Lassner MW, Zhu G.** 2007.
519 Enhanced thermostability of *Arabidopsis* Rubisco activase improves photosynthesis
520 and growth rates under moderate heat stress. *The Plant cell* **19**, 3230–3241.

521 **Kuriata AM, Chakraborty M, Henderson JN, Hazra S, Serban AJ, Pham TVT,**
522 **Levitus M, Wachter RM.** 2014. ATP and magnesium promote cotton short-form
523 ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase hexamer
524 formation at low micromolar concentrations. *Biochemistry* **53**, 7232–7246.

525 **Li J, Yokosho K, Liu S, Cao HR, Yamaji N, Zhu XG, Liao H, Ma JF, Chen ZC.**
526 2020. Diel magnesium fluctuations in chloroplasts contribute to photosynthesis in
527 rice. *Nature Plants*.

528 **Lima BP, Antelmann H, Gronau K, Chi BK, Becher D, Brinsmade SR, Wolfe**
529 **AJ.** 2011. Involvement of protein acetylation in glucose-induced transcription of a
530 stress-responsive promoter. *Molecular microbiology* **81**, 1190–1204.

531 **Ling Q, Broad W, Trösch R, Töpel M, Demiral Sert T, Lymperopoulos P,**
532 **Baldwin A, Jarvis RP.** 2019. Ubiquitin-dependent chloroplast-associated protein
533 degradation in plants. *Science* **363**, eaav4467.

534 **Mateo F, Vidal-Laliena M, Canela N, Busino L, Martinez-Balbas MA, Pagano**
535 **M, Agell N, Bachs O.** 2009. Degradation of cyclin A is regulated by acetylation.
536 *Oncogene* **28**, 2654–2666.

537 **Mischerikow N, Heck AJR.** 2011. Targeted large-scale analysis of protein
538 acetylation. *Proteomics* **11**, 571–589.

539 **Moon J, Parry G, Estelle M.** 2004. The Ubiquitin-proteasome pathway and plant
540 development. *The Plant Cell* **16**, 3181 LP – 3195.

541 **Mueller-Cajar O, Stotz M, Wendler P, Hartl FU, Bracher A, Hayer-Hartl M.**
542 2011. Structure and function of the AAA⁺ protein CbbX, a red-type Rubisco activase.
543 *Nature* **479**, 194–199.

544 **O’Leary BM, Scafaro AP, Fenske R, Duncan O, Ströher E, Petereit J, Millar**
545 **AH.** 2020. Rubisco lysine acetylation occurs at very low stoichiometry in mature

546 Arabidopsis leaves: implications for regulation of enzyme function. The Biochemical
547 journal **477**, 3885–3896.

548 **Osorio S, Ruan Y-L, Fernie AR**. 2014. An update on source-to-sink carbon
549 partitioning in tomato. Frontiers in plant science **5**, 516.

550 **Peltier J-B, Cai Y, Sun Q, Zabrouskov V, Giacomelli L, Rudella A, Ytterberg**
551 **AJ, Rutschow H, van Wijk KJ**. 2006. The oligomeric stromal proteome of
552 *Arabidopsis thaliana* chloroplasts. Molecular & Cellular Proteomics **5**, 114
553 LP – 133.

554 **Portis AR**. 2003. Rubisco activase – Rubisco’s catalytic chaperone. Photosynthesis
555 Research **75**, 11–27.

556 **Post-Beittenmiller D, Roughan G, Ohlrogge JB**. 1992. Regulation of plant fatty
557 acid biosynthesis□: analysis of acyl-coenzyme a and acyl-acyl carrier protein
558 substrate pools in spinach and pea chloroplasts. Plant physiology **100**, 923–930.

559 **Ramponi G, Manao G, Camici G**. 1975. Nonenzymic acetylation of histones with
560 acetyl phosphate and acetyl adenylate. Biochemistry **14**, 2681–2685.

561 **Robinson SP, Portis AR**. 1989. Adenosine triphosphate hydrolysis by purified
562 rubisco activase. Archives of Biochemistry and Biophysics **268**, 93–99.

