1 **Short Title:** Acclimation of *Chlamydomonas* to Sub-Lethal NO

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8 **Acclimation of *Chlamydomonas reinhardtii* to Nitric Oxide Stress Related to** 9 **Respiratory Burst Oxidase-Like 2**

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15 **One-sentence Summary:** Acclimation machinery is triggered in *Chlamydomonas*
16 *reinhardtii* cells against sub-lethal nitric oxide stress.

17

18 **Footnotes**

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33 Abstract

34 The acclimation mechanism of *Chlamydomonas reinhardtii* to nitric oxide (NO) was
 35 studied by exposure to *S*-nitroso-*N*-acetylpenicillamine (SNAP), a NO donor.
 36 Treatment with 0.1 or 0.3 mM SNAP transiently inhibited photosynthesis within 1 h,
 37 followed by a recovery without growth impairment, while 1.0 mM SNAP treatment
 38 caused irreversible photosynthesis inhibition and mortality. The SNAP effects are
 39 avoided in the presence of the NO scavenger,
 40 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO).
 41 RNA-seq, qPCR, and biochemical analyses were conducted to decode the metabolic
 42 shifts under sub-lethal NO stress by exposure to 0.3 mM SNAP in the presence or
 43 absence of 0.4 mM cPTIO. These findings revealed that the acclimation to NO stress
 44 comprises a temporally orchestrated implementation of metabolic processes: 1. trigger
 45 of NO scavenging elements to reduce NO level; 2. prevention of photo-oxidative risk
 46 through photosynthesis inhibition and antioxidant defense system induction; 3.
 47 acclimation to nitrogen and sulfur shortage; 4. degradation of damaged proteins
 48 through protein trafficking machinery (ubiquitin, SNARE, and autophagy) and
 49 molecular chaperone system for dynamic regulation of protein homeostasis. NO
 50 increased NADPH oxidase activity and respiratory burst oxidase-like 2 (RBOL2)
 51 transcript abundance, which were not observed in the *rbol2* insertion mutant. Changes
 52 in gene expression in the *rbol2* mutant and increased mortality under NO stress
 53 demonstrate that NADPH oxidase (RBOL2) is involved in the modulation of some
 54 acclimation processes (NO scavenging, antioxidant defense system, autophagy, and
 55 heat shock proteins) for *Chlamydomonas* to cope with NO stress. Our findings provide
 56 insight into the molecular events underlying acclimation mechanisms in
 57 *Chlamydomonas* to sub-lethal NO stress.

58

59 INTRODUCTION

60 Nitric oxide (NO) is a crucial signaling molecule in diverse physiological processes
 61 in plants (Durner et al., 1999), including in the green alga *Chlamydomonas*, in which
 62 NO regulates many physiological processes and stress responses such as the
 63 remodeling of chloroplast proteins by the degradation of thylakoid cytochrome *b₆f*
 64 complex and stroma ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) via
 65 FtsH and Clp chloroplast proteases under nitrogen (Wei et al., 2014) or sulfur
 66 starvation (de Mia et al., 2019). In *Chlamydomonas*, NO is also involved in cell death
 67 induced by ethylene and mastoparan (Yordanova et al., 2010), induction of oxidative
 68 stress under extreme high light (VHL, 3,000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Chang et al., 2013),
 69 interaction of NO with hydrogen peroxide (H_2O_2) for high light stress-induced
 70 autophagy and cell death (Kuo et al., 2020a), proline biosynthesis under copper
 71 stress (Zhang et al., 2008), and responses to salt stress (Chen et al., 2016). NO is
 72 responsible for the regulation of nitrogen assimilation by repressing the expression of
 73 nitrate reductase as well as nitrate and ammonium transporters (de Montaigu et al.,
 74 2010) and their enzyme activities (Sanz-Luque et al., 2013; Calatrava et al., 2017).
 75 Mitochondrial respiration by upregulation of alternative oxidase 1 is modulated by NO
 76 in *Chlamydomonas* (Zalutskaya et al., 2017).

77 Information is still lacking on the comprehensive overview of the transcriptional
 78 regulation of *C. reinhardtii* in response to NO stress. Therefore, in the present study, *C.*
 79 *reinhardtii* cells were treated with different concentrations (0.1, 0.3, and 1.0 mM) of
 80 the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) in the presence or absence of
 81 an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
 82 (cPTIO) (Mur et al., 2011). This study provides new insight into the acclimation
 83 mechanisms against NO stress in *Chlamydomonas*.

84

85

86 RESULTS

87 Physiological and Transcriptomic Changes in Response to NO Burst

88 The mechanisms that allow for the acclimation of *C. reinhardtii* cells to short-term
 89 NO burst were examined. The SNAP concentration and treatment duration were
 90 carefully chosen to allow for the monitoring of the short-term response to NO rather
 91 than cell death. Using a cell permeable NO-sensitive fluorescent dye, the fluorescence
 92 emitted from the cells (Fig. 1A) rapidly increased 0.5 h after SNAP treatment and
 93 reached a plateau after 1 h (Fig. 1B); the increase in fluorescence can be inhibited in
 94 the presence of 0.4 mM cPTIO. Both the 0.1 and 0.3 mM SNAP treatments did not
 95 affect cell viability (Fig. 1C) and growth (Fig. 1D), whereas the 1.0 mM SNAP
 96 treatment resulted in cell mortality and bleaching (Fig. 1E). The concentrations of
 97 chlorophyll *a* (Supplemental Fig. S1A), chlorophyll *b* (Supplemental Fig. S1B), and
 98 carotenoids (Supplemental Fig. S1C) were not influenced by either 0.1 or 0.3 mM
 99 SNAP treatments; however, a decrease in chlorophyll *a*, chlorophyll *b*, and carotenoids
 100 6 h after treatment with 1.0 mM SNAP was observed. Photosynthesis is relatively
 101 sensitive to NO burst, as reflected by a transient decrease in F_v'/F_m' (Fig. 2A), F_v/F_m
 102 (Fig. 2B), and the O_2 evolution rate (Fig. 2C) 0.5 h after SNAP treatment; these
 103 photosynthesis-related factors then recovered after 3–5 h in both the 0.1 and 0.3 mM
 104 SNAP treatments but not in the 1.0 mM SNAP treatment. This inhibition of
 105 photosynthesis-related factors can be suppressed in the presence of 0.4 mM cPTIO.
 106 Based on fluorescence induction kinetics (OJIP curve), the cells treated with 0.1 or 0.3
 107 mM SNAP transiently lost both the PSII acceptor-side (F_J-F_o and F_I-F_J) and
 108 donor-side (F_P-F_I and F_v/F_o) electron transfer ability, whereas 1.0 mM SNAP
 109 treatment caused irreversible inhibition, which was prevented in the presence of 0.4
 110 mM cPTIO (Supplemental Table S1). The SNAP treatments decreased the production
 111 of superoxide anion radical ($O_2^{\cdot-}$) and H_2O_2 ; the production of singlet oxygen (1O_2)

112 was significantly increased by 1.0 mM SNAP treatment (Supplemental Fig. S1D-F).
 113 Treatment with 1.0 mM SNAP also caused significant lipid peroxidation
 114 (Supplemental Fig. S1G). Overall, these results demonstrate two stages of response in
 115 *C. reinhardtii* cells in the sub-lethal NO challenge: I. NO stress together with the
 116 significant NO burst caused a sharp decline in photosynthetic activity 1 h after SNAP
 117 treatment and II. the recovery period. Therefore, *Chlamydomonas* cells treated with
 118 0.3 mM SNAP for 1 h in the presence or absence of 0.4 mM cPTIO were used for
 119 transcriptomic analyses.

120 The results of the transcriptomic analysis (Supplemental Tables S2 and S3) showed
 121 that 1,012 significant DEGs were regulated by NO (1.2 log₂FC, *P*-value of log₂FC ≤
 122 0.05) (Supplemental Table S4). Following analysis using the MapMan display mode,
 123 cellular response overview (Supplemental Fig. S2), proteasome and autophagy
 124 (Supplemental Fig. S3), photosynthesis (electron transport, Calvin cycle, and
 125 photorespiration) (Supplemental Fig. S4), and the tetrapyrrole pathway (Supplemental
 126 Fig. S5) were affected under NO stress. Using the Blast2GO suite, the analysis of all
 127 the functional DEGs and unigenes identified 45 GO terms (score ≤ 0.05) that can be
 128 assigned to 463 upregulated genes (45.75%), with 13 biological process, 30 molecular
 129 function, and two cellular component GO terms (Supplemental Fig. S6A and Table
 130 S5A). One hundred fifty-seven GO terms for 549 downregulated genes with 337
 131 known function genes (54.25%) were classified into 35 GO terms belonging to
 132 biological process, 112 to molecular function, and eight to cellular component
 133 (Supplemental Fig. S6B and Table S5B). The genes associated with amino acid
 134 catabolism, the ubiquitin-proteasome system, and the antioxidant defense system are
 135 upregulated, while those related to transcriptional and translational regulation are
 136 downregulated by NO burst (Supplemental Tables S4 and S5).

