1   # Transcriptome changes in chlorsulfuron-treated plants are caused by

2   acetolactate synthase inhibition and not induction of a herbicide

3   detoxification system in *Marchantia polymorpha*

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Abstract

A sensing mechanism in mammals perceives xenobiotics and induces the transcription of genes encoding proteins that detoxify these molecules. However, it is unclear if plants sense xenobiotics, and activate an analogous signalling system leading to their detoxification. Using the liverwort *Marchantia polymorpha*, we tested the hypothesis that there is a sensing system in plants that perceives herbicides resulting in the increased transcription of genes encoding proteins that detoxify these herbicides. Consistent with the hypothesis, we show that chlorsulfuron-treatment induces changes in the *M. polymorpha* transcriptome. However, these transcriptome changes do not occur in chlorsulfuron (CS)-treated target site resistant mutants, where the gene encoding the target carries a mutation that confers resistance to chlorsulfuron. Instead, we show that inactivation of the chlorsulfuron target, acetolactate synthase (ALS) (also known as acetohydroxyacid synthase (AHAS)), is required for the transcriptome response. These data are consistent with the changes in the transcriptome of chlorsulfuron-treated plants being caused by disrupted amino acid synthesis and metabolism resulting from acetolactate synthase inhibition. These conclusions suggest that chlorsulfuron is not sensed in *M. polymorpha* leading to induce a detoxification system.

Author Summary

Herbicide use is increasing throughout the world, however we know little about how plants respond to herbicide treatment and regulate their metabolism. Some plants have evolved resistance to herbicides such as chlorsulfuron by increasing the detoxification of the herbicide compared to sensitive plants. It has been suggested that plants can directly sense the herbicide chemical which activates a detoxification response, in a similar way to the detoxification of foreign chemicals in mammalian cells. The liverwort *Marchantia polymorpha* is an excellent system to study plant herbicide responses due to its short generation time, ease of propagation and low genetic

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43   redundancy. We show that chlorsulfuron treatment alters the expression of many genes in *M.*

44   *polymorpha*, however plants with a resistance-conferring mutation in the molecular target of

45   chlorsulfuron do not show any changes in gene expression in response to chlorsulfuron treatment.

46   This result indicates that transcriptome changes caused by chlorsulfuron depend on the inhibition of

47   the target by chlorsulfuron. This suggests that plants do not sense chlorsulfuron and activate a

48   detoxification system. This finding has implications for herbicide use and discovery.

49

50   Introduction

51   In mammals, sensing mechanisms detect the presence of xenobiotics which in turn activate a

52   signalling system that induces the expression of genes that encode proteins that function in their

53   metabolism (1,2). Genes induced by exposure to xenobiotics include those encoding cytochrome

54   P450 monooxygenases that oxidize substrates, making them more polar, which in the case of

55   xenobiotics can make them available for metabolism and inactivation (3). Genes encoding

56   cytochrome P450 monooxygenases are also transcriptionally induced – along with many other genes

57   – in plants treated with herbicides (4–19). Furthermore, transcriptional changes of genes encoding

58   cytochrome P450 monooxygenases have also been shown to confer resistance to herbicides in

59   herbicide-resistant weeds (14,20–25). Consequently, it has been hypothesized that a sensing

60   mechanism exists in plants that induces the transcription of genes encoding enzymes that detoxify

61   herbicides (26,27). An alternative hypothesis is that the detoxification of herbicides in plants is not

62   activated by a herbicide sensing mechanism.

63   Chlorsulfuron is a member of the hydroxyurea family of herbicides that are active against a

64   wide range of weeds including members of the *Poaceae* (grasses) and eudicots (broad leaf plants).

65   Its target is the enzyme acetolactate synthase (ALS) which functions in the first committed step in

66   the branch chain amino acid pathway (28,29). ALS catalyses the reaction between acetate and

67   pyruvate to produce 2-acetolactate a precursor of valine and leucine. It also catalyses the reaction

68   between acetate and 2-hydroxybutyrate to form 2-aceto-2-hydroxybutyrate, a precursor of leucine.

69   Treatment of plants with chlorsulfuron and other ALS inhibitors results in a reduction in levels of

70   branched chain amino acids in a variety of plants (30–36). However, it is unclear what causes plant

71   death following treatment with ALS inhibitors. It is likely that reduced levels of branched chain

72   amino acids contribute, but it is possible that secondary catastrophic effects that result from ALS

73   inhibition are responsible for death.

74   Resistance to chlorsulfuron has evolved in the field in response to the intense selection

75   pressure imposed by herbicide treatment (37–39). Two types of resistance have evolved to

76   chlorsulfuron; target site resistance (TSR) and non-target site resistance (NTSR). Target site

77   resistance results from mutations that cause amino acid substitutions in the channel of the ALS

78   protein leading to the active site (40). It has been shown by solving the crystal structure of the *A.*

79   *thaliana* ALS enzyme in complex with ALS-inhibiting herbicides that these mutations change the

80   shape of the channel and block access of the herbicide to the active site (40). Codons of several

81   amino acids in the channel region mutate to cause TSR (37). For example, a resistance-conferring

82   mutation that is frequently found in resistant weeds is at the codon encoding a conserved proline

83   (P197) (41–43). Non-target site resistance has evolved in weeds in the field and has been shown to

84   result from higher levels of expression of genes encoding enzymes such as cytochrome P450s

85   (14,44). It is hypothesized that the high levels of expression of these enzymes can chemically modify

86   the herbicide making it inactive and or more susceptible to degradation than in wild-type.

87   To determine if plants sense herbicides we characterized the response of the model plant *M.*

88   *polymorpha* to chlorsulfuron treatment. Our data are consistent with the hypothesis that

89   chlorsulfuron acts by inhibiting acetolactate synthase. We show chlorsulfuron-treatment changes

90   the transcriptome. However, we demonstrate that the transcriptome changes caused by

91   chlorsulfuron-treatment are not induced in plants harbouring a mutant form of ALS that cannot bind

92   to chlorsulfuron. Therefore, the presence of chlorsulfuron alone is not sufficient to induce the

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93   transcriptome change. We predict transcriptome changes induced by chlorsulfuron are not involved

94   in detoxification but result from the physiological changes to the plant caused by the inhibition of

95   the chlorsulfuron target. We test this hypothesis and demonstrate that ectopic overexpression of

96   three cytochrome P450 monooxygenase and glutathione transferase encoding genes do not increase

97   chlorsulfuron resistance. These data suggest that a xenobiotic-sensing mechanism – analogous to

98   the sensing system in mammals – that senses chlorsulfuron, does not exist in *M. polymorpha*.

99

Results

Chlorsulfuron represses the growth of *Marchantia polymorpha*

102   Since some herbicides do not effectively control *M. polymorpha,* we first tested if chlorsulfuron was

103   potent against this liverwort (45,46). To determine if chlorsulfuron (CS) represses the growth of *M.*

104   *polymorpha*, Tak-2 (wild-type female) gemmae were plated on solid nutrient media for 7 days and

105   transferred to media containing different concentrations of chlorsulfuron and grown in light for a

106   further 7 days. To quantify gemmaling growth, thallus area was calculated when imaged from above.

107   The data indicate that chlorsulfuron represses the growth of *M. polymorpha* in a dose dependent

108   manner. The concentration at which plant growth is inhibited by 50% (GR_{50}) was 3.3 nM (SD = 0.5)

109   and the lethal dose was estimated to be 300 nM (Fig 1A). These data demonstrate that chlorsulfuron

110   has herbicidal activity on *M. polymorpha* and represses growth in the nM concentration range.

111

112   **Fig 1. Chlorsulfuron represses the growth of *Marchantia polymorpha***

113   **(A)** Dose response curve of the growth of 14-day old Tak-2 gemmae after seven days on CS-treated

114   medium. Data plotted with a non-linear fit, sigmoidal, four-parameter regression curve. N=5-10.

115   Error bars are ± SD.

116   **(B)** Targeted metabolic analysis showing the accumulation of valine, leucine and isoleucine,

117   tryptophan, and isopropylmalate in untreated Tak-2 plants and in plants treated with 100 nM CS.

118   Plants were grown for 11 days, transferred to treatment plates for 24 h, then extracted. Extracts

119   were analysed using a targeted approach by LC-MS/MS. Statistical significance based on Student's t-

120   test between treatment groups for each metabolite. *=p < 0.05, ** = p < 0.01, *** = p < 0.001, n.s. =

121   not significant. N=6 except in isoleucine untreated group n=5 (one outlier was removed). Box and

122   whisker plot where whiskers are first and fourth quartiles, boxes represent second and third

123   quartiles, and the horizontal line is the median.

124   **(C)** 14-day old Tak-2 gemmae grown from day 0 on medium supplemented with 800 µM branched

125   chain amino acids and/or 100 nM CS then imaged using NightOwl imaging system which measures

126   chlorophyll autofluorescence. Plant area (mm^2) was measured using indiGo software. Statistical

127   significance based on Kruskal-Wallis test, with groups determined by Nemenyi post-hoc test. Box

128   and whisker plot where whiskers are first and fourth quartiles, boxes represent second and third

129   quartiles, and the horizontal line is the mean.

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131   Inhibition of ALS by chlorsulfuron has been shown to block the biosynthesis of branched

132   chain amino acids in several organisms including bacteria and flowering plants (30–36). To test the

133   hypothesis that chlorsulfuron has the same mode of action in *M. polymorpha* as in other organisms,

134   we determined the impact of chlorsulfuron-treatment on the levels of branched chain amino acids.

135   Plants were grown from gemmae for 11 days in constant light and then transferred to solid media

136   containing 100 nM chlorsulfuron or untreated media for 24 hours. Plants were harvested and small

137   molecules were extracted and separated by liquid chromatography before detection with mass

138   spectroscopy. The levels of the branched chain amino acids valine and leucine were significantly

139   lower in treated plants than in untreated controls. Valine accumulated to approximately 725,000

140   ions (SD = 180,000) in chlorsulfuron-treated plants compared to untreated control where the amino

6

141   acid accumulated to 2,310,000 ions (SD = 600,000) (Fig 1B). Leucine accumulated to 1,240,000 ions

142   (SD = 190,000) in chlorsulfuron-treated plants compared to untreated control where the amino acid

143   accumulated to 2,580,000 ions (SD = 890,000). There was no significant difference in the amount of

144   isoleucine between the chlorsulfuron-treated and untreated plants. These data are consistent with

145   the hypothesis that chlorsulfuron blocks the activity of the ALS enzyme, the first committed step in

146   branched chain amino acid synthesis, leading to a reduction of branched chain amino acids in the

147   plant.

148   To independently test if chlorsulfuron acts by repressing branched chain amino acid

149   synthesis, we tested if the lethality caused by chlorsulfuron-treatment could be suppressed by

150   growing plants in media supplemented with branched chain amino acids. Growing plants in 100 nM

151   chlorsulfuron entirely inhibited growth while untreated controls grew to a mean area of 33.5 (SD =

152   18.4) mm^2 (Fig 1C). Growing plants in the presence of 100 nM chlorsulfuron and supplemented with

153   800 μM valine, leucine and isoleucine restored growth to 12.6 (SD = 7.2) mm^2. The suppression of

154   the chlorsulfuron-inhibition of growth by branched chain amino acid supplementation is consistent

155   with the hypothesis that chlorsulfuron-treatment blocks the synthesis of branched chain amino acids

156   by inhibiting ALS activity. These data suggest that that the inhibition of growth by chlorsulfuron is, at

157   least in part, the result of a depletion in the pool of branched chain amino acids in chlorsulfuron-

158   treated plants.

159   Together these data are consistent with the hypothesis that chlorsulfuron inhibits *M.*

160   *polymorpha* growth by inhibiting branched chain amino acid biosynthesis as it does in other

161   organisms.

