bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

1 Running title: Double mutations in PPO2
2
3 Can double PPO mutations exist in the same allele and are such mutants functional?
4
5 Aimone Porri[1], Matheus M. Noguera[3], Michael Betz[1], Daniel Sälinger[1], Frank Brändle[4], Steven
6 J. Bowe[2], Jens Lerchl[1], Lucie Meyer[1], Michael Knapp[1] and Nilda Roma-Burgos[3]*
7
8 [1]BASF SE, Ludwigshafen, Germany
9 [2]BASF Agricultural Solutions, Research Triangle Park, NC, USA
10 [3]Department of Crop, Soil, and Environmental Sciences, University of Arkansas, Fayetteville,
11 AR, USA
12 [4]IDENTXX GmbH, Stuttgart, Germany
13
14 Aimone Porri and Matheus M Noguera are joint first authors.
15
16 *Author for correspondence: Nilda Roma-Burgos, Professor (ORCID 0000-0002-6977-6873),
17 Department of Crop, Soil, and Environmental Sciences, University of Arkansas, 1354 W
18 Altheimer Dr, Fayetteville, AR 72704
19 (E-mail: nburgos@uark.edu)
20
21
22

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

23 **Abstract**

24

25 **Background:** Resistance to PPO-inhibiting herbicides is primarily endowed by target site
26 mutations at the *PPO2* gene that compromise binding of the herbicide to the catalytic domain. In
27 *Amaranthus* ssp. *PPO2*, the most prevalent target mutations are deletion of the G210 codon and
28 the R128G and G339A substitutions. These mutations strongly affect the dynamic of the PPO2
29 binding pocket resulting in reduced affinity with the ligand. Here we investigated the likelihood
30 of co-occurrence of the most widespread target site mutations in the same *ppo2* allele.

31

32 **Results:** Plants carrying R128G+/+ ΔG210+/-, where + indicates presence of the mutation, were
33 crossed with each other. The *ppo2* of the offspring was subjected to pyrosequencing and *E. coli*-
34 based Sanger sequencing to determine mutation frequencies and allele co-occurrence. The data
35 show that R128G ΔG210 can occur only in one allele; the second allele carries only one
36 mutation. Double mutation in both alleles is less likely because of significant loss of enzyme
37 activity. The segregation of offspring populations derived from a cross between heterozygous
38 plants carrying ΔG210 G399A also showed no co-occurrence in the same allele. The offspring
39 exhibited the expected mutation distribution patterns with few exceptions.

40

41 **Conclusions:** Homozygous double-mutants are not physiologically viable. Double-mutant plants
42 can only exist in a heterozygous state. Alternatively, if two mutations are detected in one plant,
43 each mutation would occur in a separate allele.

44

45 **Nomenclature:** Palmer amaranth, *Amaranthus palmeri* S. Wats.; protoporphyrinogen oxidase,
46 PPO; tall waterhemp, *Amaranthus tuberculatus* (Moq.) J.D.Sauer

47

48 **Key words**: double mutation; fitness penalty; genotyping; herbicide resistance; PPO mutations;
49 PPO-inhibitor resistance; target-site resistance.

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

50 **1 Introduction**

51 Protoporphyrinogen IX oxidase (PPO, EC. 1.3.3.4) pertains to a highly conserved family
52 of membrane-bound enzymes in the tetrapyrrole biosynthetic pathway that can be found in
53 mammals, plants, bacteria and fungi[1]. It catalyzes the six-atom oxidation of protoporphyrinogen
54 IX to protorphyrin IX, which is the last common step in the production of chlorophylls and
55 heme. In plants, two isoforms encoded by two distinct nuclear genes are found: PPO1 in
56 chloroplasts and PPO2 in the mitochondria.[2] Some plant species can have PPO2 targeted to both
57 organelles.[3]

58 PPO-inhibitors have been used as herbicides for more than 60 years. Their high efficacy,
59 broad weed control spectrum, residual activity and the appearance of glyphosate- and ALS-
60 resistant weeds contributed to their large-scale adoption and intensive use by farmers. Eight
61 distinct chemical families of PPO-inhibiting herbicides have been commercially developed so
62 far: diphenyl ethers, triazolinones, thiadiazoles, oxadiazoles, oxazolidinedione, phenylpyrazoles,
63 pyrimidinediones, and N-phenylphthalimides. Much of the variation in herbicide efficacy across
64 PPO-inhibitors can be attributed to their chemical structures, as these molecules bind to PPO by
65 resembling its substrate.

66 Although the selection of weeds resistant to PPO-inhibitors was a slow process compared
67 to herbicides with other modes of action, biotypes from 13 species have developed resistance
68 mechanisms to these herbicides so far.[4] The involvement of herbicide metabolism has been
69 reported but not fully characterized,[5, 6] and a polymorphism in the *ppo1* gene responsible for
70 oxadiazon resistance in *Eleusine indica* was recently discovered.[7] Nevertheless, mutations in the
71 *ppo2* gene have been identified as the main resistance mechanism to PPO-inhibitors. Among
72 those, the G210 codon-deletion (ΔG210) was the first to be reported, in 2001,[8] and is so far the
73 most prevalent resistance-conferring mutation among *A. tuberculatus* and *A. palmeri* species.[9-11]
74 In 2019, a glycine to alanine substitution at the *A. palmeri ppo2* 399th position (G399A) was
75 reported,[12] and has not been found in other species yet. Interestingly, neither of the native
76 residues (G210 and G399) are directly related to protogen binding to PPO2,[13, 14] so the resistance
77 caused by mutation at these loci is unexpected. It was later understood that ΔG210 caused an
78 enlargement of the binding pocket compared to the wild-type protein, allowing concomitant
79 binding of inhibitor and substrate.[15] Protogen binding to ΔG210 PPO2 was not negatively
80 affected, which is a favorable condition to minimize related fitness costs.[15, 16] However, the same