137 We also found that the genes associated with photosynthesis are downregulated by

138 NO burst (Supplemental Tables S7). NO decreased the transcript abundances of
139 luminal PsbP-like protein (PSBP4, Cre03.g182551.t1.2), which is linked to the
140 stability of photosystem II complex assembly (Ido et al., 2014); Lhl2
141 (Cre08.g384650.t1.2) belonging to high light-induced protein (Teramoto et al., 2004),
142 Lhl3 (Cre03.g199535.t1.1); pre-apoplastocyanin (PCY1, Cre03.g182551.t1.2),
143 cytochrome c_{6A} (CYC4, Cre16.g670950.t1.2) acting as electron transfer between
144 cytochrome b_6f complex and photosystem I (Marcaida et al., 2006); CHLH
145 (Cre07.g325500.t1.1); CHLI1 (Cre06.g306300.t1.2); CHLI2 (Cre12.g510800.t1.2),
146 CHLD (Cre05.g242000.t1.2); and GENOMES UNCOUPLED 4 (GUN4,
147 Cre05.g246800.t1.2). In contrast, NO increased the transcript abundance of
148 chlorophyll *a/b* binding protein (LHCBM9, Cre06.g284200.t1.2), Lhl1
149 (Cre08.g384650.t1.2), and Lhl4 (Cre17.g740950.t1.2) (Fig. 3). For the Calvin cycle,
150 NO also decreased the transcript abundance of the Rubisco small subunit 1 (RBCS1,
151 Cre02.g120100.t1.2), ribulose phosphate-3-epimerase (RPE2, Cre02.g116450.t1.2),
152 phosphoglycerate kinase (PGK1, Cre11.g467770.t1.1), glyceraldehyde-3-phosphate
153 dehydrogenase (GAP4, Cre12.g556600.t1.2), triose phosphate isomerase (TPI1,
154 Cre01.g029300.t1.2), sugar bisphosphatase (SBP2, Cre17.g699600.t1.2), a
155 pentatrichopectide repeat protein that stabilizes Rubisco large subunit mRNA (PPR2,
156 Cre06.g298300.t1.1), a small protein for phosphoribulokinase deactivation (Howard et
157 al., 2008) (CP12, Cre08.g380250.t1.2), Rubisco large (RMT1, Cre16.g661350.t1.2
158 and a similar one, Cre16.g649700.t1.1) and small (RMT2, Cre12.g524500.t1.2)
159 subunit N-methyltransferase, and phosphoglycolate phosphatase (PGP3,
160 Cre06.g271400.t1.1) in the photorespiration (Fig. 4). In contrast, the transcript
161 abundance of Rubisco activase-like protein (RCA2, Cre06.g298300.t1.1) was
162 increased by NO (Fig. 4H). The NO-induced changes in gene expression occurred 1 h
163 after NO exposure, recovered to the same levels as the control after 3 h, and the

164 changes at 1 h can be inhibited by the presence of cPTIO.

165

166 **NO Decreases Nitrogen and Sulfur Availability**

167 The transcript abundances of the ammonium transporters AMT3
168 (Cre06.g293051.t1.1) (Fig. 5A), AMT6 (Cre07.g355650.t1.1) (Fig. 5B), and AMT7
169 (Cre02.g111050.t1.1) (Fig. 5C), and the ammonium assimilation enzymes, glutamate
170 synthase (GSN1, Cre13.g592200.t1.2) (Fig. 5D) and glutamate synthetase (GLN1,
171 Cre02.g113200.t1.1) (Fig. 5E) decreased 1 h after NO treatment and then recovered,
172 while that of glutamate dehydrogenase (GDH1, Cre09.g388800.t1.2) increased after 1
173 h (Fig. 5F).

174 Cysteine desulfurase (CSD4, Cre12.g525650.t1.2), which moves the sulfur from the
175 cysteine to sulfur-containing recipients, showed an increase in transcriptional
176 expression in the NO treatment (Fig. 5G). Cysteine can be oxidized to 3-sulfinoalanine
177 (Chai et al., 2005) by cysteine dioxygenase (CDO), and the transcript abundances of
178 CDO1 (Cre03.g174400.t1.1) (Fig. 5H) and CDO2 (Cre03.g163950.t1.1) (Fig. 5I)
179 increased after NO treatment. In the NO treatment, the transcript abundance of taurine
180 dioxygenase (TAUD1, Cre13.g569400.t1.2), which degrades taurine to sulfite and
181 aminoacetaldehyde, increased (Fig. 5J) but that of aspartate aminotransferase (AST5,
182 Cre06.g278249.t1.1), which converts 3-sulfinoalanine to pyruvate, decreased (Fig. 5K).
183 Thus, cysteine is catabolized to taurine and then degraded to sulfite instead of pyruvate.
184 Then, sulfite is oxidized by sulfite oxidase (SUOX) to avoid toxicity due to
185 accumulation (Hansch et al., 2007). However, NO did not affect the expression of
186 SUOX (Cre04.g217929.t1.1) (Supplemental Table S3).

187 The expression of genes related to amino acid degradation was transiently
188 upregulated by NO, including L-allo-threonine aldolase (ATA2, Cre10.g423550.t1.1)
189 (Fig. 5L), the aromatic amino acid hydroxylase-related protein (AAH1,

190 Cre01.g029250.t1.2) (Fig. 5M), and the catabolism of branched-chain amino acids
191 (valine, leucine and isoleucine; 2-oxoisovalerate dehydrogenase E1 component alpha
192 (BCKDHA, Cre12.g539900.t1.1) (Fig. 5N) and beta (BCKDHB, Cre06.g311050.t1.2)
193 (Fig. 5O) (Supplemental Table S6). The presence of cPTIO inhibited the changes
194 induced by SNAP treatment.

195 NO increased the transcript abundance of periplasmic arylsulfatase (ARS3,
196 Cre10.g430200.t1.2), arylsulfatase (ARS7, Cre01.g011901.t1.1), plasma membrane
197 sodium/sulfate co-transporters (SLT1, Cre12.g502600.t1.2; SLT2,
198 Cre10.g445000.t1.2), the chloroplast sulfate binding protein as the component of
199 chloroplast transporter (SULP3, Cre06.g257000.t1.2), and SNRK2.2 (SAC3,
200 Cre12.g499500.t1.1), an Snf1-like serine/threonine kinase responsible for the
201 repression of expression of genes responsible for sulfur starvation (Davies et al., 1999;
202 Gonzalez-Ballester et al., 2008). NO decreased the transcript abundance of ARS5
203 (Cre10.g431800.t1.2), ARS11 (Cre04.g226550.t1.2), ARS13 (Cre05.g239750.t1.2),
204 ARS14 (Cre05.g239800.t2.1), and ARS16 (Cre06.g293800.t1.1) (Fig. 6). The
205 presence of cPTIO inhibited these changes in response to SNAP treatment.

206

207 **NO Modulates Protein Homeostasis and Quality**

208 Based on the identified GO terms, NO induces protein degradation through
209 ubiquitination and membrane trafficking (Supplemental Fig. S6). For genes involved
210 in the ubiquitin-proteasome system (Supplemental Table S8), the transcript
211 abundances of AAA+-type ATPase (Cre16.g650150.t1.3), which is responsible for the
212 opening of the gates for substrate into the axial entry ports of the proteases (Yedidi et
213 al., 2017); ubiquitin-protein ligase E2 (Cre07.g342506.t1.2); ubiquitin-protein ligase
214 E3 A (UBE3A; Cre08.g364550.t1.3); probable E3 ubiquitin-protein ligase HERC1
215 (Cre02.g099100.t1.3); and ubiquitin fusion degradation protein (Cre03.g179100.t1.2),

216 which is similar to ubiquitin fusion degradation protein 1, were increased by NO
217 treatment (Fig. 7). In contrast, the transcript abundances of ubiquitin-conjugating
218 enzyme E2I (UBC9, Cre01.g019450.t1.1), which exhibits the activity of small
219 ubiquitin-like modifier (SUMO) E2 conjugase (Wang et al., 2008), a central enzyme in
220 SUMO conjugation for interaction with E1 to accept SUMO in the formation of a
221 SUMO~UBC9 thioester bond (Pichler et al., 2017), and ubiquitin-conjugating enzyme
222 E2 (UBC3, Cre03.g167000.t1.2) (Fig. 7D) decreased in the NO treatment (Fig. 7).
223 These changes induced by the SNAP treatment were inhibited in the presence of
224 cPTIO.