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The *ALS* P197 mutation confers resistance to chlorsulfuron

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164 If ALS is the target of chlorsulfuron in *M. polymorpha*, we hypothesized that mutations in codons

165 that encode amino acids important to chlorsulfuron binding could confer resistance to the herbicide.

166 To identify mutations that confer resistance to chlorsulfuron we mutated 10^6 spores with UV

167 radiation, grew the spores on chlorsulfuron, and selected resistant mutants after 14 days of growth

168 in light. We selected two independent mutants that were morphologically similar to wild-type and

169 verified their resistance by growing gemmae from each on 140 nM chlorsulfuron; both mutants grew

170 while wild-type plants died.

171   Once chlorsulfuron-resistance was verified, we isolated DNA from each of the two lines and

172 sequenced the Mp*ALS* gene using Sanger sequencing. There was a mutation at the codon for a

173 proline at amino acid position 197 of *A. thaliana* ALS (position 207 of *M. polymorpha* ALS protein

174 sequence) in each mutant that resulted in a P197L change in each (Fig 2A, B). P197 was unchanged in

175 wild-type parental lines. The presence of the P197L mutations in Mp*ALS* in both chlorsulfuron-

176 resistant mutants is consistent with the hypothesis that chlorsulfuron acts by binding to Mp*ALS*.

177 Mutants were designated Mp*acetolactate synthase*^{chlorsulfuronresistantP197L} (Mp*als*$^{csP197L\_7}$) and

178 Mp*als*$^{csP197L\_10}$ respectively.

179

180 **Fig 2. The P197L mutation in Mp*ALS* confers resistance to chlorsulfuron**.

181 **(A)** Diagram of the Mp*ALS* gene showing the location of the mutation in Mp*ALS* identified in lines

182 Mp*als*$^{csP197L\_7}$ and Mp*als*$^{csP197L\_10}$ (arrow head). Untranslated regions (grey), exons (yellow).

183 **(B)** Alignment of the sequenced region of Mp*ALS* showing the cysteine to thymine mutation which is

184 predicted to cause an amino acid change of a proline to a leucine at amino acid position 197 of At*ALS*

185 (position 207 in Mp*ALS*).

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186   **(C)** Growth assay of wild-type Tak-2, Mp*als*^{csP197L_7} and Mp*als*^{csP197L_10} lines grown on different

187   concentrations of CS. Error bars are ± SD. N=5-10. GR_{50} is the herbicide concentration that causes a

188   50% reduction in growth. Resistance index (RI) = GR_{50} (Resistant line)/GR_{50} (Tak-2).

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190   To quantify the degree of resistance of Mp*als*^{csP197_7L} and Mp*als*^{csP197L_10} mutant and wild-type

191   plants were grown on media containing different concentrations of chlorsulfuron. The GR_{50} values of

192   Mp*als*^{csP197L_7} and Mp*als*^{csP197L_10} were 771.9 nM (SD = 89.2) and 363.9 nM (SD = 77.4) respectively (Fig

193   2C). This was more than two orders of magnitude greater than wild-type which was 3.4 nM (SD =

194   1.0). While the lethal dose of chlorsulfuron for wild-type was 300 nM, we were unable to detect a

195   lethal dose for the mutants in this experiment because mutant plants grew at the highest

196   concentrations of chlorsulfuron applied (3 µM). Such high levels of resistance in mutants compared

197   to wild-type is typical for mutations that alter the binding of the herbicide to its target (41,47,48).

198   The demonstration that mutations at the proline 197 codon confer resistance to

199   chlorsulfuron is consistent with chlorsulfuron inhibiting ALS function in *M. polymorpha*.

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201   # Mp*acetolactate synthase* (Mp*als*) complete loss of function mutants

202   # are lethal and are rescued by branched chain amino acid supplements

203   We demonstrate that the P197L mutation in Mp*ALS* confers resistance to chlorsulfuron and the

204   lethality of chlorsulfuron in treated plants can be rescued with branched chain amino acids. This is

205   consistent with ALS being the target of chlorsulfuron in *M. polymorpha*. Since ALS inhibition is lethal

206   in *M. polymorpha*, we hypothesized that mutations in Mp*ALS* leading to a complete loss of ALS

207   activity would not survive on standard media that lacked branched chain amino acids. We also

208   predicted that Mp*als* loss of function mutants would be rescued by growing them in media with

209   branched chain amino acids, in the same way that chlorsulfuron-lethality was suppressed by growing

210   chlorsulfuron-treated wild-type plants in the presence of branched chain amino acids.

211   We generated Mp*als* mutants by CRISPR/Cas9 mutagenesis using two separate guide RNAs.

212   The first guide RNA (F) was complementary to a sequence in the second exon, 5' of the conserved

213   thiamine pyrophosphate (TPP) binding domain. The second guide RNA (W) was complementary to a

214   sequence near the beginning of the last exon. We predicted that mutations at these sites could lead

215   to a total loss of function of the encoded protein. Plants were transformed with either the F or the

216   W guide RNAs and plated on selective medium supplemented with branched chain amino acids. The

217   medium was supplemented with branched chain amino acids because it was expected that at least

218   some of the Mp*als* mutants would be auxotrophic for branched chain amino acids. 100 lines were

219   selected from the transformation and the Mp*ALS* gene was sequenced in 31 plants. 16 of the

220   sequenced transformants had mutations in Mp*ALS*. Four mutant lines – Mp*als*^{lofF12}, Mp*als*^{lofF16},

221   Mp*als*^{lofF32}, Mp*als*^{lofF84} – with mutations in the regions of the gene complementary to the F guide RNA

222   were identified. We predict that Mp*als*^{lofF16}, Mp*als*^{lofF32}, Mp*als*^{lofF84} are loss of function mutations

223   because each has deletions that result in frame shifts in the coding sequence. We predict that

224   Mp*als*^{lofF12} is either wild-type or a weak loss of function mutant because it harbours an in-frame

225   insertion. Two mutant lines – Mp*als*^{lofW20} and Mp*als*^{lofW29} – were identified in the regions of the gene

226   complementary to the W guide RNA. The mutations in the *MpALS* sequence in each of the six

227   mutants are presented in Fig 3.

228

229   **Fig 3. Six independent Mp*ALS* mutant lines were generated using CRISPR/Cas9 mediated targeted**

230   **mutagenesis.**

231   **(A, B)** Mutations in Mp*ALS* were generated using a gRNA targeting the 2nd exon (gRNAF) or the 8th

232   exon (gRNAW). The thiamine pyrophosphate binding domain is represented by grey boxes, all other

233   exons are represented by black boxes. Mutations are indicated by yellow letters, insertions by red

234   letters, and deletions by red dashes.

235   **(C)** A description of the mutations generated by CRISPR/Cas9 and predicted amino acid changes in

236   the 6 Mp*als*^{lof} mutants.

237   **(D)** 18-day-old gemmalings grown on Johnsons' medium with or without supplemented branched

238   chain amino acids (800 μM). Scale bar = 5 mm.

239

240   Different Mp*als* mutants were grown on branched chain amino acids and gemmae from

241   each line were harvested. These gemmae were then grown either in standard media (without

242   branched chain amino acids) or in media supplemented with branched chain amino acids. The line

243   containing the in-frame insertion – Mp*als*$^{lof\_F12}$ – grew in both the standard media and the branched

244   chain amino acid-supplemented media (Fig 3D). This is consistent with the hypothesis that this

245   mutant is prototrophic for branched chain amino acids as expected for a line with a wild-type Mp*ALS*

246   or weak loss of function Mp*ALS* gene. By contrast, all five of the putative strong loss of function

247   mutants grew on media containing branched chain amino acids, while none grew on standard media

248   without the branched chain amino acid supplements. This suggests that these mutants are branched

249   chain amino acid auxotrophs. These data demonstrate that loss of ALS function is lethal in *M.*

250   *polymorpha* and the lethal phenotype can be suppressed by branched chain amino acid

251   supplements. The observation that branched chain amino acid media supplementation suppresses

252   the lethality of Mp*als* loss of function mutatations and chlorsulfuron treatment, is consistent with

253   the hypothesis that chlorsulfuron inhibits ALS activity in *M. polymorpha*.

254

Chlorsulfuron-treatment induces transcriptome changes and the accumulation of some non-branched chain amino acids

To determine if the presence of chlorsulfuron induced transcriptome changes we generated transcriptomes from plants that were grown on standard media for 8 days and were then transferred to chlorsulfuron-containing media for between 2 and 48 hours. Control plants were plants grown on standard media for 8 days and then transferred to standard media (with no chlorsulfuron) for 2-48 hours. Transcriptomes for each time point and treatment were generated from two technical replicates and each replicate consisted of six plants. There was a large difference in the transcriptomes of chlorsulfuron-treated and untreated controls. Steady state levels of 3093 mRNAs were changed by chlorsulfuron treatment. 1612 mRNAs were more abundant in at least one timepoint in chlorsulfuron-treated plants than in controls. Steady state levels of 1708 mRNAs were less abundant in at least one time point in the treated plants than in controls (Fig 4A). The differences in the transcriptomes of chlorsulfuron-treated and control plants are consistent with the hypothesis that chlorsulfuron-treatment induces transcriptome changes in *M. polymorpha*.

Fig 4. Chlorsulfuron treatment induces transcriptome changes and high levels of some non-branched chain amino acids

(A) Numbers of genes with altered mRNA steady state levels 0-48 h after chlorsulfuron treatment compared to control treatments.

(B) Enriched Gene Ontology (GO) terms of mRNAs that were more abundant in chlorsulfuron-treated plants compared to untreated plants. Cut-off lines drawn at equivalents of p=0.05, p=0.01, p=0.001.

(C-E) Non-targeted metabolomic analysis of wild-type plants treated for 24 h with 100 nM chlorsulfuron and untreated plants. Samples were analysed by LC-MS/MS using hydrophilic interaction liquid chromatography (HILIC) and reversed phase chromatography (RP). Principal

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279   component analyses of control (C1-6) and herbicide-treated (H1-6) samples using HILIC **(C)** and RP

280   **(D)**. Volcano plots of differentially abundant metabolites in chlorsulfuron-treated plants compared

281   to untreated plants using HILIC **(E)** and RP **(F).** Blue box indicates significantly lower levels of

282   metabolites and red box indicates significantly higher levels of metabolites. Significance based on

283   one-way ANOVA followed by Tukey HSD post-hoc test.

284

285   To identify physiological processes that were impacted by the chlorsulfuron-induced

286   transcriptome changes we carried out an analysis of Gene Ontology (GO) terms that were enriched

287   among the 1612 mRNAs with higher steady state levels in chlorsulfuron-treated plants compared to

288   controls. This showed that GO terms associated with branched chain amino acid biosynthesis and

289   branched chain amino acid metabolism are significantly enriched at 6 hours ($p < 1.10^{-6}$ and $p < 1.10^{-7}$

290   ), 36 hours ($p < 0.001$ and $p < 1.10^{-5}$) and 48 hours ($p < 1.10^{-4}$ and $p < 1.10^{-5}$) after chlorsulfuron-

291   treatment compared to other GO terms (Fig 4B). This is consistent with the hypothesis that

292   chlorsulfuron treatment blocks branched chain amino acid synthesis in *M. polymorpha* because

293   inhibition of the pathway results in an increase in the mRNA levels of genes that code for proteins

294   involved in branched chain amino acid synthesis.

