

1 ***Chlamydomonas reinhardtii* Tubulin-Gene Disruptants for Efficient Isolation of**
2 **Strains Bearing Novel Tubulin Mutations**

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20 **ABSTRACT**

21 The single-cell green alga *Chlamydomonas reinhardtii* possesses two α -tubulin genes (*tua1*
22 and *tua2*) and two β -tubulin genes (*tub1* and *tub2*), with the two genes in each pair
23 encoding identical amino acid sequences. Here, we used an *aphVIII* gene cassette
24 insertional library to establish eight disruptants with defective *tua2*, *tub1*, or *tub2*
25 expression. None of the disruptants exhibited apparent defects in cell growth, flagellar
26 length, or flagellar regeneration after amputation. Because few tubulin mutants of *C.*
27 *reinhardtii* have been reported to date, we then used our disruptants, together with a *tua1*
28 disruptant obtained from the *Chlamydomonas* Library Project (CLiP), to isolate novel
29 tubulin-mutants resistant to the anti-tubulin agents propyzamide and oryzalin. As a result
30 of several trials, we obtained 8 strains bearing 7 different α -tubulin mutations and 24
31 strains bearing 12 different β -tubulin mutations. Some of these mutations are known to
32 confer drug resistance in human cancer cells. Thus, single-tubulin-gene disruptants are an
33 efficient means of isolating novel *C. reinhardtii* tubulin mutants.

34

35 **IMPORTANCE:** *Chlamydomonas reinhardtii* is a useful organism for the study of tubulin
36 function; however, only five kinds of tubulin mutations have been reported to date. This
37 scarcity is partly due to *C. reinhardtii* possessing two tubulin genes each for α - and
38 β -tubulin. Here, we obtained several strains in which one of the α - or β -tubulin genes was
39 disrupted, and then used those disruptants to isolate 32 strains bearing 19 mostly novel
40 tubulin mutations that conferred differing degrees of resistance to two anti-tubulin
41 compounds. The majority of the tubulin mutations were located outside of the drug-binding
42 sites in the three-dimensional tubulin structure, suggesting that structural changes underlie

43 the drug resistance conferred by these mutations. Thus, single-tubulin-gene disruptants are
44 an efficient means of generating tubulin mutants for the study of the structure–function
45 relationship of tubulin and for the development of novel therapies based on anti-tubulin
46 agents.

47

48 **Key Words:** microtubule, herbicide, anti-cancer drug

49

50 INTRODUCTION

51 Microtubules are fundamental cytoskeletal filaments that play pivotal roles in eukaryotic
52 cell functions such as cell division, intra-cellular transport, cell shape development, and
53 cilia and flagella assembly. Microtubules are produced by polymerization of α/β -tubulin
54 heterodimers. Most eukaryotic cells possess multiple genes encoding α - and β -tubulin. For
55 example, humans possess seven genes that encode α -tubulin and eight genes that encode
56 β -tubulin, with each gene encoding a slightly different amino acid sequence. The presence
57 of multiple genes for the two types of tubulin makes it difficult to study the properties of a
58 particular tubulin species by genetic analysis, because the effects arising from mutation of
59 one of the genes can be masked by the expression of the remaining intact genes.

60 The single-cell green alga *Chlamydomonas reinhardtii* is a useful experimental
61 organism for studying tubulin function because it possesses a small number of tubulin
62 genes and it produces microtubule-based organelles, flagella. In addition, there is a wide
63 range of genetic tools available and a large amount of biological data has been
64 accumulated for this species. In contrast to the majority of eukaryotes, *C. reinhardtii*
65 possesses only two genes (*tua1* and *tua2*) encoding α -tubulin and two genes (*tub1* and
66 *tub2*) encoding β -tubulin (1, 2). The two genes for each type of tubulin encode the same
67 amino acid sequence (2, 3), and the expression of all four genes is up-regulated after
68 flagellar excision (4). Whether the two genes in each pair are expressed independently of
69 each other has not yet been firmly established, but the genes do appear to be expressed
70 indiscriminately during flagella formation (4).

71 Although *C. reinhardtii* possess only two genes for each tubulin, the presence of more
72 than one gene expressing the same protein still makes it difficult to isolate tubulin mutants.

73 To date, only five kinds of tubulin mutations have been reported: a *tua1* mutation (Y24H)
74 that confers amiprofos-methyl (APM) and oryzalin resistance (upA12) (3); two kinds of
75 mutations in *tua2* (D205N and A208T) that confer colchicine hypersensitivity (*tua2-1* etc.,
76 suppressors of *uni-3-1*, a mutant lacking δ -tubulin) (5); and two mutations in *tub2* (K350E
77 and K350M) that confer colchicine resistance (*col^R4* and *col^R15*) (6). APM, oryzalin,
78 colchicine, and propyzamide are compounds that inhibit tubulin polymerization. These
79 compounds other than colchicine inhibit plant tubulin polymerization at low concentrations
80 and are used as herbicides.