225 We detected most of the genes encoding endocytosis (Supplemental Table S9A) and
226 SNAREs (Supplemental Table S9B; soluble *N*-ethylmaleimide-sensitive factor
227 attachment protein receptor), which mediate fusion events for autophagosome
228 biogenesis as the membrane trafficking pathways. NO increased the transcript
229 abundances of the vacuolar protein sorting (VPS) proteins, which sort receptors within
230 the endocytic pathway (Bishop and Woodman, 2001) including the subunits of the
231 ESCRT-I (endosomal sorting complex I required for transport) complex (i.e., VPS23
232 (Cre07.g351050.t1.2), VPS28 (Cre16.g678100.t1.2), and VPS37
233 (Cre08.g362550.t1.2)), the subunit of the ESCRT-III complex (VPS2A;
234 Cre04.g213450.t1.2), and RAB5 (Cre12.g517400.t1.2), a small rab-related GTPase
235 that regulates vesicle formation and membrane fusion. NO also increased the transcript
236 abundances of SYNTAXIN-8B-RELATED (Cre06.g293100.t1.2), a member of the
237 syntaxin family that is involved in protein trafficking from early to late endosomes via
238 vesicle fusion (Prekeris et al., 1999), and endosomal R-SNARE protein belonging to
239 the VAMP (vesicle associated membrane protein)-like family (R.III) (VMP7,
240 Cre04.g214750.t1.1), which functions in clathrin-independent vesicular transport and
241 membrane fusion events necessary for protein transport from early endosomes to late

242 endosomes (Advani et al., 1999; Prekeris et al., 1999). NO decreased the transcript
243 abundances of endoplasmic reticulum (ER) Qb-SNARE protein and Sec20-family
244 (SEC20, Cre02.g090650.t1.1) (Fig. 7). In addition, NO impacted vesicular protein
245 trafficking that is responsible for the accurate delivery of proteins to correct
246 subcellular compartments (Rosquete et al., 2018) via tethering of a transport vesicle to
247 its target membrane, which is regulated by two classes of tethering factors, long
248 coiled-coil proteins and multi-subunit tethering complexes (MTCs) (Ravikumar et al.,
249 2017). The transport protein particle (TRAPP) complex is a well-studied MTC in yeast
250 and mammals for the regulation of ER-to-Golgi and Golgi-mediated secretion
251 membrane trafficking (TRANsport Protein Particle, TRAPPI and TRAPPII) and
252 autophagy (TRAPPIII) processes (Kim et al, 2016; Vukasinovic and Zarsky, 2016).
253 Here, we detected several TRAPP genes in *Chlamydomonas*, in which the transcript
254 abundances of TRAPP I subunits (TRS20, Cre13.g571600.t1.2; TRS23,
255 Cre02.g077500.t1.2; TRS31, Cre03.g146467.t1.1; TRS33, Cre16.g663650.t1.2; BET3,
256 Cre03.g145607.t1.1; BET5, Cre06.g289700.t1.2) and a TRAPPIII subunit (TRS85,
257 Cre01.g024400.t1.1) were transiently decreased by NO burst (Fig. 8). As a catabolic
258 process for recycling cellular materials, autophagy is also induced by NO burst
259 (Supplemental Table S9C), which was reflected by a decrease in TOR1
260 (Cre09.g400553.t1.1) transcript abundance as well as an increase in the transcript
261 abundance of ATG3 (Cre02.g102350.t1.2), ATG4 (Cre12.g510100.t1.1), ATG5
262 (Cre14.g630907.t1.1), ATG6 (Cre01.g020264.t1.1), ATG7 (Cre03.g165215.t1.1),
263 ATG8 (Cre16.g689650.t1.2), and ATG9 (Cre09.g391500.t1.1) (Fig. 9). However, NO
264 decreased the transcript abundance of ATG12 (Cre12.g557000.t1.2) (Fig. 9I). ATG8
265 and ATG8-PE (phosphatidylethanolamine) proteins, which were detected using
266 western blot were also increased by NO burst. The changes in the expression of these
267 genes by SNAP were inhibited in the presence of cPTIO (Fig. 9J).

268 NO also modulates protein folding via the transcriptional control of heat shock
269 proteins (HSPs) that control protein folding and homeostasis in *Chlamydomonas*
270 (Schroda et al., 2015). An overview of how strongly NO treatment impacts expression
271 levels for each HSP gene, including DNAJ-like proteins, small HSPs, HSP60s,
272 HSP70s, HSP90s, and HSP100s, is provided in Supplemental Table S9D. HSP70D
273 (Cre12.g535700.t1.1) was significantly decreased by NO burst and CLBP3
274 (Cre02.g090850.t1.1, belonging to the HSP100 family) and small HSPs (HSP22A,
275 Cre07.g318800.t1.2; HSP22C, Cre03.g145787.t1.1; HSP22E, Cre14.g617450.t1.2;
276 HSP22F, Cre14.g617400.t1.2) were significantly increased by NO burst (Fig. 9). The
277 effect of SNAP on the expression of HSPs was inhibited in the presence of cPTIO.

278

279 **Induction of the Antioxidant Defense System by NO**

280 Through enzymatic and non-enzymatic routes, the antioxidant defense system is
281 induced by NO treatment (Supplemental Table S10). Superoxide dismutase (SOD),
282 responsible for the dismutaton of O_2^- to H_2O_2 , is among the most important
283 antioxidant defense enzymes in plants (Alscher et al., 2002). Six SODs, FSD1
284 (Cre02.g096150.t1.2), MSD1 (Cre02.g096150.t1.2), MSD2 (Cre13.g605150.t1.2),
285 MSD3 (Cre16.g676150.t1.2), MSD4 (Cre12.g490300.t1.2), and MSD5
286 (Cre17.g703176.t1.1), were detected to have the most abundant expression for FSD1
287 (Supplemental Table S10), in which FSD1 and MSD3 transcript abundances were
288 markedly increased by NO treatment (Fig. 10A-F). Furthermore, the
289 ascorbate-glutathione cycle (AGC), an important defense pathway for detoxification of
290 H_2O_2 , plays a role in the defense of photo-oxidative stress in *Chlamydomonas* (Barth
291 et al., 2014; Lin et al., 2016, 2018; Chang et al., 2017; Yeh et al., 2019; Kuo et al.,
292 2020b) and was also induced by NO. NO increased the transcript abundance of APX1
293 (Cre02.g087700.t1.2), DHAR1 (Cre10.g456750.t1.2), and GSHR1

294 (Cre06.g262100.t1.1), but decreased the transcript abundance of APX2
 295 (Cre06.g285150.t1.2), APX4 (Cre05.g233900.t1.2), and MDAR1 (Cre17.g712100.t1.1)
 296 (Fig. 10 and Supplemental Fig. S7). The transcript abundance of GSHR2
 297 (Cre09.g396252.t1.1) was not affected by NO (Supplemental Fig. S7). For glutathione
 298 (GSH) and ascorbate (AsA) biosynthesis, the transcript abundance of GSH1
 299 (Cre02.g077100.t1.2; Fig. 10J) and VTC2 (GDP-L-galactose phosphorylase,
 300 Cre13.g588150.t1.2; Fig. 10K), a key enzyme for AsA biosynthesis (Urzica et al.,
 301 2012), increased under NO treatment, while that of GSH2 (Cre17.g708800.t1.1) was
 302 not affected (Supplemental Fig. S7E). However, the activity of SOD (Fig. 10L), APX
 303 (Fig. 10M), and MDAR (Fig. 10P) was not affected by NO, whereas GR (Fig. 10Q),
 304 DHAR (Fig. 10N), and protein (Fig. 10O) did show an increase in activity. These gene
 305 expression changes under SNAP treatment were inhibited in the presence of cPTIO
 306 (Fig. 10).

307 AsA and GSH homeostasis and their redox states (AsA/dehydroascorbate (DHA)
 308 and GSH/GSSG (oxidized glutathione)) were affected in the NO treatment. Total AsA
 309 (Fig. 10R) and DHA (Fig. 10T) concentrations increased 1 h after NO treatment and
 310 AsA concentration was not affected (Fig. 10S). The AsA redox state decreased after 1
 311 h of NO treatment and then recovered (Fig. 10U). Total GSH concentration did not
 312 change in the NO treatment (Fig. 10V), while the GSH concentration decreased (Fig.
 313 10W) and the GSSG concentration increased (Fig. 10X), which in turn caused a
 314 decrease in the GSH redox state that was restored after 3 h (Fig. 10Y). These changes
 315 were inhibited in the presence of cPTIO.

316 Furthermore, the transcript abundances of a gene homologous to glutathione
 317 peroxidase (GPX5, Cre10.g458450.t1.3) and σ -class glutathione-S-transferase genes
 318 (GSTS1, Cre16.g688550.t1.2; GSTS2, Cre01.g062900.t1.3), associated with oxidative
 319 stress acclimation and detoxification response in *Chlamydomonas* (Ledford et al.,

2007; Fischer et al., 2012), also increased under NO treatment. GPX1
(Cre02.g078300.t1.1), GPX (Cre08.g358525.t1.1), GPX3 (Cre03.g197750.t1.2), and
GPX4 (Cre10.g440850.t1.1) showed a decrease in transcript abundance in the NO
treatment (Supplemental Fig. S8).