563 **Salvucci ME, Crafts-Brandner SJ**. 2004. Relationship between the heat tolerance of
564 photosynthesis and the thermal stability of Rubisco activase in plants from contrasting
565 thermal environments. Plant Physiology **134**, 1460 LP – 1470.

566 **Salvucci ME, Osteryoung KW, Crafts-Brandner SJ, Vierling E**. 2001.
567 Exceptional sensitivity of Rubisco activase to thermal denaturation in vitro and in
568 vivo. Plant physiology **127**, 1053–1064.

569 **Shen JB, Orozco EMJ, Ogren WL**. 1991. Expression of the two isoforms of spinach
570 ribulose 1,5-bisphosphate carboxylase activase and essentiality of the conserved
571 lysine in the consensus nucleotide-binding domain. The Journal of biological
572 chemistry **266**, 8963–8968.

573 **Smalle J, Vierstra RD**. 2004. The ubiquitin 26S proteasome proteolytic pathway.
574 Annual Review of Plant Biology **55**, 555–590.

575 **Spreitzer RJ, Salvucci ME**. 2002. Rubisco: structure, regulatory interactions, and
576 possibilities for a better enzyme. Annual review of plant biology **53**, 449–475.

577 **Stotz M, Mueller-Cajar O, Ciniawsky S, Wendler P, Hartl FU, Bracher A,**
578 **Hayer-Hartl M**. 2011. Structure of green-type Rubisco activase from tobacco. Nature
579 Structural & Molecular Biology **18**, 1366–1370.

580 **Striebel F, Kress W, Weber-Ban E**. 2009. Controlled destruction: AAA⁺ ATPases
581 in protein degradation from bacteria to eukaryotes. Current opinion in structural
582 biology **19**, 209–217.

583 **Tumaney AW, Ohlrogge JB, Pollard M**. 2004. Acetyl coenzyme A concentrations
584 in plant tissues. Journal of plant physiology **161**, 485–488.

585 **Wagner GR, Payne RM**. 2013. Widespread and enzyme-independent Nε-acetylation
586 and Nε-succinylation of proteins in the chemical conditions of the mitochondrial
587 matrix. The Journal of biological chemistry **288**, 29036–29045.

588 **Wang Q, Zhang Y, Yang C, et al.** 2010. Acetylation of metabolic enzymes
589 coordinates carbon source utilization and metabolic flux. *Science* **327**, 1004 LP –
590 1007.

591 **Weinert BT, Iesmantavicius V, Wagner SA, Schölz C, Gummesson B, Beli P,**
592 **Nyström T, Choudhary C.** 2013. Acetyl-phosphate is a critical determinant of lysine
593 acetylation in *E. coli*. *Molecular cell* **51**, 265–272.

594 **Wu X, Oh M-H, Schwarz EM, Larue CT, Sivaguru M, Imai BS, Yau PM, Ort**
595 **DR, Huber SC.** 2011. Lysine acetylation is a widespread protein modification for
596 diverse proteins in *Arabidopsis*. *Plant Physiology* **155**, 1769 LP – 1778.

597 **Yamori W, Masumoto C, Fukayama H, Makino A.** 2012. Rubisco activase is a key
598 regulator of non-steady-state photosynthesis at any leaf temperature and, to a lesser
599 extent, of steady-state photosynthesis at high temperature. *The Plant journal* □: for cell
600 and molecular biology **71**, 871–880.

601 **Zhang N, Portis ARJ.** 1999. Mechanism of light regulation of Rubisco: a specific
602 role for the larger Rubisco activase isoform involving reductive activation by
603 thioredoxin-f. *Proceedings of the National Academy of Sciences of the United States*
604 *of America* **96**, 9438–9443.

605 **Zhou L-J, Li Y-Y, Zhang R-F, Zhang C-L, Xie X-B, Zhao C, Hao Y-J.** 2017. The
606 small ubiquitin-like modifier E3 ligase MdSIZ1 promotes anthocyanin accumulation
607 by sumoylating MdMYB1 under low-temperature conditions in apple. *Plant, cell &*
608 *environment* **40**, 2068–2080.

