

1 Title:

2 Phytohormone inhibitor treatments phenocopy brassinosteroid and gibberellin dwarf
3 mutant interactions in maize.

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5 Authors:

6 Norman B. Best^{1,2}, Guri Johal^{3,4}, Brian P. Dilkes^{2,4}

7

8 Addresses:

9 ¹Department of Horticulture & Landscape Architecture; Purdue University; West
10 Lafayette, IN, USA

11 ²Department of Biochemistry, Purdue University; West Lafayette, IN, USA

12 ³Department of Botany & Plant Pathology; Purdue University; West Lafayette, IN, USA

13 ⁴Purdue Center for Plant Biology; Purdue University; West Lafayette, IN, USA

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20 Corresponding authors:

21 bdilkes@purdue.edu (BPD)

22

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25

26 **Abstract**

27 Phytohormone biosynthesis produces metabolites with profound effects on plant growth
28 and development. Modulation of hormone levels during developmental events, in
29 response to the environment, by genetic polymorphism, or by chemical application can
30 reveal the plant processes most responsive to a phytohormone. In many cases, chemical
31 inhibitors are applied and the levels of specific phytohormones are measured to determine
32 if, and which, phytohormone is affected by a molecule. In many cases, the sensitivity of
33 biochemical testing has determined multiple pathways affected by a single inhibitor.
34 Genetic studies are not subject to this problem, and a wealth of data about the
35 morphological impacts of hormone biosynthetic inhibition has accumulated through the
36 study of enzyme mutants. We previously identified a complex interplay between
37 brassinosteroid (BR) and gibberellin (GA) in maize, where the interdependence of the
38 two differs dependent on the developmental context. We found that: GA is required for
39 loss of BR to induce retained pistils in the tassel florets (POPIT); BR is required for the
40 loss of GA to induce tiller outgrowth; BR and GA are additive for plant height; BR has
41 no effect on the induction of anther retention in ear florets of GA mutants. In this work,
42 we sought to assess the specificity of three triazole inhibitors of cytochrome P450s by
43 determining their abilities to recapitulate the phenotype of double mutants. The GA
44 biosynthetic inhibitors uniconazole (UCZ) and paclobutrazol (PAC) were applied to the
45 BR biosynthetic mutant *na2* and all double mutant phenotypes were recovered in the
46 UCZ treatment. PAC was unable to suppress the retention of pistils in the tassels of *nana*
47 *plant2* (*na2*) mutant plants. The BR biosynthetic inhibitor propiconazole (PCZ)
48 suppressed tiller outgrowth in the GA biosynthetic mutant *dwarf5* (*d5*). All treatments
49 were additive with genetic mutants for effects on plant height. Due to additional
50 measurements done here but not in previous studies of the double mutants, we detected
51 new interactions between GA and BR biosynthesis affecting plastochron index and tassel
52 branching. These experiments, a refinement of our previous model, and a discussion of
53 the extension of this type of work are presented.

54

55 **Introduction**

56 Phytohormones control plant growth and development at very low concentrations.
57 The entire life cycle of plants are influenced by the availability and amount of these
58 metabolites. The number of identified classes of phytohormones currently includes
59 auxins, cytokinins (CK), abscisic acid (ABA), jasmonic acid, salicylic acid,
60 strigolactones, brassinosteroids (BR), and gibberellins (GA).¹⁻³ The biosynthesis,
61 transport, signaling, and responses to these phytohormones have been intensely studied in
62 many species, including *Zea mays* (maize). Both biochemistry and genetic studies
63 identified genes encoding phytohormone biosynthetic steps and mutants blocking
64 hormone biosynthesis in many genetic model systems including *Pisum sativum* (pea),
65 *Solanum lycopersicum* (tomato), *Arabidopsis thaliana* (Arabidopsis), and maize.⁴⁻⁸
66 Despite these studies, the molecular identities of many steps are as yet unidentified.
67 Furthermore, the roles of phytohormones in processes that do not occur in established
68 genetic models are less well explored. Biochemical inhibitors, particularly the triazoles,
69 have elucidated physiological effects of these phytohormones in plant systems that lack
70 the expansive genetic resources of species with vibrant genetics research communities
71 like Arabidopsis and maize. The utility of inhibitor treatments for determining the roles
72 of phytohormones in plant processes is determined by the specificity of the biochemical
73 inhibitors. A test of this specificity is available if we determine the concordance between
74 phenotypes induced by reducing hormone biosynthesis via genetic ablation of
75 biosynthetic enzymes and the phenotypes observed after chemical inhibition.

76 A number of classical maize dwarf mutants are now demonstrated to encode GA
77 and BR biosynthetic genes. Many mutants of maize affecting steps in gibberellin
78 biosynthesis have been identified, including *ent-copalyl synthase* (*anther ear1*), *ent-*
79 *kaurene synthase* (*dwarf5*; *d5*), CYP88A3 (*d3*), and GA3oxidase (*d1*).⁹⁻¹³ In addition to
80 reduced stature, GA biosynthetic mutants of maize exhibit retained anthers in the
81 normally pistillate ear florets, increased tiller outgrowth, shortened and broadened leaves,
82 and decreased primary tassel branching.¹⁰ Maize mutants in three steps of BR
83 biosynthesis are known, including disruptions of a $\Delta 24$ -sterol reductase (*nana plant2*;
84 *na2*), 5α -steroid reductase (*na1*), and CYP85A1/BR-6-oxidase (*brassinosteroid*
85 *deficient1*; *brd1*).^{8,14-17} In addition to dwarfism, the maize BR biosynthetic mutants have

86 presence of pistils in the tassel flowers (POPIT) and reduced tiller branch outgrowth.
87 Genetic interactions between BR and GA biosynthetic mutants were developmentally
88 specific and varied. Mutants in BR and GA were additive for plant height, indicating
89 independent effects on this trait. BR mutants were epistatic to GA mutants for tiller
90 outgrowth but GA mutants were epistatic to BR mutants for POPIT.

91 Specific and effective inhibition of GA and BR biosynthesis should recapitulate
92 the phenotypes of these three differing genetic interactions seen in the mutants.
93 Interpretation of inhibitor studies requires confidence in the specificity and amplitude of
94 inhibition caused by chemical treatment. Inhibitors of plant hormone biosynthesis are
95 widely used as plant growth regulators in industry and basic science to modify plant
96 metabolism and phenotype. Triazole PGRs are widely used, cheap to produce, and
97 effective on both plants and fungi. The chemical structures of three triazole inhibitors
98 containing the 1,2,4-triazole ring used in this study are shown in Figure 1. PGR
99 specificity within plants is known to be poor with wide impacts across P450s (Figure 2).
100 It is currently unknown how wide these impacts actually are.¹⁸

101 Propiconazole (PCZ) was first identified as a fungal growth inhibitor that blocked
102 the C-14 demethylation of lanosterol (Figure 1A).^{19,20} Propiconazole is a specific and
103 potent inhibitor of BR biosynthesis, as evidenced by the reversal of growth inhibition by
104 the co-application of BR.²¹ PCZ binds the CYP90D1 of Arabidopsis (Figure 2A) but it is
105 unknown whether PCZ also binds other P450s in BR biosynthesis such as
106 CYP90B1/DWF4, CYP90C1/ROT3, or CYP90A1/CPD.²² The related BR biosynthesis
107 inhibitor, brassinazole, effectively binds to CYP90B1 and the effectiveness of these two
108 inhibitors may stem from an ability to simultaneously inhibit multiple CYP90/CYP85 BR
109 biosynthetic enzymes. Co-treatment of Arabidopsis with epi-brassinolide reversed the
110 phenotypic effects of both brassinazole and PCZ, suggesting that the effects of these
111 triazoles were predominantly due to BR inhibition.^{21,23,24} PCZ is also used as a fungicide
112 in agriculture due to inhibition of ergosterol biosynthesis. It is possible that PCZ also
113 affects structural sterol biosynthesis in plants. To date, no experiments have been
114 conducted on PCZ and enzymes in other metabolic pathways to determine effects on
115 other phytohormones or sterol biosynthesis.