NO regulates the expression of methionine sulfoxide reductase (MSR), which
functions in the reversibility of the oxidization of methionine to methionine and the
control of redox homeostasis through modulating the redox status of methionine in
proteins (Branlant, 2012; Rey and Tarrago 2018). Here, transcript abundances of
MSRA3 (Cre08.g380300.t1.1), MSRA5 (Cre10.g464850.t1.1), and MSRB2.2
(Cre14.g615100.t1.2) increased under NO treatment while those of MSRA2
(Cre06.g257650.t1.2), MSRA4 (Cre01.g012150.t1.2), and MSRB2.1
(Cre14.g615000.t1.1) showed a decrease. The transcript abundance of MSRA1
(Cre13.g570900.t1.1) was not affected by NO treatment (Supplemental Fig. S8). The
presence of cPTIO inhibited the changes of MSR gene expression.

336 DISCUSSION

337 NO is a cellular messenger that mediates diverse signaling pathways and plays a
338 role in many physiological processes in plants (Lamattina and Polacco, 2007;
339 Besson-Bard et al., 2008). Studying NO burst over a short-term period provided us a
340 chance to elucidate the metabolic shift to a brief NO attack and the following
341 acclimation processes in *Chlamydomonas* cells. We recently discovered that NO
342 interacts with reactive oxygen species (ROS) to induce cell death in association with
343 autophagy in *Chlamydomonas* cells under high intensity illumination (Kuo et al.,
344 2020a). Fortunately, ROS over-production and oxidative damage were not found in the
345 0.3 mM SNAP treatment. Thus, the interference of ROS in the short-term response to
346 NO can be excluded. Moreover, the cells collected from the early exponential phase
347 are not nutrient-starved. The role of NO as a factor responsible for SNAP-induced
348 changes was confirmed with the cPTIO treatment, which allowed for a better
349 understanding of the novel components of gene networks in *Chlamydomonas* cells
350 against sub-lethal NO stress using transcriptome and physiological analyses. After 1 h
351 of NO treatment, a decrease in the expression of genes associated with transcriptional
352 and translational activity reflected an inhibition of metabolism in *Chlamydomonas*
353 cells under NO stress. However, *Chlamydomonas* growth was not impacted by
354 sub-lethal NO stress. The present data suggest that the acclimation of *Chlamydomonas*
355 cells to NO burst can be accomplished by specific metabolic pathways.

356

357 Induction of the NO Scavenging System Allows for the Acclimation of 358 *Chlamydomonas* to NO Stress

359 A transient upregulation of truncated hemoglobin (THB1, Cre14.g615400.t1.2;
360 THB2, Cre14.g615350.t1.2), flavodiiron proteins (FLVB, Cre16.g691800.t1.1), and
361 cytochrome P450 (CYP55B1, Cre01.g007950.t1.1; Supplemental Table S6 and Fig. 12)

362 was found during the period of NO burst. In *Chlamydomonas*, THB1 with
 363 dioxygenase activity that converts NO into nitrate (Calatrava et al., 2017) is
 364 upregulated by NO (Sanz-Luque et al., 2015b). Sanz-Luque et al. (2015a) showed that
 365 treatment with 0.1 mM DEANONOate, a NO donor, increased THB1 transcript
 366 abundance in the NIT2 (the nitrate assimilation-specific regulatory gene) wild type
 367 and the *nit2* mutant, while THB2 transcript abundance decreased in the NIT2 wild
 368 type and showed a slight increase in the *nit2* mutant. *Chlamydomonas* is able to reduce
 369 NO to N₂O via FLVB under light conditions (Chaux et al., 2017) or CYP55B1 in the
 370 dark (Burlacot et al., 2019). Accordingly, the present data indicate that the NO
 371 scavenging system is induced by NO burst to prevent a toxic NO effect, thus allowing
 372 the implementation of acclimation processes post NO exposure.

373

374 **NO Alters Nitrogen/Sulfur Assimilation and Primary Amino Acid Biosynthesis**

375 The availability of nitrogen and sulfur are restricted by sudden NO challenge, as
 376 reflected by a transient drop in the expression of ammonium transporters and
 377 assimilation enzymes, whereas an increased SAC3 expression was observed, which is
 378 related to the inhibition of the expression of most ARSs. Moreover, the temporal
 379 upregulation of genes involved in the degradation of several amino acids, including
 380 sulfur-containing amino acids, under NO stress suggests that NO burst decreased the
 381 synthesis of proteins and other nitrogen-containing compounds that use these amino
 382 acids as building blocks. NO also causes a reversible inhibition of high-affinity
 383 nitrate/nitrite and ammonium transport and NR activity through post-translational
 384 regulation (Sanz-Luque et al., 2013). However, time-course changes in transcription
 385 levels reveal that the impedance in nitrogen/amino acid and sulfur utilization by NO
 386 burst is relieved after 3 h. The upregulation of sulfate transporters (SLT1, SLT2, and
 387 Sulp3) and GDH1 functioning in the incorporation of ammonium in *Chlamydomonas*

388 (Muñoz-Blanco and Cárdenas, 1989) serves a purpose for improving sulfur
389 availability and amino acid synthesis under NO stress. However, the process by which
390 nitrogen uptake is limited under NO stress is not clear.

391

392 **Mechanisms to Overcome NO-Induced Protein Stress**

393 A decrease in the expression of the genes encoding the oligosaccharyltransferase
394 complex, including RNP1 (oligosaccharyltransferase complex subunit delta
395 (ribophorin II)), GTR17 (Glycosyltransferase), GTR22, GTR25
396 (oligosaccharyltransferase STT3 subunit), STT3B
397 (dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit STT3B),
398 CANX (calnexin (CANX)), and CRT2 (Calreticulin 2, calcium-binding protein), by
399 NO treatment (Supplemental Table S6) reflects the dysfunction of proteins caused by a
400 blockage of glycosylation in the ER. However, the expression of DAD1, which
401 functions in N-linked glycosylation in the ER (Kelleher and Gilmore, 1994), increased
402 in the NO treatment. The NO-induced downregulation of TRAPPI and III proteins,
403 which are related to the transport of proteins to correct compartments and the
404 formation of autophagosomes (Garcia et al., 2019; Rosquete et al., 2019), also
405 suggests that there was transient transfer of misfolded and damage proteins during NO
406 treatment. Furthermore, the downregulation of SUMO E2 conjugase, UBC9, by NO
407 reflects the inhibition of SUMOylation for post-translational modification of the
408 proteins, which is crucial for the growth of *Chlamydomonas* cells (Knobbe et al.,
409 2015). Clearly, NO burst causes protein stress in *Chlamydomonas*.

410 To overcome disorders caused by potentially misfolded proteins, the membrane
411 trafficking system is induced for the degradation of damaged proteins in the lysosome
412 and/or vacuole via autophagy, which was reflected by a transient increase in the
413 expression of E2 and E3 ubiquitin ligases, ESCRT subunits (VSP), SNAREs, and

414 ATGs by NO treatment. The increase in the expression of SYNTAXIN-8B-RELATED
415 and VAMP7, which are endosomal syntaxins that mediate the steps of endosomal
416 protein trafficking (Prekeris et al., 1999), in *Chlamydomonas* cells suggests that there
417 was fusion between autophagosomes (late endosomes) and lysosomes under NO stress.
418 The TEM images show the formation and fusion of large vesicles at 1 h, possibly
419 acting to enclose the cytosolic components for degradation (Xie and Klionsky, 2007;
420 Nakatogawa et al., 2009); the large vesicles were not visible after 6 h (Supplemental
421 Fig. S9). Using autophagy as a lysosome-mediated pathway for the degradation of
422 cytosolic proteins and organelles (Choi et al., 2013), ATG7 is involved in the two
423 ubiquitin-like systems necessary for the selective cytoplasm-to-vacuole targeting (Cvt)
424 pathway and autophagy by activation of ATG12 and ATG8 and assigns them to E2
425 enzymes, ATG10 and ATG3, respectively. During this process, ATG8 conjugates PE to
426 form lipidated ATG8 (ATG8-PE), an autophagosome membrane component and
427 binding partner for autophagy receptors, which act to recruit cargo to lysosomes for
428 catabolism in ubiquitin-dependent and independent manners for selective autophagy.
429 Although ATG12 and VTC1 (vacuolar transport chaperone-like protein,
430 Cre12.g510250 t1.2) were downregulated by NO burst, an increase in the expression
431 of other ATG genes and abundances of ATG/ATG8-PE proteins in NO-treated
432 *Chlamydomonas* cells demonstrates the induction of autophagy by working with the
433 sorting protein factors involved in the membrane trafficking system for protein
434 degradation and recycling under NO stress. Because the inhibition of TOR activity can
435 trigger autophagy, as evidenced by the increased ATG8 and ATG8-PE proteins in
436 *Chlamydomonas* (Pérez-Pérez et al., 2010), the decreased expression of TOR1 due to
437 NO treatment indicates that TOR plays a role in autophagy induction. In addition to
438 membrane trafficking, protein chaperone systems (HSP22A, HSP22C, HSP22E,
439 HSP22F, and CLBP6) are induced in *Chlamydomonas* cells under NO stress. Thus, the

440 protein trafficking system (ubiquitination, SNARE, autophagy) and HSPs are evoked
441 as a way to remove aberrantly folded or damaged proteins, allowing *Chlamydomonas*
442 cells to maintain normal functions under NO stress.