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

81 cannot be said about G399A. The authors concluded that the additional methyl group from Ala in
82 relation to Gly protruded into the binding-site, creating repulsive interactions towards the
83 inhibitor (and consequently, towards the substrate).[12] This mutation led to a 97% reduction in
84 enzyme activity compared to the WT PPO2. Lastly, mutations at the Arg128 position
85 (homologous to Arg98, in *Ambrosia artemisiifolia*) has been reported in several species, such as
86 *A. tuberculatus*,[10] *A. palmeri*,[17] *A. retroflexus*,[18, 19] *A. artemisiifolia*,[20] and *Euphorbia*
87 *heterophylla*.[21] The most common substitution is R128G (4 species), while R128M, R128I and
88 R128L are present in only one species each (*A. palmeri*, *A. tuberculatus* and *A. artemisiifolia*,
89 respectively). The importance of this residue for protogen binding relates to the salt bridge
90 formed between the positively charged Arg with the negatively charged carboxyl group of ring C
91 of protogen.[1, 13, 14, 22]
92 The continued use of PPO-inhibitors, the dioecious character of some *Amaranthus*
93 species and their hybridization capacity, has led to an accumulation of mutations at the
94 population-, plant- and allele-levels, [9, 11, 17, 23] although the latter is very rare. Computational data
95 suggested that both combinations ΔG210 + G399A and G399A + R128G would confer high
96 resistance to fomesafen, but the very low frequency of such genotypes, and the absence of
97 double homozygous plants could also indicate that the resultant protein might be inactive.[9] In
98 addition, it was predicted that the combination ΔG210 + R128G would not be viable due to the
99 loss of three-dimensional integrity of the binding pocket, which was backed up by the absence of
100 such genotype among the fomesafen-survivors. To assess the herbicide resistance risk arising
101 from double mutations , the aim of this study was to verify which mutation-combinations would
102 be tolerable in PPO2 from *A. palmeri*; and (2) study the inheritance pattern of a double-
103 heterozygous ΔG210 G399A mutant in *A. palmeri*, which was the most prevalent genotype
104 among the double-mutation carriers.
105
106 **2 Materials and Methods**
107
108 **2.1. Detection of *ppo2* target site mutations by pyrosequencing**
109 The following work was carried out in the laboratory of IDENTXX GmbH (Stuttgart,
110 Germany, www.identxx.com).

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

111 *Amaranthus palmeri* plants were sampled by collecting a 0.5 cm² leaf section and
112 individually transferring into collection microtubes (Qiagen, Hilden, Germany). The samples
113 were then homogenized in a shaking mill (TissueLyser II; Qiagen, Hilden, Germany) using steel
114 beads. The DNA extraction was carried out in the KingFisher™ Flex Magnetic Particle
115 Processors (Thermo Fisher Scientific, Schwerte, Germany) using the Chemagic Plant 400 kit
116 (Perkin Elmer, Rodgau, Germany) according to the manufacturer's instructions (modified by
117 IDENTXX GmbH). The Endpoint PCR amplification (gDNA concentration of 20-50 ng μl^{-1} per
118 sample) for each target region were performed using MyFi™ DNA Polymerase Kit (Bioline
119 GmbH, Luckenwalde, Germany) and specific primers (IDENTXX GmbH)in a PCR thermal
120 cycler (T100 PCR thermal cycler, Bio-Rad Laboratories GmbH, Germany) under the following
121 conditions: 3 min at 95°C and 42 cycles of 10 s denaturation at 95°C; 35 s annealing at 60°C and
122 45 s elongation at 72°C; and a final elongation step at 72°C for 5 min.. The successful
123 amplification (225 bp for the ΔG210, 250 bp for the G399A product) was checked per gel
124 electrophoresis on a 1.5 % agarose gel.
125 The PCR products were analyzed for SNPs at the target positions via pyrosequencing on
126 a PyroMark Q24 (Qiagen, Hilden, Germany) using specific sequencing primers (IDENTXX
127 GmbH). During the sequencing reaction, all incorporated nucleotides of a short region that
128 encompasses the SNP position of interest were detected and reported by creating a pyrogramm in
129 a pyrorun file. The created file was read by the PyroMark Q24 software (Version 2.0.7) and
130 visually evaluated for mutations, both heterozygous and homozygous.
131

132 **2.2. Verification of double mutants by cloning in *E. coli***

133 The following work was carried out in the laboratory of IDENTXX GmbH (Stuttgart,
134 Germany, www.identxx.com).
135 A 0.5 cm² of leaf sample of each plant (continuously cooled at -20°C) was transferred
136 into a collection microtubes. The samples were then homogenized at room temperature in a
137 shaking mill. The RNA extraction was carried out in the KingFisher™ Flex Magnetic Particle
138 Processors (Thermo Fisher Scientific, Schwerte, Germany) using the MagMAX™ Plant RNA
139 Isolation Kit (Thermo Fisher Scientific Inc, Schwerte, Germany) according to the manufacturer's
140 instructions (modified by IDENTXX GmbH). The cDNA transcription was done using the High-
141 Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc, Schwerte, Germany)

