

1 **The genome of *Lolium multiflorum* reveals the genetic architecture of paraquat resistance**

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Section	Count
Introduction	1003 words
Material and Methods	2200 words
Results	2019 words
Discussion	1278 words
Total	6,500 words
Figures	4
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Supporting information - Figures	5

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14 **SUMMARY**

15 - Herbicide resistance in agricultural weeds has become one of the greatest challenges for
16 sustainable crop production. The repeated evolution of herbicide resistance provides an
17 excellent opportunity to study the genetic and physiological basis of the resistance phenotype
18 and the evolutionary responses to human-mediated selection pressures. *Lolium multiflorum* is
19 a ubiquitous weed that has evolved herbicide resistance repeatedly around the world in
20 various cropping systems.

21 - We assembled and annotated a chromosome-scale genome for *L. multiflorum* and elucidated
22 the genetic architecture of paraquat resistance by performing quantitative trait loci analysis,
23 genome-wide association studies, genetic divergence analysis, and transcriptome analyses
24 from paraquat-resistant and -susceptible *L. multiflorum* populations.

25 - Results suggested that two regions of chromosome 5 were associated with paraquat
26 resistance. The regions contain candidate genes that encode cellular transport functions,
27 including a novel multidrug and toxin extrusion (MATE) protein, and a cation transporter
28 previously shown to interact with polyamines.

29 - Our results reveal the genetic architecture of paraquat resistance and identified promising
30 candidate genes for future functional studies. Given that *L. multiflorum* is a weed and a
31 cultivated crop species, the genomic resources generated will prove valuable to a wide
32 spectrum of the plant science community.

33 Keywords: Quantitative trait loci, genome-wide association studies, RNA-seq, genome
34 assembly, genome annotation, genetic divergence analysis

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40 **INTRODUCTION**

41 Agricultural activities have tremendously modified the landscape and ecological dynamics of its
42 original inhabitants (Tilman, 1999). The need to increase food production over the past 50 years
43 has led to a drastic simplification of agroecosystems (Pretty, 2018). Weed species have colonized
44 these highly disturbed areas and now compete with crops for resources. Chemical control with
45 herbicides is the main strategy used in modern agriculture, and overreliance on these agents has
46 resulted in the widespread evolution of herbicide resistance (Heap, 2023). The evolution of
47 herbicide resistance is one of the greatest challenges to sustainably produce food, fibers, and fuel
48 (Peterson et al., 2018).

49 Much remains unknown about the physiological and evolutionary mechanisms that lead to the
50 evolution of herbicide resistance. Resistance mechanisms in weeds are classified in two
51 categories: target-site and non-target-site (Gaines et al., 2020). The genetics of target-site
52 resistance has been studied extensively and involves alterations to the herbicide's target-site. By
53 contrast, the genetic basis of non-target-site resistance is believed to be more complex
54 (Suzukawa et al., 2021). Although it has been shown that non-target site resistance can be caused
55 by increased herbicide metabolism (Brunharo et al., 2019) or sequestration of the herbicide to the
56 vacuole (Brunharo & Hanson, 2017), the genetic controls remain poorly understood. Indeed,
57 basic questions about the genetic architecture of non-target site resistance, including the number
58 of loci affecting the trait, their distribution across the genome, dominance relationships, and
59 whether they involve changes in gene expression, often remain unanswered. Herbicide resistance
60 provides an excellent opportunity to study the evolutionary responses to human-mediated
61 selection pressures.

62 *Lolium multiflorum* Lam. is a winter annual species native to Europe, temperate Asia, and
63 Northern Africa, but human activities over the past 200 years have caused its spread to all
64 continents (Humphreys et al., 2009). It is a troublesome weed in agriculture, where it can cause
65 drastic reductions in yield if not controlled (Appleby et al., 1976). Management of *L. multiflorum*
66 is primarily performed with herbicides, and the recurrent use of a few herbicide chemistries has
67 resulted in the evolution of >70 herbicide-resistant populations across 14 countries.