443

444 **NO Inhibits Photosynthesis but Induces the Antioxidant Defense System for the** 445 **Prevention of Oxidative Stress upon Exposure to NO Burst**

446 A decrease in the expression of genes encoding proteins related to photosynthesis
447 and photosynthetic activity in *Chlamydomonas* by NO illustrates the NO-mediated
448 downregulation of PSII activity and the evolutionary rate of photosynthetic O₂ at the
449 transcriptional level. Because of the suppression of PSII activity and the
450 photosynthetic O₂ evolution rate by the NO treatment at the protein level in higher
451 plants (Takahashi and Yamasaki, 2002; Singh et al., 2007; Wodala et al., 2008; Ördög
452 et al., 2013), the effects of NO on photosynthesis by protein modification cannot be
453 ignored. NO is also a factor leading to the degradation of the cytochrome *b₆f* complex
454 and Rubisco through FtsH and Clp proteases in sulfur (de Mia et al., 2019) or nitrogen
455 (Wei et al., 2014) starved *Chlamydomonas* cells. However, it is not clear whether these
456 two protein complexes are degraded under NO burst in nutrient sufficient conditions.
457 We did find that the transcript abundances of the FtsH-like proteases FHL4
458 (Cre13.g568400.t1.2) and FHL6 (Cre03.g201100.t1.2), and DEG11
459 (Cre12.g498500.t1.2) were increased by NO burst, whereas the transcript abundances
460 of the ClpP proteases ClpP4 (Cre12.g500950.t1.2) and ClpP5 (Cre12.g486100.t1.2),
461 and the non-catalytic subunit of the ClpP complex, ClpR2 (Cre16.g682900.t1.2)
462 decreased (Supplemental Table S6). However, the role of FtsH-like and DEG proteases
463 responsible for chloroplast protein degradation by NO burst need to be identified.
464 Because the inhibition of photosynthesis is considered the mechanism for algae to
465 acclimate to nitrogen or sulfur limitation to avoid photo-damage (Peltier and Schmidt,

1991; Grossman, 2000; Saloman et al., 2012), our findings suggest that the transient inhibition of photosynthesis can prevent *Chlamydomonas* cells from over-producing ROS, which may occur during NO burst. ROS scavenging ability was enhanced with the increased concentration of AsA due to the upregulation of VTC2 and its regeneration rate, owing to the enhanced DHAR expression (transcript abundance, protein, enzyme activity). In addition, the enhanced GSH regeneration rate, supported by the increase in the GR activity and GSHR1 expression as well as increased GSTS1, GSTS2, and GPX5 expression, prevented the over-production of ROS. Tocopherols (Havaur et al., 2005; Krieger-Liszkay and Trebst, 2006) and carotenoids (Ramel et al., 2012) are also $^1\text{O}_2$ quenchers, but their concentrations and the transcript abundances of their biosynthetic enzymes decreased in the NO treatment (Supplemental Fig. S10). Instead, an increase in the concentration of AsA, an $^1\text{O}_2$ quencher (Kramarenko et al., 2006), was responsible for $^1\text{O}_2$ scavenging under NO stress. NO also triggers MSR expression. Although the redox of AsA and GSH as well as the thiol-based redox regulation proteins, TRX2 (thioredoxin-like protein, Cre03.g157800 t1.1), PRX1 (2-cys peroxiredoxin, chloroplastic, Cre06.g257601 t1.2), and PRX2 (2-cys peroxiredoxin, Cre02.g114600 t1.2; Supplemental Table S6), were initially decreased by NO burst, they recovered 3 h after treatment (the restoration of TRX2, PRX1, and PRX2 transcript abundance is not shown). Thus, the antioxidant defense system and MSR worked together to prevent oxidative stress and the abrupt loss in cellular redox homeostasis occurring under NO stress.

Burst Oxidase-Like 2-Dependent Regulation of the Expression of Selective NO-Inducible Genes for Coping with NO Stress

NADPH oxidase acts as an important molecular hub during ROS-mediated signaling in plants (Marino et al., 2011) and in the interplay of ROS and NO signaling

492 pathways for the regulation of plant metabolism (Suzuki et al., 2011). A previous study
493 has shown that NADPH oxidase is regulated by transcriptional and enzyme activity
494 levels in higher plants (Hu et al., 2020). The NO-mediated suppression of NADPH
495 oxidase activity is due to post-translational modification known as S-nitrosylation
496 (Wang et al., 2013). The analysis of cis-regulatory elements in the gene promoter
497 region of NADPH oxidase showed diverse expression patterns under different
498 conditions (Wang et al., 2013; Kaur and Pati, 2016). In this study, we did not examine
499 whether the S-nitrosylation of NADPH oxidase occurs in *Chlamydomonas* upon
500 exposure to NO; however, our present data did detect the constant expression of the
501 gene encoding respiratory burst oxidase-like 1 (RBOL1, Cre03.g188300.t1.1), an
502 NADPH oxidase gene (Fig. 12A). In addition, we observed the significant
503 upregulation of RBOL2 (Cre03.g188400.t1.1) (Fig. 12B) in *Chlamydomonas* after 1 h
504 of NO exposure, followed by the subsequent restoration to a baseline level after 3 h.
505 The activity of NADPH oxidase also increased after exposure to NO (Fig. 12C).
506 Therefore, it is interesting for us to examine whether NADPH oxidase mediates the
507 NO-induced changes in the expression of genes in *Chlamydomonas* using the *rbol2*
508 mutant (Fig. 13A,B). Compared to the wild type, the *rbol2* mutant had a 66% decrease
509 in NADPH oxidase activity with a low RBOL2 transcript abundance (Fig. 13C),
510 indicating a differential regulation pattern on the expression of the genes upon
511 exposure to NO (0.3 mM SNAP). In response to NO treatment (0.3 mM SNAP), the
512 transcript abundance of RBOL1 (Fig. 13Z) was not affected in the *rbol2* mutant and
513 the induction of RBOL2 expression in wild type (CC-5325) was not observed in the
514 *rbol2* mutant (Fig. 13AB). In addition, the NO-induced increase in the expression of
515 THB2 (Fig. 13D), CDO1 (Fig. 13E), CDO2 (Fig. 13F), HSP22A (Fig. 13G), HSP22C
516 (Fig. 13H), ATG4 (Fig. 13J), ATG8 (Fig. 13K), LHCBM9 (Fig. 13L), GUN4 (Fig.
517 13M), APX1 (Fig. 13P), GSHR1 (Fig. 13R), GSTS1 (Fig. 13T), GSTS2 (Fig. 13U),

518 DHAR1 (Fig. 13W), and PDS1 (Fig. 13Y) in the wild type was not observed in the
519 *rbol2* mutant. In contrast to the NO-mediated inhibition of TOR1 (Fig. 13I) and CYC4
520 (Fig. 13N) expression in the wild type, the expression of TOR1 and CYC4 showed a
521 significant increase in the *rbol2* mutant after NO treatment. The expression pattern of
522 RBCS1 (Fig. 13P), VTC2 (Fig. 13R), GSH1 (Fig. 13S), MDAR1 (Fig. 13V), and
523 MDAR5 (Fig. 13X) in the *rbol2* mutant in the NO treatment was similar to that in the
524 wild type. Compared to the wild type, the NADPH oxidase activity in the *rbol2* mutant
525 did not increase after SNAP treatment (Fig. 14A). The *rbol2* mutant showed a higher
526 sensitivity to NO stress than the wild type, as reflected by a decrease in *rbol2* mutant
527 viability (Fig. 14B) accompanied with bleaching (Fig. 14C) as the SNAP
528 concentration increased ≥ 0.3 mM. Overall, NADPH oxidase (RBOL2) is associated
529 with the expression of the genes encoding proteins for NO scavenging, antioxidant
530 defense system, autophagy, and heat shock proteins that help to resist NO stress in
531 *Chlamydomonas*.

532

533 CONCLUSIONS

534 The acclimation of *Chlamydomonas* to sub-lethal NO stress comprises a temporally
535 orchestrated implementation of metabolic processes including 1. a decrease in the NO
536 amount due to scavenging elements, 2. acclimation to nitrogen and sulfur starvation, 3.
537 upregulation of the protein trafficking system (ubiquitin, SNARE, and autophagy) and
538 molecular chaperone system for dynamic regulation of protein homeostasis and
539 function, and 4. transient inhibition of photosynthesis together with the induction of
540 the antioxidant defense system and modification of the redox state to prevent oxidative
541 stress. NADPH oxidase (RBOL2)-dependent molecular events underlie part of the
542 acclimation mechanisms in *Chlamydomonas* related to coping with NO stress. The
543 direct targets of NO and the mechanisms for the transcriptional regulation of RBOL2

544 in *Chlamydomonas* are needed to be clarified in the future.