295   The GO term enrichment analysis also demonstrates that GO terms for amino acid

296   biosynthesis and metabolism are enriched after 6 hours chlorsulfuron treatment in mRNAs that are

297   more abundant in chlorsulfuron-treated plants than controls (Fig 4B). These transcriptome profiles

298   predict that levels of some non-branched chain amino acids would be higher in chlorsulfuron-treated

299   plants than in untreated controls. To test this hypothesis, we measured the levels of amino acids

300   (and other metabolites) in chlorsulfuron-treated and untreated controls. Plants were grown for 11

301   days and treated with 100 nM chlorsulfuron for 24 hours before being extracted. Control plants

302   were transferred to standard media for 24 hours before extraction. Using two independent

303   separation methods – hydrophilic interaction liquid chromatography and reversed phase

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304   chromatography – we found that levels of threonine, tryptophan, arginine, serine, alanine, tyrosine,

305   glutamine, citrulline and phenylaniline are higher in chlorsulfuron-treated plants than in the

306   untreated controls (Fig 4 C-F). This is consistent with the transcriptome profiles observed in

307   chlorsulfuron-treated plants. This suggests that inhibition of ALS causes changes not only in the

308   branched chain amino acid pathway but in other amino acid pathways and therefore may have wide

309   ranging physiological impacts on the plant.

310   We conclude that inhibition of branched chain amino acid synthesis by chlorsulfuron results

311   in an increase in the steady state levels of mRNAs for enzymes involved in amino acid synthesis,

312   possibly due to a branched chain amino acid deficiency-induced transcriptional response. These

313   transcriptome changes may have led to increased levels of amino acids such as threonine,

314   tryptophan, arginine, serine, alanine, tyrosine, glutamine, and phenylaniline.

315

Induction of gene expression by chlorsulfuron requires the inhibition

of the herbicide-sensitive target acetolactate synthase

318   It has been hypothesized that a sensing mechanism initiates the detoxification of herbicides in plants

319   (26). According to this hypothesis, a sensing mechanism detects the herbicide, and the sensor

320   programs transcriptome changes that increase the expression of genes encoding proteins that

321   chemically modify herbicides (detoxification). An alternative hypothesis is that there is no specific

322   herbicide sensing mechanism and therefore no inducible system of herbicide resistance. If the

323   difference between herbicide-treated and untreated transcriptomes depends on a sensing

324   mechanism, the differences should also be observed in chlorsulfuron-treated plants even if

325   chlorsulfuron cannot inhibit its target, as in Mp*als^{cs}* target site resistant mutants. If, on the other

326   hand, the transcriptome changes are the result of metabolic changes caused by inhibition of ALS

327   activity, then the transcriptome changes should not be observed in Mp*als^cs* target site resistant

328   mutants.

329   To determine if the chlorsulfuron-induced transcriptome changes observed in wild-type

330   were also observed in chlorsulfuron target site resistant mutants, we generated transcriptomes from

331   chlorsulfuron-treated and untreated Mp*als^csP197L_7* and Mp*als^csP197L_10* target site resistant mutants

332   (Fig 5). While chlorsulfuron-treatment of wild-type plants resulted in large changes in the steady

333   state levels of over 3093 mRNAs, the steady state levels of most mRNAs were the same in

334   chlorsulfuron-treated and untreated Mp*als^cs* mutants (Fig 5B). 21 mRNAs were more abundant and

335   16 less abundant in chlorsulfuron-treated Mp*als^csP197L_7* plants compared to untreated controls.

336   Similarly, 2 mRNAs were more abundant and 1 less abundant in chlorsulfuron-treated Mp*als^csP197L_10*

337   plants compared to untreated controls (Fig 5B). Importantly, no single mRNA changed abundance in

338   every background – wild-type, Mp*als^csP197L_7* and Mp*als^csP197L_10* – when treated with chlorsulfuron

339   compared to untreated controls (Fig 5C). These data indicate that the chlorsulfuron-induced

340   transcriptome change observed in wild-type is not observed in Mp*als^csP197L_7* or Mp*als^csP197L_10*

341   mutants.

342

343   **Fig 5. Chlorsulfuron treatment does not alter gene mRNA steady state levels in Mp*als*^chlrP197L**

344   **mutants**

345   **(A)** Wild-type, Mp*als*^chlrP197_7 and Mp*als*^chlrP197_10 lines grown for 7 days on untreated media then

346   transferred to media containing 0 or 5 nM CS for 7 days. Scale bar = 5 mm.

347   **(B)** Differentially expressed genes (DEGs) between CS-treated and untreated plants in each

348   genotype. Blue bar represents genes with higher mRNA steady state levels, green bar represents

349   genes with lower mRNA steady state levels, and yellow bar represents genes with either higher or

350   lower mRNA steady state levels in CS-treated plants compared to untreated plants.

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351   **(C)** Number of differentially expressed genes in CS treated plants compared to untreated plants

352   between Tak-2, Mp*als^chlrP197_7* and Mp*als^chlrP197_10*.

353

354   These data are not consistent with chlorsulfuron itself being sensed by the plant and this

355   sensing mechanism initiating a transcriptome response. Instead, the data are consistent with the

356   transcriptome changes induced by chlorsulfuron treatment being the result of the inactivation of the

357   herbicide target, ALS. The change in gene expression caused by chlorsulfuron-treatment is likely a

358   result of the metabolic changes caused, directly and indirectly, by the inhibition of ALS by the

359   herbicide.

360

Ectopic overexpression of genes encoding cytochrome P450 monooxygenases and glutathione S-transferases does not confer resistance to chlorsulfuron

364   If the chlorsulfuron-induced transcriptome changes result from the inhibition of ALS and its

365   physiological consequences – and not from a chlorsulfuron-sensing mechanism that induces

366   transcriptome changes that lead to the production of enzymes that detoxify chlorsulfuron – we

367   predicted that genes encoding enzymes induced by exposure to chlorsulfuron would not form part

368   of such a sensing mechanism and would therefore not detoxify the herbicide.

369   To test this hypothesis, we ectopically overexpressed four genes whose steady state levels of

370   mRNA abundance increased upon chlorsulfuron treatment (Table 1). To maximize the chances of

371   identifying a gene with detoxifying potential, we selected members of the cytochrome P450

372   monooxygenases and glutathione transferases, because overexpression of members of these

373   families in mutant weeds confers herbicide resistance (49). To further increase the chances of

16

374 identifying members of the GST and CYP families that might detoxify chlorsulfuron, we selected

375 genes from GST and CYP clades that had previously been shown to increase herbicide tolerance

376 when overexpressed in herbicide resistant weeds (49–52). Using these criteria, we selected

377 Mp*CYP813A5* and Mp*CYP822A1* from the cytochrome P450 clan 72 and clan 71 respectively, and

378 Mp*GSTF15* and Mp*GSTF10* from the glutathione transferase ϕ clade.

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384 **Table 1. Several cytochrome P450 monooxygenases and glutathione S-transferases had higher**

385 **mRNA steady state levels in CS-treated compared to untreated plants in both RNA-Seq and qRT-**

386 **PCR experiments.**

Gene ID (Marpolbase)	Functional name	RNA-Seq up-regulation (Fold change Treated/Untreated)[a]					qRT-PCR verification (relative fold change Treated/Untreated)[b]			
		6 HAT	12 HAT	24 HAT	36 HAT	48 HAT	6 HAT	12 HAT	24 HAT	36 HAT
Mapoly0022s0192	**Mp*CYP813A5***	10.37***			10.41***	7.41***	1.09	1.13	1.62*	1.09
Mapoly0041s0103	Mp*CYP822B2*	2.70***					0.94	1.03	1.24	1.16
Mapoly0057s0084	Mp*CYP821B1*	2.40**					n.a.			
Mapoly0103s0038	Mp*CYP710A16*	2.89***			2.10**		0.96	1.01	1.26	1.08
Mapoly0163s0018	Mp*CYP73A109*	2.18**			2.46***	2.24**	1.16	0.97	1.10	1.72**
Mapoly0094s0036	Mp*CYP823A2*	2.95***	2.07*		3.50***	3.09***	0.93	0.93	1.08	1.69**
Mapoly0066s0002	Mp*CYP704AH1*			5.29*			0.70	1.11	0.87	1.07
Mapoly0010s0184	**Mp*CYP822A1***				14.20***	7.83***	1.05	1.18	2.43*	2.83**
Mapoly0067s0051	Mp*CYP829E1*				3.65***	3.50***	0.82	1.02	1.06	1.42*
Mapoly0095s0013	Mp*CYP822B1*				3.65***	3.91***	n.a.			
Mapoly0141s0021	Mp*CYP94L1*				3.51***	3.00***	0.99	1.64	1.64	2.84*
Mapoly0021s0144	Mp*CYP704N1*				2.68***	2.84***	0.86	1.70	1.47	1.82
Mapoly0081s0008	Mp*CYP73A110*				2.61***	2.75***	0.84	1.51	1.34	2.11*
Mapoly0004s0177	Mp*CYP829B1*				2.44***	2.33***	n.a.			
Mapoly0004s0182	Mp*CYP829F1*				2.13*	3.22***	n.a.			

Mapoly0002s0331	Mp*CYP817A1*			2.02*	2.26**	n.a.			
Mapoly0024s0025	Mp*CYP813B1*				6.27*	n.a.			
Mapoly0144s0022	Mp*CYP704L1*				4.90*	n.a.			
Mapoly0014s0215	Mp*CYP880A1*				2.14***	n.a.			
Mapoly0090s0052	Mp*CYP716X1*				2.02*	n.a.			
Mapoly0046s0045	**Mp*GSTF15***	11.74**		27.95***	30.15***	1.08	1.39	6.16*	7.56**
Mapoly0031s0032	Mp*GSTU1*	4.02*		16.28***	10.38***	n.a.			
Mapoly0818s0001	Mp*GSTF8*	6.39**		11.02***	6.41***	0.95	1.57	4.82	3.87*
Mapoly0177s0013	Mp*GSTF5*	5.33***	3.68*	8.46***	7.23***	n.a.			
Mapoly0193s0023	**Mp*GSTF10***	3.73**		11.46***	14.09***	0.88	1.56	3.00**	5.91*
Mapoly1812s0001	**Mp*GSTF7***	3.22**		3.77***	2.81**	0.87	1.33	1.58	2.63**
Mapoly0016s0155	Mp*GSTH1*		2.01**			0.95	1.26	0.74	1.12
Mapoly0009s0025	Mp*GSTF18*			5.94***	7.46***	n.a.			
Mapoly0185s0005	Mp*GSTF13*			3.63***	3.30***	n.a.			
Mapoly0008s0167	Mp*URE2p1*			3.46***	3.68***	n.a.			
Mapoly0020s0134	Mp*GSTT2*			2.86***	3.22***	n.a.			
Mapoly0002s0202	Mp*GSTF1*			2.66*		n.a.			
Mapoly0001s0057	Mp*GSTK2*			2.37***		n.a.			
Mapoly0141s0017	Mp*GSTF11*				2.43***	n.a.			

387 Colouring is based on level of fold change (FC) with $0 \leq FC < 1.5$ or non-significant FC: white, $2.5 \leq FC$

388 < 4: salmon, $4 \leq FC < 10$: orange, $10 \leq FC$: brick. Genes in bold were selected for functional analysis

389 by overexpression.

390 [a] Fold change is calculated using the DESeq2 package (53). Significance is calculated with an adjusted

391 *p*-value using the Benjamini-Hochberg method to control the false discovery rate.

392 [b] Relative fold change in RT-qPCR was calculated from internal control genes. Significant differences

393 are calculated from students t-tests between treated and untreated groups for each gene and

394 timepoint (n = 3). (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant).

395

396 Wild-type spores were transformed with overexpression vectors containing each gene under

397 the transcriptional control of the Mp*ELONGATIONFACTOR1α* promoter (*proEF1α*) using the *NOS*

398 terminator. Controls were generated by transforming wild-type spores with the empty vector

399 (construct without the CYP or GST coding sequence). Transformed plants were grown, RNA isolated

400 and the steady state levels of the respective CYP and GST mRNA measured using quantitative

401   reverse transcription polymerase chain reaction (RT-qPCR). Steady state levels of Mp*CYP813A5* and

402   Mp*CYP822A1* mRNA were 4000 times higher and 60 times higher in *proEF1α*:Mp*CYP813A5*_7 and

403   *proEF1α*:Mp*CYP822A1*_3 transformed plants than in empty vector controls respectively (Fig 6 A).

