Title: Inter-chromosomal linkage disequilibrium and linked fitness cost loci associated with selection for herbicide resistance

Sonal Gupta[1*], Alex Harkess[2,3*], Anah Soble[1], Megan Van Etten[4], James Leebens-Mack[5], and Regina S Baucom[1*]

[1]Ecology and Evolutionary Biology Department, 4034 Biological Sciences Building, University of Michigan, Ann Arbor, MI 48109
[2]Department of Crop, Soil, and Environmental Sciences, Auburn University, Auburn, AL 36849
[3]HudsonAlpha Institute for Biotechnology, Huntsville, AL 35806
[4]Biology Department, Pennsylvania State University, Dunmore, PA 18512
[5]Department of Plant Biology, University of Georgia, Athens, GA, 30602
[*]Corresponding authors

Abstract

The adaptation of weedy plants to herbicide is both a significant problem in agriculture and a model for the study of rapid adaptation under regimes of strong selection. Despite recent advances in our understanding of simple genetic changes that lead to resistance, a significant gap remains in our knowledge of resistance controlled by many loci and the evolutionary factors that influence the maintenance of resistance over time. Here, we perform a multi-level analysis involving whole genome sequencing and assembly, resequencing and gene expression analysis to both uncover putative loci involved in nontarget herbicide resistance and to examine evolutionary forces underlying the maintenance of resistance in natural populations. We found loci involved in herbicide detoxification, stress sensing, and alterations in the shikimate acid pathway to be under selection, and confirmed that detoxification is responsible for glyphosate resistance using a functional assay. Furthermore, we found interchromosomal linkage disequilibrium (ILD), most likely associated with epistatic selection, to influence NTSR loci found on separate chromosomes thus potentially mediating resistance through generations. Additionally, by combining the selection screen, differential expression and LD analysis, we identified fitness cost loci that are strongly linked to resistance alleles, indicating the role of genetic hitchhiking in maintaining the cost. Overall, our work strongly suggests that NTSR glyphosate resistance in *I. purpurea* is conferred by multiple genes which are maintained through generations *via* ILD, and that the fitness cost associated with resistance in this species is a by-product of genetic-hitchhiking.

Keywords: NTSR resistance, detoxification, cost, interchromosomal linkage disequilibrium, genetic hitchhiking

Introduction

Pesticide and herbicide use has reshaped ecological networks and induced strong selective pressures in the anthropogenic era. How species may adapt to strong selection is a fundamental question in evolution with great importance to the control of pesticide resistant organisms. A striking feature of pesticide resistance evolution is that there are a number of different genetic solutions that can lead to resistance[1,2]. In herbicide resistant plants, for example, resistance can be due to single gene mutations, often found in the herbicide's target protein (target site resistance, TSR), or due to changes in multiple genes, often underlying nontarget herbicide resistance (NTSR) mechanisms[3,4]. A growing body of work has produced a better understanding of resistance controlled by single genes across a variety of species[3,5,6]. However, we currently lack a deep understanding of both the genetic basis and evolutionary potential of nontarget site resistance mechanisms genome wide[7–9].

This is due in part to the broad nature of nontarget site herbicide resistance mechanisms more generally. NTSR can be caused by reduced herbicide uptake or penetration, altered translocation or sequestration, and/or herbicide detoxification[10–12] -- mechanisms that likely rely on a complex genetic basis[8,13–15]. While some investigations have pinpointed a single gene conferring NTSR[16,17], gene expression surveys or whole genome re-sequencing assays in a small handful of resistant weeds are beginning to shed light on the complexity of nontarget resistance mechanisms[18–20]. For example, in both *Amaranthus tuberculatus* and *Ipomoea purpurea*, a number of different loci found across the genome -- whether structural, regulatory, or both -- exhibit signs of selection and are thus putatively involved in resistance[18–20]. Because we lack a deep understanding of the genetic basis of NTSR in most weeds, however, we lack a firm grasp on the underlying forces that influence the maintenance of resistance in natural populations, such as the prevalence of alleles that may contribute to fitness costs of resistance, or the presence of interchromosomal linkage disequilibrium (ILD). The presence of ILD between unlinked regions of the genome would implicate the potential for epistatic interactions between alleles underlying either resistance or its cost.

Ipomoea purpurea is a common agricultural weed in the southeast and Midwest United States. Populations of this species, which have consistently been exposed to glyphosate based herbicides since the late 1990's[21,22], exhibit varying levels of herbicide resistance, with some populations exhibiting low and others high survival post-herbicide application[21]. There is a fitness cost associated with this resistance: resistant populations show lower germination and deteriorated seed quality compared to susceptible populations[23]. Further, populations from the south and midwest show evidence of genetic admixture, with both microsatellite and SNP data showing low genetic differentiation (F_{ST} = 0.11-0.14,[24] and recent genetic connectivity[24]). RADseq and exome sequencing has identified regions of the genome under selection and thus associated with herbicide

resistance. These regions are enriched for cytochrome P450s, glycosyltransferases, and ABC transporter genes, indicating a likely role of herbicide detoxification in conferring resistance[20]. Despite evidence that detoxification underlies resistance in this species, and suggestions that loci found on different chromosomes contribute to resistance, previous work relied on low-coverage RADseq sequencing without the benefit of a contiguously assembled genome. Thus, loci that may contribute to NTSR or its cost were likely missed[25], meaning that we lack a thorough understanding of NTSR, the genomic context of NTSR alleles, and the potential for relationships among NTSR alleles in this species -- all crucial to understanding the evolution of resistance more broadly.

Here, we implemented a genome-wide selection screen using whole-genome resequencing of natural populations along with a gene expression survey to characterize the genetic architecture of glyphosate resistance and its cost in *Ipomoea purpurea*. We complemented our survey with a functional assay to test the potential that resistant *I. purpurea* individuals detoxify the herbicide. Given previous evidence that multiple loci likely contribute to herbicide resistance in this species, and evidence of fitness cost of resistance, we made two main predictions regarding genome-wide patterns of selection associated with resistance in *I. purpurea*. First, we expected that regions of the genome showing high differentiation and marks of selection when comparing herbicide resistant and susceptible individuals would contain loci with strong functional links to either herbicide resistance or its cost. Second, we anticipated that linkage disequilibrium, the non-random association of alleles at different loci, should be evident among regions of the genome housing resistance loci. Although inter-chromosomal linkage disequilibrium has been identified in other systems assessing ecologically relevant traits such as mate choice and coloration[26–28], it is unknown if loci underlying herbicide resistance that are found across chromosomes exhibit long-distance or inter-chromosomal linkage disequilibrium, as would be expected if adaptation to herbicide is facilitated by multilocus genotypes favored by selection (*i.e.*, coadapted gene complexes)[29–31].

Results

A chromosome-scale genome assembly for common morning glory

We assembled a reference *I. purpurea* genome to test these hypotheses, generating the first genome sequence for this common and noxious weed. We generated a total of 48 gigabases of PacBio Sequel whole genome shotgun data (Supplemental Figure S1a). Based on a flow cytometry genome size (Benaroya Institute, Seattle, WA), this amounts to roughly 59X genome coverage for an estimated haploid genome size of 814 Mb. We used 34.79 gigabases of trimmed and self-corrected reads for assembly, scaffolding and polishing,

113 which produced a 602 Mb assembly in 402 scaffolds (434 contigs), with a scaffold N50 of
114 5.77 Mb.
115
116 We performed pseudomolecule scaffolding with Phase Genomics Hi-C map, which
117 collapsed the assembly into the expected 15 haploid chromosomes (Supplemental Figure
118 S1b). We renamed and oriented chromosomes according to a high degree of synteny with
119 the related *Ipomoea nil* genome[32] (Supplemental Figure S1c). No misjoins were identified
120 and broken based on the Hi-C linkage data. BUSCO scores on the unannotated assembly
121 show 97.5% completeness against the Viridiplantae odb10 gene set (Supplemental Figure
122 S1d).
123
124 Approximately 63% of the assembly was masked as repetitive DNA, with a significant
125 proportion of recently-expanded Long Terminal Repeat (LTR) retrotransposons
126 (Supplemental Figure S1e). Given the high degree of synteny with *I. nil* genome, the
127 discrepancy between the flow cytometry genome size (814 Mb) and the assembled size
128 (602 Mb) is likely due to young retrotransposon proliferation. We annotated 53,973 genes
129 by combining ab initio gene predictions and RNA sequencing data from leaf tissue. The
130 assembly shows a high degree of synteny with several genomes in the Convolvulaceae
131 family, including *I. nil, I. trifida, and I. triloba (*Supplemental Figure S2)*.*
132