545

546 MATERIALS AND METHODS

547 Algal Culture and SNAP Treatments

548 The green alga *Chlamydomonas reinhardtii*, strain CC-125 (mt-), was obtained from
 549 the Chlamydomonas Resource Center (USA) and photoheterotrophically cultured in
 550 Tris-acetate phosphate medium (TAP) (Harris, 1989) with a trace element solution in
 551 125 mL flasks (PYREX, Germany) and agitated on an orbital shaking incubator
 552 (model OS701, TKS company, Taipei, Taiwan) (150 rpm) under continuous
 553 illumination with white light ($50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 25°C. For chemical treatments, 50
 554 mL cultures were grown to a cell density of $3\text{--}5 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$, and after
 555 centrifugation at $1,600 \times g$ for 3 min, the supernatant was discarded. The pellet was
 556 suspended in fresh TAP medium and centrifuged again. Then, the pellet was
 557 re-suspended in fresh TAP medium to a cell density of $3 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$. Ten milliliters
 558 of culture was transferred to a 100-mL beaker (internal diameter: 3.5 cm) for
 559 pre-incubation at 25°C in $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ conditions for 1.5 h in an orbital shaker
 560 (model OS701, TKS company, Taipei, Taiwan) at a speed of 150 rpm. Then, the algal
 561 cells were subjected to treatments at 25°C. SNAP was treated in different
 562 concentrations from 0.1, 0.3, to 1.0 mM in the presence or absence of 0.4 mM cPTIO.
 563 DMSO was used as the control because SNAP was dissolved in DMSO. Each
 564 treatment included three biological independent replicates ($n=3$). For the determination
 565 of cell growth and the estimation of several physiological and biochemical parameters,
 566 the number of cells in a 1-mL sample was counted using a hemocytometer. Samples
 567 taken before (0 min) and after treatment were centrifuged at $5,000 \times g$ for 5 min, and
 568 the pellet was fixed in liquid nitrogen and stored in a -70°C freezer until analysis.

569

570 **Detection of NO Production**

571 An NO-sensitive fluorescent dye, DAF-FM diacetate (Invitrogen Life Technologies,
572 Carlsbad, CA, USA) (Kojima et al., 1998), was used to measure NO production
573 following our previous studies. DAF-FM diacetate is a pH-insensitive fluorescent dye
574 that emits fluorescence after reaction with an active intermediate of NO (Kojima et al.,
575 1998; Chang et al., 2013). The cells were pre-incubated in TAP medium containing 5
576 μM DAF-FM diacetate for 60 min at 25°C under $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ conditions, then
577 washed twice with fresh TAP medium, and transferred to $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for chemical
578 treatment. The fluorescence was detected via fluorescence microscopy and
579 spectrophotometry.

580

581 **Determination of Chlorophyll *a* Fluorescence**

582 Chlorophyll *a* fluorescence parameters were employed to determine the activity of
583 PSII using an AP-C 100 (AquaPen, Photon Systems Instruments, Brno, Czech
584 Republic). A 0.5-mL aliquot of an algal culture was diluted with TAP medium to an
585 $\text{OD}_{750} = 0.10\text{--}0.15$ with a chlorophyll *a* content of $1.5\text{--}2.2 \mu\text{g}\cdot\text{mL}^{-1}$. A 2-mL aliquot of
586 diluted algal cells was then transferred to an AquaPen cuvette and subjected to a pulse
587 of saturating light of $4,000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR to obtain the light-adapted minimal
588 fluorescence (F_t) and the light-adapted maximal fluorescence (F_m'). To determine the
589 maximum PSII activity, $F_v/F_m (=F_m - F_o/F_m)$, 2 mL of diluted algal cells in an
590 AquaPen cuvette was incubated in the dark for 20 min and then flushed with saturated
591 light ($4,000 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) to obtain the dark-adapted minimal fluorescence (F_o)
592 and dark-adapted maximal fluorescence (F_m). The active PSII activity, $F_v'/F_m' = F_m' -$
593 F_t/F_m' , and the maximum PSII activity, $F_v/F_m = F_m - F_o/F_m$, were then calculated.

594 The rapid induction of chlorophyll *a* fluorescence was also determined via the OJIP
595 test (Strasser and Strasser, 1995; Strasser et al., 2000). The inflections in the O-J-I-P
596 curve represent the heterogeneity of the process during photochemical action; peak J
597 represents the momentary maximum level of Q_A^- , $Q_A^-Q_B$, and $Q_A^-Q_B^-$; peak I
598 represents the level of $Q_A^-Q_B^{2-}$; and peak P represents the maximum level of Q_A^- , Q_B^{2-} ,
599 and PQH_2 (Stirbet et al., 1998). The fluorescence values at time intervals
600 corresponding to the O-J-I-P peaks were recorded as follows: F_o = fluorescence
601 intensity at 50 μ s; F_j = fluorescence intensity at peak J (at 2 ms); F_i = fluorescence
602 intensity at peak I (at 60 ms); F_m = maximal fluorescence intensity at the peak P; F_v =
603 $F_m - F_o$ (maximal variable fluorescence); $V_j = (F_j - F_o)/(F_m - F_o)$; $V_i = (F_i - F_o)/(F_m -$
604 $F_o)$; and F_m/F_o ; F_v/F_o ; F_j/F_m .

605

606 **Enzyme Assay and Determination of AsA and GSH**

607 SOD activity was assayed according to (Yashida et al., 2003). GR and APX activity
608 was determined according to the methods described by Lin et al. (2018) and Kuo et al.
609 (2020b), respectively. Protein concentrations were quantified using the Coomassie
610 Blue dye binding method (Bradford, 1976) using a concentrated dye purchased from
611 BioRad (500-0006, Hercules, CA, USA). AsA and DHA concentrations were
612 determined by an ascorbate oxidase based method according to Lin et al. (2016). GSH
613 and oxidized glutathione (GSSG) were extracted from the frozen algal cell pellet
614 (obtained from 5 mL samples) using 5% (w/v) trichloroacetic acid (TCA) and
615 determined at 412 nm according to Lin et al. (2018).

616

617 **Determination of NADPH oxidase activity**

618 NADPH oxidase activity was determined according to Kaundal et al.'s (2012)

method based on O_2^- production. After homogenization, centrifugation at 10,000 $\times g$, and then ultra-centrifugation at 203,000 $\times g$ for collection of total membranes, the pellet was suspended in 1 mL ice-cold 10 mM Tris-HCl (pH 7.4). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 1 mM XTT (3-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate), 1 mM NADPH, and 5 μg membrane protein. The reduction rate of XTT by O_2^- detected at 492 nm was used to calculate enzyme activity; the extinction coefficient used was $2.16 \times 10^4 M^{-1} cm^{-1}$.

RNA Isolation, cDNA Synthesis, Transcriptomic Analysis, and mRNA Quantification via Real-Time Quantitative PCR

Total RNA was extracted using the TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. The methods for cDNA library preparation, Illumina sequencing, and sequence analysis are described in Supplemental Fig. S6. For qPCR assay, the total RNA concentration was adjusted to 2.95 μg total RNA $\cdot \mu L^{-1}$ and treated with DNase (TURBO DNA-freeTM Kit, Ambion Inc., The RNA Company, USA) to remove residual DNA. Then 1.5 μg of total RNA was used for the preparation of cDNA. cDNA was amplified from the poly-(A⁺) tail using Oligo (dT)12-18 with the VersoTM cDNA Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the volume was adjusted to a concentration of 30 ng $\cdot mL^{-1}$ based on original RNA quantity in each sample. The primers for the targeted genes are listed in Supplemental Table S11. The real-time quantitative PCR was performed using a LightCycler 480 system (Roche Applied Science, Mannheim, Germany). A PCR master mix was prepared with the LightCycler 480 SYBR Green I Master Kit (Roche Applied Science, Mannheim, Germany). Each reaction was

performed in a total volume of 10 μ L, containing 1 $\times$ LightCycler 480 SYBR Green I Master Mix, the selected concentration of each primer, and cDNA corresponding to 30 or 50 ng $\cdot$ μ L⁻¹ RNA in the reverse transcriptase reaction. The amplification program consisted of an initial denaturation at 95°C for 5 min, followed by 50 amplification cycles of annealing at 60°C for 10 s, elongation at 72°C for 5 s, real-time fluorescence measurements, and finally, denaturation at 95°C for 15 s. The 2^{- $\Delta\Delta$ CT} method was used to calculate the relative change in mRNA level normalized to a reference gene (UBC, NCBI: AY062935) and the fold increase was calculated relative to the control RNA sample at 0 min. Because the results based on the EF-1 α internal control were similar to those based on UBC, the relative changes in the levels of gene transcription were expressed based on UBC.