81 Here, we isolated eight *tua2*, *tub1*, or *tub2* disruptants from an insertional library
82 comprising around 8000 clones (7). We also obtained a *tua1* disruptant from the
83 *Chlamydomonas* Library Project (CLiP) (8). We then used one of the *tub2* disruptants and
84 two double-disruptants possessing only one α -tubulin gene and one β -tubulin gene as
85 parent strains for the production of 32 mutants showing various degrees of resistance to
86 propyzamide and oryzalin. Thus, the use of single-tubulin-gene *C. reinhardtii* disruptants
87 enabled efficient isolation of a large number of tubulin mutants resistant to anti-tubulin
88 agents.

89

90 **RESULTS**

91 **Isolation of tubulin-gene disruptants**

92 A library of around 8000 clones was constructed by inserting the *aphVIII* gene cassette into
93 the genome of *C. reinhardtii* (7). Then, the library was screened by PCR using primer pairs
94 consisting of one primer targeting a consensus sequence of the four tubulin genes and
95 another primer targeting the *aphVIII* fragment. As a result, we isolated eight tubulin gene
96 disruptants: three showing *tua2* disruption (*tua2-A*, *tua2-B*, *tua2-C*), two showing *tub1*

97 disruption (*tub1-A*, *tub1-B*), and three showing *tub2* disruption (*tub2-A*, *tub2-B*, *tub2-C*).
98 Fig. S1A shows the sites of the *aphVIII* cassette insertion in the eight disruptants, and Fig.
99 S1B shows the PCR confirmation of the structure of the disrupted genes. In six of the
100 disruptants (*tua2-A*, *tua2-B*, *tua2-C*, *tub1-B*, *tub2-A*, *tub2-C*), the *aphVIII* cassette was
101 inserted into the gene. In the remaining two disruptants, the *aphVIII* cassette was inserted
102 after the open reading frame (*tub1-A*) or within an intron (*tub2-B*). In all eight disruptants,
103 *AphVIII* cassette insertion resulted in total disruption of mRNA expression of the affected
104 tubulin gene, as confirmed by northern blot analysis (Fig. S2A). For *tua1-A*, *tua2-A*,
105 *tub1-B*, and *tub2-A*, semi-quantitative real-time PCR was performed and again no
106 expression of mRNA from the tubulin genes was detected (Fig. S2B).

107 None of the disruptants exhibited any apparent defects in growth rate (data not
108 shown), tubulin expression (Fig. S2C), or flagellar regeneration after amputation (Fig.
109 S2D), suggesting that the disruptants still produced sufficient α/β -tubulin heterodimer for
110 their cellular functions via the remaining intact genes. The mean flagellar length was
111 comparable among the disruptants (see Fig. S2D). The five β -tubulin disruptants showed
112 some difference in their sensitivity to colchicine: *tub1-A* and *tub1-B* showed stronger
113 resistance while *tub2-A* showed weaker resistance than wild type (Fig. 1), although the
114 sensitivity somewhat varied among alleles (Fig. 1).

115

116 **Mutant isolation using tubulin-gene disruptants**

117 Next, we used the disruptants to isolate *C. reinhardtii* strains expressing tubulins with
118 missense mutations. Three parent strains were used: *tub2-A*, a double disruptant generated
119 by crossing *tua1-A* with *tub1-B*, and a double disruptant generated by crossing *tua2-A* and
120 *tub1-B*. As a result of 1-3 trials with each parental strain against oryzalin or propyzamide,

121 32 strains showing a total of 19 different tubulin missense mutations were isolated. Table 1
122 shows the obtained mutants classified by the gene affected, as well as the results of a
123 qualitative assessment of each strain's resistance to oryzalin and propyzamide. Most of the
124 oryzalin-resistant strains, as well as a propyzamide-resistant mutant (*pyz532*), had a
125 missense mutation in an α -tubulin gene. In contrast, most of the propyzamide-resistant
126 strains, other than *pyz532*, had mutations in a β -tubulin gene.

127 Figure 2 shows a predicted three-dimensional structure of *C. reinhardtii* α/β -tubulin
128 heterodimer labeled with the site of each missense mutation reported here and in previous
129 studies (3, 5, 6). Five of the isolates had mutations that have been reported previously: *ory2*
130 had a *tua1* Y24H mutation as did upA12 (3); *pyz8*, *pyz9*, and *pyz523* had a *tub2* K350E
131 mutation as did *col^R4* (6); and *pyz6* had a *tub2* K350M mutation as did *col^R15* (6). The five
132 mutants isolated in the present study exhibited stronger drug-resistance than the three
133 previously reported mutants (data not shown). This stronger drug-resistance may reflect the
134 fact that the mutants isolated here express only mutated α - or β -tubulin from a single gene,
135 whereas previously reported mutants express a mutated tubulin together with a wild-type
136 counterpart.