133 ***Detecting loci under selection***
134 Whole-genome analysis of 69 individuals identified twenty-one regions across the genome
135 exhibiting signals of strong genetic differentiation and signs of selection when comparing
136 herbicide resistant and susceptible populations (Supplementary TableS4). These regions
137 exhibited a G_{ST} and Md-rank-P in the top 5 percentile ($G_{ST} > 0.284$ and Md-rank-P > 5.69)
138 with the Md-rank-P being a composite test of selection that incorporates nucleotide
139 diversity, Tajima's D, Fay and Wu's H, and H12. This strategy identified 4.47 Mb of the
140 genome showing signs of selection associated with herbicide resistance. The regions under
141 selection were located across nine chromosomes, varied in size between 26kb-1272kb
142 (Figure 1), and housed 358 genes, 202 of which could be functionally annotated
143 (Supplementary TableS5).
144

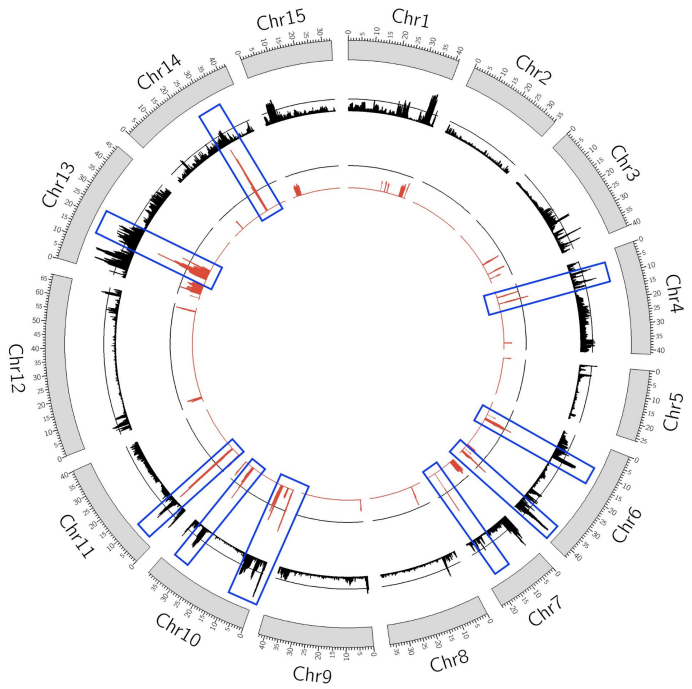

Figure 1 Circos plot depicting the regions of the genome that show signs of selection associated with herbicide resistance. The genome assembly resulted in 15 scaffolds which are represented here by grey bars. Values of G_{ST} describing the differentiation between the resistant and the susceptible populations are depicted by black bars, and the Md-rank-*P* values identifying signatures of selection are presented in red bars. Regions of the genome that exhibited both high differentiation (G_{ST} > 0.284) and a significant Md-rank-*P* value (Md-rank-P > 5.69) are identified by blue boxes. Black lines above both the G_{ST} and Md-rank-*P* represent 95% most extreme genome-wide values for each metric. Blue boxes on Chr5, Chr6, Chr10, Chr13, and Chr14 represent more than one region under selection.

The strongest signal of selection we uncovered was found within a 233kb region of chromosome 10 (average Md-rank-*P* = 7.99; average G_{ST} = 0.69, Figure 2). Within this region we identified 8 copies of cytochrome P450 genes (CYP) and 7 copies of glycosyltransferases, both of which are gene families previously implicated in herbicide detoxification. The eight cytochrome P450s belong to the 76A family (three CYP76A1 and five CYP76A2) and were present in tandem within 53kb. Four copies of the cytochrome P450s exhibited multiple non-synonymous mutations that were almost fixed in the resistant individuals (allele frequency = 0.95). Further, two of the eight cytochrome P450s (CYP76A2) in this block exhibited either a premature stop codon and/or a splice site donor variant (G->C) in the first intron (allele1 susceptible frequency = 0.68, resistant frequency = 0.05) in the majority of the susceptible individuals. The seven glycosyltransferases were found in tandem; one glycosyltransferase copy showed the loss of a stop codon (susceptible frequency = 0.64, resistance frequency = 0.05), whereas the other glycosyltransferases

exhibited multiple non-synonymous mutations close to fixation in the resistant individuals (resistant frequency = 0.05, susceptible frequency = 0.60; Supplementary Figure S3). Additionally, the block of glycosyltransferases in this region showed evidence of a hard sweep (glycosyltransferases H12 = 0.87).

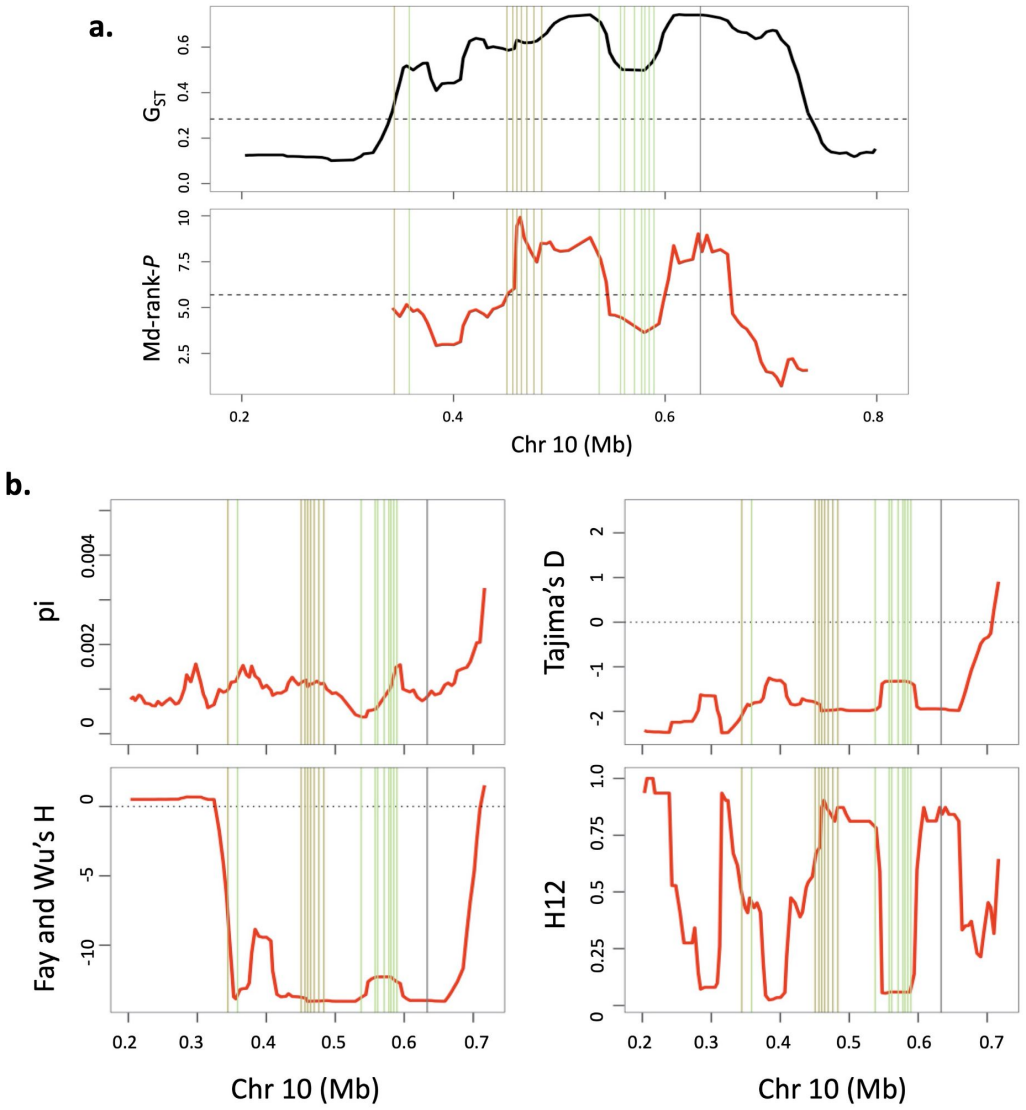

Figure 2 Region of Chromosome 10 showing signs of selection. Shown is the (a) G_{ST} (upper) and Md-rank-*P* (lower) for the resistant individuals which was estimated using statistics shown in (b) clockwise starting from upper left, pi, Tajima's D, H12 and Fay and Wu's H. Red lines indicate respective values for the resistant populations. Khaki vertical lines represent copies of glycosyltransferases, green vertical lines are the cytochrome P450, and the grey vertical line represents CTR1 (see below). The black dashed line in (a) represents 95 percentile values.