116 Paclobutrazol (PAC) was also first identified as a fungicide (Figure 1B).²⁵ In
117 fungi, it inhibits C-14 demethylation, similar to PCZ. In barley and celery cell cultures it
118 was shown to inhibit the 14 α -demethylase CYP51 required for the conversion of
119 obtusifoliol to Δ^8 -¹⁴sterol (Figure 2A).²⁶⁻²⁸ Supplementation of cell cultures with
120 stigmasterol at high concentrations, or a mix of cholesterol and stigmasterol, reversed the
121 growth retardation that resulted from PAC treatment of celery cell cultures. Ratios of
122 stigmasterol to sitosterol, a 22-desaturation carried out by CYP710A, were also altered in
123 PAC treated cells suggesting that this P450 may also be a target. The BR precursor
124 campesterol was also reduced by PAC treatment, and it may be that PAC achieves
125 growth retardation by affecting structural sterols as well as BR levels.²⁷ PAC is also
126 known to reduce GA levels by inhibiting the *ent*-kaurene oxidase/CYP701 in plants
127 (Figure 2B).¹⁸ Hedden & Graebe²⁹ showed the percent conversion of the CYP701 targets,
128 *ent*-kaurene, *ent*-kaurenol, and *ent*-kaurenal, into later intermediates was greatly inhibited
129 by 1 μ M (2RS, 3RS)-PAC in a cell-free system from *Cucurbita maxima* endosperm. The
130 different enantiomers of PAC have demonstrated differential effects on plants as
131 compared to fungi. The 2R-3R diastereoisomer of PAC affected sterol biosynthesis in
132 fungi but had no effect on plant height at 170.2 μ M while the 2S 3S diastereoisomer
133 dramatically reduced plant height at 34.0 μ M.²⁵ A number of other downstream effects
134 have been documented in PAC-treated plants including reduced accumulation of 1-
135 aminocyclopropane-1-carboxylic acid (ACC) and ethylene production in water-stressed
136 apple seedlings, however the mode of action is unknown.³⁰

137 The uniconazole (UCZ) structure differs from PAC by one desaturation leading to
138 a double bond (Figure 1C) and has been shown to inhibit GA biosynthesis by affecting
139 the same enzymes (Figure 2B).^{18,31,32} However, the height of a GA₃-insensitive DELLA
140 mutant in *Helianthus annuus* (sunflower) was further reduced by UCZ-treatment.³³ UCZ
141 has also been shown to affect structural sterol levels in *Oryza sativa* (rice) and pea
142 (Figure 2A).^{34,35} In addition, UCZ treatment reduced castasterone accumulation in pea
143 and blocked BR-induced tracheary element differentiation in *Zinnia elegans* L.
144 mesophyll cells.^{36,37} Whether these phenotypes result from BR-inhibition, the dependence
145 of BR-induced tracheary element differentiation on GA, or some other impact is
146 unknown. Recently, it has been shown that UCZ affects ABA catabolism by inhibiting

147 CYP707A in Arabidopsis and tobacco cell cultures (Figure 2D).³⁸⁻⁴⁰ UCZ also affects
148 cytokinin biosynthesis; however, there are conflicting reports depending on the species
149 tested. In Arabidopsis it inhibited trans-Zeatin accumulation (Figure 2C) but UCZ
150 treatment in rice and *Glycine max* showed higher levels of trans-Zeatin.⁴¹⁻⁴³ Izumi et al⁴¹
151 showed that ethylene levels were higher but that ABA was unaffected in UCZ-treated
152 rice. Of the three PGRs used in this study, UCZ has been the most studied. The
153 conflicting reports on hormonal levels in different species highlights the necessity of
154 studying the specificity of PGRs within the species being tested due to potential
155 differences in uptake, transport, or binding affinity by potential target P450s.¹⁸

156 We sought to test the model for BR and GA interaction in maize derived from
157 genetics experiments.¹⁷ Given the multiple interactions between the mutants it is less
158 likely that the same effects, and direction of interaction, will be generated by non-specific
159 growth retardation or general toxicity of a chemical treatment. In addition, using the
160 genetics experiments as orthogonal data confirming the utility of these inhibitors'
161 specificity in maize would permit stronger interpretation of inhibitor studies across the
162 grasses, including species without the genetic resources of maize. Orthogonal data, if
163 confirmatory, will also bolster our confidence in the interpretation of previous genetic
164 interactions between BR and GA discovered in maize.¹⁷

165 We treated the *na2-1* BR- and *d5* GA-biosynthetic mutants with each of the three
166 triazole inhibitors described above and measured phenotypes known to respond to BR
167 and GA reduction. All three inhibitors were additive with the two mutants for plant
168 height. UCZ had the strongest impact on plant height. Consistent with the expectation for
169 reduced BR biosynthesis, PCZ enhanced the POPIT phenotype of BR mutants but was
170 unable to induce POPIT in wild-type siblings or *d5* mutants at the concentrations
171 employed. PCZ application phenocopied the BR-GA interaction and suppressed the
172 outgrowth of tillers in *d5* mutants. Just like GA dwarfs, PAC treatment induced tillering
173 in wild-type plants. Unlike GA dwarfs, PAC treatment did not rescue the POPIT
174 phenotype of BR mutants nor did it induce anthers in ear florets. Only UCZ recapitulated
175 all GA dwarf phenotypes including the effects on floral organ persistence and interactions
176 with loss of BR biosynthesis, as UCZ treatment induced anther-ear in wild type and *na2-*
177 *1*.

178 **Results**

179 **Effect of hormone biosynthetic inhibitors on *na2-1* and wild-type siblings**

180 As described previously,¹⁷ *na2-1* mutants overall height and organ lengths were shorter,
181 lower leaves were more upright, mutants flowered later, and a majority of plants had
182 POPIT. Mock-treated wild type and *na2-1* are shown in Figure 3 along with a PCZ-
183 treated wild-type plant. PCZ treated wild types strongly resembled mock treated *na2-1*
184 plants (Figure 3A-C). Treatment of *na2-1* plants with PCZ displayed an enhanced
185 phenotype, indicating synergy between the loss of *na2* function and the effects of PCZ.
186 Organs and internodes were shorter in PCZ-treated plants than in mock-treated plants for
187 both *na2-1* and wild-type siblings (Table 1 and Figure 4A-B). PCZ treatment had a
188 dramatic effect on *na2-1* mutant plant height indicating either bioactive BR accumulation
189 in the mutant or non-specific inhibition of other growth-promoting metabolites by PCZ
190 application. Primary tassel branch number was 10 fold lower in PCZ treated *na2-1* than
191 in treated wild-type siblings, whereas mutants and wild types were indistinguishable in
192 the mock treated samples (Table 1). Tassel length was also non-additively affected by
193 PCZ application to *na2-1* mutants, causing a decrease by 40% in wild type and 80% in
194 *na2-1* mutants. Just as *na2-1* mutants flowered later than wild types, PCZ treatment
195 extended days to flowering in wild types. PCZ treatment of the mutants further enhanced
196 this delay (Table 1). Yet, in neither comparisons between *na2-1* and wild type nor in PCZ
197 treatments did the number of leaves per plant change. Taken together, PCZ reproduced
198 all but the POPIT phenotype in wild-type treated plants and enhanced all loss-of-BR
199 phenotypes in the *na2-1* mutant, including increased POPIT penetrance.

