

1 **DYRKP kinase regulates cell wall degradation in Chlamydomonas by inducing matrix**
2 **metalloproteinase expression**

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17 **Short title:** DYRKP regulates algal cell wall degradation

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19 **One sentence summary:** The DYRKP kinase induces the expression of matrix
20 metalloproteinases involved in the degradation of the parental cell wall, allowing prompt
21 hatching of daughter cells after cell division.

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32 dual-specificity tyrosine phosphorylation-regulated kinase (DYRKP); Cell division

33

34 **Abstract**

35 The cell wall of plants and algae is an important cell structure that protects cells from changes
36 in the external physical and chemical environment. This extracellular matrix composed of
37 polysaccharides and glycoproteins, is needed to be remodeled continuously throughout the
38 life cycle. However, compared to matrix polysaccharides, little is known about the
39 mechanisms regulating the formation and degradation of matrix glycoproteins. We report
40 here that a plant kinase belonging to the dual-specificity tyrosine phosphorylation-regulated
41 kinase (DYRK) family present in all eukaryotes regulates cell wall degradation in the model
42 microalga *Chlamydomonas reinhardtii* by inducing the expression of matrix
43 metalloproteinases (MMPs). In the absence of DYRK, daughter cells fail to degrade the
44 parental cell wall, and form multicellular structures. On the other hand, the complementation
45 line of DYRK was shown to degrade the parental cell wall normally. Transcriptomic and
46 proteomic analyses indicate a marked down-regulation of MMP expression in the *dyrk*
47 mutants. Additionally, the expression of MMP was confirmed to be consistent with the
48 expression pattern of DYRK. Our findings show that DYRK, by ensuring timely MMP
49 expression, enables the successful execution of the cell cycle. Altogether, this study provides
50 new insight into the life cycle regulation in plants and algae.

51

52 IN A NUTSHELL

53 **Background:** Plants and algae have different types of polysaccharides in their cell walls, but they
54 have glycoproteins in common. Glycoprotein synthesis and degradation must be tightly regulated to
55 ensure normal growth and differentiation. However, little is known about the regulatory mechanism of
56 glycoprotein degradation in both plants and algae. The cell cycle of *Chlamydomonas reinhardtii*
57 begins anew with the hatching of daughter cells, and the role of matrix metalloproteinases (MMPs) is
58 known to be important in this process. In our previous study, we observed that a knockout mutant of
59 the plant kinase belonging to the dual-specificity tyrosine phosphorylation-regulated kinase (DYRKP)
60 formed a palmelloid structure and failed to hatch.

61

62 **Questions:** What is the role of DYRKP in microalgae? Specifically, why does the *dyrkp* mutant form
63 a palmelloid structure? Palmelloid is usually observed in dividing cells or after exposure to stresses.
64 We therefore hypothesized that the palmelloid phenotype observed in *dyrkp* mutant could either be
65 due to a defect in cell hatching or due to an increased stress state in the mutant population.

66

67 **Findings:** We answered these questions by comparative studies in different culture conditions and by
68 examining additional *dyrkp* knockout mutants generated by CRISPR-Cas9 in various background
69 strains with more or less intact cell walls. Palmelloid formation in the *dyrkp* mutant was observed
70 under optimal growth (mixo- or auto-trophic condition) and very low light conditions. Interestingly,
71 unlike the parent strain, in which only cell wall fragments are observed in old cultures, the parental
72 cell wall of the *dyrkp* mutant remained almost intact even after the release of daughter cells. Also, the
73 cell division rate of the cell wall-less *dyrkp* mutants was similar to their background strain. These
74 results suggest that *dyrkp* mutants have a problem in degrading the parental cell walls. Indeed,
75 proteomic and transcriptomic analyses revealed reduced levels of protease families in the *dyrkp*
76 mutant, and in particular with a significantly lower amount of several key members of the MMP
77 family. Through the analysis of complementation lines, we confirmed that the DYRKP was required
78 for strong and rapid expression of MMPs.

79

80 **Next steps:** We are pursuing research to understand what the phosphorylation clients of DYRKP are
81 and how they regulate the expression of the MMPs identified in this study.

82

83 **Introduction**

84 The cell wall, one of the extracellular matrix (ECM) in plants and algae (Cosgrove, 2005;
85 Domozych and LoRicco, 2023), is a complex network of molecules surrounding cells and
86 tissues, providing them with mechanical support and transmitting regulatory cues from the
87 environment (Gu and Rasmussen, 2022). The ECM, composed of matrix polysaccharides and
88 matrix glycoproteins, is continuously synthesized and degraded during cell growth, division,
89 and differentiation (Flinn, 2008; Seifert and Blaukopf, 2010; Domozych and LoRicco, 2023).
90 Many studies about the regulatory mechanism for cell wall remodeling focus on matrix
91 polysaccharides (Bashline et al., 2014; Anderson and Kieber, 2020), but the regulation for the
92 synthesis and degradation of matrix glycoproteins has been rarely described.

93 In both animal and plant cells, glycoprotein hydrolyzing enzymes involved in ECM
94 remodeling, called ECM proteases, are known to be essential for normal growth and
95 development (Holmbeck et al., 1999; Golldack et al., 2002; Bonnans et al., 2014; Mishra et
96 al., 2021); the loss of function of ECM proteases results in growth arrest (Holmbeck et al.,
97 1999; Golldack et al., 2002), while overexpression of ECM proteases accelerates cancer
98 invasion in animal cells (Tryggvason et al., 1987) or leaf senescence in plant cells (Wu et al.,
99 2022). The control of ECM protease expression by kinase cascade (e.g., mitogen-activated
100 protein kinase (MAPK)) has been well documented in animal cells (Wagner and Nebreda,
101 2009; Kumar et al., 2010), but only limited evidence is available for plant cells; MAPK3 and
102 MAPK6 cascade induce the expression of matrix metalloproteinases (MMPs) during
103 senescence in leaves (Wu et al., 2022).

104 *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) is a photosynthetic unicellular
105 microalga that has a non-cellulosic cell wall composed of hydroxyproline-rich glycoproteins,
106 arabinose, mannose, and galactose (Baudelet et al., 2017; Goodenough and Lee, 2023). The
107 formation and degradation of cell walls occur actively during *Chlamydomonas* cell hatching
108 when daughter cells are released from the parental cell wall after mitosis (Cross and Umen,
109 2015). The MMPs (named gamete lytic enzymes) and the subtilisin-like serine proteases
110 (SUBs; named vegetative lytic enzymes) are reported to be involved in cell wall degradation
111 in *Chlamydomonas* (Kubo et al., 2009; Zou and Bozhkov, 2021). However, how cell wall
112 degradation and ECM proteases are regulated in this model organism which displays both
113 plant and ancestral eukaryotic features remains largely unknown.

114 In this study, we present the plant kinase belonging to the dual-specificity tyrosine
115 phosphorylation-regulated kinase (DYRK) as a crucial regulator for algal cell wall

116 degradation. Our comprehensive analysis demonstrates this kinase enables the successful
117 execution of the cell cycle by ensuring timely *MMP* expression.

118

119 **Results**

120 **The *dyrkp* mutant forms palmelloids independently of stress**

121 In a previous study on the *Chlamydomonas* mutant *starch degradation 1 (std1*, called
122 *dyrkp* mutant hereafter) which accumulates starch under nitrogen starvation conditions, we
123 showed that this mutant has an insertional mutation in the plant DYRK (DYRKP) (Schulz-
124 Raffelt et al., 2016). The DYRKP belongs to the DYRK kinase family which is known to be
125 regulators of cell growth and development in eukaryotes (Laguna et al., 2008; Becker, 2012;
126 Kurabayashi and Sanada, 2013). DYRK kinases are activated by autophosphorylation of
127 conserved tyrosine residues and then phosphorylates serine/threonine residues of substrates
128 (Aranda et al., 2011). In *dyrkp* mutant, we observed that cells tended to form clusters in liquid
129 culture (**Fig. 1A**) (Schulz-Raffelt et al., 2016). Investigating this aggregation phenotype
130 further, we found that the average particle size of the *dyrkp* mutant was 2–3 times bigger than
131 its background strain 137AH (**Fig. 1B**). Within the *dyrkp* mutant population, multiple cells
132 were trapped in a big cell wall (**Fig. 1C**), a structure usually called a palmelloid in literature
133 (Goodenough and Lee, 2023). Cell population analysis revealed that the 137AH strain
134 consisted of single cell mostly, whereas, more than 80% of the *dyrkp* mutant population
135 consisted of multiple cells (**Fig. 1D**).