68 Paraquat is an herbicide discovered in 1955 (Brian et al., 1958) that has been used extensively
69 around the world for non-selective weed control. It continues to be one of the most widely used
70 herbicides in the United States (EPA, 2023). Paraquat is actively taken up by plasma membrane-
71 localized transporters, where it must reach its target site in the chloroplasts (Hart et al., 1992a).

72 Once absorbed, it inhibits photosystem I by functioning as a preferential electron acceptor,
73 diverting electrons from ferredoxin to O₂ and generating reactive oxygen species (ROS)
74 (Hawkes, 2014). In plants susceptible to paraquat, this fast reaction overwhelms the endogenous
75 ROS quenching mechanisms, leading to membrane peroxidation and cell death within hours of
76 treatment (Bromilow, 2004).

77 Restricted paraquat movement has been reported in resistant weed populations (Brunharo &
78 Hanson, 2017, Soar et al., 2003, Yu et al., 2010), which has been attributed indirectly to
79 enhanced vacuolar sequestration of the herbicide by tonoplast-localized transporters (Brunharo
80 and Hanson, 2017). Enzymes in the Halliwell-Asada cycle are involved in ROS quenching, and
81 their increased activity can provide paraquat resistance. In *Conyza bonariensis*, for example,
82 increased expression of some of these enzymes was observed in resistant individuals (Ye &
83 Gressel, 2000).

84 Although much remains unknown about the genetic and physiological mechanisms that evolved
85 in natural weed populations, some insight into paraquat resistance mechanisms has been gained
86 by studying *Arabidopsis* mutants (reviewed by Nazish et al., 2022). Given that paraquat's target
87 site is localized in the chloroplasts, mechanisms that prevent or reduce its movement can limit its
88 action. Xi et al. (2012) identified a loss-of-function mutation in *pqt24-1*, a gene that encodes a
89 plasmalemma-bound ATP binding cassette (ABC), exhibiting reduced cell influx of paraquat. A
90 knockout of *mrv1* (encodes a polyamine transporter) reduced cellular paraquat uptake (Fujita et
91 al., 2012). Restriction of paraquat trafficking from the Golgi apparatus to chloroplasts has been
92 observed in *par1* mutant lines (Li et al., 2013), which contain a non-synonymous mutation in the
93 gene that encodes *AtLAT4*, another member of the polyamine transporter superfamily. Enhanced
94 paraquat tolerance can also be conferred by enhanced vacuolar sequestration and cellular efflux.
95 An amino acid substitution in *DETOXIFICATION EFFLUX CARRIER (DTX6)*, a member of the
96 multidrug and toxic compound extrusion (MATE) family, was suggested to increase affinity to
97 paraquat (Lv et al., 2021b). It should be noted that the doses of paraquat used in *Arabidopsis*

98 mutants can be six orders of magnitude lower than those that resistant weeds can withstand (0.1
99 μM in tolerant *Arabidopsis*, 0.13 M in paraquat-resistant *L. multiflorum*; (Brunharo & Hanson,
100 2017, Fujita et al., 2012), potentially suggesting that different mechanisms could be at play in
101 naturally-evolved weed populations.

102 Resistance to multiple herbicides in *L. multiflorum* has recently been documented from
103 agricultural fields in the western US (Brunharo & Tranel, 2023, Brunharo & Streisfeld, 2022,
104 Bobadilla et al., 2021, Brunharo & Hanson, 2018), with clear evidence of widespread gene flow
105 among populations, as well as repeated, independent herbicide resistance evolution. However,
106 genetic resources in *L. multiflorum* remain limited. A draft genome from a forage variety of *L.*
107 *multiflorum* has been created but remains highly fragmented (Copetti et al., 2021). Therefore, a
108 more contiguous, chromosome-level assembly would be an essential resource for dissecting the
109 genetic basis of important traits, such as herbicide resistance. In this context, the three primary
110 objectives of this study are to 1) assemble and annotate the first, full chromosome-level reference
111 genome for *L. multiflorum*, 2) elucidate the genetic architecture and gene expression changes
112 associated with paraquat resistance in *L. multiflorum*, and 3) identify the evolutionary signatures
113 of human-mediated selection pressure in the genome. To do so, we use genetic mapping, a
114 genome-wide association study (GWAS), transcriptome analyses, and population genomic scans
115 for signatures of recent selection between resistant and susceptible populations. Elucidating the
116 genetic architecture responsible for herbicide resistance can provide insights into how organisms
117 respond to strong selection pressure, and it can help initiate efforts to improve weed management
118 practices in the long term.