137 Some of the identified mutations involved the substitution of amino acids with
138 different charges. For example, the propyzamide-resistant missense strains *pyz2/pyz524*,
139 *pyz503*, and *pyz530/pyz534/pyz502/pyz525/pyz526/pyz527* expressed β -tubulins with the
140 mutations Q134H, E198L, and E198K, respectively. The isoelectric point (pI) values of
141 these β -tubulins predicted from their amino acid sequences were 4.59, 4.58, and 4.63,
142 respectively, which were greater than the pI of wild-type β -tubulin (4.55). We confirmed
143 the expression of β -tubulins with different pIs in those strains by two-dimensional

144 polyacrylamide gel electrophoresis (2D-PAGE) of axonemal proteins from the mutants and
145 wild type (Fig. 2). As expected, the spot of β -tubulin appeared at higher pH values in the
146 order *pyz530* (E198K) > *pyz2* (Q134H) > *pyz503* (E198L) > wild type. The 2D-PAGE
147 analysis also verified that each mutant expressed β -tubulin from only a single gene, since it
148 detected no β -tubulin spots with the wild-type pI in mutant samples.

149

150 **Novel tubulin mutant strains exhibited various sensitivities to anti-tubulin agents**

151 The mutant strains displayed various patterns of sensitivity to anti-tubulin agents (Table 1,
152 Fig. 1). Several strains showed high oryzalin resistance in the order *ory304* > *ory3* >
153 *ory205* > *ory313* > *ory314*. Three of these strains also showed hypersensitivity to
154 propyzamide in the order *ory304* > *ory205* > *ory3*, and one of these strains, *ory205*, was
155 also hypersensitive to colchicine. Several strains (*pyz2*, *pyz501*, *pyz503*, *pyz506*, *pyz513*,
156 *pyz529*, *pyz530*, and *pyz532*) showed strong propyzamide resistance, remaining viable on a
157 Tris–acetate–phosphate (TAP) agar plate containing more than 400 μ M propyzamide
158 whereas wild-type *C. reinhardtii* (CC-125) was barely viable at 40 μ M. Of these eight
159 mutant strains, three showed hypersensitivity to colchicine (*pyz532* > *pyz503* > *pyz2*) and
160 the remaining five showed resistance to colchicine (*pyz529*, *pyz513*, *pyz530*, *pyz501*, and
161 *pyz506*). The different sensitivities to colchicine and propyzamide in these mutants are
162 interesting because the two agents bind to almost the same position on the tubulin
163 heterodimer (9). Four of the eight propyzamide-resistant mutants exhibited oryzalin
164 resistance in the order *pyz530* > *pyz513* > *pyz2* > *pyz532*, and two, *pyz501* and *pyz529*,
165 were hypersensitive to oryzalin.

166

167 **DISCUSSION**

168 By screening an *AphVIII* insertional library, we isolated three disruptants lacking *tua2*, two
169 lacking *tub1*, and three lacking *tub2*. All were most likely null mutants (Fig. S2A).
170 Although these disruptants lacked one of their tubulin-encoding genes, their cytoplasmic
171 tubulin levels remained normal (Fig. S2C), suggesting the presence of an auto-regulatory
172 mechanism that maintains the tubulin mRNA level, as observed in other eukaryotic cells
173 (10). Indeed, in *tua1-A*, *tub1-B*, and *tub2-A*, the mRNA expression level of the remaining
174 α - or β -tubulin gene was increased approximately 2-fold compared with wild type (Fig.
175 S2B). Also, flagellar length, ability to produce flagella after amputation (Fig. S2D), and
176 overall cell growth rate did not noticeably differ from the wild-type growth rate (data not
177 shown). Thus, although *C. reinhardtii* possesses two α -tubulin genes and two β -tubulin
178 genes, a single gene for each type is enough to supply the tubulin necessary for its cellular
179 functions. However, it should be noted that the present findings do not mean that the two
180 genes for each tubulin have exactly the same function; rather, the two genes may differ
181 from each other in a subtle manner. For example, we observed that whereas the *tub1*
182 disruptants were resistant to colchicine, the *tub2* disruptants were sensitive although some
183 allele-specific variation was observed (Fig. 1). This suggests that there is some difference
184 in the regulation of gene expression that is dependent on the concentration of free tubulin
185 in the cytoplasm (11). Thus, how the two genes encoding the two tubulins differ in their
186 function and regulation warrants further investigation, and our single-tubulin-gene mutants
187 established here should be useful for such investigations.