136 During the asexual cell cycle, the particle diameter increases as palmelloid formation
137 during mitosis, and as daughter cells hatch, the particle diameter decreases and particle
138 concentration increases (**Supplemental Fig. S1A**). To measure growth, both 137AH strain
139 and *dyrkp* mutant were treated with autolysin to release single cells and inoculated with the
140 same particle number. Due to palmelloid formation, the growth pattern was expressed in
141 particle concentration indicating the number of particles in volume, including single and
142 multiple cells. The particle concentration of the *dyrkp* mutant increased slower than 137AH
143 strain and became similar 14 d after inoculation under standard light condition
144 (**Supplemental Fig. S1B**). Consistent with the slower increase in particle concentration, the
145 decrease in mean particle diameter in *dyrkp* mutant was slower than in the 137AH strain
146 (**Supplemental Fig. S1B**).

147 *Chlamydomonas* cells form palmelloids not only during mitosis but also when the cells are
148 exposed to stress conditions such as high light (**Supplemental Fig. S1A**) (Khona et al., 2016;

149 de Carpentier et al., 2019). To rule out that high light stress could be a trigger for the
150 formation of palmelloids in *dyrkp*, we investigated cell morphologies under standard and very
151 low light conditions. The particle size of the *dyrkp* mutant and 137AH strain increased in a
152 similar manner under very low light condition (**Supplemental Fig. S1C**), suggesting that the
153 palmelloid structure observed in the *dyrkp* mutant is not the consequence of stress responses.
154 Since the palmelloids were observed in the *dyrkp* mutant regardless of nutritional conditions
155 (**Supplemental Fig. S1D**), all subsequent studies were conducted under photoautotrophic
156 conditions.

157

158 **Degradation of the parental cell wall is compromised in the *dyrkp* mutant**

159 The total volume of the *dyrkp* mutant particles was up to three times bigger than that of
160 137AH (**Fig. 2A**). This phenotypical difference was also illustrated by the pellet size after
161 low-speed centrifugation, which was much larger in the *dyrkp* mutant than in 137AH (**Fig.**
162 **2B**). Many undigested parental cell walls were observed in the *dyrkp* mutant (**Fig. 2C**). The
163 undigested parental cell wall of the *dyrkp* mutant was clearly distinguished from the 137AH
164 strain. From the ‘day 25’ culture of the *dyrkp* mutant, we observed the occurrence of a
165 population with a smaller size in the cell counter, and this population disappeared after
166 autolysin treatment, indicating that this population was composed essentially of the
167 undigested parental cell wall (**Fig. 2D**). Next, we quantified and compared the total proteins
168 present in the upper phase of the culture. We observed that the protein concentration of the
169 culture medium increased rapidly in strain 137AH throughout the growth, but not of the
170 *dyrkp* mutant (**Fig. 2E**). These results indicate the absence of DYRKP resulted in a delayed
171 degradation of the parental cell wall.

172 We then investigated two complemented *dyrkp* mutant lines (DYRKP-c1 and DYRKP-c2;
173 **Fig. 3A**) driven by the light-inducible *psaD* promoter (Schulz-Raffelt et al., 2016). The
174 complemented lines formed intact and smaller size pellets than the *dyrkp* mutant (**Fig. 3B**)
175 and their particle size decreased to a single cell size within seven days of continuous light
176 conditions (**Fig. 3C**). However, unlike the 137AH strain, the particle concentration of
177 complemented lines decreased rapidly after saturating point and the decline of the DYRKP-
178 c2 line were faster than the DYRKP-c1 line (**Fig. 3D**). Based on these results, we
179 hypothesized the DYRKP involves in the degradation of the parental cell wall.

180

181 **Palmelloid formation by the *dyrkp* mutant depends on cell wall integrity**

182 137AH strain has a complete cell wall. To test our hypothesis, we sought to investigate the
183 palmelloid phenotype of the *dyrkp* mutants derived from other cell-walled or cell-wall-less
184 background strains. We obtained two mutants (*dyrkp2* and *dyrkp3*) of the CC5325 strain from
185 the Chlamydomonas mutant library (Li et al., 2019). We generated target-specific DYRKP
186 knockout mutants using the CRISPR-Cas9 method in the cell-walled strain CC125 (*dyrkp13*,
187 *dyrkp15*, and *dyrkp24*) and in two cell-wall compromised strains CC4349 (*dyrkp3*, *dyrkp9*,
188 and *dyrkp10*) and *dw15* (*dyrkp7* and *dyrkp10*). Since so-called cell wall defective strains have
189 often various degrees of defects in their cell wall, we therefore first tested the cell wall
190 integrity of the background strains using a detergent-treatment test (**Fig. 4A**). The insertion of
191 the antibiotic cassette was confirmed by PCR analysis of the respective genomic DNA
192 (**Supplemental Fig. S2**), and all mutants were confirmed to be true knockout by immunoblot
193 analysis using anti-DYRKP (**Fig. 4B**).

194 Palmelloids were not observed in the *dyrkp* mutants derived from cell-wall-less strains (**Fig.**
195 **4C**; **Supplemental Fig. S3**), supporting our hypothesis that the mutant phenotype is related
196 to the cell wall. Next, to understand the effect of DYRKP on the cell cycle, growth was
197 measured under diurnal cycles (12 h light and 12 h dark). All *dyrkp* mutants showed a similar
198 maximum cell density and maximum growth rate (**Supplemental Fig. S4**), indicating that
199 DYRKP mutation does not affect the cell cycle.

200 On the other hand, the particle size of the *dyrkp* mutants in cell-walled strains was bigger
201 than their background strains and the difference was more obvious in the CC125 background
202 than in the CC5325 background (**Fig. 4C**). The CC5325-background mutants did not show
203 large multicellular structures as much as those derived from 137AH or from CC125
204 (**Supplemental Fig. S3**; **Supplemental Fig. S5A**). The ratio of single cells in CC5325-
205 background mutants was over 50%, while CC125-background mutants showed less than 25%,
206 which was similar to the *dyrkp* mutant (**Supplemental Fig. S5B**). The changes in particle size
207 of CC125-background mutants were more distinct than that of CC5325-background mutant
208 (**Supplemental Fig. S5C-E**). Although the CC5325-background mutants formed an intact
209 pellet unlike the *dyrkp* mutant and the CC125-background mutants (**Supplemental Fig. S5F**),
210 a particle analysis showed a clearly different pattern to their background strains in common
211 (**Supplemental Fig. S5G**). Overall, the palmelloid phenotype of the *dyrkp* mutant was
212 greatly affected by the integrity of the cell wall, and the size of the palmelloid structure (i.e.
213 the extent of the phenotype) was found to be associated to the degree of the defects in the cell
214 wall of the background strains from which the mutant is derived.

215

216 **The *dyrkp* mutant secretes fewer cell wall proteins**

217 To further pinpoint the role of DYRKP in cell wall degradation, we compared the protein
218 profiles between 137AH and *dyrkp* mutant by separating extracellular proteins on SDS-PAGE
219 followed by silver staining. The proteins collected from the upper phase of the ‘day 25’
220 culture revealed distinct protein patterns between *dyrkp* mutant and the 137AH strain
221 (**Supplemental Fig. S6A**). To further understand the cause of the incomplete cell wall
222 degradation of the *dyrkp* mutant, we carried out a global proteomic analysis of cellular
223 (hereafter pellet) and extracellular proteins in the culture medium (hereafter upper phase) of
224 137AH and the *dyrkp* mutant (**Fig. 5A, Supplemental Fig. S6, B and C**). A total of 5,088
225 pellet proteins and 3,141 upper phase proteins were detected in the two strains, and after
226 elimination of redundant proteins, the remaining 2,369 pellet proteins and 1,108 upper phase
227 proteins were further analyzed (**Supplemental Fig. S6D, Supplemental Table S1 and S2**).
228 The Venn diagram showed the unique and common proteins among the two strains
229 (**Supplemental Fig. S6E**). Among protein groups with significant differences between
230 137AH and *dyrkp* mutant, 416 proteins in the pellet and 319 proteins in the upper phase were
231 identified (**Fig. 5B, Supplemental Table S3 and S4**).

232 In the extracellular medium (upper phase), 37 out of 43 cell wall-related proteins
233 commonly detected in both strains were more abundant in the 137AH strain (**Fig. 5C,**
234 **Supplemental Fig. S7**). This includes the well-known cell wall proteins of *Chlamydomonas*,
235 i.e. glycoproteins (GP1, GP2, and GP3) and pherophorin-*Chlamydomonas* homolog 4 (PHC4)
236 (Goodenough and Lee, 2023). These results therefore show that the low protein concentration
237 in the culture medium of *dyrkp* mutant could explain the delay or defective degradation of the
238 parental cell wall.