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

142 according to the manufacturer's instructions (modified by IDENTXX GmbH). The Endpoint
143 PCR amplification for the target region encompassing both SNPs positions were performed using
144 MyFi™ DNA Polymerase Kit and specific primers (IDENTXX GmbH) in a PCR thermal cycler
145 under the following conditions: 5 min at 95°C and 42 cycles of 20 s denaturation at 95°C; 35 s
146 annealing at 60°C and 90 s elongation at 72°C; and a final elongation step at 72°C for 5 min. The
147 successful amplification of the 1850 bp PCR product was checked per gel electrophoresis on a
148 1.5 % agarose gel.
149 PCR products were cloned using StrataClone PCR Cloning Kit (Agilent, Waldbronn,
150 Germany). Positive white colonies were randomly picked and verified with colony PCR. For
151 each clone, 10 positive PCR fragments were randomly selected and verified via Sanger
152 sequencing (SeqLab-Microsynth, Göttingen, Germany). Sequences were analyzed using
153 Geneious software v. 9.1.8 (Biomatters, Auckland, New Zealand).
154
155 **2.3. Evaluation of mutant PPO enzyme activity**
156 The complete description of expression and purification of Amaranthus PPO2 variant
157 proteins and the enzymatic assay to determine protein activity was referenced to the method
158 described by Rangani et al.[12] To calculate the percentage of remaining protein activity, the
159 enzyme activity of WT PPO2 was divided by the activities of R128G, ΔG210 and R128G ΔG210
160 PPO2 variants and multiplied by 100. This was further normalized to the amount (ng) of protein
161 used in the assay.
162
163 **2.4 Arabidopsis transgenics growth and herbicide treatment**
164 **2.4.1 Donor plant material and growth conditions**
165 *Arabidopsis thaliana* seeds (stock MC24, from the Max Planck Institute for Molecular
166 Plant Physiology at Golm) were sown into a substrate composed of GS90 soil + 5% sand. Plants
167 were subjected to stratification for 5 days at 4°C, followed by a short-day growth period of 10 d
168 (10/14 h of day/night intervals at 20/18°C ± 1°C, ~120 μmol PAR). After that, plants were
169 transplanted into 8X8 cm pots filled with GS90 soil and cultivated under the same conditions for
170 14 d, under long-day growth conditions (16/8 h day/night at 20/18°C ± 1°C, ~200 μmol PAR)
171 and maintained until seed harvest. Plants were fertilized with 0.3% Hakaphos Blau (15-10-15

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

172 NPK) twice a week until flowering. Relative humidity was not controlled, but kept between 40-
173 70% during all growth stages, except during stratification.
174
175 **2.4.2 Transgene preparation**

176 To prepare the transgene, the mutant *ppo2* was inserted into RTP6557 transformation
177 vector, which was then inserted into *Agrobacterium tumefaciens* strain C58C1pMP90. The gene
178 insert also included an acetolactate synthase-herbicide-resistance trait as a selectable marker to
179 identify transformed *Arabidopsis* seedlings. This would ensure that plants eventually tested for
180 resistant to PPO herbicide all expressed the transgene.
181
182 **2.4.3 Bacterial culture and dipping medium**

183 *Agrobacterium* culture containing the plasmid was prepared a day before dipping by
184 inoculating 1 ml of glycerol stock into 250 ml of YEB medium (1 g L^{-1} yeast, 5 g L^{-1} beef
185 extract, 5 g L^{-1} peptone, 5 g L^{-1} sucrose, 0.49 g L^{-1} $MgSO_4*7H_2O$) + appropriate antibiotic. The
186 bacteria were cultured for 12 h at 28°C with continuous agitation at 150 rpm. The next day, after
187 adjusting the *Agrobacterium* culture density to OD600 = 1.0 (with YEB medium), the culture
188 was collected by centrifugation at 4000 rpm for 10 min and re-suspended in 150 ml *infiltration*
189 *medium* composed of 2.2 g L^{-1} MS (Murashige & Skoog medium), 50 g L^{-1} sucrose, 0.5 g L^{-1}
190 MES hydrate, 10µl L^{-1} BAP (Benzylaminopurin, 1 mg ml^{-1}). The pH was then adjusted to 5.7-
191 5.8.
192
193 **2.4.4 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* by floral dip**

194 Plant transformation was performed following a previously established protocol. [24]
195 Briefly, plants with immature floral buds were dipped in the bacterial suspension for 10 sec after
196 adding 75 µl of Silwet-L77 per 150 ml of infiltration medium to a jar. After dipping, plants were
197 kept overnight in a cabinet under high humidity and low light intensity, and were grown under
198 long-day conditions until maturity. When siliques turned yellow, plants were placed inside paper
199 bags to collect the seeds. T1 seeds were then transferred to falcon tubes and stored at 4°C.
200
201 **2.4.5 Selection of putative transformants with Imazamox**