188 Next, we used the disruptants to obtain mutants with resistance to two anti-tubulin
189 agents, propyzamide and oryzalin. Several rounds of trials to isolate mutants resistant to
190 one or both of the agents afforded 8 mutants with 7 different α -tubulin gene missense
191 mutations and 24 mutants with 12 different β -tubulin gene missense mutations. The

192 number of mutations obtained was much larger than the total number that has been
193 reported previously (i.e., 3 kinds of α -tubulin mutations and 2 kinds of β -tubulin
194 mutations)(3, 5, 6). In addition, we found that about one-third of the colonies picked from
195 the screening plates harbored a mutation in a tubulin gene (data not shown). Together,
196 these findings suggest that our approach of using single-tubulin-gene disruptants is a
197 highly efficient means of obtaining tubulin mutant strains.

198 How the sensitivity to anti-tubulin agents varied in the mutants is an important issue
199 that warrants clarification. Some of the tubulin mutants that conferred resistance to the
200 anti-tubulin agents had a mutation near to where the anti-tubulin agents bind to the
201 α/β -tubulin heterodimer (Fig. 3). The binding site of oryzalin, inferred from that of an
202 analogous compound, tubulysin M, is at the intra-dimer interface (12) close to the
203 α -tubulin mutation F351L (in strain *pyz532*). Other *ory* mutants whose mutation sites
204 occur independently of the tubulysin M-binding site may confer oryzalin resistance by
205 modulating the three-dimensional structure of α -tubulin. Likewise, a propyzamide-like
206 compound, 2RR, is known to bind at the inter-dimer interface (13) close to several of the
207 identified β -tubulin mutation sites: E198K/L (in
208 *pyz530/pyz534/pyz502/pyz525/pyz526/pyz527* and *pyz503*), I236N (in *pyz513*),
209 K350N/E/M (in *pyz528*, *col^R4/pyz8/pyz9/pyz523*, and *col^R15/pyz6*), and I368F (in
210 *pyz501/pyz504/pyz514/pyz535*). Other mutations may confer propyzamide resistance
211 through some structural change in α/β -tubulin. Another interesting observation is that the
212 β -tubulin mutation E198K conferred colchicine resistance whereas the E198L mutation
213 conferred high colchicine sensitivity. This suggests that the electric charge of E198 is a
214 critical determinant of colchicine sensitivity.

215 Several of the mutations detected in the present study are similar to those reported in
216 other organisms (Table S1). For α -tubulin, mutation F49C in *ory314*, F52L in *ory3*, and
217 S165A in *ory205/ory505* have been reported in a *Toxoplasma gondii* oryzalin-resistant
218 mutants (14, 15). For β -tubulin, mutation Q134H in *pyz2/pyz524* has been reported in a
219 *Beauveria bassiana* benzimidazole-resistant mutant (16); mutations E198K/L in
220 *pyz530/pyz534/pyz502/pyz525/pyz526/pyz527* and *pyz503* are found in fungi and
221 nematodes that confer benzimidazole resistance and phenylcarbamate hypersensitivities
222 (17-22); I236N in *pyz513* corresponded to the mutation responsible for resistance to the
223 anti-cancer drug 2-methoxyestradiol in human epithelial cancer cells (23); K350N in
224 *pyz528* corresponded to the mutations responsible for colcemid and vinblastine resistance
225 in Chinese hamster ovary (CHO) cells (24) and indanocine resistance in human leukemia
226 cells (25). Mutations Y24N, F138V, and F351L in α -tubulin (*ory304*, *ory313*, and *pyz532*)
227 and mutations L165Q, F266C and I368F in β -tubulin (*pyz506*,
228 *pyz529/pyz531/pyz533/pyz536*, and *pyz501/pyz504/pyz514/pyz535*) are being reported here
229 for the first time; further investigations are needed to examine whether these mutations are
230 responsible for altered drug sensitivity in other organisms.

231 Although the present study selected mutants based only on their resistance to two
232 anti-tubulin agents, use of other agents such as the microtubule-stabilizing agent paclitaxel,
233 or screening for other properties such as hypersensitivity to drugs, resistance to low
234 temperature, or deficiency in flagellar formation and motility will lead to the isolation of a
235 greater variety of mutants. Detailed analyses of many such mutants will deepen our
236 understanding of the structure–function relation of tubulins. Since some of the tubulin
237 mutations identified in the present study corresponded to mutations found in human
238 tubulins that confer drug resistance in cancer cells, we expect that studies of

239 *Chlamydomonas* tubulin mutants will contribute to the development of improved cancer

240 therapies.