655

656 **Western Blots**

Soluble protein was extracted according to Kuo et al. (2020a). For each sample, 30 μ g of protein was loaded into each lane, resolved on a 15% SDS-PAGE gel, and transferred to a polyvinylidene fluoride membrane for antibody binding with rabbit polyclonal anti-ATG8 antibody (ab77003; Abcam, Cambridge, UK), anti-CrDHAR (LKT BioLaboratories Ltd., Taoyuan, Taiwan), or a mouse monoclonal antibody against α -tubulin (ab11304; Abcam, Cambridge, UK). Following the incubation with horseradish peroxidase-conjugated secondary antibodies (MD20878; KPL, Gaithersburg, MD, USA), the immunoblots were visualized and the relative abundance of ATG8, ATG8-PE, or CrDHAR1 protein was estimated based on α -tubulin intensity.

666

667 **Statistics**

Three independent biological replicates were performed and all experiments were repeated at least three times. Because the replications showed similar trends, only

the results from one replicate were shown in this paper. Statistical analyses were performed using SPSS (SPSS 15.0, Chicago, IL, USA). Significant differences between means were analyzed using Student's *t*-test or Scheffe's test following significant analysis of variance for the controls and treatments at $P < 0.05$.

Accession Numbers

The transcriptome sequences can be accessed from the Sequence Read Archive (SRA) website using the BioProject accession number: PRJNA629395 (<https://www.ncbi.nlm.nih.gov/books/NBK158898/>) and the BioSample accessions SAMN14775313, SAMN14775314, SAMN14775315, SAMN14775316, SAMN14775317, SAMN14775318, SAMN14775319, SAMN14775320, SAMN14775321, and SAMN14775322.

Supplemental Data

The following supplemental materials are available:
Supplemental Tables S1–S11.
Supplemental Figures S1–S10.

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Figure Legends

Figure 1. Microscopic observation (A) and spectrophotometric determination (B) of nitric oxide fluorescence (DAF-FM), cell viability (C), cell growth (D), and

696 appearance (E) in *Chlamydomonas reinhardtii* in response to 0.1, 0.3, or 1.0 mM
697 SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$
698 SD (n = 3). Different symbols indicate significant differences between treatments
699 (Scheffe's test, $P < 0.05$).

700

701 **Figure 2.** The active (F_v'/F_m') (A) and maximum (F_v/F_m) (B) PSII activity,
702 photosynthetic O₂ evolution rate (C), and respiration rate (D) in *C. reinhardtii* after
703 exposure to 0.1, 0.3, or 1.0 mM SNAP in the presence or absence of 0.4 mM cPTIO.
704 Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant
705 differences between treatments (Scheffe's test, $P < 0.05$).

706

707 **Figure 3.** Time-course changes in the transcript abundances of PSBP4 (A), LHCBM9
708 (B), Lhl1 (C), Lhl2 (D), Lhl3 (E), Lhl4 (F), PCY1 (G), CYC4 (H), CHLH (I), CHLI1
709 (J), CHLI2 (K), CHLD (L), and GUN4 (M) in *C. reinhardtii* upon exposure to 0.3 mM
710 SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$
711 SD (n = 3). Different symbols indicate significant differences between treatments
712 (Scheffe's test, $P < 0.05$).

713

714 **Figure 4.** Time-course changes in the transcript abundances of RBCS1 (A), RPE2 (B),
715 PGK1 (C), GAP4 (D), TPI1 (E), SBP2 (F), PPR2 (G), RCA2 (H), CP12 (I), RMT1 (J),
716 RMT2 (K), and PGP3 (L) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the
717 presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3).
718 Different symbols indicate significant differences between treatments (Scheffe's test, P
719 < 0.05).

720

721 **Figure 5.** Time-course changes in the transcript abundances of AMT3 (A), AMT6 (B),

722 AMT7 (C), GSN1 (D) GLN1 (E), GDH1 (F), CSD4 (G), CDO1 (H), CDO2 (I),
723 TAUD1 (J), AST5 (K), ATA2 (L), BCKDHA (N), and BCKDHB (O) in *C. reinhardtii*
724 in response to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are
725 expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences
726 between treatments (Scheffe's test, $P < 0.05$).

727

728 **Figure 6.** Time-course changes in the transcript abundances of SAC3 (A), SUOX (B),
729 ARS3 (C), ARS7 (E), ARS5 (D), ARS11 (G), ARS13 (H), ARS14 (I), ARS16 (J),
730 SLT1 (K), SLT2 (L), and SULP3 (M) in *C. reinhardtii* after exposure to 0.3 mM SNAP
731 in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n
732 = 3). Different symbols indicate significant differences between treatments (Scheffe's
733 test, $P < 0.05$).

734

735 **Figure 7.** Time-course changes in the transcript abundances of AAA+-type ATPase
736 (A), ubiquitin-protein ligase E2 (B), UBC9 (C), UBC3 (D), UBE3A (E), probable E3
737 ubiquitin-protein ligase HERC1 (F), ubiquitin fusion degradation protein (G), VPS23
738 (H), VPS28 (I), VPS37 (J), VPS2A (K), RAB5 (L), SEC20 (M),
739 SYNTAXIN-8B-RELATED (N), and VAMP (O) in *C. reinhardtii* after exposure to 0.3
740 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the
741 mean $\pm$ SD (n = 3). Different symbols indicate significant differences between
742 treatments (Scheffe's test, $P < 0.05$).

743

744 **Figure 8.** Time-course changes in the transcript abundances of TRS20 (A), TRS23 (B),
745 TRS31 (C), TRS33 (D), TRS85 (E), BET3 (F), and BET5 (G) in *C. reinhardtii* after
746 exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are
747 expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences

748 between treatments (Scheffe's test, $P < 0.05$).

749

750 **Figure 9.** Time-course changes in the transcript abundances of autophagy-related
751 genes and heat-shock proteins and the protein abundances of ATG8 in *C. reinhardtii*
752 upon exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. A.
753 TOR1; B. ATG3; C. ATG4; D. ATG5; E. ATG6; F. ATG7; G. ATG8; H. ATG9; I.
754 ATG12; J. ATG8 (square symbol) and ATG8-PE (circle symbol) protein abundances;
755 K. HSP70D; L. CLBP3; M. HSP22A; N. HSP22C; O. HSP22E; P. HSP22F. Data are
756 expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences
757 between treatments (Scheffe's test, $P < 0.05$).

758

759 **Figure 10.** Time-course changes in the transcript abundances of FSD1 (A), MSD1 (B),
760 MSD2 (C), MSD3 (D), MSD4 (E), MSD5 (F), APX1 (G), DHAR1 (H), GSHR1 (I),
761 GSH1 (J), and VTC2 (K); the activity of APX (L), DHAR (M), MDAR (N), and GR
762 (O); the concentrations of total AsA (P), AsA (Q), and DHA (R); the ratio of
763 AsA/DHA (S); the concentrations of total GSH (T), GSH (U), and GSSG (V); and the
764 ratio of GSH/GSSG (W) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the
765 presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3).
766 Different symbols indicate significant differences between treatments (Scheffe's test, P
767 < 0.05).

768

769 **Figure 11.** Time-course changes in the transcript abundances of THB1 (A), THB2 (B),
770 FLVB (C), and CYP55B1 (D) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the
771 presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3).
772 Different symbols indicate significant differences between treatments (Scheffde's test,
773 $P < 0.05$).

774

775 **Figure 12.** Time-course changes in the transcript abundances of RBOL1 (A) and
776 RBOL2 (B) and the activity of NADPH oxidase (C) in *C. reinhardtii* after exposure to
777 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the
778 mean $\pm$ SD (n = 3). Different symbols indicate significant differences between
779 treatments (Scheffe's test, $P < 0.05$).

780

781 **Figure 13.** Isolation of the *Chlamydomonas* rbol2 mutant and its transcript abundances
782 of several genes in response to 0.3 mM SNAP in the presence or absence of 0.4 mM
783 cPTIO. (A) Schematic representation of insertion sites of the APHVIII cassettes in the
784 genomic sequence of RBOL2. Tall boxes denote exons, gray boxes indicate protein
785 coding regions and filled boxes show 5' and 3' untranslated regions (UTRs) and the
786 promoter region. Arrows indicate primer locations used to detect APHVIII cassette
787 insertions. (B) Genotyping of the rbol2 mutant. Genomic DNA fragments were
788 amplified by PCR using the primer sets indicated in (A). (C) The rbol2 mutant showed
789 low RBOL2 transcript abundance and NADPH oxidase activity but a normal RBOL1
790 expression compared to the CC-5325 wild type. The data are expressed as the mean $\pm$
791 SD (n = 3) from three independent biological replicates, and * indicates a significant
792 difference between CC-5325 and the rbol2 mutant using *t*-test ($P < 0.05$). The
793 transcript abundance of THB2 (D), CDO1 (E), CDO2 (F), HSP22A (G), HSP22C (H),
794 TOR1 (I), ATG4 (J), ATG8 (K), LHCBM9 (L), GUN4 (M), CYC4 (N), RBCS1 (O),
795 APX1 (P), VTC2 (Q), GSHR1 (R), GSH1 (S), GSTS1 (T), GSTS2 (U), MDAR1 (V),
796 DHAR1 (W), MSAR5 (X), PDS1 (Y), RBOL1 (Z), and RBOL2 (AB). The data are
797 expressed as the mean $\pm$ SD (n = 3), in which different symbols indicate significant
798 differences between treatments (Scheffe's test, $P < 0.05$).