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

202 After 14 or more days of storage at 4°C, T1 seeds were sown to select putative transgenic
203 *Arabidopsis* plants. Sowing and stratification was performed as described previously. Following
204 that, seeds were treated with a 20 ppm imazamox solution and cultivated under short-day growth
205 conditions for 12-14 days, when resistant seedlings (4-leaf stage) were transplanted into 6 cm
206 pots filled with GS90 soil and grown for another 10 days. One day prior to herbicide application,
207 growth conditions were set to 'long-day' and maintained throughout the duration of the test.
208 Herbicide treatments consisted of two concentrations of saflufenacil (Kixor, BASF Corporation,
209 at 10 and 25 g ai ha^{-1}) foliar-applied when plants reached the 10-leaf stage, using a spray
210 chamber calibrated to deliver 375 L ha^{-1} of spray solution. Herbicide efficacy was visually
211 assessed after 7 days from herbicide treatments.
212
213 **2.5 Verifying PPO double mutants from heterozygous dG210 x heterozygous G399A**
214 In a previous study we learned that ΔG210 -/+ G399A -/+ was the most common
215 genotype among the double mutant plants identified.[9] The double mutants referred to here were
216 plants carrying two *ppo2* mutations, but without confirmation on whether the two mutations
217 occurred in one allele. To study the inheritance pattern of this genotype, seeds from the selected
218 Palmer amaranth population were sown in a plastic tray containing potting mix (Sunshine LC1;
219 SunGro Horticulture, Agawam, MA, USA). After one week, 400 seedlings were transplanted to
220 50-cell trays at one seedling per cell. When plants reached the 6- to 8-cm stage, fomesafen (264
221 g ai ha^{-1}, Flexstar® 1.88 EC, Syngenta Crop Protection, Greensboro, NC, USA) was applied in a
222 spray chamber, equipped with two flat-fan 100 0065 nozzles, calibrated to deliver 187 L ha^{-1} of
223 spray mix at a speed of 3.6 km h^{-1}. At 21 d after application, leaf tissues from around 200
224 survivors were individually collected into 1.5-mL microtubes (VWR International LLC, Radnor,
225 PA, USA) and stored at -80 □ until processing. DNA was extracted and the plants were
226 genotyped via pyrosequencing as previously described. Four males and 14 females of the same
227 genotype (ΔG210 -/+ G399A -/+) were individually transplanted to 8-L pots and grown together
228 to interbreed. Seeds were harvested separately from each female plant and cleaned. The
229 germination capacity of 14 F1 lines was evaluated. Six F1 lines with the higher germination
230 capacity were selected, and up to 100 plants from each female were selected for genotyping via
231 pyrosequencing.

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

232 Since Palmer amaranth is diploid, a plant that is heterozygous for both mutations can
233 either: a) have one allele containing two mutations, while the other allele is WT; or b) have each
234 allele carrying one mutation only. The Palmer amaranth parent population used in this study
235 exhibited the latter condition. A cross between two double-heterozygous is expected to result in
236 25% of the offspring carrying a homozygous ΔG210, 25% carrying a homozygous G399A and
237 50% of the offspring having the same parental genotype. A Pearson's chi-squared test was used
238 to confirm this hypothesis, where the calculated chi-squared value (χ^2 Calc, as shown in
239 Equation 1) is compared to a tabulated value (χ^2 Tab)
240
241 Equation 1. $$\chi^2 = \sum \frac{(Oi - Ei)^2}{Ei}$$
242
243 where *Oi* = observed number of plants of a genotype and *Ei* = expected number of plants
244 of a genotype.
245 Lastly, the occurrence of double mutants was verified by cloning the *ppo2* gene into *E.*
246 *coli* as previously described. A total of 26 samples were chosen for this procedure, and around 10
247 clones from each sample were Sanger-sequenced.
248

249 3 Results and Discussion

250 3.1. PPO mutation profile of R128G+/+ ΔG210-/+ offspring.

251 A male and a female plant LIH11640A and LIH11640F carrying R128G+/+ ΔG210+/-
252 (where + indicates presence of the mutation), were detected in a large biotype screening of
253 putative resistant *A. palmeri*. The R128G+/+ ΔG210+/- plants carried homozygous R128G,
254 indicating that double mutations ΔG210 and R128G can occur on the same allele. This finding
255 answers the question we posed in a previous study,[9] where this genotype was not observed
256 among the double mutants. In that study, we hypothesized that: a) either the occurrence of this
257 genotype was too rare and would require a bigger sample size to be detected; or b) the resultant
258 protein would be inactive and, therefore, the fitness cost associated with this double-mutation
259 would be lethal.
260 To determine whether homozygous double mutants R128G ΔG210 plants are viable,
261 LIH11640A was crossed with LIH11640F and the *ppo2* of LIH11640F1 offspring was subjected

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

262 to pyrosequencing for the positions R128 and G210. If the R128G ΔG210 PPO is functional, and
263 inheritance follows a Mendelian pattern, it is expected that two parents having the R128G+/+
264 ΔG210+/- genotype would result in 25% of the offspring being double-homozygous, 25% being
265 R128G+/+ G210 and 50% having the parental genotype (Fig 1).