241

242 MATERIALS AND METHODS

243 Isolation of tubulin-gene disruptants

244 Eight tubulin-gene disruptants were isolated from a library of mutants generated by
245 inserting the *aphVIII* gene (paromomycin resistance gene) into the genome of *C.*
246 *reinhardtii* (7). Disruptants *tua2-B*, *tua2-C*, *tub1-B*, *tub2-A*, *tub2-B*, and *tub2-C* were
247 isolated by performing PCR on the pooled transformants using primers targeting the
248 *aphVIII* sequence (PSI103-F2 and RB02) and two tubulin consensus sequences
249 (3'-Tus1891g and 3'-Tus1803g). A disruptant, *tub1-A*, was isolated using two alternative
250 tubulin consensus primers (5'-Tus1082c and 5'-Tus1596g). A disruptant, *tua2-A*, was
251 isolated using RB02 and an alternative consensus primer (3'-TuA2-3254g). Supplementary
252 Information 1 shows the primers used in the present study. After screening, the disruptants
253 were sequenced in the vicinity of their disrupted tubulin gene (Macrogen Japan Co.,
254 Japan).

255 In addition, a *tua1* disruptant (LMJ.RY0402.158052; referred to as *tua1-A* in the
256 present study) was obtained from the *Chlamydomonas* Library Project (8); this disruptant
257 has a long insertion composed of two facing paromomycin-resistant CIB1 cassettes
258 immediately before the stop codon in *tua1*.

259 The disruptants were backcrossed with wild-type *C. reinhardtii* (CC-125) and
260 selected for tubulin-gene disruption by PCR before use. Double disruptants were
261 constructed by standard methods (26), and selected from tetrads by PCR analysis.

262

263 Isolation of anti-tubulin drug resistant mutants

264 *C. reinhardtii* strains whose *tub1* or *tub2* was disrupted with or without *tua2* disruption
265 were grown to the mid-log phase and then irradiated by ultraviolet light until about 50% of

266 the cells were killed. The culture was spread on TAP-agar plates containing 20 μ M
267 propyzamide or 10 μ M oryzalin, kept in the dark for 12 h, and then incubated under light
268 for 5–10 days. Colonies that appeared were transferred to liquid TAP medium in 96-well
269 plates containing the same concentration of propyzamide or oryzalin. From each culture
270 that grew, genomic DNA was extracted and subjected to PCR using the following primers:
271 5'-ChlaTuA1_long969 and 3'-TuA6260 (for *tua1*), 5'-Tua2-10g and 3'-TuA2-3288g (for
272 *tua2*), 5'-tub1-33c and 3'-tub1-1667c (for *tub1*), and 5'-EcoTuB2-upper and
273 3'-XhoTuB2-lower (for *tub2*). The PCR products were processed for DNA sequencing
274 (Macrogen Japan Co.).

275

276 **Drug-resistance test**

277 *C. reinhardtii* strains were grown in liquid TAP medium until the mid-log phase, and then
278 diluted or concentrated to 5×10^6 cells/mL. Then, 3 μ L of culture was spotted on a
279 TAP-agar plate containing Propyzamide Reference Material (0–400 μ M), Oryzalin
280 Standard (0–40 μ M), or colchicine (0–8000 μ M) (all from Fujifilm Wako Pure Chemical
281 Co., Japan) and cultured for a week at 26°C under 12-h light/12-h dark conditions. A
282 wild-type strain (CC-125) and a colchicine-resistant mutant strain (*col^R4* (6)) were used as
283 references.

284

285 **Three-dimensional structure prediction of *C. reinhardtii* α/β -tubulin heterodimer**

286 The three-dimensional structure of the *C. reinhardtii* α/β -tubulin heterodimer was
287 predicted by using FAMS software (27) based on a known tubulin tetramer structure
288 obtained from the Protein Data Bank (PDB ID: 1Z2B (28)). To determine the amino acids

289 that most likely interacted with the examined drugs, *in silico* molecular docking analyses
290 were performed using the ChooseLD program (29).

291

292 **2D-PAGE of isolated axonemes**

293 Axonemes were isolated from the *C. reinhardtii* strains by using standard procedures (30).

294 A small aliquot of axonemal precipitate (~2 or 10 μ g) was extracted with a buffer

295 containing 5 M urea and 2 M thiourea and analyzed by 2D-PAGE as described previously

296 (31). Since α - and β -tubulin are modified post-translationally, the loading amount was

297 adjusted so that their major forms only were detectable by silver staining. The predicted pI

298 values of the wild-type and mutant tubulins were calculated by using the EMBOSS

299 database and the Sequence Manipulation Suite, which is a collection of JavaScript

300 programs for examining short protein sequences

301 (https://www.bioinformatics.org/sms2/protein_iep.html) (32).