119

120 **MATERIAL AND METHODS**

121 ***Lolium multiflorum* genome assembly and annotation**

122 A *L. multiflorum* individual was grown from seed in autoclaved sand from a previously
123 characterized susceptible population (population Gulf; Brunharo & Streisfeld, 2022) and
124 hydroponically irrigated with half-strength Hoagland's solution. A tiller was clone-propagated to
125 a larger pot filled with potting soil and grown to maturity. Leaf tissue was collected from plants

126 for high-molecular weight DNA extraction (Wizard HMW DNA Extraction, Promega, Madison,
127 WI). We used flow cytometry to estimate genome size relative to *Conyza canadensis* and
128 *Solanum lycopersicum*. Genomic DNA was sheared with a Megaruptor 3. Sheared DNA was
129 converted to a sequencing library with the SMRTbell Express Template Prep kit 3.0, prior to
130 sequencing on 11 SMRTcell 8M for generation of long-read PacBio HiFi reads on a Sequel IIe
131 platform with 30 h movie time. The combined yield was 244 Gb of raw HiFi data. RNA was
132 extracted from root, leaf, pistil, and anther tissue with the RNeasy Plant Mini Kit (Qiagen,
133 Germantown, MD), and individual libraries were generated with the Iso-Seq SMRTbell prep kit
134 3.0, generating 23 Gb of HiFi data. To improve the assembly, we used proximity ligation (Hi-C)
135 to capture the 3D structure of chromosomes with the restriction enzymes DpnII, DdeI, HinFI,
136 and MseI and generated 138 Gb of Illumina paired-end sequences.

137 We generated an initial haploid assembly using *Hifiasm* (v. 0.19.2) (Cheng et al., 2021). HiFi
138 reads were assembled in the integrated mode along with the Hi-C data. This initial assembly was
139 further refined with the *purge_dups* pipeline. Scaffolding was performed with *YaHS* (Zhou et al.,
140 2022) after processing of HiC reads, following the Arima genomics pipeline
141 (https://github.com/ArimaGenomics/mapping_pipeline). Manual curation was performed with
142 *Juicebox* (Durand et al., 2016). Finally, we conducted another round of scaffolding with *RagTag*
143 (Alonge et al., 2022) using the *scaffold* module to order scaffolds onto the genome assembly of
144 the closely related *L. perenne* (Frei et al., 2021).

145 Full-length transcript sequences were individually processed following the *Iso-Seq* and *tama*
146 pipelines (Kuo et al., 2020). Briefly, sequencing primer removal and demultiplexing was
147 performed with *lima*, poly(A) tail trimming and concatemer identification and removal with
148 *refine*, and clustering of reads and polishing with *cluster*. Processed reads from each tissue were
149 aligned to the *L. multiflorum* reference genome with minimap2 (Li, 2018) and individually
150 collapsed with *tama_collapse*, followed by merging with *tama_merge*.

151 Repetitive elements were identified with *EDTA* (v2.1.0; Ou et al., 2019). The coding sequences
152 generated in the previous step were provided to *EDTA* to improve repetitive element detection.
153 *RepeatModeler* (Flynn et al., 2020) was also used to identify any remaining transposable
154 elements missed by *EDTA*.