239

240 **Expression of ECM proteases is reduced in the *dyrkp* mutant**

241 In *Chlamydomonas*, the parental cell wall is degraded by ECM proteases, which are
242 transported through cilia (Long et al., 2016; Zou and Bozhkov, 2021) and secreted by
243 ectosomes at the ciliary tips (Wood et al., 2013; Long et al., 2016). Cilia are essential for
244 ectosome secretion because they are the only organelles not surrounded by a cell wall (Wood
245 et al., 2013). Indeed, the delay or failure of cell hatching is observed in the cilia-assembly
246 mutants of *Chlamydomonas* (Brown et al., 2015; Wang et al., 2022). Thus, we investigated
247 the specific proteins associated with cilia and ectosome based on the previous proteome data

248 (Long et al., 2016). Cilia-component proteins such as flagella-associated proteins (FAPs) and
249 intraflagellar transport proteins (IFTs) were less abundant or even absent in the *dyrkp* mutant
250 (**Supplemental Fig. S8A**). When the ciliary length was measured after autolysin treatment, it
251 was observed that the *dyrkp* mutant had shorter cilia or even no cilia comparing to the 137AH
252 strain (**Supplemental Fig. S8b**). Nevertheless, in the *dyrkp* mutant, a ciliary movement was
253 observed from some daughter cells inside and outside of the parent cell wall (**Supplemental**
254 **Fig. S8C, Supplemental Movie S1 to S5**), suggesting that lack of DYRKP delays cilia
255 assembly but without affecting cell motility. For the ectosomal proteins, most of them were
256 detected in both strains but were less abundant in the *dyrkp* mutant (**Supplemental Fig. S9**),
257 indicating the delayed cilia assembly might have affected ectosome secretion.

258 The parental cell wall was still not degraded even after prolonged cultivation (**Fig. 2**),
259 suggesting that there is likely a defect in the synthesis or transport of ECM proteases. Indeed,
260 MMP1 and VLE1, major known ECM proteases of *Chlamydomonas* (Kubo et al., 2009; Zou
261 and Bozhkov, 2021), were found in both strains but were more abundant in the 137AH strain
262 than in the *dyrkp* mutant (**Fig. 5D**). Except for MMP14 and the protein product encoded by
263 the locus Cre05.g232600, all other 13 MMP1 homologs were more abundant in the 137AH
264 strain than *dyrkp*. On the other hand, of the six VLE1 homologs, only SUB11 and the protein
265 product encoded by the locus Cre03.g145827 were enriched in the 137AH strain. Taken
266 together, proteomic analysis indicates that the DYRKP affects cilia assembly as well as the
267 amount of ECM proteases.

268 To identify difference in gene expression levels, transcriptomic analysis was performed.
269 Compared to the 137AH strain, 687 up-regulated genes and 1568 down-regulated genes were
270 identified in the *dyrkp* mutant (**Supplemental Fig. S10A, Supplemental Table S5**).
271 Validation of transcriptome data by qPCR showed high accuracy (**Supplemental Fig. S10B**).
272 Consistent with proteomics analysis, many hydrolytic enzymes for glycoproteins like MMPs
273 and SUBs were down-regulated in the *dyrkp* mutant (**Fig. 6A**). Particularly, MMP and SUB
274 showed a high positive correlation between transcriptome and proteome (**Supplemental Fig.**
275 **S10C**). Among ECM proteases identified in the transcriptome and proteome, *MMP1*, *MMP3*,
276 *MMP13*, *MMP19*, *VLE1*, and *SUB11* were selected and qRT-PCR further validated their
277 differential expression pattern. As a result, the overall expression of the *MMP* family was
278 significantly decreased in the *dyrkp* mutant but the expression of *VLE1* and *SUB11* did not
279 show statistically significant differences (**Fig. 6B**). Similar patterns were confirmed in other
280 cell-walled DYRKP knockout mutants (**Supplemental Fig. S11A**). The decreased expression

281 of *MMP1*, *MMP3*, and *MMP13* were also observed in the cell-wall-less or -compromised
282 DYRKP knockout mutants (**Supplemental Fig. S11B**).

283 In addition to ECM proteases, down-regulated genes in the *dyrkp* mutant include those
284 related to protein phosphorylation, kinase activity and phosphotransferase activity. Among
285 207 kinase genes, 177 were down-regulated in the *dyrkp* mutant. Among 22 phosphatase
286 genes, 14 were down-regulated in the *dyrkp* mutant (**Supplemental Fig. S10D**,
287 **Supplemental Table S5**).

288

289 **DYRKP positively regulates the expression of *MMP1*, *MMP3* and *MMP13***

290 Our multi-omics analysis indicated that DYRKP likely plays a role in regulating MMP
291 expression. According to the gene expression database under light/dark conditions (Proost
292 and Mutwil, 2018), *DYRKP* expression is associated with the life cycle of *Chlamydomonas*.
293 In detail, the *DYRKP* expression increases gradually during the cell growth and peaks at the
294 end of the light and dark phases which correspond to G1 and G0 phases (**Supplemental Fig.**
295 **S12A**). Indeed, the expression in the 137AH strain increased once in each light and dark
296 phase (**Fig. 6C**). However, DYRKP expression in the two complemented *dyrkp* mutants
297 driven by the light-inducible promoter increased only in the light phase.

298 We then confirmed the expression patterns of *MMP1*, *MMP3*, *MMP13* and *VLE1* under
299 light/dark conditions (**Fig. 6C**, **Supplemental Fig. S12B**). In the *dyrkp* mutant, the
300 expression levels of *MMPs* were lower than the 137AH strain at almost all sampling points.
301 In the 137AH strain, the expression of *MMP1* and *MMP3* increased during both light and
302 dark phases while the expression of *MMP13* increased only in the dark phase. Similar to our
303 results, gene expression databases indicated that the expression of *MMP1* and *MMP3*
304 gradually increased and peaked at the end of each phase, and the expression of *MMP13*
305 increased only in the dark phase. In the complemented lines, the expression of *MMP1*, *MMP3*,
306 and *MMP13* was restored to a similar and even higher level than that in the 137AH strain,
307 and their expression increased in the light phase and decreased in the dark phase, similar to
308 the expression pattern of *psaD* gene. As expected, the expression of *VLE1* did not show a
309 clear association with *DYRKP* expression.

310

311 **Discussion**

312 Although the importance of cell hatching in the *Chlamydomonas* cell cycle is evident, little
313 is known about its regulation. Here, we demonstrate that DYRKP plays an important role in

314 cell hatching by regulating ECM degradation (**Fig. 7**). We further point out that this
315 regulation is achieved through positively modulating the expression of ECM-associated
316 MMPs. Genetic knockouts of DYRKP delayed the degradation of ECM components during
317 cell division, and sustained expression of DYRKP in complemented lines restored the
318 phenotype of the background strain. Moreover, many *MMPs* were repressed in the *DYRKP*
319 knockout mutants, and the expression of *MMPs* altered consistent with the expression pattern
320 of *DYRKP*.

321 MMPs are important for a balanced and regulated degradation of ECM proteins during
322 growth, morphogenesis and development in multicellular organisms (Holmbeck et al., 1999;
323 Golldack et al., 2002; Page-McCaw et al., 2007). For example, the *At2-mmp* mutant of
324 *Arabidopsis thaliana* is defective in tissue development, and its leaf cells are smaller than the
325 wild-type and unevenly distributed (Golldack et al., 2002). Interestingly, the DYRKP
326 knockout mutants of *M. polymorpha* failed to decompose the cilia-like structure usually
327 degraded in wild-type strains (Furuya et al., 2021). In the case of *Chlamydomonas*, it has also
328 been observed that the mutant defected in MMP32 exhibits a spontaneous aggregation
329 phenotype (de Carpentier et al., 2022). The phenotypes of MMP-defective mutants are
330 similar to those defected in DYRKP, further supporting our finding that DYRKP regulates
331 cell hatching by controlling MMP expression.

332 Regulation of cell cycle progression by members of the DYRK family has been observed
333 in animal cells at different stages of the cell cycle (Becker, 2012). For example, DYRK1A
334 and DYRK1B regulate the length of the G1-phase as well as the decision between the entry
335 and exit of the cell cycle; DYRK2 is a negative regulator of S-phase entry and is down-
336 regulated in tumor cells; DYRK3 regulates the cell cycle of erythroblasts. On the other hand,
337 DYRKP does not directly affect cell division (Furuya et al., 2021) but is involved in the last
338 step of a cell cycle, i.e., cell hatching. In contrast to our study, DYRKs are found to act as a
339 negative regulator of the cell cycle, highlighting the complexity and difference in the
340 evolutionary trajectory of DYRKs in different kingdoms of life. Nevertheless, these
341 observations collectively establish the conserved role of DYRKs in regulating the cell cycle
342 in eukaryotes.