302

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311

312 **COMPETING INTERESTS**

313 The authors have no competing interests to declare.

314

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318

319

320 **AUTHOR CONTRIBUTIONS**

321 TKM and RK designed and conducted the research and wrote the paper. YO performed the
322 real-time polymerase chain reaction analysis. TY and HF produced a temporal library of
323 *Chlamydomonas reinhardtii* carrying the *aphVIII* gene.

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Table 1 Tubulin missense mutants isolated in this study.

Gene altered	Strain	Mutation (DNA)	Mutation (protein)	Resistance to		
				oryzalin	propyzamide	colchicine
<i>tua1</i>	<i>ory2</i>	TAC->CAC	Y24H	+++	+/-	+/-
	<i>ory3</i>	TTC->CTC	F52L	++++	--	+
	<i>ory205, ory505</i>	TCC->GCC	S165A	++++	--	--
	<i>pyz532</i>	TTC->TTA	F351L	+	+++	---
<i>tua2</i>	<i>ory304</i>	TAC->AAC	Y24N	++++	---	+/-
	<i>ory314</i>	TTC->TGC	F49C	+++	-	+/-
	<i>ory313</i>	TTC->GTC	F138V	++++	+/-	+/-
<i>tub1</i>	<i>pyz530, pyz534</i>	GAG->AAG	E198K	+++	+++	++
	<i>pyz529, pyz531, pyz533</i>	TTC->TGC	F266C	---	+++	+++
<i>tub2</i>	<i>pyz2, 524</i>	CAG->CAT	Q134H	++	+++	-
	<i>pyz506</i>	CTG->CAG	L165Q	+/-	+++	+
	<i>pyz502, pyz525, pyz526, pyz527</i>	GAG->AAG	E198K	N.E.	N.E.	N.E.
	<i>pyz503</i>	GAG->TTG	E198L	+/-	+++	--
	<i>pyz513</i>	ATC->AAT	I236N*	+++	+++	+++
	<i>pyz536</i>	TTC->TGC	F266C	N. E.	N.E.	N.E.
	<i>pyz8, pyz9, pyz523</i>	AAG->GAG	K350E	+	+	++++
	<i>pyz6</i>	AAG->ATG	K350M	N.E.	N.E.	N.E.
	<i>pyz528</i>	AAG->AAT	K350N**	N.E.	N.E.	N.E.
	<i>pyz501, pyz504, pyz514, pyz535</i>	ATC->TTC	I368F	---	+++	+

438 N.E., not examined; +, resistant; -, sensitive; +/-, wild-type level. The number of + and -
 439 symbols represents the qualitative difference among the strains. *, mutation found in
 440 human cancer cells resistant to 2-methoxyestradiol(23).**, mutation found in human
 441 cancer cells resistant to indanocine and 2-methoxyestradiol(25, 33).

442 **Figure 1.** Drug sensitivities of tubulin-gene disruptants and missense mutant strains.
443 Cell growth on TAP agar plates containing different concentrations of anti-tubulin drugs.
444 Three plates each inoculated with nine strains were used for each condition, as summarized
445 in panel (A). Sensitivity to colchicine (B), oryzalin (C), and propyzamide (D) was
446 examined.

447

448 **Figure 2.** Two-dimensional polyacrylamide gel electrophoresis analysis of axonemes from
449 strains *pyz2*, *pyz503*, and *pyz530*.

450 Protein extracts of axonemes of wild type (CC-125), *pyz2*, *pyz503*, and *pyz530* were loaded
451 on a two-dimensional polyacrylamide gel and stained with silver. pH range: 4.0–7.0. (A)
452 Electrophoresis pattern of wild-type axoneme (~10 μ g loaded). (B) Portions of
453 polyacrylamide gels showing the major spots of α - and β -tubulin. Upper panel shows a
454 close-up of the area indicated by the box in (A). The lower four panels show the
455 polyacrylamide gels after loading approximately 2 μ g of axoneme. The predicted pIs of the
456 wild-type and three mutant α - and β -tubulins are indicated to the right of the panels.

457

458 **Figure 3.** Predicted three-dimensional structure of *Chlamydomonas reinhardtii*
459 α/β -tubulin heterodimer showing the mutations reported in the present and previous
460 studies.