155 Genome annotation was performed with *Maker* (Campbell et al., 2014). We included the merged
156 transcripts generated from the *Iso-Seq/tama* pipeline, as well as protein sequences from
157 *Brachypodium distachyon*, *Hordeum vulgare*, and *L. perenne* obtained from Ensembl Plants for
158 annotation based on protein homology. Repeats were masked with RepeatMasker, including the
159 species-specific library created with *EDTA*. We employed Augustus (Stanke et al., 2006) and
160 SNAP (Korf, 2004) *ab initio* gene predictors to train and predict genes, in addition to transcript,
161 protein, and repeat alignments. Functional annotation was performed by querying the protein
162 dataset generated against the Uniprot and interproscan databases (Jones et al., 2014).

163 We studied the phylogenetic relationships of *L. multiflorum* with other related taxa in the
164 Poaceae family. We used *OrthoFinder* (v2.5.4; Emms & Kelly, 2019) to identify single-copy
165 orthologs from *L. perenne*, *B. distachyon*, *Triticum aestivum*, *H. vulgare*, *Setaria viridis*,
166 *Echinochloa crus-galli*, and *Oryza sativa*, and we produced alignments with *MAFFT* (Katoh et
167 al., 2002). A phylogenetic analysis was performed with *RAxML-NG* (Kozlov et al., 2019), and
168 plotted with *ggtree* (Xu et al., 2022) and *treeio* (Wang et al., 2019). We used the CoGe
169 (<https://genomevolution.org/coge/>) platform to obtain the pairwise synonymous mutation rates
170 (K_s) between *L. multiflorum* and *L. perenne*, *H. vulgare*, *T. aestivum*, and *B. distachyon*.
171 Divergence time was calculated based on a mutation rate of 5.76174×10^{-9} (De La Torre et al.,
172 2017).

173

174 **Quantitative trait locus (QTL) mapping of paraquat resistance**

175 We used QTL mapping to identify the genomic locations contributing to paraquat resistance. An
176 outcrossed F_2 population segregating for resistance was generated. To phenotype paraquat
177 resistance, plants from all generations were treated with 1682 g a.i. ha^{-1} of paraquat. Individuals
178 were scored as dead or alive 14 days after treatment. Prior to paraquat treatment, leaf tissue was
179 sampled from individual plants for DNA extraction. We isolated genomic DNA from 47
180 susceptible and 48 resistant F_2 individuals and created individually barcoded nextRAD libraries
181 (Russello et al., 2015) and sequenced on a Novaseq 6000 (2×150 bp reads).

182 Paired-end reads were trimmed with *trimmomatic* (Bolger et al., 2014). Reads were aligned to
183 the reference *L. multiflorum* genome with *bwa*, removed PCR duplicates with *samblaster* (Faust
184 and Hall, 2014), and sorted with *samtools* (Li et al., 2009). Sequencing data from resistant and
185 susceptible individuals were pooled separately, and we then used *freebayes* (Garrison and Marth,
186 2012) to identify SNPs. We used the QTL-seq method, as implemented in *QTLseqr* (Mansfeld
187 and Grumet, 2018), to identify QTL regions associated with paraquat resistance (Takagi et al.,
188 2013). A positive Δ SNP-index that exceeds the 95% confidence interval suggests that identified
189 alleles are significantly associated with the resistance phenotype.

190

191 **Resequencing data generation and analysis**

192 While QTL mapping can provide information on the loci involved in the evolution of resistance,
193 the large linkage blocks present in an F_2 population can make it difficult to narrow down the
194 genomic location(s) involved in the trait of interest. To complement the QTL analysis and to
195 further explore the genetic architecture of paraquat resistance, we performed a GWAS from 94
196 individuals collected from six populations (two agricultural fields in Oregon and one in
197 California that are resistant to paraquat, two from fields from Oregon that are susceptible, and a
198 known susceptible cultivated variety from Oregon). At the 3-tiller growth stage, plants were
199 sprayed with lethal doses of paraquat (1682 g a.i. ha^{-1}) as described above. Survival was recorded
200 two weeks after treatment. Genomic DNA from 94 individuals was sequenced on a Novaseq6000
201 in 2x150bp mode to generate 10 \times coverage.