343 Expression of *MMP1*, *MMP3* and *MMP13* responded rapidly to DYRKP expression, thus it
344 may be interesting to understand how this kinase regulates them simultaneously. In this
345 regard, it is worth mentioning that many protein kinases and phosphatases were down-
346 regulated in the *dyrkp* mutant. Among them, the aurora kinases which are the family of

347 serine/threonine kinases, perform essential functions during cell division (Carmena and
348 Earnshaw, 2003). In breast cancer cells, the siRNA-mediated silencing of aurora kinases
349 markedly decreases the MMP-9 expression levels induced by the protein kinase C pathway
350 (Noh et al., 2015). On the other hand, MAPKs induce the expression of MMPs during ECM
351 remodeling and degradation in metastatic cancer cells (e.g. p38 MAPKs) (Wagner and
352 Nebreda, 2009; Kumar et al., 2010) or senescence in leaves (e.g. MAPK3/MAPK6) (Wu et
353 al., 2022). Another possibility is the phosphorylation of transcription factors. In animal cells,
354 DYRK1 and DYRK2 are known to be involved in cancer initiation and progression such as
355 proliferation and invasion by phosphorylating the nuclear factor of activated T cells (NFAT)
356 (Shou et al., 2015). Direct regulation of MMP3 by NFAT1 promotes the growth and
357 metastasis of melanoma tumors (Shoshan et al., 2016). However, the relationship between
358 DYRK family and MMP in plants is still unknown, and it has not been confirmed whether
359 DYRKP has the same phosphorylation client as DYRK1 and DYRK2. Therefore, further
360 studies on direct DYRKP targets through phosphoproteomics or kinase client assays (Huang
361 and Thelen, 2012) can help to understand its regulatory mechanism more clearly.

362 Taken together, our data broaden the context of DYRKP activity by demonstrating that this
363 kinase has an impact on cell wall remodeling. Future work on candidates for DYRKP
364 phosphorylation will provide a better understanding of how DYRKP is involved in regulating
365 various cellular processes in plants and algae.

366

367 **Materials and Methods**

368 **Strains and growth conditions**

369 Background strains of *Chlamydomonas* were selected into two groups; cell-walled strains
370 (137AH, CC5325, and CC125) and cell wall-less or cell wall-compromised strains (CC4349
371 and *dw15*). The DYRKP knockout mutant, named *std1* (*starch degradation 1*), was obtained
372 from our mutant library (Schulz-Raffelt et al., 2016). All mutants and their background
373 strains are shown in the **Supplemental Table S6**. Cells were cultured in 20 mL of
374 photoautotrophic culture medium (MOPS-buffered minimal medium; MM-MOPS) in a 100
375 mL flask. The cells were cultured photoautotrophically in incubator shakers (Infos HT) with
376 natural dissolving of 2% CO₂ by continuous shaking (120 rpm) under continuous illumination
377 ($80 \pm 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at $25 \pm 1^\circ\text{C}$ unless otherwise stated.

378

379 **Cell wall integrity assay**

380 To confirm the cell wall integrity of background strains, 1 mL of 137AH, CC5325, CC125,
381 CC4349, and *dw15* cultures was harvested by centrifugation at 1,000 g. The pellet was
382 resuspended with 0.05% (w/v) Triton X-100 or with MM-MOPS medium by vortexed
383 vigorously for 30 s then incubated in dark for 30 min. The size of the cell population and the
384 number of intact cells were measured using a Coulter Counter (Multisizer 4; Beckman
385 Counter, Brea, CA, USA).

386

387 **Generation of knockout mutants by CRISPR-Cas9 RNP method**

388 To obtain *dyrkp* mutants, the CRISPR-Cas9 RNP method was used with a few
389 modifications (Kim et al., 2020). The single guide RNA (sgRNA) sequence for the specific
390 target sequence on the DYRKP gene was designed by Cas-Designer
391 (<http://www.rgenome.net/cas-designer>) and selected as 5'- TCCTCGCAGCAAGTCTGCGC
392 AGG -3' (**Supplemental Fig. S2**). To increase the selection efficiency, insertion and
393 expression of hygromycin-resistance (HygR; aphVII) gene cassette was combined. The
394 purified Cas9 protein (100 µg, Cas9 expression plasmid: pET-NLS-Cas9-6xHis (Plasmid
395 #62934)) and 70 µg of sgRNA synthesized by using GeneArt™ Precision gRNA Synthesis
396 Kit (ThermoFisher, MA, USA), were mixed to form the sgRNA and Cas9 protein (RNP)
397 complex. The RNP complex and aphVII gene expression cassette were co-transformed with
398 electroporation (600 V, 50 µF, infinity resistance). After 12 hours of incubation to allow
399 recovery from electroporation shock, cells were plated on a TAP medium containing 1.5%
400 agar and hygromycin (25 µg mL⁻¹; ThermoFisher). To screen *dyrkp* mutants, the colonies
401 grown onto the selection plate were confirmed by genomic PCR using the target-specific
402 primer sets (**Supplemental Table S7**).

403

404 **Microscopy analysis**

405 For the light microscopic observation, the Leica DMRXA microscope (Leica Microsystems,
406 Wetzlar, Germany) was used. To measure the ciliary length, cells were fixed with 0.2 % (w/v)
407 glutaraldehyde added to the medium. Images and videos were obtained by using the Spot
408 Insight 4 software (Diagnostic Instruments Inc., Sterling Heights, USA).

409 For the Confocal microscopic observation, the ZEISS LSM 780 Confocal microscope
410 (Carl-ZEISS, Oberkochen, Germany) was used. To visualize cell wall structures, cells were
411 stained with Concanavalin A (ConA) – Alexa Fluor™ 594 conjugates (ThermoFisher) for 10
412 min at room temperature. The ConA was excited with a 561 nm laser and an emitting signal

413 was collected between 582-635 nm. Chlorophyll autofluorescence was excited with a 488 nm
414 laser and emission was collected between 648-683 nm. Data analysis was performed with the
415 FIJI program. To quantify the cell population, all particles were classified into three groups; a
416 single cell, 2-4 cells, and more than 8 cells. The range of chlorophyll autofluorescence signal
417 area was determined based on more than 50 particles. To make sure our criteria of data
418 analysis, the ratio was double-checked in the transmitted image. Afterward, all the particle
419 signal from five independent images was classified into three groups and were expressed as a
420 ratio.

421

422 **Quantitative and qualitative assays for extracellular proteins**

423 Cell culture (2 mL) was collected from the culture after 0, 3, 5, 10, and 25 days of
424 inoculation. To separate extracellular proteins and cellular proteins, cell cultures were
425 centrifuged at 2,000 g for 5 min and the upper phase was carefully collected. Additionally, the
426 collected culture medium was filtered with a 0.45 μm polyethersulfone (PES) membrane
427 (VWR International, Radnor, PA, USA) to avoid contamination by the cellular proteins. Then,
428 proteins in the filtered culture medium were precipitated by adding 5 volumes of ice-cold 100%
429 acetone and were collected after centrifugation (10 min, 16,000 g, 4°C). After washing pellets
430 three times with ice-cold 80% acetone, it was resuspended in 2 mL of 0.1% (w/v) SDS buffer
431 and used for quantitative and qualitative assays.

432 For the quantitative assay, the total proteins in the samples were measured through the
433 BiCinchoninic acid Assay using a kit (Sigma-Aldrich, Saint Louis, MO, USA). The assay
434 was conducted according to the method provided by the manufacturer. For the qualitative
435 assay, 100 ng of total proteins in 'day 25' were separated on a 10% (w/v) Bis-Tris gel
436 (Invitrogen, Waltham, MA, USA) run for 1 h at 190 V in Novex Nupage (Invitrogen) and
437 stained by the Silver staining method.

438

439 **Protein extraction and immunoblot analysis**

440 Soluble cell extracts were prepared as follows: 2 mL of *C. reinhardtii* cell culture in the
441 exponential phase (eq. to 5×10^6 cells mL^{-1} or $0.8 \text{ mm}^3 \text{ mL}^{-1}$) were harvested by
442 centrifugation for 2 min at 1,789 g and resuspended in 0.5 mL lysis buffer (20 mM HEPES–
443 KOH pH 7.2, 10 mM KCl, 1 mM MgCl_2 , 154 mM NaCl, 0.1 \times protease inhibitor cocktail).
444 Cells were sonicated on ice for 30 s with an alternating cycle of 1 s pulse/1 s pause. After
445 centrifugation (10,000 g 10 min 4°C), soluble proteins in the supernatant were precipitated

446 with ice-cold 80% (v/v) acetone (-20°C, 1 h). Samples were then centrifuged for 10 min,
447 16,000 g at 4°C. The protein pellet was resuspended with Novex Nupage LDS buffer 1×
448 (Invitrogen), and proteins were then denatured at 70 °C for 20 min. Protein extracts (30 µg
449 protein) were loaded on 3-8% Novex Nupage Tris-Acetate gel (Invitrogen) and run for 1 h at
450 190 V in Novex Nupage (Invitrogen). Proteins were transferred onto nitrocellulose using a
451 semidry transfer technique. Immunodetection of DYRKP was performed following the
452 process described in our previous work (Schulz-Raffelt et al., 2016).