200 As expected if PAC primarily acts via the inhibition of GA biosynthesis, all
201 organs and overall plant height were reduced by PAC treatment in both *na2-1* and wild-
202 type siblings (Table 1 and Figure 4A,C). Treatment of wild-type siblings with PAC
203 resulted in a significant increase in tiller outgrowth similar to the GA biosynthetic mutant
204 *d5*. The mock-treated *na2-1* mutants did not tiller and *na2-1* suppressed the tiller
205 outgrowth affected by PAC-treatment. Thus, PAC treatment phenocopied the *na2-1/d5*
206 double mutants, where *na2* was required for tillering induced by a loss of GA. Unlike
207 *na2-1/d5* double mutants, PAC did not suppress POPIT in *na2-1* mutants indicating that
208 PAC treatment was insufficient to cause all of the changes in plant growth affected by

209 genetic loss of GA biosynthesis. Similar to the phenotypes of *d5* mutants,¹⁷ PAC
210 treatment of wild-type siblings caused the lower leaf angle to become more upright.
211 Contrastingly, the upper leaf angles of wild-type siblings went the opposite direction and
212 were less upright following PAC treatment. As was the case for tillering, *na2-1*
213 suppressed the effects of PAC on leaf angle and no discernable difference between mock
214 and PAC treatments were observed. Similar to PCZ, primary tassel branch numbers of
215 *na2-1* plants were reduced by PAC treatments. Flowering time was increased by PAC
216 treatment of both *na2-1* and wild types. Surprisingly, PAC treatment reduced the number
217 of leaves before the top ear and the total number of leaves per plant in *na2-1* but not wild-
218 type siblings. This demonstrates that PAC reduced the plastochron index in *na2-1*
219 mutants. For all phenotypes except suppression of POPIT and induction of anther-ear,
220 PAC recapitulated the phenotypes observed in *d5* and *d1* mutants as well as the genetic
221 interactions of *d5* and *na2*.

222 Treatment with UCZ reproduced all of the effects of a loss of GA biosynthesis
223 and the genetic interactions between *d5* and *na2*. The effect of UCZ on organ length and
224 plant height of *na2-1* and wild-type siblings was similar to what was observed in *na2-1*/
225 *d5* double mutants, PAC, and PCZ treatments (Table 1 and Figure 4A, D). Consistent
226 with a strong inhibition of GA biosynthesis, UCZ caused ears of both *na2-1* and wild-
227 type siblings to exhibit florets with persistent anthers. Similar to *d5* mutants and PAC
228 treatment, UCZ treated wild types profusely tillered. Similar to the *na2-1/d5* double
229 mutants, and consistent with the genetic BR and GA interactions, *na2-1* strongly
230 suppressed UCZ-induced tillering. UCZ treatment reduced the frequency of POPIT in
231 *na2-1* by over two fold, similar to the suppression of POPIT in *na2-1/d5* double mutants,
232 but this difference was not statistically significant from mock treated *na2-1* mutants.
233 UCZ treatments had a synergistic effect on leaf width in combination with *na2-1* and
234 caused *na2-1* leaves to become narrower. UCZ resulted in a dramatic decrease in primary
235 tassel branch number, similar to the other inhibitors. UCZ treatment delayed the number
236 of days before tassel emergence in both mutants and wild-type siblings. Similar to PAC
237 treatment, both the number of leaves before the top ear and the total number of leaves per
238 plant were dramatically reduced in *na2-1*, but not wild-type siblings, by UCZ treatment.
239 Thus, for UCZ treatments this extreme delay without additional production of leaves

240 resulted in an increase in plastochron index in *na2-1* UCZ treated plants. Taken together,
241 UCZ treatments reproduced all of the phenotypes present in GA biosynthetic mutants and
242 consistently affected the same interactions with loss of BR biosynthesis observed in *na2-*
243 *1/d5* double mutants.

244

245 **Effect of hormone biosynthetic inhibitors on *d5* and wild-type siblings.**

246 Similar to what was found previously,¹⁷ *d5* mutants flowered later, were shorter, had
247 shorter organs, exhibited persistent anthers in the ear florets, had reduced primary tassel
248 branch numbers, and tillered profusely. Shown in Figure 5 are mock-treated wild-type
249 and *d5* plants compared to PAC-treated and UCZ-treated wild-type siblings. Similar to
250 what was observed for the wild-type siblings of *na2-1*, PAC treatment largely
251 recapitulated the phenotypes affected by loss of function mutants in GA biosynthesis
252 (Figure 5A-C). At the concentrations employed, one out of sixteen wild types exhibited
253 anther-ear and the reduction in tassel length was less in PAC treated wild types than in
254 mock-treated *d5* mutants. PAC further reduced the height and organ lengths of *d5* plants
255 (Table 2 and Figure 6A, C). Similar to the effects of PAC on primary tassel branch
256 number in *na2-1* mutants, PAC reduced primary tassel branch number to zero in *d5*. The
257 *d5* mutants had greater leaf widths than wild-type controls. PAC treatment increased leaf
258 widths of wild types but PAC treated *d5* mutants had narrower leaves than mock treated
259 mutants. The *d5* mutants had the same number of nodes and, unlike PAC-treated *na2-1*,
260 treatment of *d5* mutants with PAC did not affect node number. PAC treatment delayed
261 flowering in wild-type siblings and further delayed flowering in *d5* mutants as compared
262 to mock treatments. Thus, a delay without increased leaf numbers suggests an increase in
263 plastochron index, though not as dramatic as the synergistic effects of inhibitor treatment
264 in *na2-1* mutants. All PAC treated phenotypes were consistent with a loss of GA, but the
265 low frequency of anther-ear, similar to the lack of suppression of POPIT in *na2-1*,
266 suggest that at this concentration PAC is a weak GA inhibitor in maize.

267 As was observed in applications of UCZ to *na2-1* and wild types, application of
268 UCZ to *d5* and wild-type siblings recapitulated all loss-of-GA phenotypes (Figure 5A-B,
269 D). Application of UCZ decreased plant height and the lengths of all organs and
270 internodes for both *d5* and wild-type siblings (Table 2 and Figure 6A, D). Similar to the

271 effects of UCZ on *na2-1*, UCZ narrowed both the upper and lower leaves of *d5* mutants.
272 Unlike PAC treatment, UCZ induced anthers in the ear florets of all wild-type siblings,
273 consistent with it effectively inhibiting GA biosynthesis at the concentration employed.
274 UCZ substantially reduced primary tassel branch number in *d5* mutants, just as it had for
275 *na2-1*. While not affecting an increase in the number of leaves produced per plants, UCZ
276 treatment increased days to tassel emergence for both *d5* and wild-type siblings. Thus,
277 UCZ also appears to increase flowering time though no synergism affecting node number
278 was observed between *d5* and UCZ, consistent with the mode of action affecting the
279 same pathway.