609
610

611

612 **Figures legends**

613 **Fig. 1** Dark and high temperature increase Rubisco activase (RCA) acetylation in
614 tobacco.

615 **(A)** RCA acetylation level changes in a circadian pattern. The tobacco plants were
616 grown under a photoperiod of 11/13h light/dark. Three-week old fully expanded
617 tobacco leaves were collected in the dark period and 2 h interval during the
618 daytime. For each sample, three leaves were pooled together. In total, around ~3 µg
619 of soluble proteins were loaded for each sample. For the western-blot analysis, the
620 antibodies raised against acetylation and the RCA were used in dilution of
621 1:10,000. The acetylated tobacco RCA was examined by western-blot with an
622 anti-AcK. The RCA levels were estimated as the signal strength (band size)
623 obtained with the RCA antibody.

624 **(B)** The RCA acetylation level increases at high temperature (45 °C). Fully expanded
625 tobacco leaves were treated for 30 min at either 23 °C (control, CK) or 45 °C,
626 therefore, intact chloroplasts were isolated, and subsequently the total chloroplastic
627 proteins were extracted by SDS protein extract buffer. The total soluble proteins in
628 stroma were also isolated from the intact chloroplast. Then, the acetylated tobacco
629 RCA was examined by western-blot with an anti-AcK. The RCA levels were
630 estimated as well by western-blot using the RCA specific antibody.

631 **(C)** A schematic simplified illustration (mapping) of the RCA secondary structure
632 with its different functional domains, including N domain exhibited from 1 to 68th
633 amino acids (AA), α/β subdomain, α-helical subdomain and C-ext domain which
634 were depicted in pink, green, yellow and grey, respectively. The AAA⁺ module
635 includes α/β subdomain, α-helical subdomain (green and yellow regions).

636 **(D-E)** Mass spectrum of the acetylated lysine residues in tobacco RCA. The RCA
637 from tobacco leaves was extracted, followed by separation with SDS-PAGE and
638 digestion. The acetylated lysine residues were thereafter processed (analyzed) by
639 LC-MS/MS and two acetylated residues were identified to K313 **(D)** and K360 **(E)**.
640 **(F-H)** RCA site-directed mutations at the acetylation sites changed their ATPase
641 activities. K126R, K164R and D299A **(F)**, K313R and K313D **(G)**, K360R and
642 K360D **(H)** RCA proteins were expressed in *E.coli*. The ATPase activity for each
643 mutant was determined and compared to that of the wild type (WT) RCA which
644 was purified following the same protocol as for the mutants. Each bar data
645 represents the average of triplicate assays (±SD). The different alphabetic letters
646 adjacent to the histogram bars reflect the significance level at a *P*-value < 0.05
647 based on one-way ANOVA statistical analysis.

648 **Fig. 2** RCA acetylation by different acetyl group donors.

649 **(A-C)** The tobacco RCA protein expressed in *E. coli* was acetylated by Ac-CoA **(A)**,
650 ACP (acetyl phosphate; **B**) and Ac-AMP (Ac-AMP; **C**). The RCA was firstly

651 incubated with Ac-CoA, ACP and Ac-AMP for 30 min at 25 °C, and then the RCA
652 acetylation level was assessed by western-blot analysis.

653 **(D)** A pre-treatment with RuBP inhibits the RCA acetylation by the Ac-CoA. Indeed,
654 RCA was incubated with RuBP for 5 min at 25 °C before its (RCA) incubation with
655 Ac-CoA (0.5 mM) for 30 min.

656 **(E)** The RCA acetylation by the substrates such as Ac-CoA and Ac-AMP adversely
657 affects the RCA ATPase activity. The RCA was acetylated by 0.5 mM Ac-CoA or
658 10 mM Ac-AMP, therefore RCA was incubated for 30 min at 25 °C, and then the
659 ATPase activity was determined.