180 On chromosome 11, we found a 6.4-7.1Mb region to show signs of selection (average Md-
181 rank-*P* = 8.91; average G_{ST} = 0.392) with six copies of a phosphate transporter gene (*PHO1*),
182 an ABC transporter gene (*ABCB19*), and a sugar transporter gene (*ERD6*), all containing
183 almost fixed non-synonymous mutations in resistant individuals (resistant frequency =
184 0.99). This region also contained two copies each of a glycosyltransferase and a cytochrome
185 P450 gene (CYP736A12 family, Supplementary TableS5).

186 Another region of note showing strong signals of selection was found on chromosome 6
187 (average Md-rank-*P* = 6.55; average G_{ST} = 0.782, Figure 1), with evidence of strong
188 differentiation continuing further upstream and downstream (40.23Mb - 40.81Mb; mean
189 G_{ST} = 0.727). Within the extended downstream region, we found ethylene responsive
190 transcription factor (*ERF4*) and multiple copies of serine/threonine kinases, genes that are
191 involved in the signal transduction in response to various biotic and abiotic stresses[33–37].
192 Within this region we also identified loci that are likely related to the cost of resistance in
193 this species, expanded upon further in 'Signs of selection on potential cost loci' below.

194 Across the other regions exhibiting signs of selection, we found multiple environmental
195 stress response genes (Supplemental TableS4): serine/threonine-protein kinase CTR1
196 (chr10) involved in stress signalling, *LOG3* (chr 4), associated with drought stress
197 response[38], the GT-3B transcription factor (chr 6) which is responsible for inducing
198 response to salt[39], tubby-like F-box protein 5 (TULP5), AP2-like ethylene-responsive
199 transcription factor PLT1, AT-hook motif nuclear-localized protein 24 (AHL24)[40], and
200 several homologs of FRS related sequence (FRS) and E3 ubiquitin-protein ligases, genes
201 involved in response to oxidative stress[34,37]. Of special note, we uncovered a gene involved
202 in the shikimate acid pathway on chromosome 10 -- the bifunctional 3-dehydroquinate
203 dehydratase/shikimate dehydrogenase (DHD/SHD) gene, which is responsible for
204 converting dehydroquinate to shikimate[41]. This latter gene is notable in that it is a part of
205 the shikimic acid pathway, which is the biochemical pathway inhibited by glyphosate[42].

206 Overall, our selection screen using a WGS resequencing approach identified highly
207 differentiated regions under selection, with these regions containing genes involved in
208 herbicide detoxification (cytochromeP450s, glycosyltransferases, ABC and phosphate
209 transporters), the shikimate acid pathway (DHD/SHD), environmental sensing
210 (serine/threonine kinases), and stress response genes (ERFs, PLT1, E3 ubiquitin-protein
211 ligase, FRSs, TULP5, AHL24, LOG3, GT-3B transcription factor). Thus, our study expands on
212 our previous work which found detoxification genes to be under selection[20] by providing
213 strong evidence that glyphosate resistance in *I. purpurea* is controlled by a polygenic NTSR
214 mechanism likely involving herbicide detoxification, response to environmental stimuli and
215 stress, and components of the shikimate acid pathway, which is the biochemical pathway
216 inhibited by glyphosate.

Gene expression differences implicate herbicide detoxification

We compared gene expression between herbicide treated resistant and susceptible plants and found support for the idea that herbicide detoxification, plant signalling, and stress response underlies resistance. Of the 250 differentially expressed genes (111 upregulated and 139 downregulated; Supplementary TableS6), we found cytochrome P450s, glycosyltransferases, and glutathione S-transferase genes (Figure 3a) to be differentially regulated between resistant and susceptible plants. Two copies of the cytochrome P450 family CYP82D7 were significantly upregulated in the resistant individuals (logFC: 2.05 and 1.35), along with two copies of UDP-glycosyltransferases (UGT87A2 and UGT88B1) and a glutathione S-transferase (GST). We additionally found a cytochrome P450 (CYP82C4) and a glycosyltransferase (UGT89B2) downregulated in the resistant individuals.

We likewise uncovered differences in the expression of genes associated with environmental stress responses. Among notable genes were ethylene responsive transcription factors (ERF003, ERF107, TINY[43,44]), serine/threonine kinase BLUS1[45], E3 Ubiquitin protein ligase PUB23[34], NAC domain containing protein 72[46], and WRKY transcription factors (WRKY4, WRKY31, WRKY75[47–49]) (Figure 3a). Homologs of these genes (ERF4, PLT1, CTR1, PRP4, HT1, B120, RHC1A, RF298, NAC92, NAC25, WRKY22; Supplementary TableS5) were also under selection when comparing herbicide resistant and susceptible populations.

In the control (non-herbicide) environment, we found 623 differentially expressed genes when comparing resistant and susceptible individuals (319 upregulated and 304 downregulated; Supplementary TableS7). We identified multiple copies of cytochrome P450s, glycosyltransferases, and ABC transporters that were differentially expressed, indicating that glyphosate resistance through detoxification is constitutive, and not induced, in this species. Interestingly, the specific cytochrome P450s and glycosyltransferase genes that exhibited signs of selection from our whole-genome scan were not the same as those that exhibited differential expression, which could be due to the non-simultaneous nature of the gene transcription response to glyphosate[50], or could represent a transcriptional sampling stage caveat.