280 The BR inhibitor PCZ reduced overall plant height, internode lengths, and the
281 lengths of all measured organs for both *d5* and wild-type siblings (Table 2 and Figure
282 6A-B). Primary tassel branching decreased dramatically in both PCZ treated *d5* mutants
283 and their wild-type siblings. PCZ application at this concentration did not reproduce the
284 *na2-1* effect on POPIT in the wild-type controls and as expected from the *d5* suppression
285 of POPIT in *na2-1/d5* double mutants no POPIT was observed in PCZ treated *d5* plants.¹⁷
286 Consistent with the inability of *na2-1* to suppress anther-ear in *d5*,¹⁷ PCZ treatment failed
287 to suppress persistence of anthers in ear florets of *d5* mutants. The *d5* mutants exhibited
288 increased tillering, which we previously demonstrated was dependent on BR.¹⁷ PCZ
289 treatment suppressed tiller outgrowth induced by the loss of *d5*, just as *na2-1* suppressed
290 tiller outgrowth in *na2-1/d5* double mutants.¹⁷ PCZ treatment increased the angle of the
291 upper leaf in *d5* mutants and the angle of the lower leaf in wild types. Days to tassel
292 emergence were greater in PCZ treated *d5* and wild-type siblings consistent with the
293 flowering delay in double mutants and PAC treatment of *na2-1* plants. Thus, PCZ
294 treatment of *d5* mutants and wild-type siblings delayed flowering, but no discernable
295 difference was observed in the total number of nodes. Different from treatment of *na2-1*
296 treated with PAC, synergism was not detected when *d5* was combined with PCZ
297 treatment at this concentration for effect of total node number.

298

299 **Discussion**

300 We report here the effects of PCZ, PAC, and UCZ treatment on the phenotypes of
301 *na2-1*, *d5*, and wild-type siblings at maturity. PCZ-treatment of wild-type plants was able

302 to recapitulate the phenotype of *na2-1*, except for the POPIT phenotype (Table 1 and
303 Figure 3B-C). However, PCZ at twice the concentration (500 μ M) used in our study
304 induced POPIT in wild-type siblings of the *nal-1* mutant, which was in a unknown
305 mixed-parentage *Mu*-active genetic background.⁸ Off-target and non-specific effects of
306 inhibitors are a greater concern as concentrations go up and we sought to minimize these
307 with lower concentrations. PCZ-treatment did decrease plant height and increase POPIT
308 in *na2-1* (Table 1). These results, and our unpublished observations that *brd1* mutants
309 and *nal/na2* double mutants are more severely dwarfed than *nal* or *na2* single mutants
310 suggests that disruption of this gene may not be a complete knock-out of brassinosteroid
311 biosynthesis. Alternative bioactive BRs can accumulate in rice brassinosteroid
312 biosynthetic mutants due to the existence of a bypass pathway to C29/28 and C27
313 bioactive BRs and the same alternative BR pathway may exist in maize.⁴⁴ Targeted
314 metabolic analysis of these alternative BRs in the maize mutants is required to determine
315 if this is the case.

316 UCZ treatment to wild-type plants was able to phenocopy *d5* mock-treated plants
317 and UCZ treated *na2-1* plants displayed all the interactions predicted for loss of both GA
318 and BR biosynthesis from *na2-1/d5* double mutant studies (Table 1 and 2). UCZ reduced
319 plant height, promoted tiller outgrowth, induced anther persistence in the ear, and
320 suppressed the *na2-1* mutant dependent POPIT (Table 1 and 2). These phenotypic results
321 are consistent with UCZ affecting maize growth via specific inhibition of GA
322 biosynthesis at the concentrations employed. However, the suppression of tiller formation
323 induced by a loss of *na2* was the least effective of all treatment and genetic combinations
324 observed (Table 1). Our experiments do not rule out inhibition of P450 enzymes outside
325 of GA biosynthesis. Indeed it was established that UCZ inhibits ABA catabolism in
326 *Arabidopsis* at concentrations lower than employed here and that specific inhibition of
327 recombinant CYP707A occurs in with an IC50 of 68nM (Figure 2D).⁴⁵ This reaction
328 contributes to the formation of phaseic acid which has been demonstrated to have
329 additional biological activities of its own (Figure 2D).^{46,47} The perfect agreement of the
330 developmental phenotypes caused by UCZ and the GA mutants was somewhat surprising
331 in light of these additional modes of action (Figure 2A-D). It is possible that the weak
332 suppression of tiller formation by *na2-1* in UCZ treatments may result from inhibition of

333 other P450s. It is formally possible, though we have no evidence indicating this is the
334 case, that UCZ might inhibit P450s in strigolactone biosynthesis or that ABA might
335 enhance tillering in maize. The simplest interpretation of our results is that UCZ affects
336 these changes in development via inhibition of GA, for which there is ample evidence.¹⁸
337 Further research, particularly work comprehensively evaluating the binding and catalytic
338 inhibition by these compounds on isolated or recombinant P450s, as was done for ABA
339 catabolism and UCZ, is needed to test these hypotheses and establish the specificity of all
340 triazole inhibitors.

341 PAC treatment was unable to phenocopy all of the effects of GA deficient
342 mutants in maize. PAC treatment of wild type and *na2-1* did not promote anther retention
343 in the ear florets nor did it suppress POPIT in *na2-1* plants (Table 1). This contrasts with
344 both UCZ treatments and our previous genetic results.¹⁷ The concentration of PAC used
345 was the same as UCZ, but it may be that PAC is a weaker inhibitor as has been
346 demonstrated in sunflower, *Impatiens wallerana*, *Salvia splendens*, *Tagetes erecta* L.,
347 and *Petunia hybrid*.^{48,49} If PAC is effectively inhibiting GA biosynthesis, this would
348 contradict maize double mutant analyses where GA biosynthetic mutants were epistatic
349 to BR biosynthetic mutants for the expression of POPIT.¹⁷ More likely the discordance
350 between PAC and genetic ablation resulted from incomplete inhibition of GA
351 biosynthesis by our repeated root drenches with 60 μ M PAC. The effects of treatment on
352 height and stimulation of tiller outgrowth in wild-type plants, and suppression of
353 outgrowth by *na2-1*, matched the effects of reduced GA biosynthesis (Table 1 and 2).
354 The inability of PAC treatment to induce anther-ear in any genotype, except for *d5*, or
355 suppress POPIT in *na2-1* may be due to the concentration used, unspecific inhibition of
356 other pathways besides GA biosynthesis (Figure 2), or combination of the two. The
357 observation that PAC growth arrest can be reversed by application of sterols raises the
358 possibility that PAC achieves some height reduction by affecting both BR and GA
359 levels.²⁷ If BR biosynthesis is inhibited by PAC treatment this could increase the
360 penetrance of POPIT in the *na2-1* mutant, as was observed in PCZ treatment, and require
361 greater reduction in GA to prevent floral organ persistence. However, the lack of anthers
362 in ear florets in PAC treatments, which were observed in all maize GA mutants,
363 suggested that PAC might simply be a weaker GA biosynthesis inhibitor.

364 One unexpected finding was the effect of GA biosynthesis on tassel branch
365 numbers. The *d5* mutants had fewer primary tassel branches than wild-type siblings but
366 *na2-1* mutant primary tassel branch numbers were unaffected in both the results of this
367 study and our previous work (Table 1 & Table 2).¹⁷ Treatment of wild-type plants with
368 PAC or UCZ also reduced primary tassel branch numbers. Unlike the *na2-1* mutants,
369 PCZ treatment reduced tassel branch number, however the interpretation of these results
370 is complicated by the potential for genetic background effects. The wild-type background
371 of the *d5* mutants, but not the wild-type siblings of *na2-1*, had fewer primary tassel
372 branches following PCZ treatment. The *na2-1* mutants did not have fewer branches in
373 mock treatments but PCZ treatment of mutants did reduce tassel branch numbers. The
374 inability of PCZ to reduce tassel branching in *na2-1* wild-type siblings casts some doubt
375 into whether the full tassel branching phenotype of *na2-1* might be different in the
376 genetic background of our *d5* mutants. Residual BR biosynthetic activity in all *na2* alleles
377 is suggested by the stronger phenotype of *brd1* mutants and *na1/na2* double mutants
378 (Dilkes and Best, data not shown) and it may be that the residual BRs are sufficient to
379 maintain normal tassel branching in *na2-1* mutants.¹⁶ Alternatively, PCZ might have
380 effects on regulators of tassel branching encoded by P450s outside of BR biosynthesis.
381 All triazoles affected branching in *na2-1* and *d5* mutants. In our previous work,
382 increasing GA by exogenous application to *na2-1*, *d5*, and wild type growing apices did
383 not alter tassel branch numbers.¹⁷ Additional work determining the physiological basis of
384 triazole application on tassel branching may uncover as yet unknown impacts of GA in
385 tassel architecture, or implicate triazoles in the inhibition of other branch-promoting
386 signals.