404   Steady state levels of MpGSTF15 and MpGSTF10 from mRNA were 2.5 times higher and 300 times

405   higher in *proEF1α*:Mp*GSTF15*_5 and *proEF1α*:Mp*GSTF10*_17 transformed plants than in empty

406   vector controls respectively (Fig 6 A).

407

408   **Fig 6. Ectopic overexpression of genes encoding cytochrome P450 monooxygenases and**

409   **glutathione S-transferases does not confer resistance to chlorsulfuron**

410   **(A)** Relative mRNA abundance in 12-day-old gemmalings of Mp*CYP813A5*, Mp*CYP822A1*, Mp*GSTF10*

411   and Mp*GSTF15* over-expressing lines and wild-type (Tak-1 and Tak-2) was compared to relative

412   mRNA abundance in empty vector controls (dotted line, n = 3). mRNA abundance is given as x-fold

413   change. Statistical significance based on student t-test is given for each line compared to empty

414   vector control. *=$p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, n.s. = not significant. Error bars are ± SD.

415   **(B)** 14-day-old gemmalings of an empty vector line (B, a-c) and of Mp*CYP813A5*, Mp*CYP822A1*,

416   Mp*GSTF10* and Mp*GSTF15* over-expressing lines (B, d-l) grown on untreated solid medium (Control),

417   30 nM CS and 200 nM CS. Images taken on a Berthold NightOwl. Chlorophyll autofluorescence is

418   used to measure gemmaling area (mm^2) which is treated as a proxy for growth. Images of whole

419   120x120 mm square petri dish.

420   **(C)** Box and whisker plot where whiskers are first and fourth quartiles, boxes represent second and

421   third quartiles, and the horizontal line is the median. N = 18. Statistical significance is measured by a

422   Kruskal-Wallis test. Significant groups determined by a Nemenyi post-hoc test. * = p<0.05, ** = p <

423   0.01, *** = $p < 0.001$, n.s. = not significant. Error bars are ± SD. The same data for the 30 nM CS

424   treatment is shown on a smaller y axis in **(D).**

425

426   Gemmae from control and transformed plants were grown on 0 mM (control), 30 nM and

427   200 nM chlorsulfuron. The growth of all transformed genotypes was reduced slightly compared to

428   the empty vector control when grown on media without chlorsulfuron (Fig 6 B, C). Therefore, to

429   control for within-genotype growth differences, plant area was presented as percentage of area of

430   untreated plants for each genotype (Fig 6 D). No genotypes grew on media containing 200 mM

431   chlorsulfuron, indicating that the plants transformed with the CYP and GST overexpressing

432   constructs were sensitive to the herbicide (Fig 6 B, C). There was almost no growth of any genotype

433   on 30 mM, but *proEF1α*:Mp*CYP822A1*_3 transformed plants grew slightly more than the control ($p <$

434   0.001) (Fig 6B, C, D). The *proEF1α*:Mp*CYP822A1*_3 transformed plants grew to 3.4% (SD = 1.2) of the

435   area of untreated plants, while the empty vector grew to 1.1% (SD = 0.8) area of untreated plants.

436   However, this percentage growth is very small compared to each genotype grown in the absence of

437   chlorsulfuron, therefore Mp*CYP822A1* overexpression does not restore growth of chlorsulfuron-

438   treated plants to untreated levels

439   We conclude that ectopic overexpression of two CYP-encoding genes and two GST-encoding

440   genes did not confer chlorsulfuron tolerance. These data are consistent with the hypothesis that

441   genes whose steady state mRNA levels increase on chlorsulfuron-treatment are not involved in

442   herbicide detoxification.

443

Discussion

445   We show that the herbicide chlorsulfuron induces a transcriptome response in sensitive *M.*

446   *polymorpha* plants. However, this transcriptome response does not occur in target-site-resistant

447   mutants (*Mpals^cs^*) treated with chlorsulfuron. The observation that the transcriptome change does

448   not occur in mutants where the chlorsulfuron cannot inhibit the activity of its target, the ALS

449   enzyme, suggests that inhibition of ALS is required for the transcriptome response. It also suggests

450   that the presence of chlorsulfuron alone is not sufficient to induce the transcriptome response. If the

451   presence of chlorsulfuron is insufficient to induce a transcriptome response, it suggests that the

452   herbicide molecule is not sensed by the plant. We conclude that a mechanism that senses

453   chlorsulfuron does not exist in *M. polymorpha*, and the observed transcriptome response is the

454   result of direct and or indirect effects of the inhibition of ALS by chlorsulfuron. If this observation is

455   holds true for other plant species and with other herbicides, it suggests that a herbicide-sensing

456   system that induces herbicide detoxification mechanisms does not exist in plants. We hypothesize

457   that a herbicide-sensing system that induces transcriptional changes that promote the production of

458   proteins active in herbicide detoxification does not exist in *M. polymorpha*, nor perhaps in other

459   plants.

460   There is abundant evidence in the literature that treatment of plants with herbicides results

461   in changes in steady state mRNA levels that are consistent with our data. However, the underlying

462   cause of these herbicide-induced transcriptome changes has remained unclear. The transcriptome

463   changes could have been due to the existence of a sensing mechanism that perceives the presence

464   of a herbicide, which then activates a signalling pathway resulting in changes in gene expression as

465   observed in the transcriptomes. Alternatively, the transcriptome changes could also have been the

466   result of the inhibition of the target protein by the herbicide. In the research reported here, we have

467   been able to distinguish between these possibilities. The data demonstrate that the chlorsulfuron-

468   induced changes only occur if the herbicide target, the ALS enzyme, is sensitive to the herbicide. If

469   the herbicide target is insensitive to the enzyme, the transcriptome change does not occur. Our data

470   are therefore consistent with transcriptome data reported in the literature, but go a step further to

471   show that the transcriptome change depends on the defective function of the herbicide target.

472   While we report that a chlorsulfuron sensing system does not exist in *M. polymorpha* and we

473   hypothesize that herbicide sensing mechanisms do not exist in plants, since it has been shown that

474 transcriptome responses to ALS inhibitors are highly conserved across plant species (54), it is

475 formally possible that such a sensing mechanism may exist in some plants for some herbicides. If so,

476 we expect the presence of herbicides would activate a signalling pathway that would cause

477 transcriptome changes and the production or activation of proteins that chemically modify the

478 herbicide and reduce its toxicity. Such a herbicide-plant interaction remains to be demonstrated.

479 Our conclusion – that a herbicide sensing mechanism that induces herbicide detoxification

480 does not exist – is consistent with observations from the field. Resistance to herbicides has evolved

481 many times in the field. Non target site resistance (NTSR) is a form of herbicide tolerance which

482 prevents the active herbicide from coming into contact with its target, either through the chemical

483 modification of the compound or the sequestration of the compound so that it no longer has access

484 to the herbicide target, and therefore cannot inhibit its biological function. NTSR is often the result

485 of gene overexpression. These genes may encode proteins that oxidise the herbicide (e.g.

486 cytochrome P450 monooxygenases), conjugate the herbicide to glutathione (e.g. glutathione

487 transferases) or to saccharides (e.g. glycosl-transferases) or transport the herbicide across a

488 membrane (e.g. ABC transporters). Their overexpression is the result of a mutation that increases

489 their expression levels compared to "wild-type", herbicide sensitive weeds. These overexpressing,

490 mutant alleles, would arise rarely in the field. However, these rare mutations would be selected for

491 in the presence of intense herbicide selection. They could become dominant alleles in populations

492 undergoing prolonged herbicide selection. While these resistance-conferring alleles contribute to

493 resistance in the field, our data suggest that they are not part of an inducible mechanism that

494 detoxifies herbicides.

495

496 ## General Conclusion

497 These data indicate that while chlorsulfuron-treatment results in transcriptome changes, these

498 changes are not the result of a herbicide-sensing mechanism. Instead, the transcriptome changes

499   depend on the inactivation of the chlorsulfuron target. This is consistent with the hypothesis that

500   transcriptome changes result from the disrupted physiology caused by the inhibition of ALS activity

501   and are not specifically related to herbicide detoxification.

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Materials and methods

Plant materials and growth conditions

511   *M. polymorpha* accessions Takaragaike-1 (Tak-1, male) and Takaragaike-2 (Tak-2, female) were used

512   as wild-type plants (55). Mp*als*^{csP197L}, Mp*als*^{lof}, Mp*CYP813A5*^{GOF}, Mp*CYP822A1*^{GOF}, Mp*GSTF10*^{GOF} and

513   Mp*GSTF15*^{GOF} lines were generated as described below. Plants were propagated asexually from

514   gemmae and grown in light cabinets under 30 μmolm^{-2}s^{-1} continuous white light at 23 °C.

515   Plants used in the metabolomic analyses, transcriptome analysis, quantitative real time PCR

516   verification of transcriptome data and chlorsulfuron dose response assay were grown on 100x21 mm

517   round petri dishes on top of autoclaved cellulose discs (A.A. Packaging Ltd, Preston, UK) placed on

518   the surface of 30 ml Johnson's medium (56) with 0.8% agar. Plants were transferred from the

519   herbicide-free medium to herbicide-containing medium by lifting the discs on which plants were

520   growing from one plate to another. Plants used in the other experiments were grown on 120x120

23

521   mm square petri dishes with 60 mL half strength Gamborg medium (57) with 1% sucrose and 0.8%

522   agar.

523   To generate spores for transformations, Tak-1 and Tak-2 plants were grown on a 1:3 mixture

524   of vermiculite and John Innes No.2 compost and incubated in a growth chamber under a 16 h light:8

525   h dark photoperiod at 23°C. Gametophore production was induced under far red light irradiation

526   (58).

527

Generation of Mp*als^csP197L* lines

529   Mp*als^csP197L* mutant *M. polymorpha* plants were generated via UV-B mutagenesis. 10^6 spores were

530   mutated under a UV-B wavelength of 302 nm and grown on 140 nM (0.05 ppm) chlorsulfuron under

531   continuous light. Resistant plants were selected after 14 days.

532

Sequencing Mp*ALS* in wild-type and Mp*als^csP197L* plants

534   To determine the mutations in the ALS gene of Mp*als^csP197L* lines, whole plant DNA was extracted

535   from two mutants (Mp*als^csP197L_7* and Mp*als^csP197L_10*) and wild-type (Tak-2) using the CTAB method

536   adapted from (59). DNA concentration was measured on a Nanodrop ND-1000 spectrophotometer.

537   A 140 bp region of the *M. polymorpha ALS* gene that contains the most commonly found

538   mutations conferring resistance to ALS-inhibitors was amplified by polymerase chain reaction (PCR)

539   using the PCRBIO HiFi polymerase and buffer (PCR Biosystems, Cat. No. PB10.41-02). The

540   amplification was carried out using a BIO-RAD Dyad Peltier thermal cycler. Each reaction was

541   conducted in a 50 μl mixture, consisting of 10 μl reaction buffer, 2 μl of forward primer, 2 μl of

542   reverse primer, 5 μl of DNA, 0.5 μl of HiFi Polymerase, and 30.5 μl of DNase free milli-Q H2O, with

543   three replicates per sample. The PCR program consisted of 1 min initial denaturation phase at 95 °C,

24

544   followed by 32 cycles, with each cycle consisting of a 15 s incubation time at 95 °C, a 15 s incubation

545   at 56 °C annealing temperature, and a 30s extension time at 72 °C. Amplified DNA was extracted

546   from the gel using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The quantity of

547   extracted DNA was measured on a Nanodrop ND-1000 spectrophotometer. Samples were

548   sequenced by Sanger sequencing (Source Bioscience, Oxford, UK). The primers used for PCR

549   amplification and sequencing are listed below.