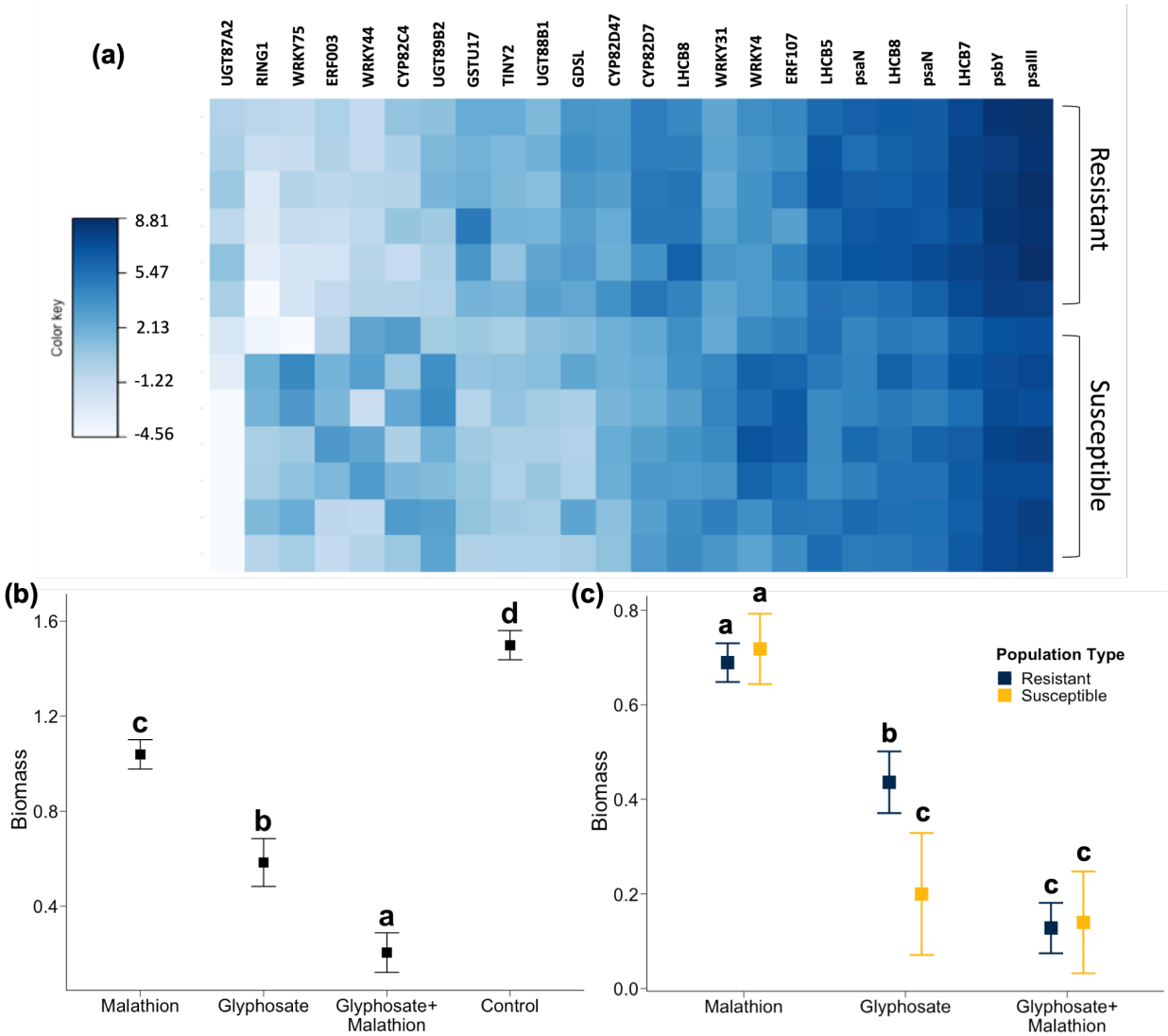

Figure 3 Gene expression variation associated with herbicide resistance, and results of a functional assay supporting the idea that resistance in *I. purpurea* is due to detoxification. (a) Loci associated with glyphosate resistance identified by differential expression analysis with P-value < 0.0005. Color key represents log2 fold-change values. (b) Least square means of above-ground biomass according to treatment (malathion, glyphosate, glyphosate plus malathion, and a control (no treatment)) and (c) summarized according to resistance type (R/S). Letters in (b) and (c) indicate significant differences between treatment environments. The addition of the cytochrome P450 inhibitor malathion reverses glyphosate resistance (glyphosate vs glyphosate+malathion, contrast estimate = 0.379, t-ratio = 2.946, p-value = 0.019), with the resistant individuals showing the same phenotype as the susceptible individuals in the presence of glyphosate and malathion but not in the presence of glyphosate only.

Functional assay supports herbicide detoxification as a mechanism of resistance

We performed an assay to determine if the functional mechanism of resistance in *I. purpurea* was herbicide detoxification (following[17,51–54]). We applied malathion, a pesticide

265 that inhibits cytochrome P450s, to multiple resistant and susceptible *I. purpurea*
266 individuals from the same populations used in the WGS re-sequencing and gene expression
267 studies. The expectation that malathion would act to inhibit *I. purpurea* cytochrome P450s
268 was met; we found a significant overall treatment effect (F-value = 59.33, df = 3, $p < 0.0001$;
269 Fig 3b) with individuals treated with both glyphosate and malathion showing lower
270 biomass compared individuals treated with either malathion, glyphosate, or untreated
271 controls (Fig 3b; Supplemental TableS8).

272 As expected, resistant individuals showed significantly greater biomass compared to the
273 susceptible individuals in the presence of glyphosate (F-value = 4.81, df = 1, P-value = 0.03;
274 Figure 3c). However, the biomass of resistant individuals in the presence of both malathion
275 and glyphosate was significantly lower than that of resistant individuals treated only with
276 glyphosate (resistant plants, malathion+glyphosate *vs* glyphosate: t = 3.65, df = 78, p-value
277 = 0.001), indicating that the presence of malathion reduces the resistance response. In fact,
278 the presence of both malathion and glyphosate led to similar (and low) remaining biomass
279 of both resistant and susceptible individuals (malathion+glyphosate treatment: resistant vs
280 susceptible plants: t = 0.15, df = 32, p-value = 0.88). This shows that the presence of a
281 cytochrome P450 inhibitor lowers the level of glyphosate resistance in *I. purpurea* plants,
282 supporting the idea that modification to the detoxification pathway underlies glyphosate
283 resistance in this species.

284

285 ***Role of long-distance and interchromosomal linkage disequilibrium in maintaining***
286 ***NTSR alleles***
287 Our whole-genome scan identified regions under selection containing genes involved in
288 environmental sensing, stress responses, and herbicide detoxification. This broad scan
289 implicates a polygenic basis of resistance in *I. purpurea* and shows that multiple regions of
290 the genome likely contribute to resistance. We thus sought to determine if there was
291 evidence of linkage disequilibrium between these regions, which would potentially suggest
292 either epistatic interactions among alleles or the inheritance of coadapted gene
293 complexes[29,55]. We calculated a measure of linkage disequilibrium (r^2) between long-
294 distance and interchromosomal SNPs that showed the most extreme level of differentiation
295 and selection (98th percentile, $G_{ST} > 0.39$) -- regions on Chromosome 4, 6 (two regions,
296 hereon referred to as 6.1 and 6.2), 10 and 11, and compared it to the whole-genome
297 measure. We found that the five regions under selection showed islands of elevated
298 interchromosomal linkage disequilibrium (Supplementary TableS9) in a backdrop of nearly
299 zero genome-wide ILD (background interchromosomal r^2 mean = 0.00096; Figure 4).
300 Additionally, the five regions with high differentiation under selection also showed higher
301 linkage (99th percentile ILD = 0.23 ± 0.0004 SE) in comparison to the five random highly
302 differentiated regions of the same size that are not under selection (99th percentile ILD =

303 0.13 ± 0.0003 SE). The region under selection on Chr10 exhibited the strongest linkage to
304 other chromosomal regions under selection (99% ILD Chr4-Chr10 = 0.256, Chr6.1-Chr10 =
305 0.257, Chr6.2-Chr10 = 0.22, Chr11-Chr10 = 0.17).

306 Interestingly, the highest r^2 values (within the top 1 percentile) within these regions was
307 observed for putative resistance genes identified above. For instance, multiple
308 glycosyltransferases and cytochrome P450s under selection on Chr10 showed high ILD
309 with SNPs on Chr11 (Supplementary TableS10). Multiple cytochrome P450 genes
310 (*CYP76A2*) on Chr10 showed a high value of ILD with an uncharacterized protein and a
311 region upstream of GT-3B on Chr6.1 (range of r^2 = 0.256-0.278, Supplementary TableS10)
312 as well as the intergenic region between the transcription factors *SPL1* and *DOF1.4*, both of
313 which are responsible for plant growth and development, on Chr6.2 (range of r^2 = 0.249-
314 0.288), perhaps indicating that this region on Chr6 may influence the regulation of the
315 *CYP76A2* on Chr10 in some way. Furthermore, the highest linkage between Chr4 and other
316 chromosomes (range of r^2 = 0.167-0.274) was observed for a SNP just upstream of *LOG3* on
317 Chr4 (Supplementary TableS9). Thus, the identified resistance alleles within these five
318 highly differentiated regions show signs of linkage and perhaps evidence of epistatic
319 selection.

320 Local regions of strong long-distance linkage disequilibrium and ILD within species might
321 be aided by demographic processes like population structure[55,56], genetic drift[57], or could
322 be due to other processes like selection[28,58]. Furthermore, epistatic interactions among loci
323 wherein adaptive alleles at two independent loci will be inherited together can generate
324 linkage disequilibrium[28,58–60]. Given that our sampling design included multiple resistant
325 and susceptible populations from varied locations, low population differentiation among
326 populations, and evidence of recent migration between them (Supplementary Figure S4), it
327 is unlikely that the observed ILD is due entirely to demographic processes. Moreover, we
328 observed the strongest ILD between regions under selection harboring resistance
329 associated genes, indicating the potential role of selection in maintaining the observed ILD.
330 Thus, our finding suggests that the highly differentiated regions under selection containing
331 candidate loci for glyphosate resistance are linked, indicating the potential role of epistatic
332 selection in maintaining resistance.