453

454 **Protein extraction and *in-solution* digestion**

455 For proteomic analysis, the ‘day 5’ cultures of the 137AH strain and the *dyrkp* mutant were
456 harvested by centrifugation (2,000 g) for 10 min. To ensure data reliability, samples were
457 prepared from five biologically independent cultures. After centrifugation, the upper phase
458 and pellet were carefully separated as the samples of extracellular proteins and cellular
459 proteins, respectively. To avoid the contamination of cellular proteins and extracellular
460 proteins, the isolated culture medium was filtered using a 0.45 µm PES membrane.

461 The pellet samples were extracted by adding 2.5 mL of Tris pH 8.8 buffered phenol and 2.5
462 mL of extraction media (0.1 M Tris- HCl pH 8.8, 10 mM EDTA, 0.4% (v/v) 2-
463 mercaptoethanol, 0.9 M sucrose) and agitating for 30 min at 4°C. The phenol phase was
464 removed, and the aqueous phase was back extracted with 2.5 mL of extraction media added
465 with 2.5 mL of phenol. Phenol-extracted proteins were precipitated by adding 5 volumes of
466 0.1 M ammonium acetate in 100% methanol. On the other hand, the upper phase samples
467 were precipitated right away without the phenol extraction process.

468 Precipitated proteins were collected after centrifugation (10 min, 16,000 g, 4°C). Pellets
469 were washed twice with 0.1 M ammonium acetate in methanol, and three times with ice-cold
470 80% acetone. An aliquot was removed and centrifugated at 16,000 g, 4°C for 5 min followed
471 by pellet resuspension in urea buffer (6M urea, 2M thiourea, 100 mM ammonium
472 bicarbonate). Protein content was quantified by Bradford assay with Bovine Gamma Globulin
473 as standard.

474 Before mass spectrometry analysis, 10 µg of each sample was aliquoted and normalized to
475 the same concentration and volume (40 µL) in urea buffer (6M urea, 2M thiourea, 100mM
476 ammonium bicarbonate). Reduction (10mM DTT in 10 mM ammonium bicarbonate) was
477 performed at 30°C for 30 min and alkylation (40 mM iodoacetamide in 10mM ammonium
478 bicarbonate) for 1 hour at room temperature in the dark. Samples were diluted to a final urea

479 concentration of 0.8 M before tryptic digestion. Protein digestion was performed by adding a
480 volume of trypsin aiming at an enzyme/protein ratio of 1:50 at 37 °C for 16 h. To achieve
481 optimal digestion, a second addition of trypsin was done, and samples were incubated for 4 h.
482 After digestion, the tryptic peptides were dried under vacuum centrifugation. Subsequently,
483 samples were loaded onto Evotips according to the manufacturer's instructions.

484

485 **Mass spectrometry data acquisition (UHPLC-MS/MS)**

486 The acquisition of mass spectrometric data after tryptic digestion was performed on an
487 ultra-high-performance liquid chromatography (UHPLC) EvoSep system that was used for
488 reverse-phase liquid chromatography over a 44 min gradient at 250 nL min⁻¹ flow. Peptides
489 were separated on a C18 analytical column (PepSep C18 Bruker Daltonics, 15cmx150µm,
490 1.5µm particle size). Acquisition of mass spectra was carried out concomitantly to the
491 chromatographic separation and peptides were analyzed through a Data Dependent
492 Acquisition (DDA) method using a TimsTOF Pro 2 (Bruker Daltonics, Billerica, MA, USA).
493 The instrument was operated in positive-ion, data-dependent PASEF mode over a m/z range
494 of 100 to 1700. PASEF and TIMS were set to "on" for PASEF and TIMS. One MS and ten
495 PASEF frames were acquired per cycle of 1.17sec. Target MS intensity was set at 10,000 with
496 a minimum threshold of 2500 and from 20 to 59 eV, and a charge-state-based rolling collision
497 energy table was employed. An active exclusion/reconsider precursor method with release
498 after 0.4min was used. A second MS/MS spectra were obtained if the precursor had a four-
499 time increase in signal strength in subsequent scans (within a mass width error of 0.015 m/z).

500

501 **Protein identification and data analysis for global proteomics**

502 The PEAKS Studio 10.0 (Bioinformatics Solutions, Waterloo, Ontario, Canada) software
503 was used to provide automated *de novo* sequencing from MS/MS spectra. PEAKS *de novo*
504 sequencing was performed with precursor and fragment error tolerance values of 20 ppm and
505 0.01 Da, respectively. Trypsin was used as a protease with 3 maximum missed cleavage
506 allowed. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed
507 and variable modifications, respectively. A maximum of four variable modifications per
508 peptide was allowed. PEAKS DB, which is a database search module in PEAKS Studio, was
509 used in the second step to identify peptide spectrum matches (PSMs) from existing protein
510 databases (Zhang et al., 2012). The *C. reinhardtii* (19,526 entries) was used as the reference
511 database. The target-decoy method known as "decoy fusion" that is included in PEAKS was

512 utilized to estimate the 1% FDR of the PEAKS DB result for establishing a confidence
513 threshold for PSMs.

514 Data processing was performed using Perseus Software (version 2.0.9.0) with default
515 settings as workflow depicted in **Supplemental Fig. S6**. Intensity values were log₂-
516 transformed, and the quantitative profiles were filtered for missing values as identified
517 proteins had to be present in at least 70% of samples for further analysis. Data normalization
518 was performed by dividing each protein intensity by the total intensity sum of all identified
519 proteins in its sample, respectively. Significantly different protein groups were assessed by
520 pairwise comparison analyses with Student's T-test with 0.05 probability level with
521 Benjamini-Hochberg FDR correction. Hierarchical clustering was based on Euclidean
522 distance and on the average linkage of 300 clusters for both row and column trees. The mass
523 spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via
524 the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier
525 PXD047255 (pellet) and PXD047263 (upper phase).

526

527 **RNA extraction and quantitative real-time PCR**

528 Five milliliter of cell cultures were harvested by centrifugation (1 min, 20°C, 1,790 g) and
529 flash frozen in liquid nitrogen and conserved at -80°C. Total RNA was extracted using the
530 Direct-Zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA) and treated with the
531 RNase-free DNase Set (Zymo Research) according to the manufacturer's instructions.

532 In order to quantitative real-time PCR (qRT-PCR), complementary DNA (cDNA) was
533 synthesized from 1 µg of total RNA using SuperScript VILO Master Mix (Invitrogen). qRT-
534 PCR was performed on a 480 LightCycler thermocycler (Roche, Indianapolis, IN, USA)
535 following the manufacturer's instructions with TB Green Premix Ex Taq II (Takara, Shiga,
536 Japan) and the primers listed in **Supplemental Table S7**.

537

538 **RNA sequencing**

539 The RNA-seq analysis was carried out on 4 replicates for the 137AH strain and the *dyrkp*
540 mutant. A cDNA library was constructed from 1 µg of total RNA and Illumina HiSeq 2500
541 sequencing was performed at the Biopuces et Sequencage platform (Illkirch, France). In
542 general, between 28 to 37 million 50-nt single-end reads were generated for each replicate
543 (**Supplemental Table S5**). We processed the fastq files with trimgalore in search of adapters
544 and to trim low-quality nucleotides and remove reads shorter than 20 bp after removing

545 adapters and low-quality bases. Then, we used ribodetector to detect and remove any rRNA
546 that might have survived polyA selection/ribodepletion. The remaining reads were aligned
547 onto the *C. reinhardtii* genome assembly version v6.0 using the Bowtie2 software. HTSeq
548 count was used to identify uniquely mapped reads. For each replicate, between 19 and 33
549 million reads were mapped on the *C. reinhardtii* genome. DESeq2 package was used to
550 analyze differential gene expression. Only genes with a *p*-value adjusted ≤ 0.01 and an
551 absolute log₂ fold change (log₂FC) ≥ 1 or ≤ -1 were kept for further analysis. Of 16,288
552 identified transcripts, 2,139 were categorized as Differentially Expressed Genes (DEGs)
553 (**Supplemental Table S5**). The raw data of RNA-Seq was submitted to NCBI under the
554 accession number (BioProject: PRJNA1042620). The gene ontology term annotation for
555 molecular function and biological process as well as enrichment analysis of DEGs was
556 conducted using the functional enrichment analysis of the STRING database (version 10.0)
557 with default parameters (Szklarczyk et al., 2014). The gene annotation was cross-checked by
558 searching manually on the Phytozome server (*C. reinhardtii* v5.6).

559

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565 genome assembly.

566

567 **Authors contributions:**

568 M.K. and Y.L.-B. conceived the study. M.K. and Y.L.-B. designed the experiments and
569 interpreted the results. M.K., S.C., M.B., G.L.J., J.J.T., M.M., M.A., and J.-S.Y. performed
570 experiments. M.K., S.C., M.A., and M.B. visualized the data. Y.L.-B. took part in funding
571 acquisition and administrated the study. F.B., G.P., and Y.L.-B. supervised and commented on
572 the study. M.K. and Y.L.-B. wrote the manuscript with comments from other authors.