387 We repeatedly treated plants with PGRs and this continuous treatment was
388 required to observe the dramatic decrease of plant height. This requirement suggests that
389 maize can catabolize or inactivate these PGRs over the course of a few days. Reduction
390 in the concentrations of bioactive PGRs should permit the biosynthesis of phytohormones
391 to return and promote growth in responsive cells. We ceased treatment at tassel
392 emergence during the elongation of the uppermost internodes and the lengths of these
393 internodes were not as affected in our treated plants (Figure 4). We propose that PGR

394 inactivation rather than phytohormone-independent growth underlies these differences in
395 internode responses.

396 Days to tassel emergence was significantly longer for *na2-1* and *d5* (Tables 1 and
397 2) compared to their wild-type siblings similar to our earlier work.¹⁷ Wild-type siblings
398 treated with PCZ, PAC, or UCZ had greater days to tassel emergence than controls.
399 Every mutant-triazole combination further increased days to tassel emergence. When the
400 *na2-1* mutant was combined with PAC or UCZ treatment, this resulted in a synthetic
401 interaction producing fewer nodes even though plants took 2-3 weeks longer to flower.
402 The plastochron index is the rate of lateral organ initiation from the meristem. We
403 quantified total number of nodes and time to tassel emergence, so it is possible that we
404 could overestimate the effect of these treatments as decreased elongation may delay
405 emergence by a few days. However, the average increase of days to tassel emergence of
406 *na2-1* or *d5* treated with any one inhibitor was an average of 19.0 days or 13.4 days,
407 respectively. These results could be due to synergistic effects between BR and GA or
408 inhibition of another cytochrome P450 by the triazoles. Overexpression of the maize
409 *plastochron1* gene, which encodes cytochrome P450 CYP78A1, increases leaf number
410 but decreased plastochron index.⁵⁰ Loss of function of CYP78A1 exhibited slower leaf
411 elongation, but interpretation is limited by the presence of multiple CYP78A paralogs in
412 the maize genome.

413 The ability to combine chemical and genetic inhibition of phytohormone
414 biosynthesis to assess interactions and combinatorial effects is limited only by resource
415 availability. The discovery of compounds with additional modes of action would enable
416 work on the effects of all phytohormones on plant development. An additional triazole
417 was identified by Ito et al⁵¹ that inhibits strigolactone (SL) biosynthesis. This inhibitor,
418 TIS13, effectively reduced SL levels in roots and root exudates of rice and increased tiller
419 outgrowth of the second tiller. In these tests, PAC and UCZ had no effect on SL levels in
420 root or root exudates, eliminating off-target inhibition of SL biosynthesis as a possible
421 side-effect of PAC and UCZ treatments in rice. SLs control tiller outgrowth in maize.⁵² If
422 SL also affects tassel branching, we would expect treatment of maize with TIS13 or
423 GR24, a synthetic SL, to cause a decrease or increase of primary tassel branching,
424 respectively. It may be that loss of GA affects tillering and reduced tassel branching via

425 SL accumulation or signaling. A similar study to this one, using TIS3 application to GA
426 and BR biosynthetic mutants of maize as well as UCZ, PCZ, and PAC treatment of the
427 *carotenoid cleavage dioxygenase8* mutant of maize should provide a test of this
428 hypothesis.⁵² Similarly, identification of specific inhibitors of cytochrome P450s in other
429 metabolic pathways would permit combinatorial assessment with available mutants to
430 explore metabolite function in maize.

431 In conclusion, PCZ treatment of wild-type siblings largely phenocopied *na2-1*
432 mutants and PAC or UCZ treatment predominantly phenocopied *d5* mutants. PAC did
433 not affect sexual organ persistence, unlike GA biosynthetic mutants or UCZ treatment.
434 There was no strong evidence of non-specific growth inhibition by these three triazole
435 inhibitors in maize. Unexpectedly, primary tassel branch number was reduced in *na2-1* or
436 *d5* treated with PCZ, PAC, or UCZ. Thus, either all three triazoles another pathway
437 controlling tassel branching or BR and GA biosynthetic pathways interact to affect tassel
438 branching. Plastochron index was synergistically increased by PCZ, PAC, or UCZ
439 treatment of both *na2-1* and *d5*. The mechanism for this is unknown, and we did not
440 measure flowering time in *na2-1/d5* double mutants in our previous study.¹⁷ The
441 similarity of the effects on plant height, tiller outgrowth, and floral organ persistence in
442 the mutant analyses and inhibitor treatments were consistent with the genetic model
443 proposed previously.¹⁷ The concordance of phenotypes increased the confidence of
444 interpretation for UCZ and PCZ as GA and BR inhibitors, respectively. Using these
445 inhibitors in the greenhouse via simple repeated pot drench will allow for rapid
446 assessment of phytohormone function, even in the absence of genetic resources.
447 Confidence in the interpretation of phenotypes resulting from inhibitor application should
448 encourage exploration of BR and GA effects on growth and development in grass species
449 such as *Sorghum bicolor*, *Panicum virgatum*, *Saccharum officinarum*, *Miscanthus*
450 *sinensis*, and *Setaria viridis*.

451

452 **Materials and Methods**

453 **Plant growth and genetic materials**

454 Maize was planted in the winter of 2013 at the Purdue Horticulture Plant Growth Facility
455 in 2 gallon pots with 2:1 mixture of Turface MVP:peat germinating mix and irrigated
456 with fertilizer (MiracleGro Exel at 200ppm). Supplemental light was provided by sodium
457 lamps for a L:D cycle of 16:8 with target temperatures of 27 °C Day 21 °C Nighttime. All
458 experiments were planted as complete randomized blocks, controlling for genotype and
459 treatment. Maize genotypes *na2-1*, *d5*, and their respective wild types were described
460 previously.¹⁷ Both mutants are recovered from segregating families. As such all
461 comparisons are between mutants and wild-type siblings.

462 **Inhibitor treatments**

463 All inhibitors were supplied to plants as a soil drench. Inhibitors were dissolved in
464 methanol and added to irrigant to a final concentration of 0.15% methanol, 250 μM
465 propiconazole (94% purity; ORICO Global, Zhuhai, China), 60 μM uniconazole (95%
466 purity; Seven Continent, Zhangjiagang, China), or 60 μM paclobutrazol (0.4%;
467 formulated as *Bonzi*, Syngenta, Basel, Switzerland). Inhibitor concentrations were chosen
468 based on soilless media inhibition of efficacy and to closely phenocopy respective genetic
469 mutants.⁵³ A mock treatment consisting of 0.15% methanol was used as the control for all
470 experiments. Plants were treated first at 10 days after germination (DAG) and on every
471 subsequent 3rd or 4th day until tassel emergence. At the time of tassel emergence,
472 treatments were stopped.