266 Out of 100 plants genotyped from the LIH11640F1 population, only 29% of the offspring
267 carried the parental genotype; 71% of the plants were R128G +/+ G210; and none were
268 homozygous double mutants (Table 1). The calculated chi-squared value for this population was
269 three times higher than the tabulated (χ^2 Calc = 18.46 and χ^2 Tab = 5.991), indicating that
270 observed and expected ratios do not agree. Due to the odd inheritance pattern in LIH11640F1,
271 additional R128G+/+ ΔG210+/- male and female plants from this population were selected and
272 crossed to generate another three independent LIH11640F2 populations, which were also
273 pyrosequenced (Table 1). The mutation inheritance pattern among the three F2 populations
274 conformed to that of the F1 population, where no homozygous double mutants were detected and
275 the majority of the offspring carried a single, homozygous R128G mutation (R128G +/+ G210).
276 All calculated chi-squared values were higher than the tabulated. These results indicate that,
277 although rare individuals containing both R128G ΔG210 mutations can occur, their co-
278 occurrence on both alleles is not possible. In addition, the percentage of F1 and F2 plants
279 carrying R128G+/+ ΔG210+/- was significantly lower than the expected ratio (Table 1),
280 suggesting that having R218G and ΔG210 in one allele has a significant impact on fitness cost.
281

282 **3.2. Verification of double mutant allele combination by cloning in *E. coli* and Sanger**
283 **sequencing**

284 For further verification of the pyrosequencing results**,** the plants coded as LIH11640A
285 and LIH11640F were used for RNA extraction, cDNA synthesis, and amplification of the whole-
286 length *PPO2* gene, which was later cloned into *E. coli*. For each plant, 10 clones were selected
287 for Sanger sequencing.

288 As expected, all clones from both plants carried the R128G mutation. The number of
289 clones containing double mutations was similar for both plants studied: four and five out of 10
290 clones for plants LIH11640A and LIH11640F, respectively (Table 2). Since the *ppo2* fragments
291 inserted into *E. coli* were derived from RNA of the parent plants, the balanced distribution of
292 double- and single-mutants suggests that there is no difference in the expression of those alleles.

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

293 In other words, LIH11640A and LIH11640F plants do not favor the expression of one allele over
294 the other, and the amount of double- and single-mutant proteins produced *in vivo* are equivalent.
295

296 **3.3. Evaluation of double-mutant R128G ΔG210 PPO2 enzymatic activity**

297 The segregation of mutations in the offspring suggests that the double mutant (R128G
298 ΔG210) protein activity is either severely compromised or non-existent, and plants carrying such
299 double-mutant allele rely exclusively on the other, single-mutant allele to survive. To confirm
300 this hypothesis, the WT, single-mutant and double-mutant enzymes were heterologously
301 expressed in *E. coli*, the PPO2 protein was purified, and the enzyme activity was quantified.
302 Enzyme activity data are shown as percentages relative to the WT protein (Table 3). Of the three
303 mutants tested, the R128G variant showed the highest remaining activity, followed by the ΔG210
304 and the double-mutant variants. The enzyme activity of the double mutant was well below the
305 detection limit, indicating that it was practically inactive. The inactivity of the double-mutant
306 enzyme agrees with our previous study,[9] where we deduced that a R128G ΔG210 protein would
307 lose its binding pocket integrity.

308 The effect of ΔG210 and R128G mutations on PPO activity has already been investigated
309 and our results somewhat agrees with previous studies.[15, 16, 18, 25] These mutations are known to
310 affect PPO activity and substrate binding differently. While ΔG210 did not affect substrate
311 binding (no differences in Km between WT and mutant), the mutation reduced enzyme activity
312 by a factor of 10.[15] The deletion of G210 causes an enlargement of the substrate binding pocket
313 and displaces G207, which has an important role in positioning protogen at the ideal distance and
314 orientation from the co-factor FAD, favoring the rate-limiting hydride abstraction.[15] The loss of
315 that interaction results in lower protein activity. In contrast, R128G impairs protein binding
316 efficacy, but doubles its activity.[18] The decrease in substrate binding is caused by the loss of the
317 salt bridge interaction between R128 and protogen. However, this also facilitates the release of
318 proto from the binding site. In addition, the substitution of the bulky, charged Arg to the small,
319 non-polar Gly, increases the opening of the binding pocket, further speeding the proto egress.[18,
320 25]

321 To confirm the double-mutant viability, *Amaranthus palmeri* PPO2 carrying R128G
322 ΔG210 was overexpressed in *Arabidopsis* and the resulting transgenic plants were treated with
323 two rates of saflufenacil (10 and 25 g ha^{-1}). If active, transgenic plants expressing R128G ΔG210

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

324 should be resistant to saflufenacil. The R128G ΔG210 *Arabidopsis* transgenics did not survive
325 the field dose of saflufenacil, indicating that protein activity of this variant is too low to confer
326 herbicide tolerance (Fig 2). The absence of a significant tolerance of the transgenic line suggests
327 that R128G ΔG210 strongly impairs PPO2 protein function.
328

329 **3.4. PPO mutation profile of crosses between double heterozygous ΔG210G399A**

330 Among field populations of Palmer amaranth, the most common *ppo2* mutation is ΔG210
331 and the most common mutations detected in the same population are ΔG210 and G399A.[9]
332 Therefore, it is only logical to assume that due to the obligate outcrossing behavior of this specie,
333 the co-existence of single-mutant plants (regardless of zygosity) in one population would only
334 increase the frequency of ΔG210 G399A double mutants with time. This could then become the
335 most common resistant genotype that would challenge current and future PPO-inhibiting
336 herbicides. That is, if such double mutant does not carry fitness penalty. Hence this follow-up
337 experiment. We expected that a cross between two double-heterozygous ΔG210 G399A would
338 result in 50% of the offspring carrying a single homozygous mutation (either ΔG210 or G399A),
339 while the other 50% would carry both mutations concomitantly as heterozygous. To check if the
340 observed values agreed with the expected inheritance pattern, a chi-squared test was done. The
341 χ^2 Calc values for individual F1 lines ranged from 1.02 to 4.73, and were lower than the
342 tabulated threshold (χ^2 Tab = 5.991), confirming that the observed inheritance pattern agrees
343 with the expected (Table 4). The same result was obtained when considering samples across all
344 F1 lines (N=376), where χ^2 Calc = 2.33.