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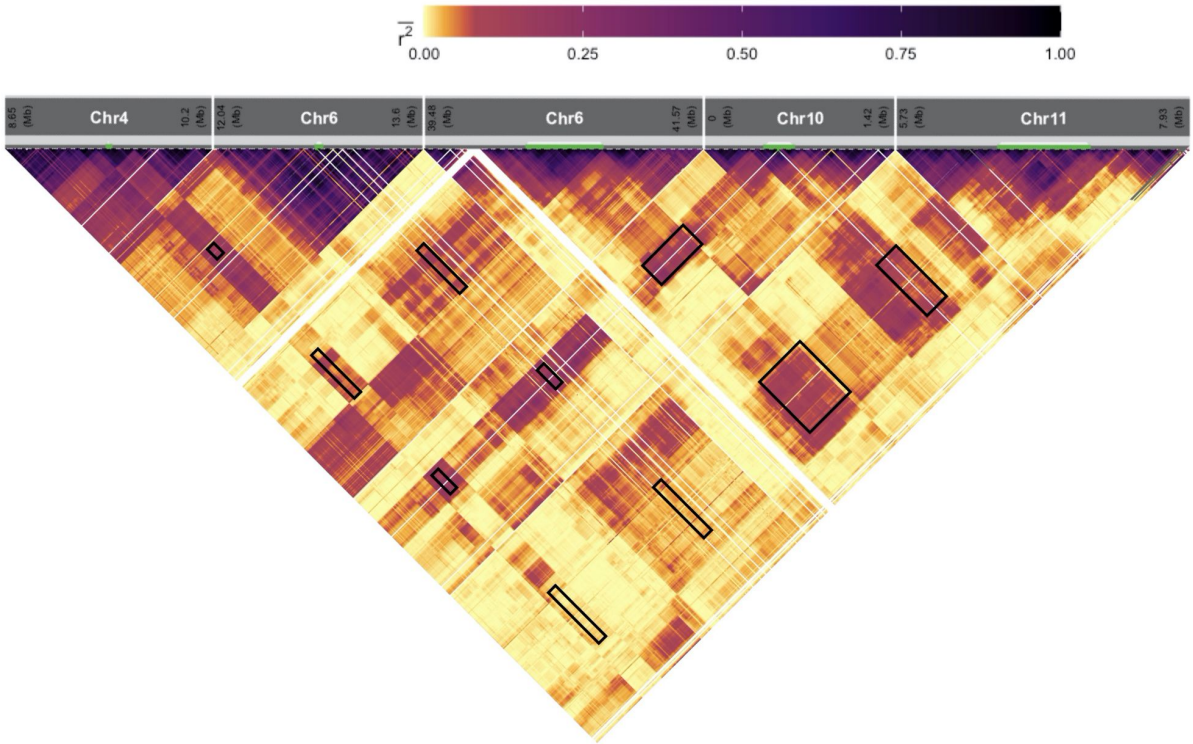

Figure 4 Long-distance linkage disequilibrium and ILD among the five highly differentiated (G_{ST} > 0.39) regions under selection associated with glyphosate resistance. The five intervals displayed islands of increased linkage disequilibrium as estimated by r^2 for SNPs separated by at least 1 kb in and between broad regions under selection. The white lines represent absence of SNPs (missing data) whereas the black boxes represent linkage between the five selection intervals. r^2 values are averaged over two-dimensional bins of 10 x 10 kb.

Signs of selection on potential cost loci

Our scan of regions associated with herbicide resistance, paired with a transcriptome survey, identified potential alleles with strong functional connections to the previously identified fitness cost in resistant *I. purpurea*. We found alternate alleles close to fixation in each population type within the 585kb highly differentiated region on chromosome 6 (40.23Mb - 40.81Mb; mean G_{ST} = 0.727, Figure 5a). This region contained the nuclear fission defective 6 (*NFD6*) and NAC transcription factor 25 (*NAC25*) genes, both of which function in seed development. *NAC25,* a gene that is required for normal seed development and morphology [61], exhibited two missense variants in the resistant individuals (mutant allele resistant frequency = 0.91, susceptible frequency = 0.23). Additionally, *NFD6*, a protein required for nuclear fusion in the embryo sac during the production of the female gametophyte[62], contained six missense variants in the resistant individuals (mutant allele resistant frequency = 0.88, susceptible frequency = 0.21). The resistant haplotype of this gene also contained 10 SNPs in the promoter region which could potentially alter its expression. Indeed, we found this protein to be downregulated in the presence of the herbicide, with a log-fold change of -3.52 in resistant individuals as compared to the

susceptible individuals (Figure 5b). Thus, our data suggest that these genes may be responsible for the lower and abnormal germination leading to the observed fitness cost in this species [23]).

Interestingly, this highly differentiated region containing these seed development genes is strongly linked to other regions under selection that harbor resistance alleles (99% ILD value = 0.22; Figure 4), indicating the potential role of linkage disequilibrium in maintaining the cost. More specifically, *NFD6* is within 83 kb from, and thus physically linked to (r^2 = 0.70), the potential regulatory region on Chr6.2 that exhibits interchromosomal long distance linkage disequilibrium with the CYP76A2 gene on Chr10 (ILD = 0.27). Further, the NAC25 gene is found in close proximity to serine/threonine kinases on Chr6.2 (i.e., 82 kb away), indicating another potential gene involved in the cost phenotype is in physical linkage (r^2 = 0.67) with potential NTSR loci.