573

574 **Data and material availability**

575 All biological material described in this study is available upon request. All data are available
576 in the main text or the supplementary materials.

577

578 **Figure legends**

579 **Figure 1. The *Chlamydomonas reinhardtii* *dyrkp* mutant formed palmelloid structure. A)**

580 Cell culture images. **B)** Distribution of particle size in the culture 3 days after inoculation.
581 The mean value of particle size was calculated from all particles. **C)** Confocal microscopic
582 images. The merged images indicate the cell wall stained with Concanavalin A conjugating
583 fluorescent dye at 594 nm (ConcA; cyan) and the chloroplast with chlorophyll
584 autofluorescence at 488 nm (Chl; red). The Z-stack images clearly showed that the *dyrkp*
585 mutant cells were trapped inside three parental cell walls surrounded by another big cell wall
586 (white arrows). **D)** Cell population distributions. The composition of particles classified into a
587 single cell and the number of cells inside the parental cell wall. The sample was measured in
588 the culture 3 days after inoculation.

589

590 **Figure 2. The *dyrkp* mutant showed an impairment in the digestion of the parental cell**

591 **wall. A)** Changes in the total particle volume under standard light condition ($80 \pm 5 \mu\text{mol}$
592 $\text{photons m}^{-2} \text{s}^{-1}$). **B)** Morphology of pellets after low-speed (500 g) centrifugation. The same
593 volume (10 mL) was collected from the culture of 5 days and 25 days after inoculation. **C)**
594 Confocal microscopy images of the upper phase and pellet after low-speed centrifugation.
595 The 25-day-old cultures were harvested and the samples were stained with the solution of
596 concanavalin A conjugating fluorescent dye (ConcA; cyan) without filtration. Cells and
597 empty parental cell walls can be distinguished by the presence of chlorophyll
598 autofluorescence (Chl; red). The ConA and Chl signals were obtained at 594 nm and 488 nm,
599 respectively. The yellow scale bar indicates 20 μm . **D)** Distribution of particle size in the 25-
600 day-old cultures. The population was compared between the autolysin treatment (+Autolysin)
601 and no treatment (Control) groups after 30 min of treatment. The black arrow points to the
602 population of undigested parental cell wall debris. **E)** The amount of extracellular proteins in
603 the culture medium. All experiments were performed at least in three biological replicates (\pm
604 S.D).

605

606 **Figure 3. The complemented lines of *dyrkp* mutant (DYRKP-c1 and DYRKP-c2)**
607 **appeared the restoration of parental cell wall digestion ability. A)** Immunoblot of 137AH

608 strain, *dyrkp* mutant, and two complemented lines. The upper and below panels indicated
609 DYRKP antibody (α -DYRKP) and loading control, respectively. **B)** Morphology of pellets
610 after low-speed (500 g) centrifugation. The same volume (10 mL) was collected from the

611 culture of 25 days after inoculation. **C)** Changes in mean particle diameter under standard
612 light condition. **D)** Changes in particle concentration under standard light condition; All
613 experiments were performed at least in three biological replicates (\pm S.D).

614

615 **Figure 4. The *dyrkp* mutants generated from other cell-walled background strains**
616 **formed palmelloid structures. A)** Cell wall integrity was tested by detergent (Triton X-100)
617 treatment in cell-walled (CW) and cell-wall-less (CWL) background strains. **B)** Immunoblot
618 to DYRKP antibody (α -DYRKP) in the upper panel and loading control in the below panel. **C)**
619 Distribution of particle size in the culture 3 days after inoculation.

620

621 **Figure 5. Cell wall proteins and ECM proteases were less abundant in the *dyrkp* mutant.**

622 **A)** Schematic diagram of the proteomic analysis workflow. The detailed analysis procedure is
623 shown in **Supplemental Fig. S6**. **B)** Hierarchical clustering based on Euclidean distance.
624 Five biological replicates in each group have high similarity to each other. **C)** The cell wall
625 proteins detected in the upper phase. The cell wall proteins in the upper phase were assumed
626 to be the cell wall proteins released from the parental cell walls. Venn diagram represents the
627 number of commonly or uniquely found proteins between the 137AH strain and the *dyrkp*
628 mutant. The heatmap shows the protein abundance of major cell wall proteins. Details are
629 shown in **Supplemental Fig. S7**. **D)** Heatmap of the matrix metalloproteinases and serine
630 proteases detected in the pellet and upper phase. Statistical analysis was performed using the
631 non-parametric Mann-Whitney test; * $p < 0.05$ (\pm S.D).

632

633 **Figure 6. *MMP* expression showed a positive correlation to *DYRKP* expression. A)** The
634 differentially expressed genes encoding the matrix metalloproteinases and serine proteases. **B)**
635 Gene expression levels of selected matrix metalloproteinases and serine proteases in 137AH
636 strain and *dyrkp* mutant. **C)** Gene expression pattern of *DYRKP*, *MMP1*, *MMP3*, and *MMP13*
637 in 137AH strain, *dyrkp* mutant, and two complemented lines (DYRKP-c1 and DYRKP-c2)
638 under light/dark conditions. The value was normalized by the reference gene (*RACK1*). All
639 experiments were performed at least in three biological replicates. Statistical analysis was
640 performed using the non-parametric Mann-Whitney test; * $p < 0.05$ (\pm S.D). The ‘ns’
641 indicates no significance.

642

643 **Figure 7. Schematic diagram of the role of DYRKP in cell hatching.** DYRKP induces
644 increased transcription of *MMP1*, *MMP3*, and *MMP13* genes located in nuclear DNA through
645 phosphorylation of unknown protein kinases, protein phosphatases, or transcription factors.
646 Afterward, the translated protein in the pre-activated form of MMPs (pre-MMPs) is delivered
647 through cilia (Long et al., 2016; Zou and Bozhkov, 2021) and secreted in the form of an
648 ectosome from the cilia membrane (Wood et al., 2013; Long et al., 2016). Pre-MMPs are
649 activated by other proteases (Wilkinson et al., 2017); for example, pre-MMP1 is activated by
650 VLE1 (Kubo et al., 2009; de Carpentier et al., 2022). Activated MMPs then hydrolyze and
651 degrade the hydroxyproline-rich glycoprotein structure of the parental cell wall (Goodenough
652 and Lee, 2023), causing fragmentation of the parental cell wall. When the degradation of the
653 parental cell wall progresses sufficiently, the daughter cells are released (cell hatching), and
654 the remaining parental cell wall is completely degraded by MMPs that remain active.

655

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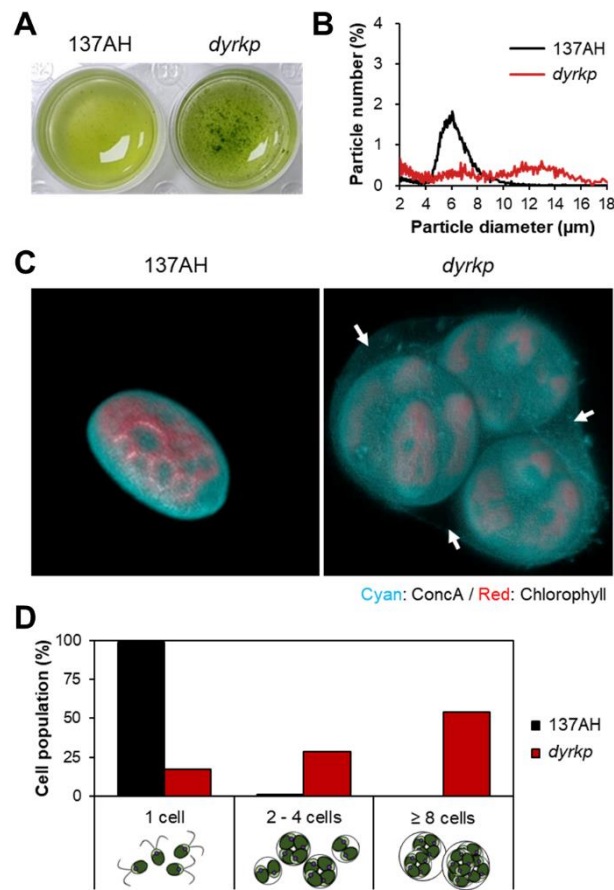
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776 molecular mechanisms. *Journal of Experimental Botany* **72**, 7680-7693.