473 **Phenotypic measurements and statistical analysis**

474 In total, 256 plants were phenotyped for an array of growth and developmental
475 parameters. Plant height was measured as the distance from the soil to the uppermost leaf
476 collar. The width at the widest point and the length from the leaf collar to the tip of the
477 blade was determined for the second uppermost leaf (one below the flag leaf) and the leaf
478 below the uppermost ear. These are described as the upper leaf and lower leaf in the
479 tables. Leaf angle was measured as the difference from a 90° projection from the stem
480 and the abaxial side of the leaf; greater values indicate a more upright leaf. The number
481 of nodes on each plant, the length of each node, the node of the uppermost ear, the
482 number of tassel branches, and number of tillers per plant were all recorded at maturity

483 after stripping the plant of leaves. Tassels were inspected for POPIT and given a binary
484 yes/no score. Ears were inspected for anthers and given a binary yes/no score. Flowering
485 time was measured as DAG to visible tassel emergence from the leaf whorl. Statistical
486 analysis of differences between genotypes and between treatments for continuously
487 variable traits were performed by ANOVA with post-hoc pairwise tests by the Holm-
488 Sidak method implemented in Daniel's XL Toolbox add-in (ver. 7.2.6,
489 <http://xltoolbox.sourceforge.net>) for Microsoft Excel (Redmond, WA). For the binary
490 traits, differences were tested by Fisher's exact tests using a Bonferroni correction for
491 multiple tests that sets a corrected $p < 0.05$ at the nominal $p < 0.002$ as implemented in the
492 Excel add-in Real Statistics Resource Pack (ver. 3.3.1, <http://real-statistics.com>).⁵⁴

493

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502 is offered in memory of Sewall Wright for his stunning breakthrough in biochemical
503 genetics one century ago.

504

505

506 **Figure 1. Structures of chemicals used in this study.**

507 Chemical structure of (A) propiconazole, (B) paclobutrazol, and (C) uniconazole.

508

509 **Figure 2. All three plant growth regulators are known to affect more than one**

510 **pathway.** (A) Brassinosteroid, (B) gibberellin, (C) cytokinin, and (D) ABA metabolic

511 pathways. Metabolites are indicated in black text and enzymes by black arrows with

512 respective names in grey text. Functionally characterized enzymes in maize are indicated

513 in parentheses. Previously identified enzymes inhibited by PCZ (yellow), PAC (purple),

514 and UCZ (green) are indicated by colored ovals.

515

516 **Figure 3. Effects of *na2-1* or PCZ on maize plant architecture.** Mock treated (A) wild-

517 type plant and (B) *na2-1* plant. (C) PCZ-treated wild-type plant.

518

519 **Figure 4. Effects of plant growth regulators and genotype on internode length.** Mean

520 internode lengths in cm and SD of (A) mock-treated, (B) PCZ-treated, (C) PAC-treated,

521 and (D) UCZ-treated *na2-1* and wild-type siblings.

522

523 **Figure 5. Effects of *d5*, PAC, and UCZ on maize plant architecture.** Mock treated (A)

524 wild-type plant and (B) *d5* plant. (C) PAC-treated and (D) UCZ-treated wild-type plant.

525

526 **Figure 6. Effects of plant growth regulators and genotype on internode length.** Mean

527 internode lengths in cm and SD of (A) mock-treated, (B) PCZ-treated, (C) PAC-treated,

528 and (D) UCZ-treated *d5* and wild-type siblings.

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Table 1. Morphometric analysis of *na2-1* and heterozygous wild-type siblings treated with PCZ, PAC, and UCZ.

	Mock +/na2-1	<i>na2-1/na2-1</i>	+PCZ +/na2-1	<i>na2-1/na2-1</i>	+PAC +/na2-1	<i>na2-1/na2-1</i>	+UCZ +/na2-1	<i>na2-1/na2-1</i>
<i>n</i>	18	14	17	9	15	17	17	13
plant height ^a	175.9 ±22.1a	30.6 ±4.8b	24.5 ±6.3c	7.1 ±2.0d	39.4 ±11.9e	9.7 ±2.8f	18.2 ±5.3g	6.5 ±2.2d
days to tassel ^a	57.8 ±3.6a	67.9 ±2.8b	64.8 ±5.5b	86.6 ±13.9cd	67.3 ±4.6b	80.9 ±7.1c	80.2 ±5.1c	93.3 ±9.2d
tillers per plant ^a	0.3 ±0.7a	0 ±0a	0 ±0a	0 ±0a	3.1 ±0.7b	0.1 ±0.2a	4.5 ±0.8c	1.0 ±1.0a
plants with POPIT ^b	0 (0%)a	9 (64%)bc	0 (0%)a	9 (100%)b	0 (0%)a	13 (76.5%)bc	0 (0%)a	3 (23%)ac
plants with anthers in ears ^b	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	17 (100%)b	13 (100%)b
tassel branches ^a	14.6 ±4.1a	12.4 ±4.7a	12.5 ±4.3a	1.1 ±1.5b	3.8 ±1.7c	0.7 ±1.4b	0.2 ±1.0b	0 ±0b
total nodes ^a	15.7 ±1.6a	14.9 ±1.2ab	15.9 ±1.2a	13.9 ±1.8abc	15.7 ±1.6a	13.1 ±1.2c	16.1 ±2.1a	13.4 ±1.5bc
node of top ear ^a	10.6 ±1.2a	10.2 ±1.5ab	10.5 ±1.4a	10.1 ±1.8ab	11.3 ±1.7a	8.9 ±1.3b	12.0 ±2.0a	8.9 ±1.4b
tassel length ^a	50.3 ±5.2a	28.4 ±5.9bc	31.3 ±6.6c	4.9 ±1.7d	39.8 ±7.3e	13.3 ±6.1f	22.5 ±7.1b	8.8 ±4.5df
angle of upper leaf ^a	45.0 ±27.0ab	65.7 ±17.4a	64.1 ±17.9a	89.4 ±1.4c	26.0 ±16.6b	62.9 ±24.4ac	54.1 ±22.7a	59.6 ±15.6a
length of upper leaf ^a	48.3 ±11.5a	21.6 ±11.9bc	20.0 ±8.4bc	13.1 ±4.0cd	25.3 ±7.2b	11.1 ±3.3d	13.5 ±3.5cd	5.7 ±1.0e
width of upper leaf ^a	5.9 ±1.1a	4.3 ±2.0abc	3.6 ±1.3bc	2.5 ±0.6cd	5.9 ±1.6a	3.4 ±1.3cd	4.8 ±1.3ab	2.2 ±0.7d
angle of lower leaf ^a	44.2 ±10.2a	64.6 ±10.3b	75.6 ±6.1c	67.2 ±4.4b	61.3 ±10.6b	59.4 ±15.1b	62.9 ±12.8b	59.6 ±16.0b
length of lower leaf ^a	99.8 ±9.3a	59.5 ±7.6b	56.9 ±8.4bc	18.4 ±2.9d	51.1 ±4.5c	26.3 ±5.9e	32.1 ±7.9e	16.4 ±6.2d
width of lower leaf ^a	8.7 ±1.0a	10.1 ±1.0a	9.1 ±1.6a	4.7 ±0.9b	11.4 ±1.1c	8.3 ±2.1ad	9.9 ±2.1a	6.5 ±1.4d

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^aData are presented as means with SD. Lowercase letters indicate connecting letter report as determined by ANOVA with post-hoc analysis using the Holm-Sidak algorithm with $P < 0.05$. ^bNumber of plants with tassel seeds or anthers in ear florets, with the percentage of plants in parentheses. Lowercase letters indicate connecting letter report as determined by Fisher's exact test with $P < 0.01$.