550

Chlorsulfuron effect on *M. polymorpha* growth

552   A whole plant dose-response experiment was carried out to determine the sensitivity of wild-type

553   and target-site resistant plants to chlorsulfuron. Chlorsulfuron, 1-(2-chlorophenylsulfonyl)-3-(4-

554   methoxy-6-methyl-1,3,5-triazin-2-yl)urea, was supplied by Sigma Aldrich and diluted in milli-Q H2O,

555   then filter sterilized with a 0.2 μM sterile syringe filter. Chlorsulfuron was pipetted into melted

556   medium and mixed thoroughly before pouring into plates. The female *M. polymorpha* accession

557   (Tak-2) was chosen as the susceptible biotype to chlorsulfuron because it tends to grow flatter on

558   the medium than Tak-1 making it easier to image. Tak-2, Mp*als^{csP197L}_7* and Mp*als^{csP197L}_10* gemmae

559   were grown for 7 days then transferred onto herbicide treated plates and replaced in the growth

560   cabinet for 7 days before imaging. Tak-2 plants were treated with ten herbicide doses: 0 nM, 0.1 nM,

561   0.2 nM, 0.5 nM, 1 nM, 1.5 nM, 2.5 nM, 5 nM, 10 nM, and 30 nM chlorsulfuron. Mp*als^{csP197L}* plants

562   were treated with ten herbicide doses: 1 nM, 2 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM and 300

563   nM chlorsulfuron. There were two replicate plates per herbicide dose and five plants grown per

564   plate. The plants were imaged using a Panasonic Lumix camera (model no. DMC-FS7) on day 7 after

565   transfer to herbicide plates. Lateral growth was quantified using Image J. Data were expressed as %

566   of the average area of untreated plants and were plotted with a non-linear fit, sigmoidal, four-

567   parameter regression curve using GraphPad Prism 9.1.0 (GraphPad Software, Inc., San Diego, CA,

568   USA). The herbicide dose resulting in 50% growth reduction (GR$_{50}$) was calculated as follows:

25

569
$$y = C + \frac{D - C}{1 + \left(\frac{ED50}{x}\right)^{b}}$$

570   In which C is the lower limit, D is the upper limit, ED_{50} is the effective dose which reduced growth by

571   50%, and b is the slope at ED_{50}. Resistance index was then calculated as follows: $RI = \frac{GR50(S)}{GR50(R)}$

572

Branched chain amino acid growth assay

574   Tak-2 gemmae were grown in petri dishes on solid medium supplemented with 800 μM branched

575   chain amino acids, 800 μM branched chain amino acids and 100 nM CS, with 100nM CS only or on

576   unsupplemented medium and grown for 14 days. Whole plates were imaged using a Berthold

577   NightOwl II LB 983 *In Vivo* imaging system (Berthold, Bad, Wildbad, Germany) which detects

578   chlorophyll autofluorescence (560 nm). Images were taken after exposing the plants to 120 s white

579   light. The area (mm^2) of living tissue was measured using indiGo™ software (Berthold, Bad Wildbad,

580   Germany). Statistical significance was based on Kruskal-Wallis test, with groups determined by

581   Nemenyi post-hoc test.

582

Sample extraction for metabolomic analysis

584   11-day old Tak-2 plants were transferred to medium containing 100 nM CS or untreated medium for

585   24 hours, then extracted. Approximately 150 mg of fresh plant tissue per sample was collected and

586   frozen immediately in liquid N2. 6 biological replicates were collected per treatment group.

587   Metabolite extraction was as follows. 500 μL methanol:acetonitrile:H_2O (2:2:1, v/v) (stored at -20 °C)

588   was added to each sample. Plant material was then homogenised for 2 min in a tissuelyser (Qiagen)

589   using adaptors kept at -70 °C. Samples were incubated for 1 h at -20 °C followed by centrifugation at

590   14,000 rpm at 4 °C. The supernatant was removed and placed in a new Eppendorf tube, and stored

591   at -20 °C. 400 μL of 80% methanol:water (stored at -20 °C) was added to the precipitate then

592   vortexed for 60 s at 4-8 °C. Samples were incubated for 1 h at -20 °C then centrifuged for 14,000 rpm

593   at 4 °C. The supernatant from the first extraction was combined with the second extraction, and the

594   samples were stored for 2 h at -20 °C. Samples were centrifuged one final time at 14,000 rpm for 10

595   min at 4 °C. The supernatant was transferred to a new Eppendorf tube, frozen in liquid N2 then

596   stored at -80 °C.

597

598   Targeted metabolomic analysis

599   Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of samples from

600   chlorsulfuron-treated and untreated plants was performed by the Metabolomics Facility at Vienna

601   BioCenter Core Facilities (VBCF), member of the Vienna BioCenter (VBC), Austria and funded by the

602   City of Vienna through the Vienna Business Agency. For the analysis of branched chain amino acids

603   and their precursors, 1 μL of the metabolite extract was injected on a Kinetex C8 column (100 Å, 100

604   x 2.1 mm), employing a flow rate of 80 μL/min. A 5-min-long gradient from 95% A (0.1 % formic acid

605   in water) to 60% B (0.1 % formic acid in acetonitrile) was used for the separation. The HPLC (RSLC

606   ultimate 3000; Thermo Fisher Scientific) was directly coupled to a TSQ Altis mass spectrometer

607   (Thermo Fisher Scientific) via electrospray ionization. The following SRM (selected reaction

608   monitoring) transitions were used in the positive ion mode: m/z 118.1 to m/z 72 (valine), m/z 132.1

609   to m/z 86.1 (isoleucine and leucine), m/z 205.1 to m/z 188 (tryptophan). Isopropylmalate wash was

610   measured in the negative ion mode using the transitions m/z 175.1 to m/z 115 and m/z 118.1 to m/z

611   113. Optimal collision energies and retention times were determined and validated for each

612   metabolite with the respective authentic standard. Other amino acid precursor metabolites (e.g. 2-

613   ketobutyric acid) were targeted using the respective SRMs but only detected in the mixture of

614   authentic standards. The data was manually interpreted using Trace Finder (Thermo Fisher

615   Scientific).

616

Non-targeted metabolomic analysis

618 For the non-targeted detection of metabolites in chlorsulfuron treated and untreated plants,

619 samples were analysed by LC-MS/MS using the hydrophilic interaction liquid chromatography (HILIC)

620 the reversed phase chromatography (RP) separation methods. 1 µL of each sample was injected

621 independently onto two different phase systems, on a SeQuant ZIC-pHILIC HPLC column (Merck, 100

622 x 2.1 mm; 5 µm) and on a C18-column (Waters, ACQUITY UPLC HSS T3 150 x 2.1; 1.8 µm). 6 µL of

623 each sample was pooled into a quality control (QC) sample used for normalization. Separation was

624 performed with a flow rate of 100 µl/min employing an Ultimate 3000 HPLC system (Thermo Fisher

625 Scientific, Germany). In HILIC (hydrophilic interaction liquid chromatography) a 25 min gradient from

626 10% to 80% B was used (A: acetonitrile (ACN); B: 25 mM ammonium bicarbonate in water) and in

627 reversed phase chromatography (RP) a gradient from 1% to 90% B in (A: 0.1% formic acid (FA) in

628 water; B: 0.1% FA in ACN). The HPLC was coupled via electrospray ionization to the mass

629 spectrometer. Metabolites were ionized via electrospray ionization in polarity switching mode,

630 acquiring high-resolution tandem mass spectrometry data on a Q-Exactive Focus (Thermo Fisher

631 Scientific, Germany) in data-dependent acquisition mode. Data sets were processed by Compound

632 Discoverer (Thermo Fisher Scientific), searching our in-house library and publicly available spectral

633 libraries with a mass accuracy of 3 ppm for precursor masses and 10 ppm for fragment ion masses.

634

gRNA design and vector construction for the generation of Mp*als* loss of function alleles

637 The generation of mutations in the Mp*ALS* gene using the CRISPR/Cas9 system followed the method

638 developed by (60). Two short guide RNAs (gRNAs) gRNA_W (GTTCTGCCTATGATTCCTGG,

639 complementary to nucleotides 1962 to 1984 of the coding sequence) and gRNA_F

28

640   (CTCTGAGGGGACATGCATAC, complementary to the reverse strand of nucleotides 133 to 153 of the

641   coding sequence) were designed to target two different locations in the Mp*ALS* DNA sequence.

642   gRNAs must be upstream (5') of a PAM sequence (NGG) and contain a protospacer or 'target'

643   sequence consisting of 20 nucleotides that are unique to Mp*ALS*. gRNAs were designed using the

644   CRISPR-P website (61). Individual gRNAs were cloned in the MpU6-1pro:gRNA expression vector

645   using BsaI overhangs. MpU6-1pro:gRNA was combined with the Cas9 expression vector, MpGE010

646   via LR recombination. Vectors were transformed into *Escherichia coli* One Shot OmniMAX 2 T1

647   (ThermoFisher Cat# C854003). CRISPR-Cas9 expression vectors carrying gRNA_W or gRNA_F were

648   transformed in *Agrobacterium tumefaciens* (GV3101). *Agrobacterium*-mediated spore

649   transformation followed previously described methods (55), with the additional step of

650   supplementing 800 µM isoleucine, leucine and valine to the *Agrobacterium* and spore liquid cultures

651   and to the medium in the selection plates.

652

Selection and genotyping of transformants

654   Approximately 100 transformants were grown to adult size on medium containing the hygromycin

655   selection and 800 µM isoleucine, leucine and valine. One gemma was taken from each of 100

656   transformants and placed on medium supplemented with 800 µM branched chain amino acids,

657   while a gemma from the same gemma cup was placed on medium without branched chain amino

658   acids. Plants from which the gemma died or grew smaller on the medium lacking branched chain

659   amino acids were then chosen as possible Mp*ALS* mutants and genotyped. Plants were genotyped

660   using the Phire Plant Direct PCR kit (Thermo Fisher) using a 0.5 mm diameter piece of plant tissue

661   and the manufacturer's recommended 3-step cycling protocol. Primers used for genotyping can be

662   found listed below.

663

Sample preparation and RNA extraction

665 Total RNA was extracted from Tak-2 and target-site resistant mutants Mp*als$^{csP197L\_7}$* and

666 Mp*als$^{csP197L\_10}$* across eight timepoints: 0h, 2h, 4h, 6h, 12h, 24h, 36h and 48h after transferring plants

667 to herbicide-treated (5 nM) or untreated plates. The criteria for the selection of the herbicide dose

668 was a dose causing a toxic effect without killing the plant, and was chosen based on the results from

669 the dose response curve. There were two replicate samples per timepoint, and six plants per sample.

670 Total RNA was extracted from 90 samples.

671 Gemmae were grown for 8 days on cellulose discs on untreated medium then moved onto

672 chlorsulfuron-treated medium or untreated control plates. Plants were collected at the different

673 timepoints after transfer onto chlorsulfuron and immediately frozen in liquid N2 and kept at -80°C.

674 Samples were pulverised with a tissuelyser (Qiagen) and total RNA was extracted using RNeasy plant

675 mini kits (Qiagen, Hilden, Germany). To remove DNA, RNA samples were DNase treated using the

676 DNA-free kit by Ambion (Life Technologies) following manufacturer instructions. The quantity of RNA

677 was measured on a Nanodrop ND-1000 spectrophotometer and RNA integrity was measured on an

678 Agilent 2100 BioAnalyser (Agilent Technologies). The RNA integrity number (RIN) provided by the

679 BioAnalyzer software ranges from 1 (a very degraded sample) to 10 (a mostly intact sample).

680 Samples with RIN numbers ≥8 were suitable for cDNA library preparation following the Illumina RNA

681 prep protocols (Welcome Trust Oxford Genomics Centre, Oxford). Illumina sequencing of 75 bp

682 paired-end reads was conducted on the 90 cDNA libraries by an Illumina HiSeq4000. Samples were

683 run over five lanes, and coverage was approximately 12 million reads per sample.