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779

780 **Figure 1. The *Chlamydomonas reinhardtii* dyrkp mutant formed palmelloid structure. A)**

781 Cell culture images. **B)** Distribution of particle size in the culture 3 days after inoculation.

782 The mean value of particle size was calculated from all particles. **C)** Confocal microscopic

783 images. The merged images indicate the cell wall stained with Concanavalin A conjugating

784 fluorescent dye at 594 nm (ConcA; cyan) and the chloroplast with chlorophyll

785 autofluorescence at 488 nm (Chl; red). The Z-stack images clearly showed that the dyrkp

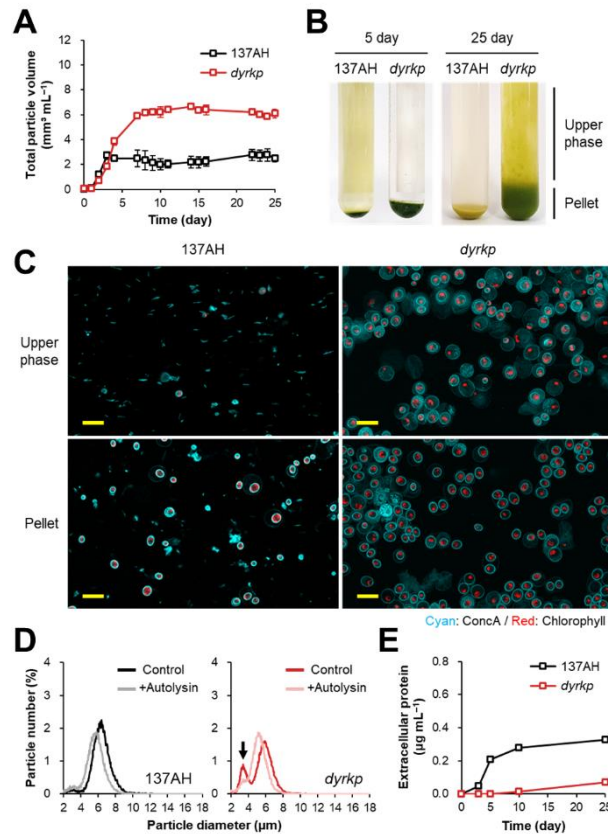
786 mutant cells were trapped inside three parental cell walls surrounded by another big cell wall

787 (white arrows). **D)** Cell population distributions. The composition of particles classified into a

788 single cell and the number of cells inside the parental cell wall. The sample was measured in

789 the culture 3 days after inoculation.

790



791

792 **Figure 2. The *dyrkp* mutant showed an impairment in the digestion of the parental cell**

793 **wall. A)** Changes in the total particle volume under standard light condition ($80 \pm 5 \mu\text{mol}$

794 $\text{photons m}^{-2} \text{s}^{-1}$). **B)** Morphology of pellets after low-speed (500 g) centrifugation. The same

795 volume (10 mL) was collected from the culture of 5 days and 25 days after inoculation. **C)**

796 Confocal microscopy images of the upper phase and pellet after low-speed centrifugation.

797 The 25-day-old cultures were harvested and the samples were stained with the solution of

798 concanavalin A conjugating fluorescent dye (ConcA; cyan) without filtration. Cells and

799 empty parental cell walls can be distinguished by the presence of chlorophyll

800 autofluorescence (Chl; red). The ConA and Chl signals were obtained at 594 nm and 488 nm,

801 respectively. The yellow scale bar indicates 20 μm . **D)** Distribution of particle size in the 25-

802 day-old cultures. The population was compared between the autolysin treatment (+Autolysin)

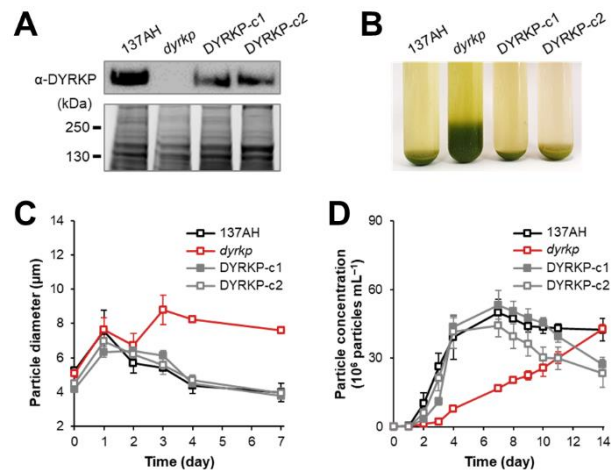
803 and no treatment (Control) groups after 30 min of treatment. The black arrow points to the

804 population of undigested parental cell wall debris. **E)** The amount of extracellular proteins in

805 the culture medium. All experiments were performed at least in three biological replicates (\pm

806 S.D).

807



808

809 **Figure 3. The complemented lines of *dyrkp* mutant (DYRKP-c1 and DYRKP-c2)**

810 **appeared the restoration of parental cell wall digestion ability. A)** Immunoblot of 137AH

811 strain, *dyrkp* mutant, and two complemented lines. The upper and below panels indicated

812 DYRKP antibody (α -DYRKP) and loading control, respectively. **B)** Morphology of pellets

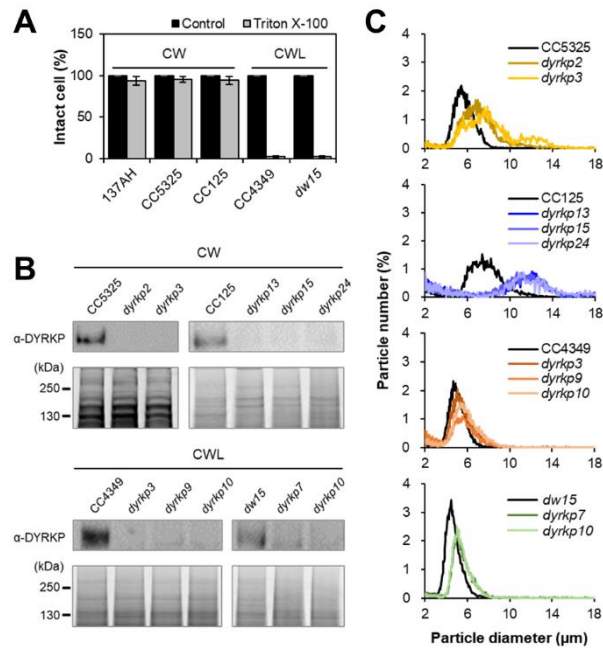
813 after low-speed (500 g) centrifugation. The same volume (10 mL) was collected from the

814 culture of 25 days after inoculation. **C)** Changes in mean particle diameter under standard

815 light condition. **D)** Changes in particle concentration under standard light condition; All

816 experiments were performed at least in three biological replicates (\pm S.D).

817



818

819 **Figure 4. The *dyrkp* mutants generated from other cell-walled background strains**

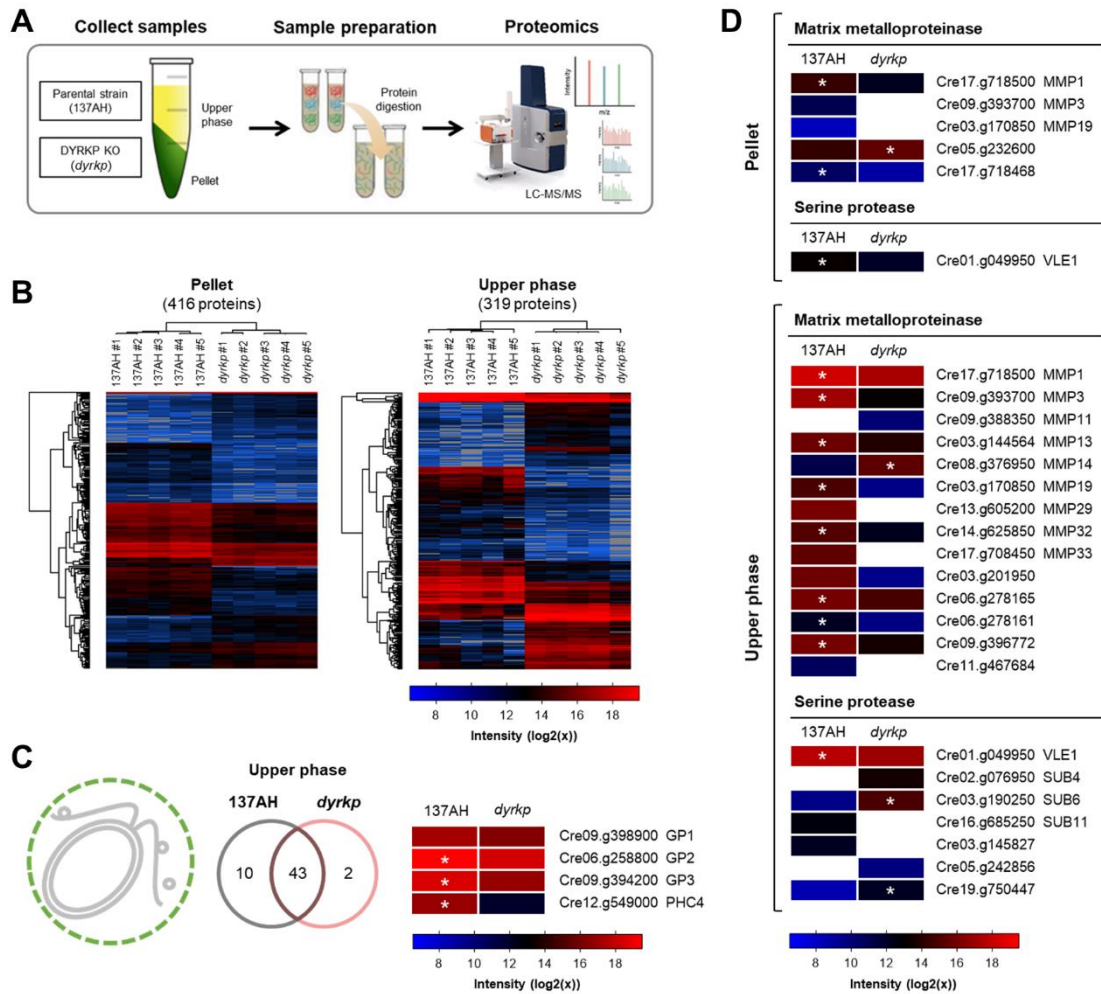
820 **formed palmelloid structures. A)** Cell wall integrity was tested by detergent (Triton X-100)