660 **(F)** The Ac-CoA modifies the RCA but did not influence the Rubisco activation
661 process (activity). The RCA was incubated with 0.5 mM Ac-CoA for 30 min at 25
662 °C and the ATPase initial activity was immediately measured before any potential
663 pre-activation. For panels E and F, each data bar represents the mean of three
664 independent replicates ($n = 3$) $\pm$ SD, and the significance level between the control
665 (CK) and the treated sample was calculated via the Student's *t*-test, where $P < 0.01$
666 (***) means very significant and $P < 0.001$ (****) signifies highly significant; ns
667 stands for non-significant ($P > 0.05$).

668 **(G-H)** The interaction between the RCA and Ac-CoA was performed by the surface
669 alkaline resonance method. The RCA was directly immobilized to the sensor chip
670 and the different concentrations of Ac-CoA (0.08 mM, 0.16 mM, 0.32 mM, 0.64
671 mM and 2 mM) were injected at a flow rate of 20 $\mu\text{L}\cdot\text{min}^{-1}$ **(G)**. The affinity
672 constant of dissociation (K_D) was thus determined by simulating the kinetic curves
673 and deduced accordingly **(H)**.

674 **Fig. 3** Evaluation of RCA non-enzymatic acetylated by Ac-CoA using
675 deuterium-labeled Ac-CoA (D^2 -Ac-CoA).

676 **(A-B)** Mass spectrum of the acetylated lysine residues in tobacco RCA. The latter
677 (RCA) was initially incubated with 0.5 mM D^2 -Ac-CoA for 30 min at 25 °C, then
678 digested and loaded to the LC-MS/MS for further fragmentation (decomposition),
679 analysis and detection. **(A)** Modification of the K17 peptide. **(B)** Modification of
680 the K116 and K126 peptides. The sequences fragments are displayed on the top of
681 each panel.

682 **Fig. 4** The lysine acetylation adversely affects the RCA stability and activity.

683 **(A)** The lysine acetylation proteins of RCA induced by HDACi for different hours.

684 **(B)** The RCA protein expression levels due to HDACi induced acetylation effects.

685 **(C)** The RCA protein expression levels due to either Ac-AMP or Ac-CoA induced
686 acetylation effects.

687 **(D)** The RCA protein expression levels in E-RCA (expressed in *E. coli*) due to
688 Ac-CoA induced acetylation effects.

689 Intact tobacco chloroplast was treated with HDACi inhibitor, 50 nM TSA, 2 mM
690 NAM and 2 mM NAB (see material and methods for full inhibitors names), then the
691 stroma total soluble proteins were collected at different time-points including 0, 3 and
692 6 h. The intact tobacco chloroplast was treated with Ac-AMP (10 mM) and Ac-CoA
693 (0.5 mM; **C**) and E-RCA (expressed by *E. coli*) (**D**). For all samples containing
694 HDACi inhibitors, the stroma proteins were collected at 0, 1 and 3 h. The RCA
695 protein levels were determined by western-blot analysis. Data were reproducible and
696 representative of at least 3 independent experiments.

697 **Fig. 5** A simplified schematic representative working model depicting the biological
698 relevance and the regulatory mechanisms of the RCA acetylation.

699 The RCA can be acetylated in presence of Ac-CoA *in vivo* and its (RCA) acetylation
700 level could be inhibited by light as well as by RuBP. Thus, the acetylated RCA can
701 slow down its ATPase activity and decreases its protein amount. Therefore, the
702 degraded protein, as reflected by the decreased RCA protein level, may eventually
703 lead to a lower Rubisco activity. Ultimately, we could deduce that the ubiquitination
704 process does not represent the direct reason standing behind the RCA protein
705 degradation rather it is very likely its acetylation.

706

Fig. 1, Yang et al. 2020

Fig. 2, Yang et al. 2020

Fig. 3, Yang et al. 2020

Fig. 4, Yang et al. 2020

Fig. 5, Yang et al. 2020