684

Read pre-processing and mapping to reference transcriptome

686 Raw reads were returned as fastq.gz files. These were unzipped and their quality was viewed using

687 FastQC v.0.11.7. Illumina adaptors and low-quality tails were quality trimmed using Trimmomatic-

688   0.38 (62). Paired-end reads were interleaved, and ribosomal RNA was removed using Sortmerna-2.1

689   (63) and error corrected using BayesHammer (SPAdes-3.10.0) (64) with setting–only-error-

690   correction. Clean reads were mapped to a reference transcriptome (JGI 3.1, obtained from

691   https://marchantia.info/download/v31/) using Salmon (65).

692

Differential expression analysis

694   The differences in expression of each gene per transcript between sample pairs were calculated

695   using the DESeq 2 v.1.21.3 package (53) in the statistical software R. P-values were adjusted

696   following the Benjamini-Hochberg procedure for controlling the false discovery rate (66). The criteria

697   for differential gene expression was an adjusted p-value < 0.05 and a $|log2(fold\text{-}change)| \geq 1$ (a fold-

698   change of expression ≥ 2) between compared groups. Volcano plots were produced for each

699   comparison. Expression differences were compared between untreated and treated Tak-2 2-48

700   hours after chlorsulfuron treatment.

701

Gene Ontology term enrichment analysis

703   The VISEAGO R package (67) was used to identify GO terms significantly enriched in differentially

704   expressed genes between treated and untreated plants, using the classic algorithm and Fisher's

705   exact test. The functional annotations obtained from Marpolbase

706   (https://marchantia.info/download/MpTak_v6.1/) for the MpTakv6.1 transcriptome were used to

707   create a custom GO database, because the transcriptome used for the RNA-Seq analysis (JGI 3.1) is

708   poorly annotated with a large number of obsolete GO annotations. Takv6.1 annotations were

709   associated with JGI 3.1 gene names using the gene correspondence table provided by Marpolbase.

710   Multidimensional plots were generated showing clusters of GO terms into functional groups based

711   on Wang's semantic similarity distance and ward.D2 aggregation criterion.

712

Real time quantitative PCR validation of candidate genes

714   The RNA-Seq experimental setup was repeated with fewer timepoints for real time quantitative PCR

715   (RT-qPCR) validation. Plants were transferred to control or herbicide treated plates eight days after

716   growth and collected 6, 12, 24, and 36 hours after treatment. Samples were collected and RNA was

717   extracted and DNase treated as described above. 1 µg RNA was converted into cDNA using

718   ProtoScript II RT (NEB), oligo(dT)s and Murine RNAse inhibitor (NEB) in a 20 µL reaction volume

719   according to manufacturer instructions. Specific primers for the Mp*CYP* and Mp*GST* genes identified

720   in the transcriptome analysis were designed using the Primer3Plus tool

721   (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Mp*APT* and Mp*ACT* were selected as

722   reference genes (68). The list of primers used for RT-qPCR can be found below. Primer efficiencies

723   were calculated for each primer pair by building a standard curve with a 1:5 dilution series of pooled

724   cDNA (50, 10, 2, 0.4, 0.08 ng) and ranged between 1.8-2.2.

725   RT-qPCR experiments were performed with three biological replicates using a QuantStudio 7

726   cycler (Applied Biosystems). The 10 µl reaction mixture contained 5 µl 2X SYBR Green/ROX master

727   mix (Applied Biosystems), 2 µl of 1:20 diluted cDNA, and 0.6 µl each of the forward and reverse

728   primer, and 2.4 µl of RNAse free water. Each biological replicate was run in two technical replicates.

729   The QuantStudio7 cycling method was as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of

730   95°C for 15s and 60°C for 1 min. A melt curve analysis was conducted after PCR amplification to

731   check primer specificity, with temperatures ranging from 60°C to 95°C increasing in 0.05°C s^{-1}

732   increments. Relative expression was calculated using an adapted 2-(ΔΔCt) method that accounts for

733   differences in primer efficiencies between the gene of interest and the reference gene(s), and

734   incorporates multiple control genes (69). Technical replicate Ct values were averaged for each

735   biological replicate. Each biological replicate Ct was then subtracted from the average Ct of the 3

736   untreated biological replicates to give ΔCt values. The calculated primer efficiency of each primer

32

737   pair to the power of ΔCt gave the relative quantity (RQ). Expression of the gene of interest in each

738   sample was calculated by dividing the RQ of that gene by the geometric mean of the RQ of the two

739   reference genes in the same sample to give the normalised relative quantity (NRQ) (70). Significant

740   differences between treated and untreated groups for each gene and timepoint were calculated by

741   Student's t-tests.

742

743   **Vector construction for the generation of Mp*CYP* and Mp*GST* overexpression alleles**

744   DNA was extracted from the Tak-2 wild-type line using a CTAB extraction method adapted from (59).

745   Each of the up-regulated genes (Mp*GSTF7*, Mp*GSTF10*, Mp*GSTF15*, Mp*CYP813A5*, Mp*CYP822A1*) was

746   PCR amplified from genomic Tak-2 DNA using CloneAmp HiFi Premix (Takara Bio). The primers used

747   to amplify gene sequences are listed below. PCR products were run on 1% agarose gel and

748   fragments of the correct size were extracted and purified using the GeneJET Gel Extraction Kit

749   (ThermoFisher) following manufacturer instructions. The purified products were individually cloned

750   into a directional entry vector pENTR D-TOPO (ThermoFisher) and transformed into *E. coli*. The

751   plasmid was extracted and purified using a Minprep Kit (Qiagen), and then cloned by LR reaction into

752   destination vector pMpGWB403 carrying the constitutive endogenous elongation factor alpha

753   (Mp*EF1α)* promoter (71). The final vectors were transformed into *Agrobacterium tumefaciens*

754   (GV3101) by electroporation. Agrobacterium-mediated transformation of *M. polymorpha* spores

755   followed previously described methods (55).

756

757   # Real time quantitative PCR of Mp*GST* and Mp*CYP* overexpression

758   # transformants

759   RNA was extracted from 14-day old gemmalings from five independent lines transformed with an

760   overexpression construct containing one of five chlorsulfuron-induced genes (Mp*GSTF7*, Mp*GSTF10*,

33

761   Mp*GSTF15*, Mp*CYP813A5*, Mp*CYP822A1*). RNA was extracted using the RNeasy plant mini kit

762   (Qiagen, Hilden, Germany). RNA samples were DNase treated using the DNA-free kit by Ambion (Life

763   Technologies) according to manufacturer instructions. RNA was converted to cDNA as described in

764   the transcriptome analysis. The primers used for RT-qPCR are listed below. RT-qPCR experiments

765   were performed with three biological replicates using a QuantStudio7 cycler (Applied Biosystems).

766   Each biological replicate sample was run in two technical replicates. The reaction mixture, cycling

767   method and relative expression calculations were conducted as described in the transcriptome

768   analysis.

769

770   Herbicide sensitivity assay of Mp*GST* and Mp*CYP* overexpression lines

771   To characterise the sensitivity of Mp*GST*- and Mp*CYP*- over-expressing plants to chlorsulfuron,

772   gemmae over-expressing one of the four candidate genes (Mp*GSTF7*, Mp*GSTF10*, Mp*CYP813A5* and

773   Mp*CYP822A1*) and from an empty vector control were grown on medium supplemented with 30nM

774   chlorsulfuron (non-lethal dose), 200 nM chlorsulfuron (lethal dose) or no herbicide. Significant

775   differences in growth between each line and the empty vector control within each treatment were

776   calculated by Student's t-tests.

777

778   Primers

Gene	Experiment	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product size (bp)
Mp*ALS*	PCR amplification	TCATTGGTGTTATAATCGTTGAAGTGG	AGGTAATTGTGCTTGGTGATCG	291
	Sequencing	TTGGTCCGACTCATCATTTG		
	CRISPR-Cas9 gRNA_F	CTCGCTCTGAGGGGACATGCATAC	AAACGTATGCATGTCCCCTCAGAG	
	CRISPR-Cas9 gRNA_W	CTCGTTCTGCCTATGATTCCTGG	AAACCCAGGAATCATAGGCAGAA	
	Genotyping gRNA_F	TATTGCTATCTCGCGCTGTGT	CTGGAACGTGTAAGAGCCTG	648
	Genotyping gRNA_W	TGTCAGGGATGGACAGTATGG	GGACAAGGCTACCTCTTCAATGT	537
Mp*ACT*	qPCR	AGGCATCTGGTATCCACGAG	ACATGGTCGTTCCTCCAGAC	108
Mp*APT*	qPCR	CGAAAGCCCAAGAAGCTACC	GTACCCCCGGTTGCAATAAG	146

Mp*CYP73A109*	qPCR	GCAAACAACCCCGAACACTG	ACAGCGGAATGGCGATGATG	154
Mp*CYP73A110*	qPCR	GAAAGACCAGGGGCAATCGA	GAATCACCATCAGCGTTGCC	145
Mp*CYP94L1*	qPCR	AGCGATGGTGCTGACATTCA	GTACCAGTTTGCTCTCGGCT	122
Mp*CYP704AH1*	qPCR	ACCTGCGACGGAAACATCTT	CAGCTGCCGTTGATTCACAC	125
Mp*CYP704N1*	qPCR	CCGGATGGCACCCGTATTAA	CTGGTTGGAAGACTCCGTCC	139
Mp*CYP710A16*	qPCR	TCCAATGACCATGCGGCTAC	GCTTCGTGGTATGCAAAGCC	177
Mp*CYP813A5*	qPCR	CGATGGTCACGGTGGTCTTG	AATCAGAAGTGCTGACGCGA	129
	Overexpression	CACCTCGGGTAAGACTGGTC	TAGCAGTCAGGCCAGGATAT	2892
Mp*CYP822A1*	qPCR	CCACACGTGAACATGGAGGA	GCTTGAGCACAAAAGGGTCG	127
Mp*CYP822B2*	qPCR	GTTGTCGGTGTAGGGAAGCA	GTGAGAACGGGGATAGCTCG	110
	Overexpression	CACCATGGAGAATGCTACGTTT	CGGCTGTGCCAATGAATTCTA	2113
Mp*CYP823A2*	qPCR	ACAAGGACGCGATGGACTTC	CCTGCCAAAACCATGTCGTG	109
Mp*CYP829E1*	qPCR	TGAGTGTGTGGCAGCCTTAG	TTTCGTCGTGGGCTCAATGA	94
Mp*GSTF7*	qPCR	ATACAACGGCCAAGGCAAGA	TCTGTCACGCCCTAGATGGA	92
	Overexpression	CACCATGGCGATCAAGATTCAT	AGCTTCAATTCGACAGTGATTTCA	1189
Mp*GSTF8*	qPCR	TCACTATCGAATGGTAGTTGCA	ATGCAGGAAAGCCCTCAAGG	142
	Overexpression	CACCATGGCGATCAAGATTCAT	CACAAAGAGTCTTAGCGGCCG	1246
Mp*GSTF10*	qPCR	GGGTCAGGGTACTCCTCTGT	TTCCTGAGTTCCTCCCCGAT	123
	Overexpression	CACCATGGCGAACACGATTTTT	CGATCGATGTCAATAATTGTTGGTGTA	1093
Mp*GSTF15*	qPCR	ATCGGGATATTTCCAGGGCATG	GTTCAAGAGGCGTGCTGAAAG	105
	Overexpression	CACCATGGCCATTCAGATCTAT	TCATGGGATATGTCCTCCCAG	1544
Mp*GSTH1*	qPCR	CGCTCAGACTGTATGGCGAT	TCTGCGTCCACTCTTTGTCC	114

779   The overhangs for CRISPR-Cas9 and Gateway cloning are underlined. Start and stop codons are in

780   bold.