345 A total of 26 plants double-heterozygous from all the F1 lines were selected for cloning
346 of the *PPO2* gene into *E. coli*. At least 10 clones per plant were submitted for Sanger
347 sequencing. In this case, half of the clones were expected to contain the G210 deletion, while the
348 other half would carry G399A. This distribution was tested using the Pearson's chi-squared
349 goodness-of-fit test. Four out of six F1 lines had the observed distribution equal to the expected,
350 but the lines LIH19626 and LIH19628 did not fit this pattern (Table 5). In these two cases, the
351 higher number of clones containing the ΔG210 mutation indicates an unequal expression of this
352 allele in comparison to the allele containing G399A. The mechanism or reason for this
353 phenomenon is not yet understood.
354

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

355 **4 CONCLUSIONS**

356 The co-occurrence of two mutations in a same *ppo2* allele, although rare, is possible.
357 However, the coded protein has a severely reduced activity, which is supported by the fact that
358 no double-homozygous plants were detected, and that its insertion in Arabidopsis did not result
359 in resistance to PPO-inhibitors. Plants carrying a double-mutant allele must rely on the
360 alternative allele to produce a functional enzyme. The fitness cost in plants carrying a double-
361 mutant allele is yet to be determined.
362

363 **5 ACKNOWLEDGEMENTS**

364 We are grateful to Daniel Sälinger for providing the PPO enzyme substrate and we thank
365 Hardy Schön and Sarah Heyn for the Arabidopsis service transformation.
366

367 **6 CONFLICT OF INTERESTS**

368 Authors affiliated with BASF have contributed to the planning and implementation of
369 research activities. All other authors declare no conflict of interest.
370

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

7 REFERENCES

1. Hao G-F, Zuo Y, Yang S-G and Yang G-F, Protoporphyrinogen oxidase inhibitor: an ideal target for herbicide discovery. *CHIMIA International Journal for Chemistry* **65**: 961-969 (2011).
2. Lermontova I, Kruse E, Mock H-P and Grimm B, Cloning and characterization of a plastidal and a mitochondrial isoform of tobacco protoporphyrinogen IX oxidase. *Proceedings of the National Academy of Sciences* **94**: 8895-8900 (1997).
3. Watanabe N, Che F-S, Iwano M, Takayama S, Yoshida S and Isogai A, Dual Targeting of Spinach Protoporphyrinogen Oxidase II to Mitochondria and Chloroplasts by Alternative Use of Two In-frame Initiation Codons *. *J Biol Chem* **276**: 20474-20481 (2001).
4. Heap I, International Survey of Herbicide Resistant Weeds. www.weedscience.org [accessed Jan 7 2021].
5. Obenland OA, Ma R, O'Brien SR, Lygin AV and Riechers DE, Carfentrazone-ethyl resistance in an Amaranthus tuberculatus population is not mediated by amino acid alterations in the PPO2 protein. *PloS one* **14**: e0215431 (2019).
6. Varanasi VK, Brabham C and Norsworthy JK, Confirmation and Characterization of Non–target site Resistance to Fomesafen in Palmer amaranth (*Amaranthus palmeri*). *Weed Sci* **66**: 702-709 (2018).
7. Bi B, Wang Q, Coleman JJ, Porri A, Peppers JM, Patel JD, et al., A Novel Mutation A212T in Chloroplast Protoporphyrinogen Oxidase (PPO1) Confers Resistance to PPO Inhibitor Oxadiazon in Eleusine indica. *Pest Manage Sci* 2019).
8. Patzoldt WL, Hager AG, McCormick JS and Tranel PJ, A codon deletion confers resistance to herbicides inhibiting protoporphyrinogen oxidase. *Proc Natl Acad Sci USA* **103**: 12329-12334 (2006).
9. Noguera MM, Rangani G, Heiser J, Bararpour T, Steckel LE, Betz M, et al., Functional PPO2 mutations: co-occurrence in one plant or the same ppo2 allele of herbicide-resistant Amaranthus palmeri in the US mid-south. *Pest Manage Sci* 2020).
10. Nie H, Mansfield BC, Harre NT, Young JM, Steppig NR and Young BG, Investigating target-site resistance mechanism to the PPO-inhibiting herbicide fomesafen in waterhemp and interspecific hybridization of Amaranthus species using next generation sequencing. *Pest Manage Sci* **75**: 3235-3244 (2019).
11. Copeland JD, Giacomini DA, Tranel PJ, Montgomery GB and Steckel LE, Distribution of PPX2 Mutations Conferring PPO-Inhibitor Resistance in Palmer Amaranth Populations of Tennessee. *Weed Technol* **32**: 592-596 (2018).
12. Rangani G, Salas-Perez RA, Aponte RA, Knapp M, Craig IR, Mietzner T, et al., A Novel Single-Site Mutation in the Catalytic Domain of Protoporphyrinogen Oxidase IX (PPO) Confers Resistance to PPO-Inhibiting Herbicides. *Fron Plan Sci* **10**2019).
13. Heinemann Ilka U, Diekmann N, Masoumi A, Koch M, Messerschmidt A, Jahn M, et al., Functional definition of the tobacco protoporphyrinogen IX oxidase substrate-binding site. *Biochem J* **402**: 575-580 (2007).
14. Koch M, Breithaupt C, Kiefersauer R, Freigang J, Huber R and Messerschmidt A, Crystal structure of protoporphyrinogen IX oxidase: a key enzyme in haem and chlorophyll biosynthesis. *The EMBO Journal* **23**: 1720-1728 (2004).
15. Dayan FE, Daga PR, Duke SO, Lee RM, Tranel PJ and Doerksen RJ, Biochemical and structural consequences of a glycine deletion in the α-8 helix of protoporphyrinogen oxidase. *Biochim Biophys Acta* **1804**: 1548-1556 (2010).