538 Table 2. Morphometric analysis of *d5* treated with PCZ, PAC, and UCZ.

539

	Mock		+PCZ		+PAC		+UCZ	
	+ <i>d5</i>	<i>d5/d5</i>	+ <i>d5</i>	<i>d5/d5</i>	+ <i>d5</i>	<i>d5/d5</i>	+ <i>d5</i>	<i>d5/d5</i>
<i>n</i>	16	16	16	16	16	16	16	16
plant height ^a	196.3±17.8a	33.8±7.3b	26.4±5.2c	12.2±2.7d	45.5±12.8e	10.1±1.9f	26.4±7.1c	6.5±1.3g
days to tassel ^a	52.5±4.4a	63.3±4.0bc	58.3±5.8d	72.9±5.6e	60.0±5.5bd	78.8±6.0e	66.4±4.5c	78.3±13.5e
tillers per plant ^a	0.3±0.4ab	3.1±1.4cd	0±0a	0.6±0.6b	2.9±1.2c	3.1±1.5c	5.2±2.3d	3.9±1.7cd
plants with POPIT ^b	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a	0 (0%)a
plants with anthers in ears ^b	0 (0%)a	16 (100%)b	0 (0%)a	15 (94%)b	1 (6%)a	16 (100%)b	16 (100%)b	16 (100%)b
tassel branches ^a	23.9±6.1a	8.9±4.1b	15.8±7.8c	3.8±3.0d	5.1±4.7d	0±0e	1.6±1.5f	0±0e
total nodes ^a	14.1±1.2a	14.4±2.1a	14.2±0.8a	14.9±2.0a	14.7±1.2a	15.0±2.3a	14.9±1.7a	13.9±2.2a
node of top ear ^a	8.6±1.3a	8.5±1.3a	9.6±1.1a	9.3±1.8a	9.8±0.8a	8.4±2.3a	9.5±2.0a	7.8±2.5a
tassel length ^a	63.3±4.7a	30.5±2.1b	41.2±12.9c	21.4±3.7d	55.5±7.0e	15.1±4.4f	37.3±6.9c	10.8±3.4g
angle of upper leaf ^d	52.0±23.2abc	39.7±16.3a	49.7±17.2ab	74.4±7.7d	46.3±14.2a	66.9±16.9bcd	51.6±25.0abc	72.5±12.9cd
length of upper leaf ^d	53.2±7.4a	28.9±6.5bc	29.4±10.8bc	19.6±5.2d	34.5±6.2b	10.0±2.5e	22.6±6.2cd	7.0±2.3f
width of upper leaf ^d	7.2±1.0abc	7.7±1.6ab	5.9±1.5c	6.5±1.3bc	8.0±1.0a	4.0±1.3d	6.2±1.3bc	3.6±1.0d

angle of lower leaf ^a	50.0±10.0a	63.8±6.5b	70.6±8.1b	71.6±8.3b	68.1±6.8b	69.7±7.6b	68.1±7.0b	68.8±11.2b
length of lower leaf ^a	107.9±8.7a	56.5±8.0b	57.3±9.4b	34.7±7.2c	52.3±9.3b	16.3±2.0d	34.4±7.1c	11.9±1.7e
width of lower leaf ^a	7.8±0.8a	10.0±1.3bc	9.8±0.8bc	10.2±1.4bc	10.6±0.9c	7.3±1.0a	9.2±1.4b	5.9±1.1d