799

800 **Figure 14.** The effects of SNAP treatment on NADPH oxidase activity (A), the
 801 viability of *Chlamydomonas* after 4 days of culture on an agar plate including 6 h
 802 SNAP treatment (B) and the appearance of cells 6 h after SNAP treatment. SNAP was
 803 applied at 0, 0.1, 0.3, or 1.0 mM concentrations. Data are expressed as the mean $\pm$ SD
 804 ($n = 3$) for NADPH oxidase activity. Different symbols indicate significant differences
 805 between treatments (Scheffe's test, $P < 0.05$).
 806
 807

1 **Acclimation of *Chlamydomonas reinhardtii* to Nitric Oxide Stress Related to**

2 **Respiratory Burst Oxidase-Like 2**

3 Eva YuHua Kuo and Tse-Min Lee

4

A. DAF-FM fluorescence observed by fluorescence microscope (1 h)

B. Spectrometric determination of NO level

C. Viability

D. Cell density

E. Appearance (24 h)

Figure 1. Microscopic observation (A) and spectrophotometric determination (B) of nitric oxide fluorescence (DAF-FM), cell viability (C), cell growth (D), and appearance (E) in *Chlamydomonas reinhardtii* in response to 0.1, 0.3, or 1.0 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

1
2 **Figure 2.** The active (F_v'/F_m') (A) and maximum (F_v/F_m) (B) PSII activity,
3 photosynthetic O₂ evolution rate (C), and respiration rate (D) in *C. reinhardtii* after
4 exposure to 0.1, 0.3, or 1.0 mM SNAP in the presence or absence of 0.4 mM cPTIO.
5 Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant
6 differences between treatments (Scheffe's test, $P < 0.05$).

Figure 3. Time-course changes in the transcript abundances of PSBP4 (A), LHCBM9 (B), Lhl1 (C), Lhl2 (D), Lhl3 (E), Lhl4 (F), PCY1 (G), CYC4 (H), CHLH (I), CHLI1 (J), CHLI2 (K), CHLD (L), and GUN4 (M) in *C. reinhardtii* upon exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD ($n = 3$). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

Figure 4. Time-course changes in the transcript abundances of RBCS1 (A), RPE2 (B), PGK1 (C), GAP4 (D), TPI1 (E), SBP2 (F), PPR2 (G), RCA2 (H), CP12 (I), RMT1 (J), RMT2 (K), and PGP3 (L) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD ($n = 3$). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

Figure 5. Time-course changes in the transcript abundances of AMT3 (A), AMT6 (B), AMT7 (C), GSN1 (D), GLN1 (E), GDN1 (F), CSD4 (G), CDO1 (H), CDO2 (I), TAUD1 (J), AST5 (K), ATA2 (L), BCKDHA (N), and BCKDHB (O) in *C. reinhardtii* in response to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

Figure 6. Time-course changes in the transcript abundances of SAC3 (A), SUOX (B), ARS3 (C), ARS7 (E), ARS5 (D), ARS11 (G), ARS13 (H), ARS14 (I), ARS16 (J), SLT1 (K), SLT2 (L), and Sulp3 (M) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

Figure 7. Time-course changes in the transcript abundances of AAA+-type ATPase (A), ubiquitin-protein ligase E2 (B), UBC9 (C), UBC3 (D), UBE3A (E), probable E3 ubiquitin-protein ligase HERC1 (F), ubiquitin fusion degradation protein (G), VPS23 (H), VPS28 (I), VPS37 (J), VPS2A (K), RAB5 (L), SEC20 (M), SYNTAXIN-8B-RELATED (N), and VAMP (O) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD ($n = 3$). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

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Figure 9. Time-course changes in the transcript abundances of autophagy-related genes and heat-shock proteins and the protein abundances of ATG8 in *C. reinhardtii* upon exposure to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. A. TOR1; B. ATG3; C. ATG4; D. ATG5; E. ATG6; F. ATG7; G. ATG8; H. ATG9; I. ATG12; J. ATG8 (square symbol) and ATG8-PE (circle symbol) protein abundances; K. HSP70D; L. CLBP3; M. HSP22A; N. HSP22C; O. HSP22E; P. HSP22F. Data are expressed as the mean $\pm$ SD (n = 3). Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

1

2

3 **Figure 10.** Time-course changes in the transcript abundances of FSD1 (A), MSD1 (B),
4 MSD2 (C), MSD3 (D), MSD4 (E), MSD5 (F), APX1 (G), DHAR1 (H), GSHR1 (I),
5 GSH1 (J), and VTC2 (K); the activity of APX (L), DHAR (M), MDAR (N), and GR
6 (O); the concentrations of total AsA (P), AsA (Q), and DHA (R); the ratio of
7 AsA/DHA (S); the concentrations of total GSH (T), GSH (U), and GSSG (V); and the
8 ratio of GSH/GSSG (W) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the
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10 Different symbols indicate significant differences between treatments (Scheffe's test,
11 $P < 0.05$).

1
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3 FLVB (C), and CYP55B1 (D) in *C. reinhardtii* after exposure to 0.3 mM SNAP in the
4 presence or absence of 0.4 mM cPTIO. Data are expressed as the mean $\pm$ SD (n = 3).
5 Different symbols indicate significant differences between treatments (Scheffde's test,
6 $P < 0.05$).

1
2 **Figure 12.** Time-course changes in the transcript abundances of RBOL1 (A) and
3 RBOL2 (B) and the activity of NADPH oxidase (C) in *C. reinhardtii* after exposure to
4 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. Data are expressed as
5 the mean $\pm$ SD ($n = 3$). Different symbols indicate significant differences between
6 treatments (Scheffe's test, $P < 0.05$).

(V) Schematic representation of insertion sites of the APHVIII cassettes in the genomic sequence of RBOL2.

(W) Genotyping of the *rbol2* mutant (c) RBOL gene expression and enzyme activity

Figure 13. Isolation of the *Chlamydomonas rbol2* mutant and its transcript abundances of several genes in response to 0.3 mM SNAP in the presence or absence of 0.4 mM cPTIO. (A) Schematic representation of insertion sites of the APHVIII cassettes in the genomic sequence of RBOL2. Tall boxes denote exons, gray boxes indicate protein coding regions and filled boxes show 5' and 3' untranslated regions

1 (UTRs) and the promoter region. Arrows indicate primer locations used to detect
2 APHVIII cassette insertions. (B) Genotyping of the *rbol2* mutant. Genomic DNA
3 fragments were amplified by PCR using the primer sets indicated in (A). (C) The
4 *rbol2* mutant showed low RBOL2 transcript abundance and NADPH oxidase activity
5 but a normal RBOL1 expression compared to the CC-5325 wild type. The data are
6 expressed as the mean $\pm$ SD ($n = 3$) from three independent biological replicates, and
7 * indicates a significant difference between CC-5325 and the *rbol2* mutant using *t*-test
8 ($P < 0.05$). The transcript abundance of THB2 (D), CDO1 (E), CDO2 (F), HSP22A
9 (G), HSP22C (H), TOR1 (I), ATG4 (J), ATG8 (K), LHCBM9 (L), GUN4 (M), CYC4
10 (N), RBCS1 (O), APX1 (P), VTC2 (Q), GSHR1 (R), GSH1 (S), GSTS1 (T), GSTS2
11 (U), MDAR1 (V), DHAR1 (W), MSAR5 (X), PDS1 (Y), RBOL1 (Z), and RBOL2
12 (AB). The data are expressed as the mean $\pm$ SD ($n = 3$), in which different symbols
13 indicate significant differences between treatments (Scheffé's test, $P < 0.05$).

A. NADPH oxidase activity 6 h after SNAP treatment

B. Viability (6 h after SNAP treatment and then plating on agar plate for 4 days of growth)

C. Appearance after 6 h of SNAP treatment

Figure 14. The effects of SNAP treatment on NADPH oxidase activity (A), the viability of *Chlamydomonas* after 4 days of culture on an agar plate including 6 h SNAP treatment (B) and the appearance of cells 6 h after SNAP treatment. SNAP was applied at 0, 0.1, 0.3, or 1.0 mM concentrations. Data are expressed as the mean $\pm$ SD (n = 3) for NADPH oxidase activity. Different symbols indicate significant differences between treatments (Scheffe's test, $P < 0.05$).

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