821 treatment in cell-walled (CW) and cell-wall-less (CWL) background strains. **B)** Immunoblot

822 to DYRKP antibody (α -DYRKP) in the upper panel and loading control in the below panel. **C)**

823 Distribution of particle size in the culture 3 days after inoculation.

824



825

826 **Figure 5. Cell wall proteins and ECM proteases were less abundant in the *dyrpk* mutant.**

827 **A)** Schematic diagram of the proteomic analysis workflow. The detailed analysis procedure is

828 shown in **Supplemental Fig. S6**. **B)** Hierarchical clustering based on Euclidean distance.

829 Five biological replicates in each group have high similarity to each other. **C)** The cell wall

830 proteins detected in the upper phase. The cell wall proteins in the upper phase were assumed

831 to be the cell wall proteins released from the parental cell walls. Venn diagram represents the

832 number of commonly or uniquely found proteins between the 137AH strain and the *dyrpk*

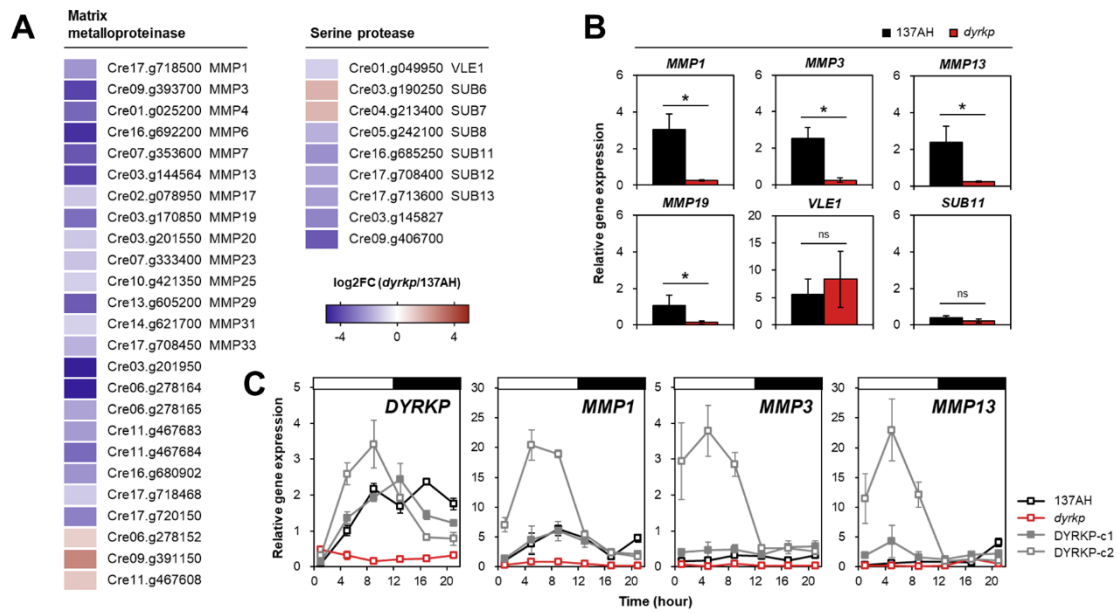
833 mutant. The heatmap shows the protein abundance of major cell wall proteins. Details are

834 shown in **Supplemental Fig. S7**. **D)** Heatmap of the matrix metalloproteinases and serine

835 proteases detected in the pellet and upper phase. Statistical analysis was performed using the

836 non-parametric Mann-Whitney test; * $p < 0.05$ (\pm S.D).

837



838

839 **Figure 6. MMP expression showed a positive correlation to DYRKP expression.** A) The

840 differentially expressed genes encoding the matrix metalloproteinases and serine proteases. B)

841 Gene expression levels of selected matrix metalloproteinases and serine proteases in 137AH

842 strain and *dyrkp* mutant. C) Gene expression pattern of *DYRKP*, *MMP1*, *MMP3*, and *MMP13*

843 in 137AH strain, *dyrkp* mutant, and two complemented lines (DYRKP-c1 and DYRKP-c2)

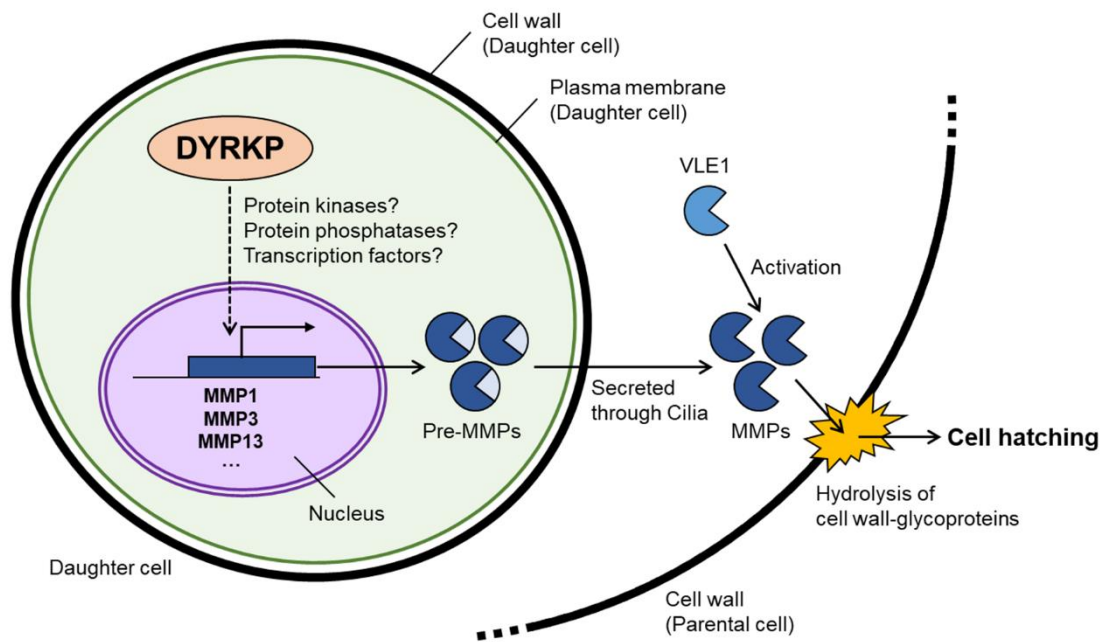
844 under light/dark conditions. The value was normalized by the reference gene (*RACK1*). All

845 experiments were performed at least in three biological replicates. Statistical analysis was

846 performed using the non-parametric Mann-Whitney test; * $p < 0.05$ (\pm S.D). The 'ns'

847 indicates no significance.

848



849

850 **Figure 7. Schematic diagram of the role of DYRKP in cell hatching.** DYRKP induces
851 increased transcription of *MMP1*, *MMP3*, and *MMP13* genes located in nuclear DNA through
852 phosphorylation of unknown protein kinases, protein phosphatases, or transcription factors.
853 Afterward, the translated protein in the pre-activated form of MMPs (pre-MMPs) is delivered
854 through cilia (Long et al., 2016; Zou and Bozhkov, 2021) and secreted in the form of an
855 ectosome from the cilia membrane (Wood et al., 2013; Long et al., 2016). Pre-MMPs are
856 activated by other proteases (Wilkinson et al., 2017); for example, pre-MMP1 is activated by
857 VLE1 (Kubo et al., 2009; de Carpentier et al., 2022). Activated MMPs then hydrolyze and
858 degrade the hydroxyproline-rich glycoprotein structure of the parental cell wall (Goodenough
859 and Lee, 2023), causing fragmentation of the parental cell wall. When the degradation of the
860 parental cell wall progresses sufficiently, the daughter cells are released (cell hatching), and
861 the remaining parental cell wall is completely degraded by MMPs that remain active.

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