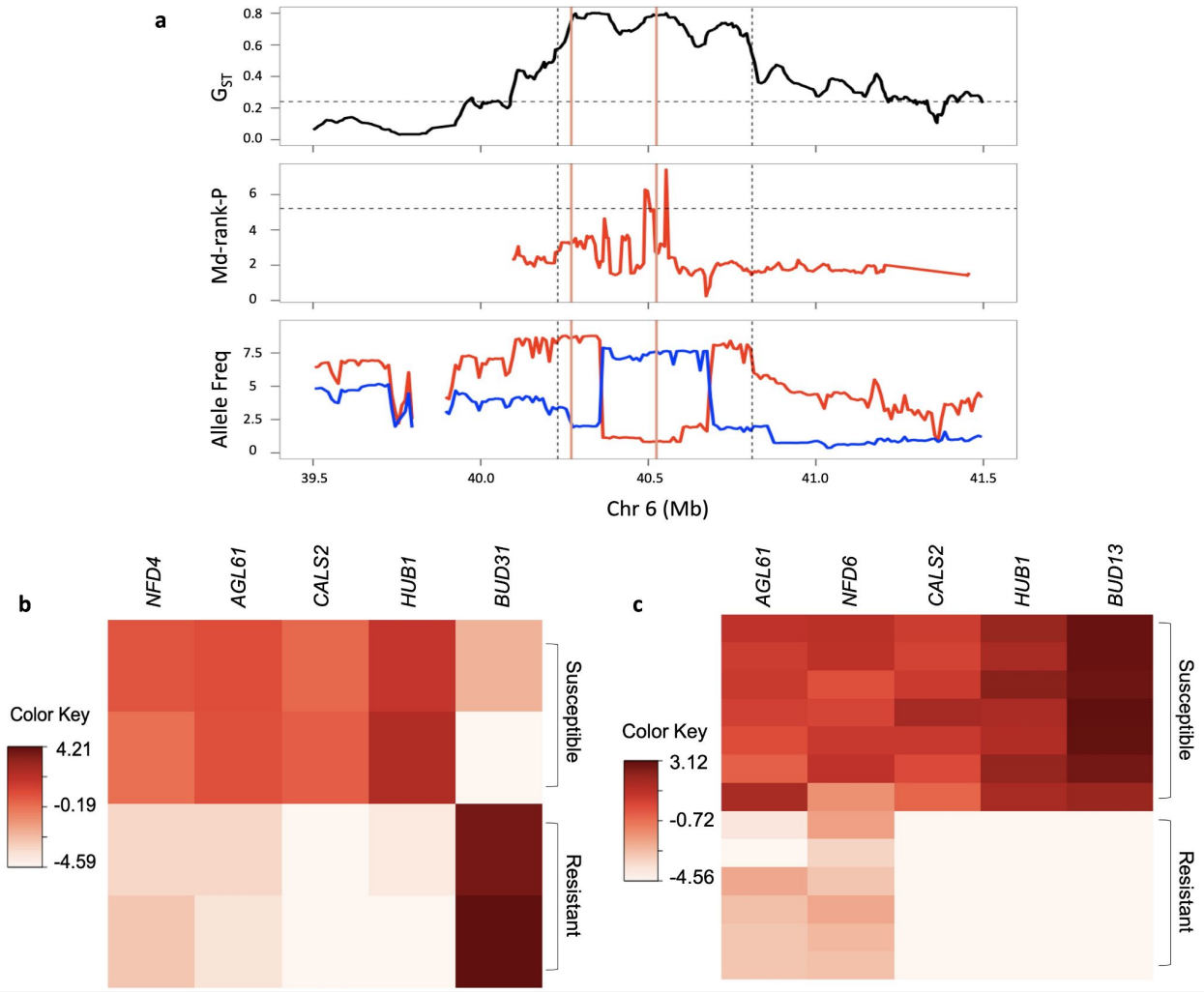

Figure 5 Loci associated with the cost of glyphosate resistance identified by the (a) whole-genome selection-scan and differential expression analysis in the (b) absence and (c) presence of herbicide.

373 Top panel of (a) represents G_{ST} between the resistant and the susceptible populations, mid panel is
374 the Md-rank-*P* value, and the lower panel represents the allele frequency. Salmon vertical lines
375 represent NFD6 and NAC25, in that order. Red and blue represent resistant and susceptible
376 populations, respectively. Black horizontal dotted lines represent 95 percentile values while
377 vertical lines represent regions with G_{ST} above 0.6. The differentially expressed cost genes shown
378 here were chosen based on their functional annotation and had FDR < 0.005 and P-value < 0.00005.
379 Color key represents log2 fold-change values.

380 In addition to the potential cost loci identified from the WGS screen, our gene expression
381 analyses in the absence of herbicide (*i.e.* the environment in which fitness costs are
382 assessed) comparing resistant and susceptible individuals identified five differentially
383 expressed gene that play a role in fertilization and seed maturation and are thus potentially
384 related to the cost (Supplementary TableS6). Of special interest, the bud-site selection
385 protein 31 (*BUD31*) was found to be highly upregulated (logFC = 7.22) in resistant plants in
386 the absence of herbicide, whereas its homologue *BUD13* was highly significantly
387 downregulated in resistant individuals in the presence of herbicide (logFC = -11.39).
388 *BUD13* is involved in pre-mRNA splicing in embryos and is critical for early embryo
389 development[63].

390 In the control environment, two genes downregulated in the resistant individuals -- *NFD4*
391 (logFC = -3.61) and Agamous-like MADS-box protein AGL61 (*AGL61*; logFC = -4.98) -- are
392 involved in megagametogenesis. The *NFD4* gene, like *NFD6*, is responsible for ovule polar
393 nuclei fusion during female karyogamy[62], whereas *AGL61* is required for the central cell
394 development and differentiation[64]. A loss of function mutation in *AGL61* has been shown to
395 cause abnormal morphology and over 50% seed abortion upon fertilization in
396 Arabidopsis[64]. We also identified a callose synthase 2 (*CALS2*) to be strongly
397 downregulated in resistant individuals (logFC = -8.72); another member of the callose
398 synthase family (*CALS5*) has been shown to be responsible for pollen viability[65]. Finally, we
399 also found that E3 ubiquitin-protein ligase BRE1 (*HUB1*), a protein involved in seed
400 germination, was strongly downregulated among the resistant individuals (logFC = -8.27).
401 *HUB1* has been shown to control chromatin remodeling during seed development and
402 leads to alterations in seed dormancy[66].

403 Interestingly, three of these five candidate genes (*AGL61*, *CALS2*, *HUB1*), and homologues of
404 other two (*BUD13* and *NFD6)* were also significantly downregulated in the resistant
405 populations in the presence of herbicide (Supplementary TableS6). These candidate cost
406 genes are all essential for plant reproduction and are highly downregulated (except
407 *BUD31*) in the resistant population in both the absence and presence of the herbicide, and
408 thus could potentially explain the phenotypic costs of glyphosate resistance in *I. purpurea*
409 seen by Van Etten and colleagues[23].

410

411 **Discussion**

412 While there is an increasing appreciation for the role of nontarget site mechanisms
413 underlying herbicide resistance in agricultural weeds[12,67–69], there are strikingly few
414 comprehensive whole genome assays of resistant weeds suggesting that the entirety of the
415 NTSR response is rarely captured. Our study using a sequenced and assembled genome,
416 whole genome resequencing of natural populations, and a gene expression survey offers a
417 unique opportunity to identify loci associated with NTSR and to further investigate the
418 evolutionary forces that underlie the maintenance and expected evolutionary trajectory of
419 resistance alleles in natural populations.

420 Our results show detoxification underlies resistance in *I. purpurea*. Detoxification is
421 hypothesized to involve three steps -- uptake of the herbicide by phosphate transporters,
422 chemical modification (*i.e.* the addition of an OH and sugar group by cytochrome P450s and
423 glycosyltransferases, respectively), and transport to vacuoles by ABC transporters and
424 other sugar transporters where the molecule is stored and/or inactivated[12,18]. We found
425 evidence of selection on all genes involved in this pathway. We also found evidence of
426 selection (and in some cases, differential expression) of genes involved in plant signalling
427 and environmental stress (i.e., serine/threonine kinases, ERFs, E3-Ubiquitin Ligases, FRSs,
428 LOG3, GT-3B, TULP5) as well as genes involved in the shikimate acid pathway (DHD/SHD).
429 Our results thus expand what we currently know about the detoxification NTSR mechanism
430 in this species to include plant signalling and stress responses, both of which are either
431 hypothesized [7] or shown to be involved in herbicide resistance[70–73]. While we do not
432 currently have functional genomics resources for this species, our study using a
433 cytochrome P450 inhibitor verifies that resistant *I. purpurea* individuals have the ability to
434 detoxify the herbicide. The next step in understanding resistance in *I. purpurea* involves
435 determining the contribution of each of the candidate loci under selection (and/or showing
436 differential regulation) to both resistance and its associated cost. With future development
437 of genome editing protocols for *I. purpurea*, we will be able to experimentally test the
438 function of loci hypothesized to be contributing to herbicide resistance.

439 Due to the involvement of multiple genes involved in the herbicide detoxification pathway,
440 and evidence for selection on regions of the genome found on separate chromosomes, we
441 hypothesized that multiple loci would show evidence of ILD, perhaps indicating epistatic
442 selection between alleles or co-inheritance. Our results support this hypothesis. Foremost,
443 in contrast to low background ILD, long-distance linkage disequilibrium and ILD were high
444 among intervals under selection, and consistently differentiated between the resistant and
445 susceptible types across multiple populations. The strongest linkage was observed
446 between putative resistance genes that exhibited signs of selection. This linkage could
447 quickly become very steep in the presence of epistatic interactions among loci[74], as would
448 be the case if genes underlying NTSR worked in concert to produce the resistance

449 phenotype. Indeed, we found high ILD values between regulatory regions and resistance
450 alleles, and between intervals harboring genes involved in the same molecular pathways
451 (e.g. detoxification, and stress signaling and response).

452 Although linkage should become decoupled over time due to recombination and gene flow,
453 the ongoing selection for herbicide resistance could slow down this decoupling between
454 these functionally interacting genes[75]. Even given gene flow between these populations [24],
455 co-adaptation and epistasis could lead to the fixation of the resistance alleles given strong
456 selection[76] or weaker recombination rate [77]. Thus, long-distance linkage disequilibrium and
457 ILD aided with epistatic selection could act to maintain resistance through generations in
458 natural populations.