461 Light gray, α -tubulin; dark gray, β -tubulin. Altered amino acids are shown as sphere
462 representations. The binding sites of tubulysin M (red, an oryzalin-like compound) and
463 2RR (blue,

464 3-[(4-{1-[2-(4-aminophenyl)-2-oxoethyl]-1H-benzimidazol-2-yl}-1,2,5-oxadiazol-3-yl)a

465 mino]propanenitrile, a propyzamide-like compound) were determined by applying the
466 alignment command in MacPyMol software to the tubulin structures 4ZOL and 4O2A
467 reported in the presence of these compounds (12, 13). The orange stick representations
468 show GTP (in α -tubulin) and GDP (in β -tubulin). Mutations identical to previously
469 reported mutations are marked with asterisks: *, (3); **, (5); and ***, (6).

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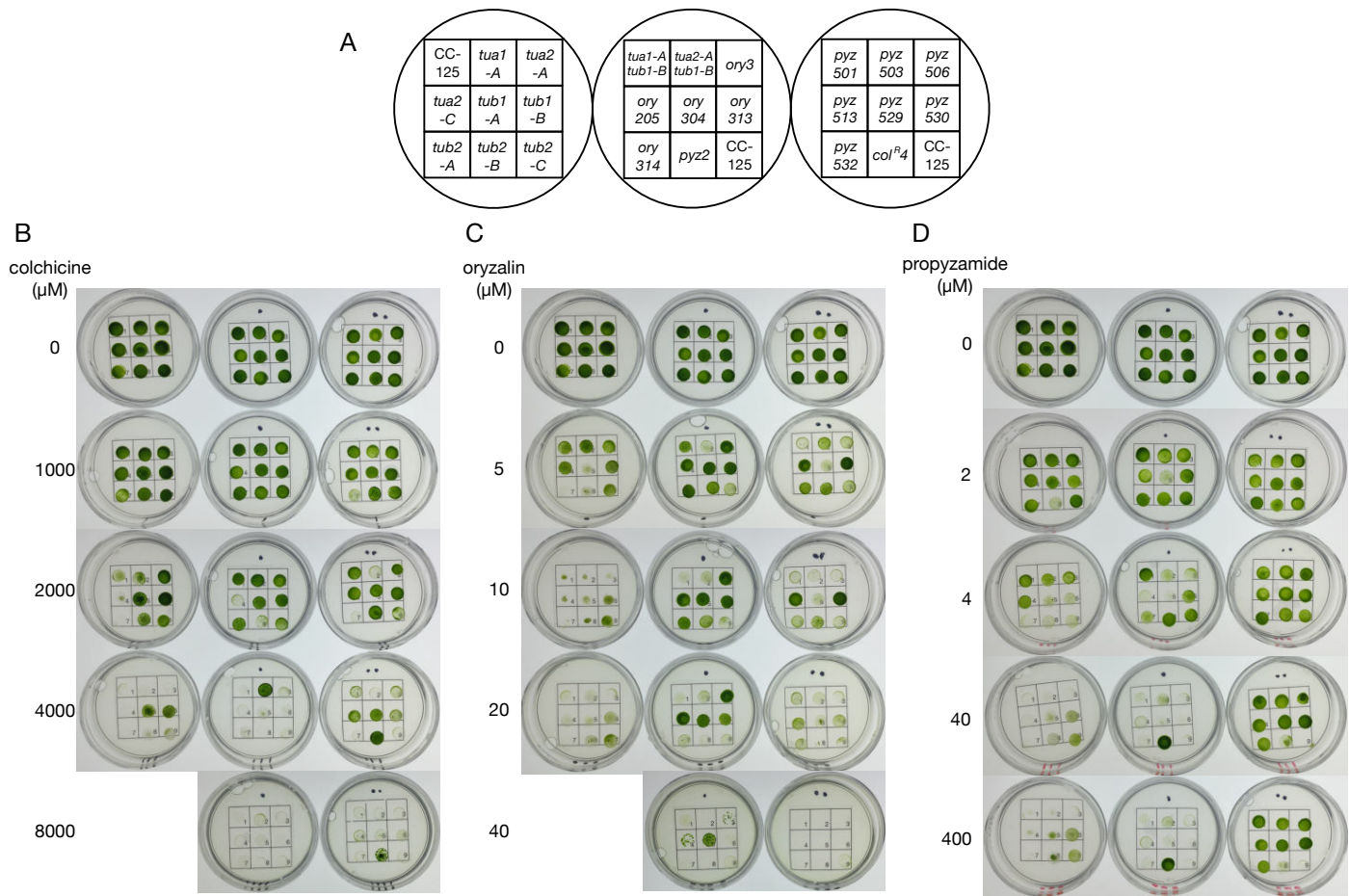