202 Paired-end sequences were initially processed with *HTStream*
203 (<https://github.com/s4hts/HTStream>). Processed files were aligned to the *L. multiflorum* genome
204 with the *minimap2* module for short-read sequences, and PCR duplicates were removed with the
205 *MarkDuplicates* tool of *gatk* (Poplin et al., 2018). Variant detection was performed with
206 *freebayes* (Garrison & Marth, 2012). We used *bcftools* (Danecek et al., 2021) to retain biallelic
207 variants with depths between 10 and 250 in at least 75% of the samples. To obtain an overview
208 of the structural genetic diversity among *L. multiflorum* populations, we identified small variants
209 with *manta* (Chen et al., 2016). Genome-wide association analysis was performed with *GAPIT*

210 (Lipka et al., 2012), with the Enriched Compressed Mixed Linear Model (ECMLM) (Li et al.,
211 2014) and correcting for population structure with PCA eigenvalues and a kinship matrix. Upon
212 identification of statistically significant markers, annotated genes within 2 Mb upstream and
213 downstream of the marker with lowest significance were identified with *bedtools* intersect
214 (Quinlan & Hall, 2010).

215

216 **RNA-seq data generation and analysis and weighted correlation network analysis**

217 To assess the effects of differences in transcription level conferring paraquat resistance, we
218 compared gene expression levels among individuals from two independently generated F₃
219 populations segregating for paraquat resistance that originated from population PRHC (Brunharo
220 & Hanson, 2018) and population pop60 (Bobadilla et al., 2021). At the 2-leaf growth stage,
221 plants were treated with a lethal rate of paraquat (1682 g a.i. ha⁻¹). Leaf tissue was collected from
222 independent F₃ plants at multiple time points: 0 (immediately before application), 3, 6, 12, and 24
223 hours after treatment. Tissue was snap frozen in liquid nitrogen. At each timepoint, leaf tissue
224 was collected from four resistant and four susceptible individuals from each of the F₃
225 populations. Plants were scored 7 d after application as dead or alive, and chlorophyll
226 fluorescence was measured with a portable fluorometer (OS1p+, Opti-Sciences, Hudson, NH) at
227 each collection time. RNA was extracted from samples with a commercial kit (RNase Plant
228 Mini Kit), and 3'-Tag-RNA-seq sequencing libraries were generated with the QuantSeq FWD kit
229 (Lexogen GmbH, Vienna, Austria). Sequencing was performed on an Illumina Novaseq 6000 in
230 2 × 150bp. Forward sequences were filtered using *BBDuk*
231 (<https://sourceforge.net/projects/bbmap/>). Filtered reads were aligned to the reference genome
232 with *STAR* in *quantMode* (Dobin et al., 2012). Differential gene expression was quantified with
233 the R package *edgeR* (Robinson et al., 2009) and *limma* (Ritchie et al., 2015).

234 To determine if differentially expressed genes between resistant and susceptible individuals
235 tended to be co-expressed with genes with similar functions, we performed a Weighted Co-
236 Expression Network Analysis (WGCNA) with the *WGCNA* package (Langfelder & Horvath,
237 2008) using the chlorophyll fluorescence as the response variable. To identify any outlier