540 ^aData are presented as means with SD. Lowercase letters indicate connecting letter report as determined by ANOVA with post-hoc
541 analysis using the Holm-Sidak algorithm with $P < 0.05$. ^bNumber of plants with tassel seeds or anthers in ear florets, with the
542 percentage of plants in parentheses. Lowercase letters indicate connecting letter report as determined by Fisher's exact test with $P <$
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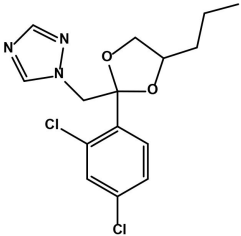
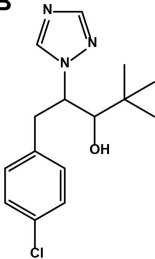
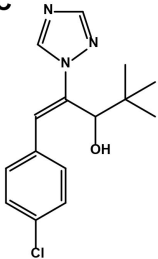
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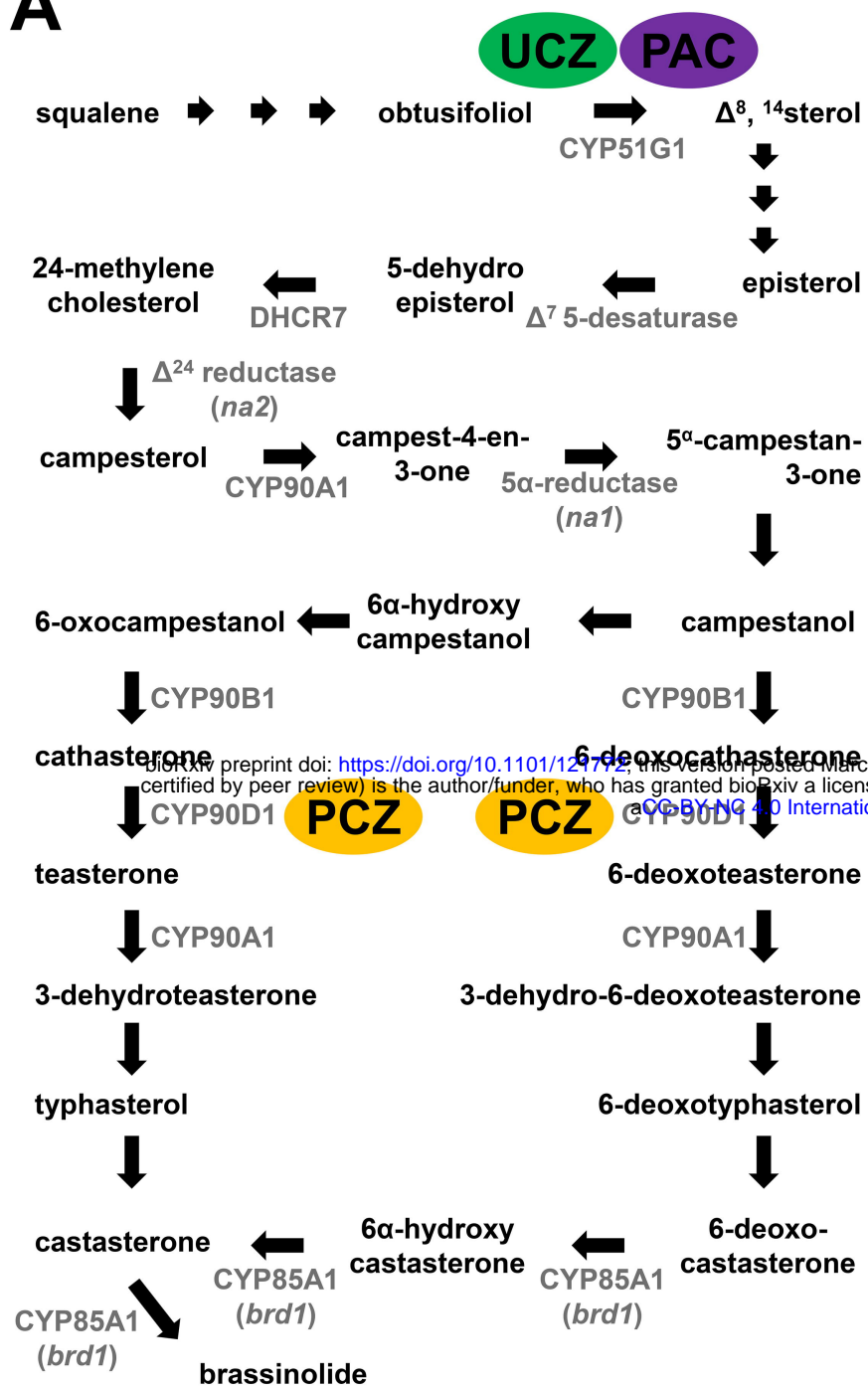
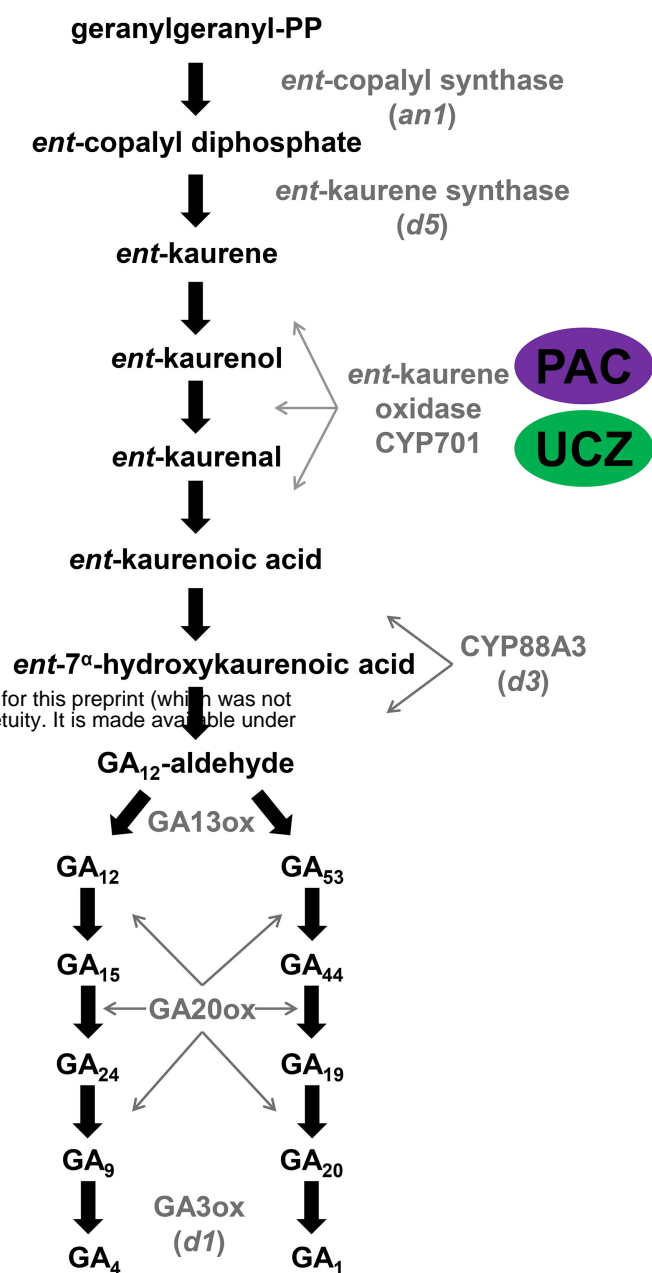
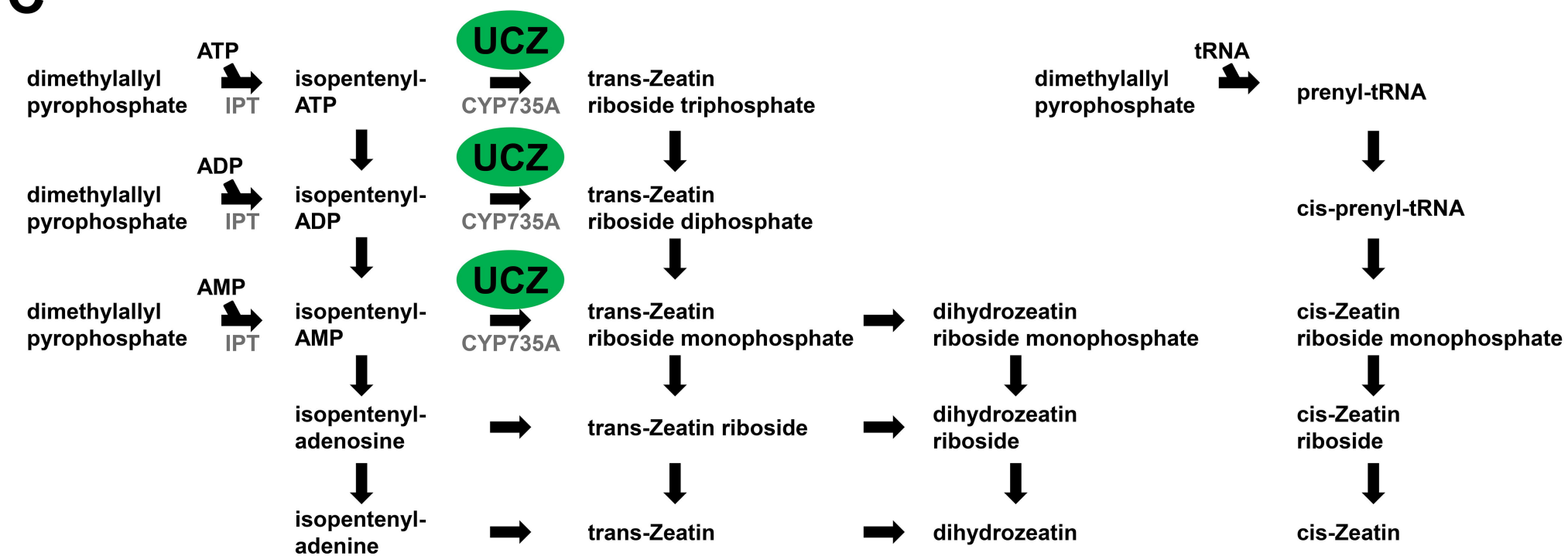
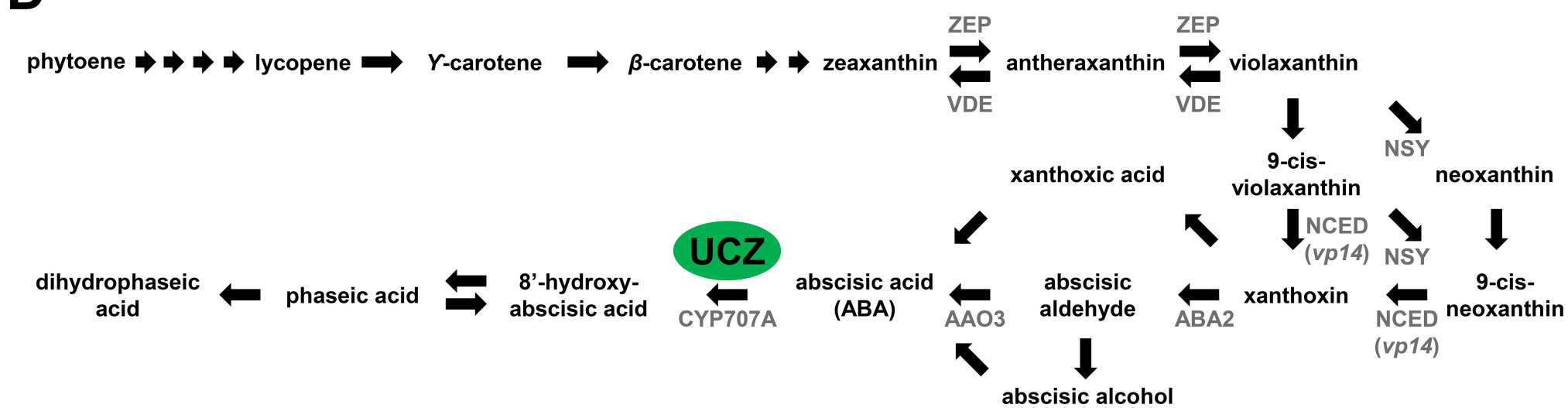
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