459 One evolutionary force that should counteract the continued evolution of resistance is the
460 potential for fitness costs of resistance, either due to the pleiotropic effects of resistance
461 alleles themselves or due to negative fitness effects of loci that are linked to resistance loci.
462 While costs are central to theories of resistance evolution[78–81], there are currently no
463 examples, to our knowledge, in which the loci underlying fitness costs of nontarget site
464 resistance have been identified. Our results suggest candidate loci associated with the
465 previously identified cost of glyphosate resistance. Specifically, we found a highly
466 differentiated region on Chr6 that exhibited alternate alleles in resistant and susceptible
467 populations, and found this region to contain loci required for normal seed development
468 and maturation (*NAC25, NFD6*). One of these genes, *NFD6*, was differentially regulated in
469 the resistant individuals, further supporting its role in the low seed quality, and thus fitness
470 cost, that we have previously described[23].

471 Additionally, our results strongly suggest genetic hitchhiking may act to maintain the cost
472 in this species. Both *NAC25* and *NFD6* are physically linked on chromosome 6 to the regions
473 under selection containing serine-threonine kinase genes and a regulatory region that is
474 itself exhibiting ILD to the *CYP76A2* gene on chromosome 10. Although recombination
475 should decouple cost alleles that are physically linked to resistance alleles, these loci would
476 not completely decouple if the recombination rate (c) is much lower than the selection
477 coefficient (s) (i.e., c<<s,[82]. The requirement that c<<s is not improbable given the close
478 proximity of cost and resistance loci (< 85kb) and the strong ongoing selection for
479 herbicide resistance. Furthermore, if the ratio $c/s < 10^{-4}$, the hitchhiking would almost be
480 complete and the cost alleles could become fixed in the populations[83]. Alternatively, it is
481 possible that new compensatory mutations arising in the population could increase in
482 frequency over time due to selection, decoupling the cost and resistance alleles and thus
483 reducing fitness cost associated with glyphosate resistance[84,85].

484 Overall, our work identified the potential genetic basis of NTSR glyphosate resistance in *I.*
485 *purpurea* -- our whole genome and transcriptome assays strongly support the role of

486 detoxification conferring herbicide resistance in this species, and we additionally identified
487 a role for plant sensing and stress along with components of the shikimate acid pathway.
488 Interestingly, we show that NTSR glyphosate resistance in *I. purpurea* is conferred by
489 multiple loci which are maintained through generations via ILD. We also provide strong
490 evidence to support the idea that fitness costs may be due to loci in strong linkage with
491 resistance loci. Our work highlights the importance of multi-level, multi-population study
492 in identifying the genetic mechanisms underlying polygenic defense traits, and for
493 understanding the complex genetic-interplay between defense and cost.
494
495 **Methods**
496 *Genome sequencing, assembly, and annotation:* We used an *I. purpurea* line originally
497 sampled from an agricultural field in Orange County, NC, in 1985 by M. Rausher (*i.e.*, prior
498 to the widespread use of glyphosate) and selfed for >18 generations in the lab for genome
499 sequencing (seeds of this line 'Fred/C' are available upon request). High molecular weight
500 DNA was isolated from flash-frozen leaf tissue using a modified large-volume CTAB
501 protocol[86] and sequenced on a PacBio Sequel at the University of Georgia. Raw PacBio
502 subreads from 9 cells of Sequel chemistry were error-corrected with Canu (v1.7.1)[87] with
503 default parameters for raw PacBio reads (--pacbio-raw). The corrected and trimmed reads
504 from Canu were assembled with Flye (v2.4-release)[88] and anchored onto pseudomolecules
505 by nearly 81 million read pairs of Phase Genomics Hi-C (Seattle, WA) of leaf tissue using
506 Sau3AI cutsites. Within-genome and across-genome synteny was visualized using the CoGE
507 SynMap platform[89], with DAGChainer options "-D20 -A 5", as well as with jcvi with default
508 parameters (https://github.com/tanghaibao/jcvi). *Ipomoea purpurea* pseudomolecules
509 were numbered and oriented according to chromosome synteny against *Ipomoea nil*
510 pseudomolecules (Supplemental Figure S2).
511 Raw 50nt single-end RNA-seq reads were aligned using STAR (v.2.7.0)[90] with
512 default single-pass parameters. Repetitive elements were first annotated with
513 RepeatModeler (v1.0.11). Long Terminal Repeat (LTR) retrotransposons were annotated
514 with LTRharvest (v1.6.1) with options -similar 85 -mindistltr 1000 -maxdistltr 15000 -
515 mintsd 5 -maxtsd 20". RepeatModeler annotations were combined with all Viridiplantae
516 repeats from Repbase and used as a species-specific repeat database built using
517 RepeatModeler with default options.
518 Genome annotation was performed using a diverse set of evidence. First, a set of 12
519 RNA-seq libraries from leaf tissue was aligned with STAR (v2.7.0), and transcripts
520 assembled with Stringtie (v2.1.3). MAKER2[91] was initially run with evidence from the RNA-
521 seq alignments, as well as peptides from *I. trifida, I. triloba,* and *I. nil*. The resulting gene set
522 was used to train SNAP (v2013-11-29). AUGUSTUS (v3.3.2) was trained with evidence from
523 BUSCO (v4.1.0) against the eudicot odb10 set. with default options. MAKER2 was re-run
524 with the *ab initio* SNAP and AUGUSTUS training sets, in addition to the homologous protein
525 and RNA-seq evidence, to build a final gene annotation set.

526
527 *Sampling and sequencing:* We selected eight populations to investigate the genetic basis of
528 glyphosate resistance and its cost following[20]-- 4 low resistance, from here on referred to
529 as the susceptible population (S: <20% population survival at 1X the field dose of
530 RoundUp) and 4 high resistance populations (R: >70% population survival at 1X the field
531 dose of RoundUp), from here on referred to as the resistant populations (Supplementary
532 TableS1). Seeds from 10 maternal lines per population were germinated, except for one
533 susceptible population (RB), wherein 9 maternal lines were used. We extracted DNA from
534 leaf tissue using the Qiagen Plant DNeasy kit. 150 paired-end sequencing was performed
535 using Illumina HiSeq4000 and NovaSeq6000 using three and two lanes, respectively. We
536 sequenced two populations at high coverage (at least 25X) and the remaining six
537 populations at low coverage (10X). Two populations (WG, resistant and RB, susceptible)
538 were run on one lane of HiSeq6000 and NovaSeq6000 each whereas the other lane had the
539 remaining six populations. This yielded a total of 3,300,397,148,700 bases with average
540 coverage of 28.84X for WG and RB. Coverage of the other six populations has an average of
541 14.66X.
542
543 *Variant calling:* We aligned the reads to our draft genome using BWA mem v0.7.15[92] with
544 parameter -M. Since the same sample was sequenced using multiple platforms (HiSeq and
545 NovaSeq), the alignment files were merged and duplicate reads were marked using the
546 MarkDuplicate tool of Picard v2.8.1 (http://broadinstitute.github.io/picard). Next, we
547 prepared a database of true known variants, required for base recalibration. This database
548 was created using data from the top eighteen individuals with the highest read counts,
549 upon which variant call was performed using the HaplotypeCaller tool of GATK v4.1[93]. Low
550 confidence variants were filtered out using the VariantFiltration tool of GATK v4.1[93] (15 <
551 DP < 60; ReadPosRankSum < -8.0; QD < 2.0; FS > 60.0; SOR > 3.0; MQ < 40.0; MQRankSum <
552 -12.5) and only the high confidence variants were used in the dataset. This was used to
553 recalibrate base qualities using GATK v4.1 tools BaseRecalibrator and ApplyBQSR[93].
554 Variants were called individually on all the individuals using the HaplotypeCaller tool of
555 GATK v4.1[93] using parameters -ERC GVCF --min-pruning 1 --min-dangling-branch-length 1.
556 The variants from each individual were combined to one variant file (a raw cohort variant
557 file) using the tools GenomicsDBImport, GenotypeGVCFs, and GatherVcfs[93], with invariants
558 included. Next, multiple rounds of filtration were performed on this variant dataset to filter
559 out potential false positives. First, using the GATK v4.1 tools VariantFiltration and
560 SelectVariants we filtered the variants using the parameters QD<1.5, DP<10 and DP>2000,
561 FS>80, SOR>5, MQ<40, MQRankSum< -6 and MQRankSum>6, and ReadPosRankSum< -4
562 and ReadPosRankSum> 4[93]. For the next round of filtration, we removed variants that had
563 genotype depth more than twice the
564 average and heterozygosity more than 0.8 using the het packages from VCFtools v0.1.15[94].
565 In the third round of filtration, we filtered variants that had quality above 20, had no