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

417 16. Wu C, Goldsmith M-R, Pawlak J, Feng P, Smith S, Navarro S, et al., Differences in efficacy,
418 resistance mechanism and target protein interaction between two PPO inhibitors in Palmer
419 amaranth (Amaranthus palmeri). *Weed Sci* **68**: 105-115 (2020).
420 17. Giacomini DA, Umphres AM, Nie H, Mueller TC, Steckel LE, Young BG, et al., Two new PPX2
421 mutations associated with resistance to PPO-inhibiting herbicides in *Amaranthus palmeri*. *Pest*
422 *Manage Sci* **73**: 1559-1563 (2017).
423 18. Du L, Li X, Jiang X, Ju Q, Guo W, Li L, et al., Target-Site Basis for Fomesafen Resistance in Redroot
424 Pigweed (Amaranthus retroflexus) from China. *Weed Sci* **69**: 290-299 (2021).
425 19. Huang Z, Cui H, Wang C, Wu T, Zhang C, Huang H, et al., Investigation of resistance mechanism
426 to fomesafen in Amaranthus retroflexus L. *Pestic Biochem Physiol* **165**: 104560 (2020).
427 20. Rousonelos SL, Lee RM, Moreira MS, VanGessel MJ and Tranel PJ, Characterization of a common
428 ragweed (*Ambrosia artemisiifolia*) population resistant to ALS-and PPO-inhibiting herbicides.
429 *Weed Sci* **60**: 335-344 (2012).
430 21. Mendes RR, Takano HK, Adegas FS, Oliveira RS, Gaines TA and Dayan FE, Arg-128-Leu target-site
431 mutation in PPO2 evolves in wild poinsettia (Euphorbia heterophylla) with cross-resistance to
432 PPO-inhibiting herbicides. *Weed Sci* **68**: 437-444 (2020).
433 22. Hao G-F, Tan Y, Xu W-F, Cao R-J, Xi Z and Yang G-F, Understanding Resistance Mechanism of
434 Protoporphyrinogen Oxidase-Inhibiting Herbicides: Insights from Computational Mutation
435 Scanning and Site-Directed Mutagenesis. *J Agric Food Chem* **62**: 7209-7215 (2014).
436 23. Lillie KJ, Giacomini DA, Green JD and Tranel PJ, Coevolution of resistance to PPO inhibitors in
437 waterhemp (Amaranthus tuberculatus) and Palmer amaranth (Amaranthus palmeri). *Weed Sci*
438 **67**: 521-526 (2019).
439 24. Clough SJ and Bent AF, Floral dip: a simplified method for Agrobacterium -mediated
440 transformation of Arabidopsis thaliana. *The Plant Journal* **16**: 735-743 (1998).
441 25. Hao G-F, Tan Y, Yang S-G, Wang Z-F, Zhan C-G, Xi Z, et al., Computational and Experimental
442 Insights into the Mechanism of Substrate Recognition and Feedback Inhibition of
443 Protoporphyrinogen Oxidase. *PLOS ONE* **8**: e69198 (2013).
444

Internal

445 **8 TABLES**

446 Table 1. Segregation of mutant alleles in F1 and F2 generations of PPO-herbicide-resistant *Amaranthus palmeri* genotypes.

F1 line	Number of plants							χ^2 Calc
	Genotyped	Expected			Observed			
		128G +/+ Δ210 -/-	128G +/+ Δ210 +/-	128G +/+ Δ210 +/+	128G +/+ Δ210 -/-	128G +/+ Δ210 +/-	128G +/+ Δ210 +/+	
LIH11640 F1	100	25	50	25	71	29	0	118.46
LIH11640 F2-1	100	25	50	25	65	35	0	93.50
LIH11640 F2-2	100	25	50	25	73	27	0	127.74
LIH11640 F2-3	100	25	50	25	70	30	0	114.00
Grand total	400	100	200	94	279	121	0	451.62

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

447 Table 2. Confirmation of the occurrence of R128G and ΔG210 mutants in PPO2 clones in *E. coli*
448 from PPO-herbicide-resistant *Amaranthus palmeri* plants.