781

Acknowledgments

788

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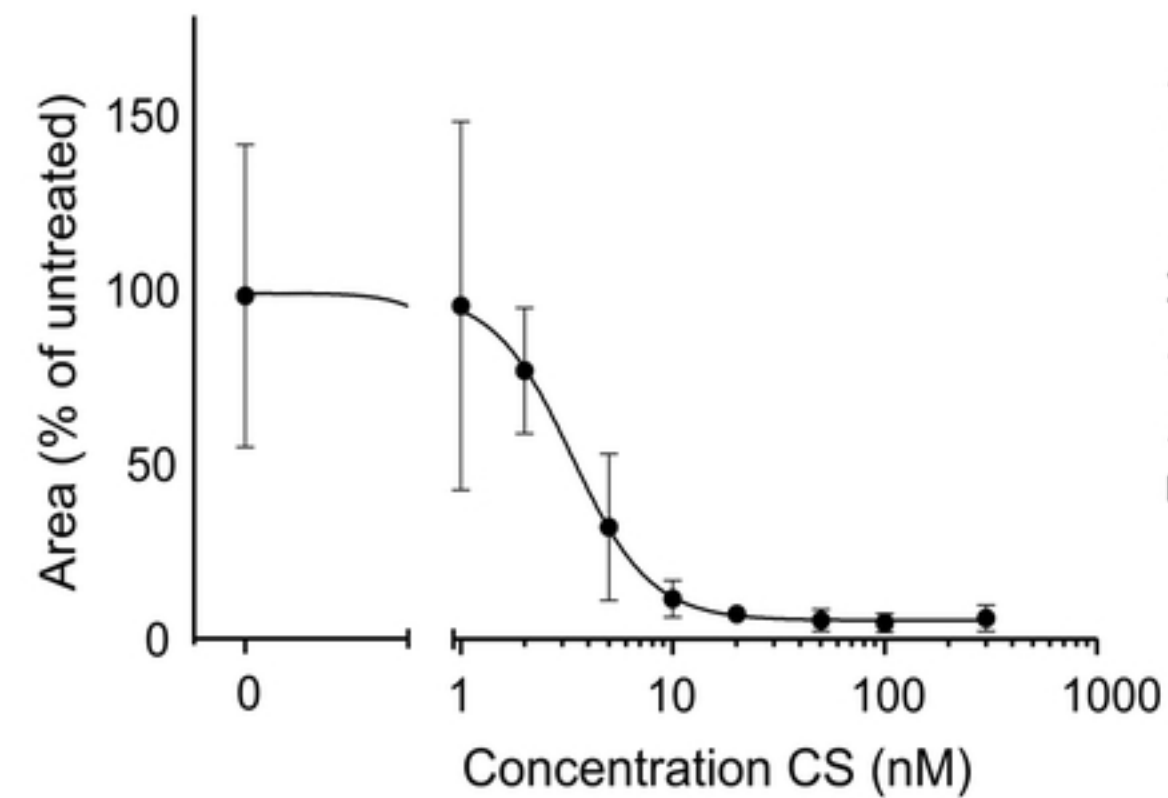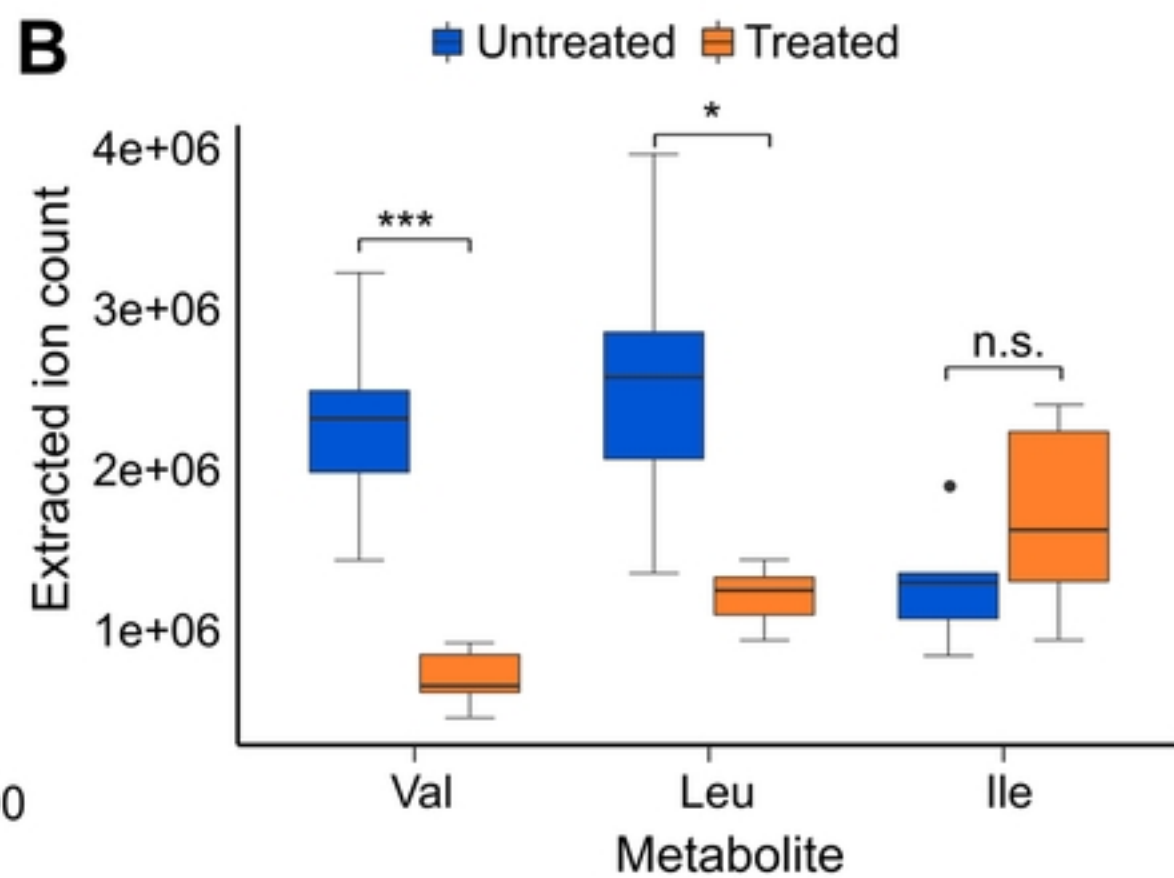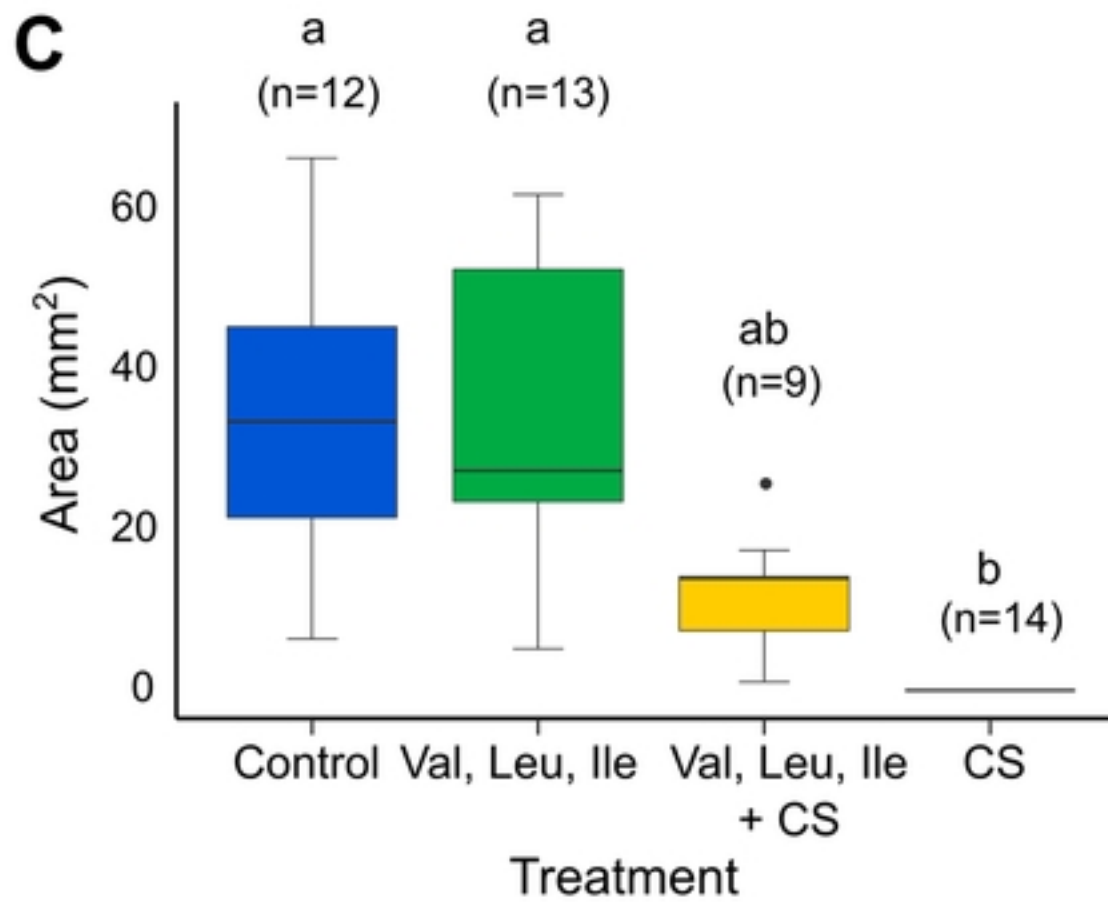
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Supporting Information

981 **Table S1. List of metabolites detected in both RP and HILIC untargeted metabolomics analyses of**

982 **chlorsulfuron-treated *M. polymorpha* (Fig 4C-E).** Showing metabolite name, molecular weight

983 (MW), retention time (RT), normalized peak area, Log_2(fold change), *p*-value and adjusted *p*-value for

984 each sample. C1-C6 are control samples. H1-H6 are herbicide-treated samples.