566 missing information, a minor allele frequency of 0.05, and a minimum mean depth of 10
567 (vcftools --minQ 20 --max-missing 1.0 --maf 0.05 --min-meanDP 10)[94]. Finally, we filtered
568 using BCFtools (v1.7)[95] to keep only bi-allelic SNPs (bcftools view -m2 -M2 -v snps). This
569 gave us a total of 3,942,549 high confidence SNPs. These SNPs were used for downstream
570 analyses.
571
572 We performed a PCA analysis using the allele frequencies of all the SNPs to investigate the
573 population structure using the package bigsnpr v.1.4.4[96] in R, and found that the
574 populations did not segregate into two separate genetic clusters (Supplementary Figure
575 S4a-b). Further, we repeated this analysis for SNPs from regions under selection (see
576 below) to test whether we observe the same population structure patterns. We observed
577 that these separated into distinct resistant and susceptible groups, with the exception of a
578 resistant population, BI, which clustered between the susceptible and other resistant
579 populations (Supplementary Figure S4c). Thus for the purposes of this study, we dropped
580 the BI population from further analysis.
581
582 *Selection analysis:* We split the high confidence variant dataset obtained into 'resistant' and
583 'susceptible' variant dataset using vcf-subset of VCFtools v0.1.15[94]. The 'resistant' and
584 'susceptible' variant datasets contained 30 and 39 individuals, respectively (Table S1). We
585 then used these datasets to calculate diversity and selection statistics G_{ST}[97], pi, Tajima's D[98],
586 Fu and Way's H[99] using a custom script from [100] in a 300SNP window, for both the dataset.
587 Furthermore, to detect hard sweep we phased the variants using beagle version 5.1[101]
588 which was then used to calculate the haplotype homozygosity statistic (H12, a measure of
589 haplotype homozygosity that detects both hard and soft sweeps) using the scripts
590 provided[102]. For regions above 95 percentile G_{ST}, we calculated a composite rank based
591 statistic (Md-rank-*P*) which was computed as the Mahalanobis distance on the negative
592 log10 transformation of raw statistics into rank P-values[103]. This Md-rank-P was calculated
593 using pi, Tajima's D, Fu and Way's H, and H12. To identify potential regions of selection we
594 chose bins with greater than 95 percentile Md-rank-*P*.
595
596 *Linkage analysis:* We calculated linkage disequilibrium (r^2) at three different levels. First, to
597 estimate the background genome-wide long-distance (and interchromosomal) linkage
598 disequilibrium (ILD), we calculated r^2 values for 5842 SNPs separated by at least 100kb
599 using VCFtools v0.1.15[94] (--thin 100000 --interchrom-hap-r2). Second, we estimated the r^2
600 for SNPs separated by at least 1kb in and between broad regions (0.75Mb upstream and
601 downstream) around the five focused regions (with $G_{ST} > 0.39$) under selection using
602 VCFtools v0.1.15[94]. Lastly, since one would expect higher linkage between regions with
603 high differentiation, we also randomly chose five regions with high differentiation
604 (showing no signs of selection) of similar lengths as the five focused regions above and
605 compared its linkage values to those regions.

606
607 *RNA-Seq:* To identify transcripts associated with glyphosate resistance and its potential
608 cost, we sequenced transcriptomes of 17 individuals belonging to four different treatments;
609 resistant control (Rc), susceptible control (Sc), resistant herbicide sprayed (Rh), and
610 susceptible herbicide sprayed (Sh). Each treatment had multiple individuals (Rc-2, Sc-2,
611 Rh-6, Sh-7; Supplementary TableS2). The seeds were grown in a controlled environment
612 (growth chamber) to reduce variation due to environmental differences. 20 days after
613 planting, we sprayed glyphosate (concentration of 1.52 kg ai/ha) on the Rh and Sh
614 treatment plants and collected the second and fourth leaf for RNA extractions 8 hours post-
615 spray. These were flash frozen using liquid nitrogen and stored at -80°C. We extracted RNA
616 using Qiagen RNeasy Plant mini kit with the optional DNase digestion step. This was then
617 sequenced using Illumina NovaSeq 6000 at 150bp paired-end sequencing. A total of
618 132,551,535,000 bp were obtained.
619
620 *Differential gene expression* -- We processed the raw reads obtained to remove adapters
621 using cutadapt v1.18[104] and then mapped them to the de-novo assembled genome (--
622 sjdbOverhang 149 --outSAMtype BAM SortedByCoordinate Unsorted) using STAR v2.7.5[90].
623 Next, using HTSeq v0.11.1[105], we counted read counts for each gene. These read counts
624 were then used to filter out lowly expressed transcripts using the Bioconductor package
625 edgeR version 3.18.1[106] such that transcripts were retained only if they had greater than
626 0.5 counts-per-million in at least two samples (Rc vs Sc) and four samples (Rh vs Sh). The
627 libraries were then normalized in edgeR (using the trimmed mean of M-values method)
628 followed by differential gene expression analysis using the classic pairwise comparison of
629 edgeR version 3.18.1. We extracted the significance of differentially expressed transcripts
630 (DETs) with FDR <= 0.05. This was done for two contrasts, Rh vs Sh (total sample size = 13;
631 Rh = 6, Sh = 7) and Rc vs Sc (total sample size 4; Rc = 2, Sc = 2). The first contrast informs us
632 of the genes that are regulated in response to the herbicide, and how this gene regulation
633 differs between the resistant and the susceptible populations, whereas the latter informs
634 us of the baseline expression difference due to glyphosate resistance between the two
635 populations.
636
637 *Malathion Experiment:* On May 15th, 2019, we planted a total of 180 replicate seeds from
638 multiple resistant and susceptible populations (Supplementary TableS3) in Cone-Tainers
639 (Stewe and Sons). These were allowed to grow for 30 days, after which we subjected them
640 to one of the four treatment environments--malathion (7.81 ml/L according to
641 manufacturer's recommendations), glyphosate (3.4 kg ai/ha), glyphosate and malathion,
642 and a control. Twenty-five days post treatment spray, we recorded death as a metric trait
643 (dead/almost dead or green and healthy), and harvested the plants. These were dried for 3
644 days at 70C and weighed for an estimate of dry above ground-biomass.
645

Using this data, we assessed whether biomass was significantly altered by the different treatments. First, we normalized the above-ground biomass using the transformTukey function from Rcompanion v.2.0.0[107]. We then used a generalized linear model (lm function[108] with as normalized biomass as the dependent variable and population Type (R/S) and treatment as the independent variables. We assessed the significance of the variables using the Anova function of the car package v.3.0.10[109], and performed a pairwise comparison between groups using the lsmeans function from package lsmeans v2.30.0[110], adjusted for multiple tests using tukey correction. Using the same general model, we also compared whether biomass was significantly different between treatments for each population type. To control for the differences in the plant size we standardized the biomass of the individuals by the average biomass of the respective maternal line in the control treatment, and then normalized it as above.

Data Availability

The datasets generated during and/or analysed during the current study have been deposited in GeneBank database under the project XXX , which are publicly accessible at XXX.

The *I. purpurea* genome assembly and annotation are available in the CoGe platform - https://genomevolution.org/coge/GenomeInfo.pl?gid=58735 .

Code Availability

The custom codes used in this study are deposited in GitHub (https://github.com/gsonal802/IP_GS.git).

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Footnotes

[1]S.G. and A.H. contributed equally to this work.

[2]Author contributions: R.S.B., and S.G. designed the project; R.S.B., M.L.V.E., and S.G. designed the experiments; A.H. and J.L.M. assembled and annotated the genome; A.S. performed the growth room experiment; M.L.V.E. performed the WGS experiment, S.G. analyzed whole genome resequencing, RNAseq data, and performed linkage analysis; and R.S.B., S.G., A.H., and J.L.M. wrote the paper.

The authors declare no competing interest.

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