Figure 1
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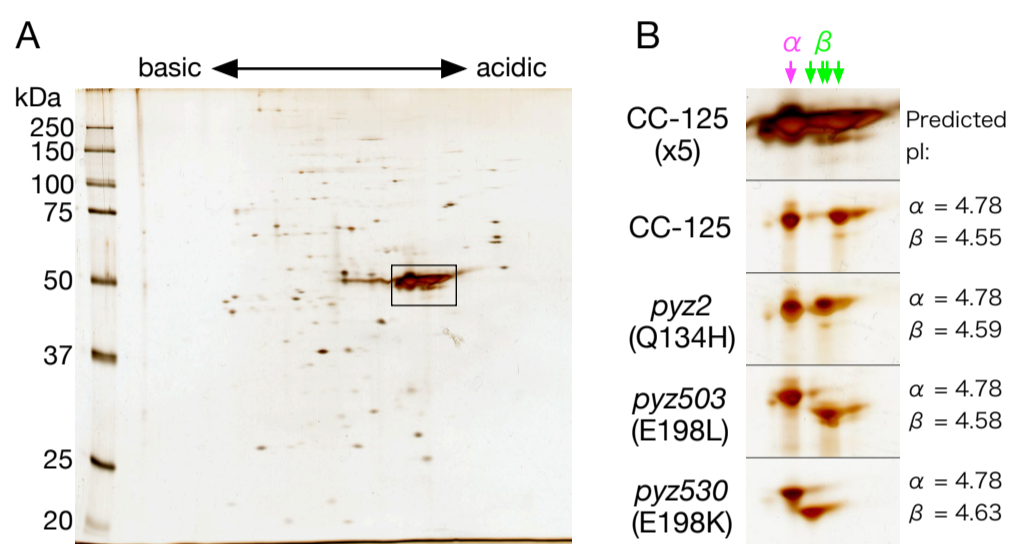


Figure 2
Kato-Minoura, T et al.

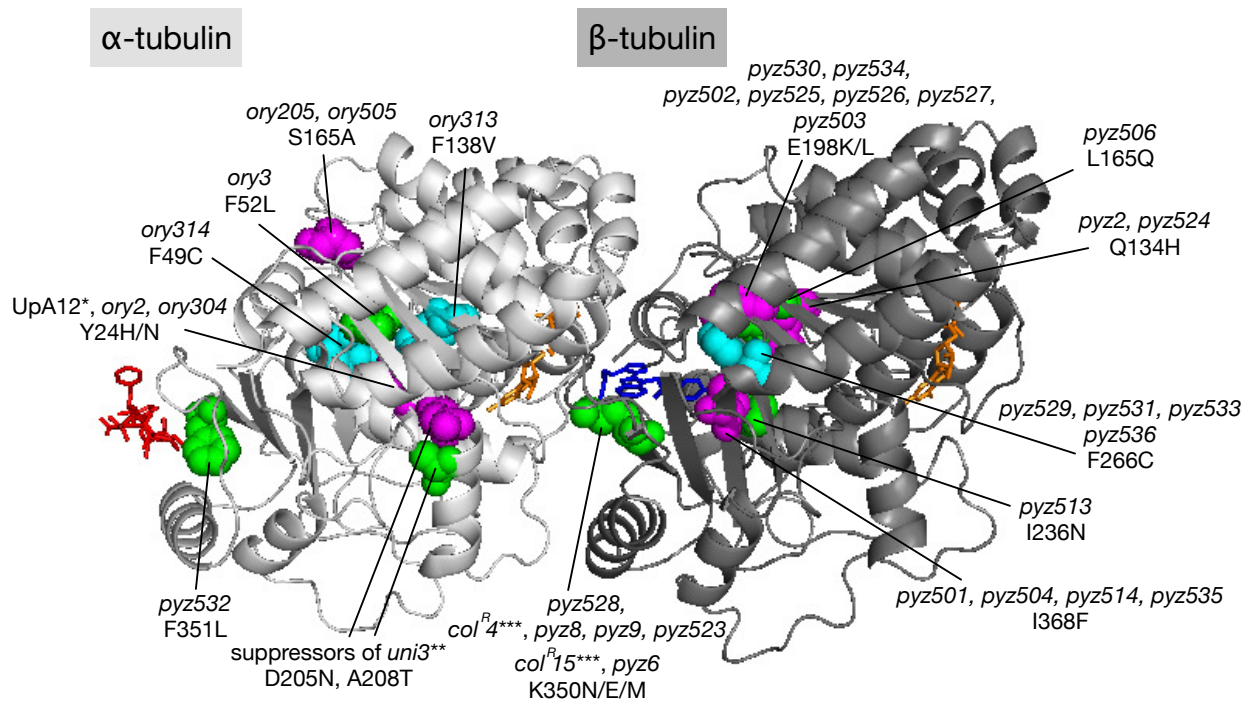


Figure 3
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