449
450

Plant ID	**Clone ID**	**R128G**	**ΔG210**
AMAPA LIH11640A	Clone 1	+	+
	Clone 2	+	-
	Clone 3	+	+
	Clone 4	+	-
	Clone 5	+	-
	Clone 6	+	-
	Clone 7	+	+
	Clone 8	+	-
	Clone 9	+	-
	Clone 10	+	+
AMAPA LIH11640F	Clone 1	+	+
	Clone 2	+	-
	Clone 3	+	-
	Clone 4	+	+
	Clone 5	+	-
	Clone 6	+	-
	Clone 7	+	+
	Clone 8	+	-
	Clone 9	+	+
	Clone 10	+	+

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

451 Table 3. Enzyme activity assay of *Amaranthus palmeri* PPO2 mutants.

PPO Variant	FU/min	% Remaning activity
WT PPO2 (25ng)	584	100
ΔG210 (500ng)	1500	11
R128G (25ng)	250	50
R128G ΔG210 (500 ng)	15	0.12

452

Internal

453 Table 4: Genotyping of Palmer amaranth *ppo2* mutations via pyrosequencing from six F1 lines produced by a cross between double-

454 heterozygous ΔG210/G399A parents

F1 line	**Number of plants**							**χ^2 Calc**
	Genotyped	**Expected**			**Observed**			
		ΔG210 -/- G399A +/+	ΔG210 +/- G399A +/-	ΔG210 +/+ G399A -/-	ΔG210 -/- G399A +/+	ΔG210 +/- G399A +/-	ΔG210 +/+ G399A -/-	
LIH19623	52	13	26	13	14	19	19	4.73
LIH19624	93	23.3	46.5	23.3	16	51	26	3.02
LIH19625	97	24.3	48.5	24.3	21	56	20	2.34
LIH19626	20	5	10	5	3	10	7	1.60
LIH19627	14	3.5	7.0	3.5	3	10	1	3.14
LIH19628	100	25	50	25	28	51	21	1.02
Grand total	376	94	188	94	85	197	94	2.33

455

456

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

457 Table 5: Sanger sequencing of *E. coli* clones expressing the *ppo2* gene from six F1 *Amaranthus*
458 *palmeri* lines

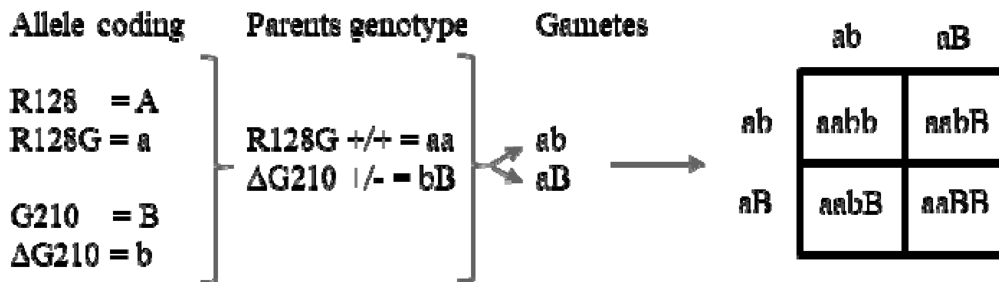
F1 line	# of plants cloned	Number of clones					χ^2 Calc
		Sequenced	Expected		Observed		
			dG210	G399A	dG210	G399A	
LIH19623	6	62	31	31	30	28	0.067
LIH19624	3	30	15	15	17	17	0.267
LIH19625	3	30	15	15	17	13	0.267
LIH19626	5	51	25.5	25.5	35	16	7.078
LIH19627	3	36	18	18	15	21	1.000
LIH19628	6	62	31	31	40	22	5.225

Internal

bioRxiv preprint doi: https://doi.org/10.1101/2022.01.21.477186; this version posted January 25, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-NC-ND 4.0 International license.

459 ## 9 FIGURES AND FIGURES LEGENDS

460



461

462 Figure 1. The expected segregation pattern of the offspring from a cross of homozygous R128G
463 mutant (R128G+/+) and heterozygous ΔG210 mutant (ΔG210+/-).

464

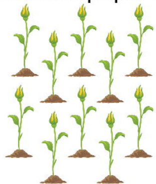
465

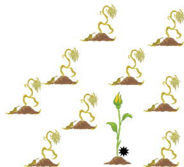
466

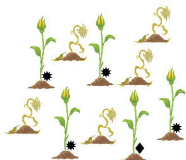
467 Figure 2. T1 35S:AMAPA PPO2 R128G ΔG210 Arabidopsis lines sprayed with Saflufenacil.
468 Pictures were taken 7 days after treatment. The bottom 2 pots contain wild-type plants, the upper
469 5 pots contain independent transgenic events (T1 plants, selected with Imazamox by confirming
470 presence of resistance gene AHAS).

Sensitive population

Resistant population



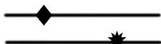




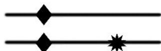
Time

Recurrent selection with PPO-inhibiting herbicides and the outcrossing behaviour of *Amaranthus palmeri* produces resistant populations carrying multiple *ppo2* gene mutations.

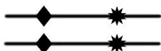
R128G ΔG210



Double-heterozygous: most common; two different mutations, one in each allele



One heterozygous and one homozygous mutation: rare; plants rely on the single-mutant allele to grow



Double-homozygous: double mutation in both alleles; not viable, inactive protein is lethal