A MpALS 0 — 7 kb

B

P197L

intron   G   Q   V   R   R   M

Tak-2   1 CCCCTCGTCTAGTTCATTTCCGTCTAACTATGTTTCTTTTCCATTGCAGGTCAAGTACC CAGAAGGATGA 70

Mp*als*csP197L_7   1 CCCCTCGTCTAGTTCATTTCCGTCTAACTATGTTTCTTTTCCATTGCAGGTCAAGTACT CAGAAGGATGA 70

Mp*als*csP197L_10   1 CCCCTCGTCTAGTTCATTTCCGTCTAACTATGTTTCTTTTCCATTGCAGGTCAAGTACT CAGAAGGATGA 70
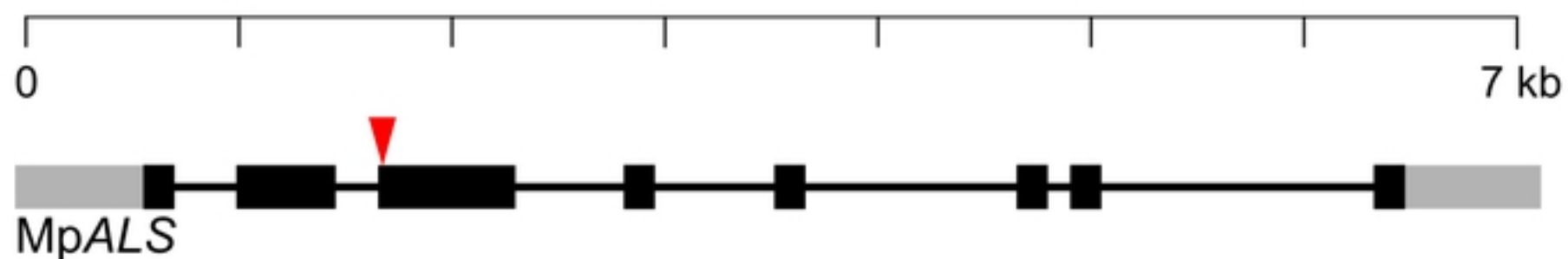
C
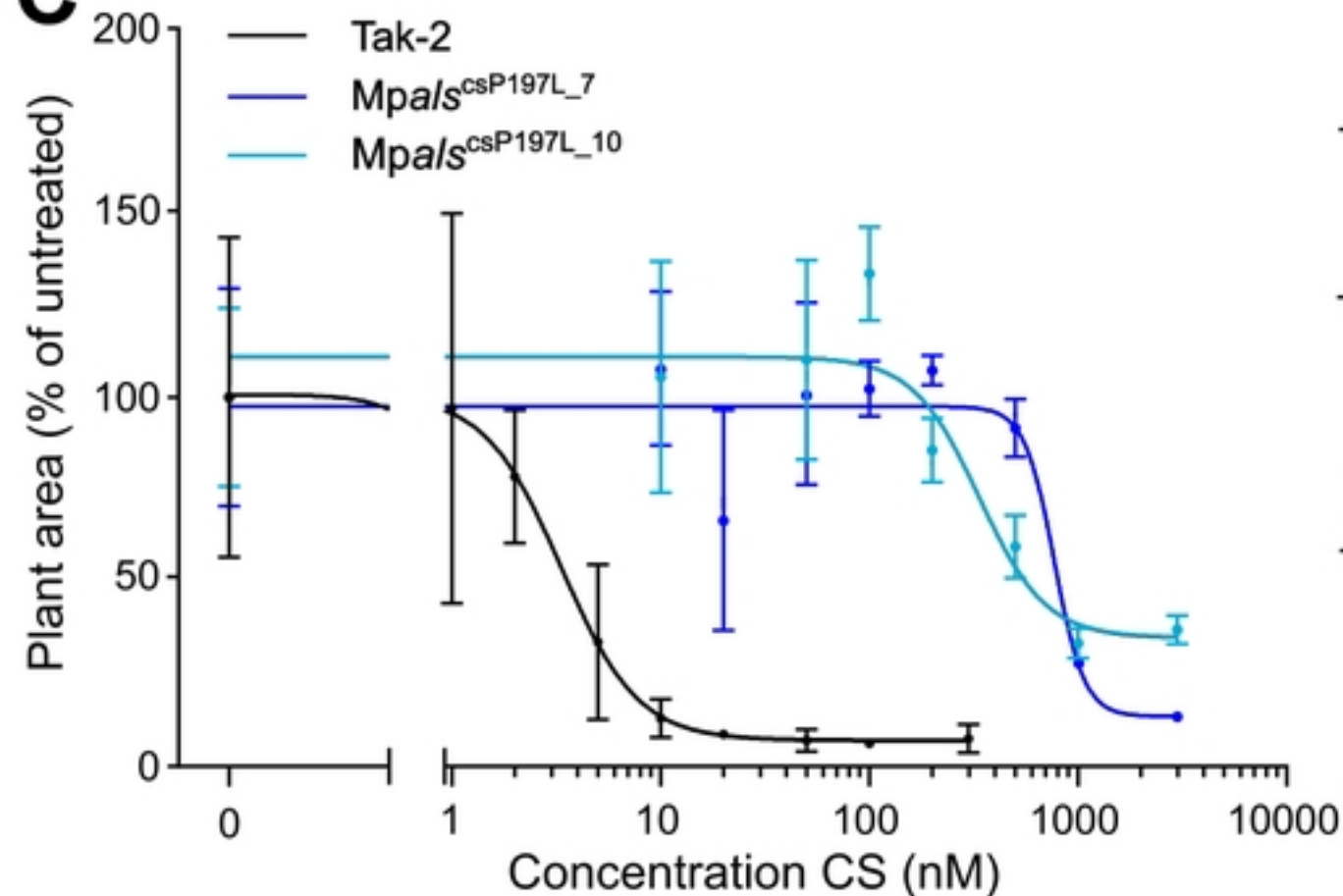
Genotype	GR$_{50}$ (nM)	RI (GR$_{50}$[R/S])
Tak-2	3.3 ± 0.5	1
Mp*als*csP197L_7	771.9 ± 89.2	233.9 ± 69.4
Mp*als*csP197L_10	363.9 ± 77.4	100.6 ± 57.0

A

gRNAF

MpALS

| | WT | 5'-TCGGGAGCCGCTCTGAGGGGACATGCATACAACACC-3' |
| | | S G A A L R G H A Y N T |

Mpals_F12lof TCGGGAGCCGCTCCCGAGAATACATGTTGTACCCTCATGTTCCCCATGAGGGGACATGCAT
              S G A A P E N T C C T L M F P M R G H A

Mpals_F16lof TCGGGAGCCGCTC   CG-----GACATGCAT
              S G A A   A   D M H

Mpals_F32lof TCGG---------   --AGGGGACATGCAT
              S   E   G T C

Mpals_F84lof TCGGGAGCCGCTC   -------ACATGCAT
              S G A A   H   M H

B

gRNAW

MpALS

| | WT | 5'-GAGCACGTTCTGCCTATGATTCCTGGGGGGAGCAAGT-3' |
| | | E H V L P M I P G G A S |

Mpals_W20lof GAGCACGTTCTGCCTATGATAGGCATAT-GGGGAGCAAGT
              E H V L P M I G I W G A S

Mpals_W29lof GAGCACGTTCTGCCTATGATTC-T GGGGGAGCAAGT
              E H V L P M I L   G E Q

C

Mpalslof line	DNA sequence change	Protein sequence change
F12	36bp insertion in 2nd exon	12 aa insertion (in frame)
F16	T>C mutation, 4bp deletion in 2nd exon	Frameshift, truncation 17 aa downstream
F32	11bp deletion in 2nd exon	Frameshift, truncation 12 aa downstream
F84	7bp deletion in 2nd exon	Frameshift, truncation 14 aa downstream
W20	4bp insertion, 1bp deletion, four mutations in 8th exon	Pro>Gly, Gly>Trp substitutions, insertion of Ile
W29	1bp deletion in 8th exon	Frameshift, loss of stop codon

D

JM + Ile, Leu, Val | JM

Mpals_F12lof | Mpals_F12lof

Mpals_F16lof | Mpals_F16lof

Mpals_F32lof | Mpals_F32lof

Mpals_F84lof | Mpals_F84lof

Mpals_W20lof | Mpals_W20lof

Mpals_W29lof | Mpals_W29lof

A Tak-2 CS Tr vs U

B Gene_ratio 0.2 0.4 0.6 | Gene_count ○ ○ ○ ◯ 5 10 15 20

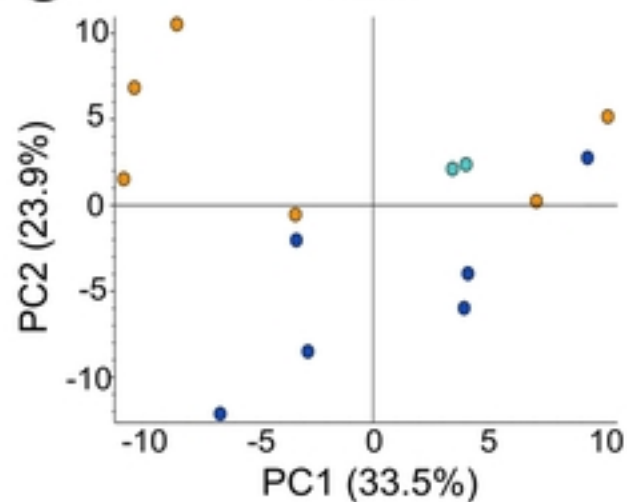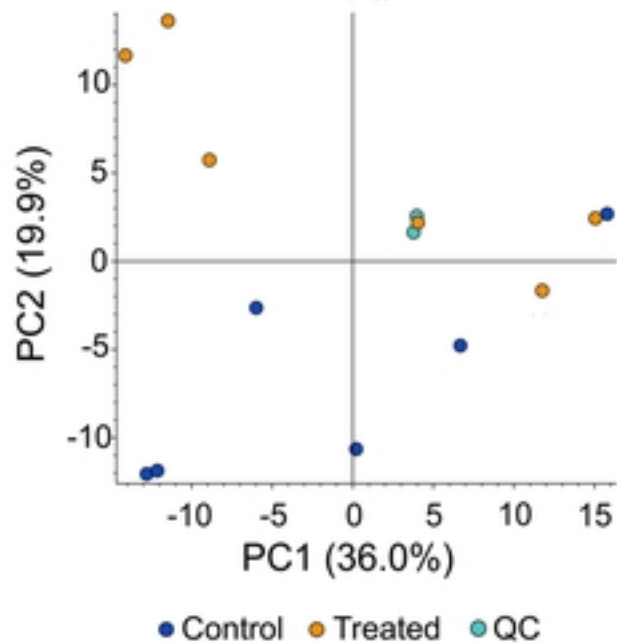6 HAT | 24 HAT | 36 HAT | 48 HAT

branched−chain amino acid metabolic process GO:0009081
branched−chain amino acid biosynthetic process GO:0009082
arginine metabolic process GO:0006525
glutamine biosynthetic process GO:0006542
glutamine metabolic process GO:0006541
cellular amino acid metabolic process GO:0006520
cellular amino acid biosynthetic process GO:0008652
arginine biosynthetic process GO:0006526
glutamine family amino acid metabolic process GO:0009064
glutamine family amino acid biosynthetic process GO:0009084
alpha−amino acid metabolic process GO:1901605
L−phenylalanine catabolic process GO:0006559
erythrose 4−phosphate/PEP family amino acid catabolic process GO:1902222

−Log$_{10}$(*p*-value)

C HILIC

PC2 (23.9%) / PC1 (33.5%)

D RP

PC2 (19.9%) / PC1 (36.0%)

● Control ● Treated ● QC

E HILIC

Serine
Arginine
Tryptophan
Citrulline
Glutamine
Tyrosine
Leucine

−Log$_{10}$(*p*-value) / Log$_2$(Fold Change)

F RP

Threonine
Tryptophan
Citrulline
Glutamine
Alanine
Methionine
Tyrosine
Phenylalanine

−Log$_{10}$(*p*-value) / Log$_2$(Fold Change)

A

	Tak-2	Mp*als*csP197L_7	Mp*als*csP197L_10
0 nM CS			
5 nM CS			

B

□ Higher □ Lower □ DE

Gene number

3000

2000

1000

0

Tak-2   Mp*als*csP197L_7   Mp*als*csP197L_10

Genotype

C

Tak-2

3090

3   0

0

18   3   0

Mp*als*csP197L_7   Mp*als*csP197L_10

A

MpCYP813A5

Relative expression

n.s. n.s. ***

Tak-1 Tak-2 Empty vector proEF1α:MpCYP813A5_7

MpCYP822A1

n.s. ** ***

Tak-1 Tak-2 Empty vector proEF1α:MpCYP822A1_3

MpGSTF10

* n.s. **

Tak-1 Tak-2 Empty vector proEF1α:MpGSTF10_17

MpGSTF15

* ** *

Tak-1 Tak-2 Empty vector proEF1α:MpGSTF15_5

B

Control 30nM CS 200nM CS

Empty vector

proEF1α:MpCYP813A5_7

proEF1α:MpCYP822A1_3

proEF1α:MpGSTF10_17

proEF1α:MpGSTF15_5

C

% of control area

Control 30nM CS 200nM CS

ac ab b c ad

Empty vector proEF1α:MpCYP813A5_7 proEF1α:MpCYP822A1_3 proEF1α:MpGSTF10_17 proEF1α:MpGSTF15_5

Genotype

D

% of control area

*** ** *** * *

Empty Vector proEF1α:MpCYP813A5_7 proEF1α:MpCYP822A1_3 proEF1α:MpGSTF10_17 proEF1α:MpGSTF15_5

Genotype