238 samples in our dataset, we employed hierarchical clustering, and subsequently used the constant-
239 height tree-cut function to eliminate the outliers. The appropriate soft-threshold power was
240 identified by performing the approximate scale-free topology criterion. We then derived a signed
241 adjacency matrix through bi-weight mid-correlation and a signed topological overlap matrix
242 through dissimilarity calculations. Genes were grouped into modules using hierarchical
243 clustering, and we employed the dynamic tree-cutting algorithm to partition genes into distinct
244 modules. Next, we computed module eigengenes, which allowed us to merge similar modules
245 and pinpoint modules associated with paraquat resistance. To identify genes that exhibit a strong
246 correlation with genes in modules linked to paraquat resistance (i.e., hub genes), we conducted
247 an intra-modular analysis. Hub genes were identified based on their module membership
248 (ranging from 0 to 1, indicating overall connectivity) and their gene-trait significance
249 (determined by the Pearson correlation between expression and the trait). Finally, to gain insights
250 into the biological functions associated with these significant modules, we conducted a Gene
251 Ontology (GO) term enrichment analysis for each of them using *TopGO* R package.

252

253 **Population genomic analyses of selection on resistance**

254 To complement the genetic mapping approaches described above, we used population genomic
255 information from the different susceptible and resistant populations to determine the role of
256 human-mediated selection on the evolution of paraquat resistance. The application of paraquat in
257 agricultural fields is expected to confer a strong selective pressure for resistance, resulting in
258 signatures of a selective sweep surrounding the loci involved in resistance. However, if the
259 genetic variation responsible for resistance existed in the population prior to the onset of
260 paraquat application, functional variants would likely occur on multiple haplotypes, which might
261 obscure the signatures of selection. We calculated levels of genetic divergence (*Fst*) between the
262 resistant and susceptible populations across the genome, with the expectation that locally
263 elevated patterns of genetic divergence between susceptible and resistant populations would be
264 associated with resistance. We estimated *Fst* using the Weir method (Weir and Cockerham,
265 1984) at each biallelic SNP after removing indels, as implemented in *VCFtools* (Danecek et al.,

266 2011). These were plotted across the genome, and regions of exceptionally elevated Fst were
267 considered as potentially selected loci.

268 We also asked if the three resistant populations we sampled were differentiated in the same
269 regions of the genome. Elevated Fst could be caused by only one or two resistant populations
270 being differentiated from the susceptible populations, but this would not indicate a region that is
271 always involved in resistance. To test this, we calculated Fst in each 25 kb window across the
272 genome between each pair of resistant and susceptible populations using the *popgenwindows.py*
273 script (https://github.com/simonhmartin/genomics_general). For each of the nine pairwise
274 comparisons, we counted the number of times each window was found in the top 1% of windows
275 across the distribution of Fst values, because the more often a window is found in the top 1% of
276 Fst values across comparisons, the more likely the same resistance haplotype is diverged from
277 susceptible haplotypes.

278 Fst can be considered as a relative measure of sequence divergence because its value is
279 influenced by levels of genetic diversity within populations (Cruickshank & Hahn, 2014).
280 Although a region of locally elevated Fst could be caused by reduced diversity in either (or both)
281 susceptible or resistant groups, we expect that selection occurred in resistant individuals.
282 Therefore, we expect to find locally reduced genetic diversity and fewer segregating sites (i.e.,
283 polymorphism) in resistant relative to susceptible individuals. In addition, we expect resistant
284 individuals to show a higher average frequency of alternate alleles relative to the reference. The
285 reference assembly is derived from a susceptible individual, so a higher frequency of alternate
286 alleles in resistant individuals would be consistent with selection increasing the frequency of
287 alleles responsible for resistance. Based on overlap between the genetic mapping and Fst results,
288 we focused these analyses on two regions on chr5 (see Results). Finally, we calculated the site-
289 specific extended haplotype homozygosity statistic. Due to a recent selective sweep, a rapid
290 increase in the frequency of a beneficial mutation will result in elevated linkage disequilibrium,
291 leading to extended patterns of homozygosity within haplotypes (Sabeti et al., 2002, Smith &
292 Haigh, 1974, Voight et al., 2006). However, in the absence of selection, we expect haplotypes to
293 break down over time due to new mutations and recombination. We compared haplotypes from
294 susceptible and resistant individuals by focusing on two sites that had the highest Fst in each of
295 the two regions on chr5. Haplotype and marker information was extracted from each VCF file

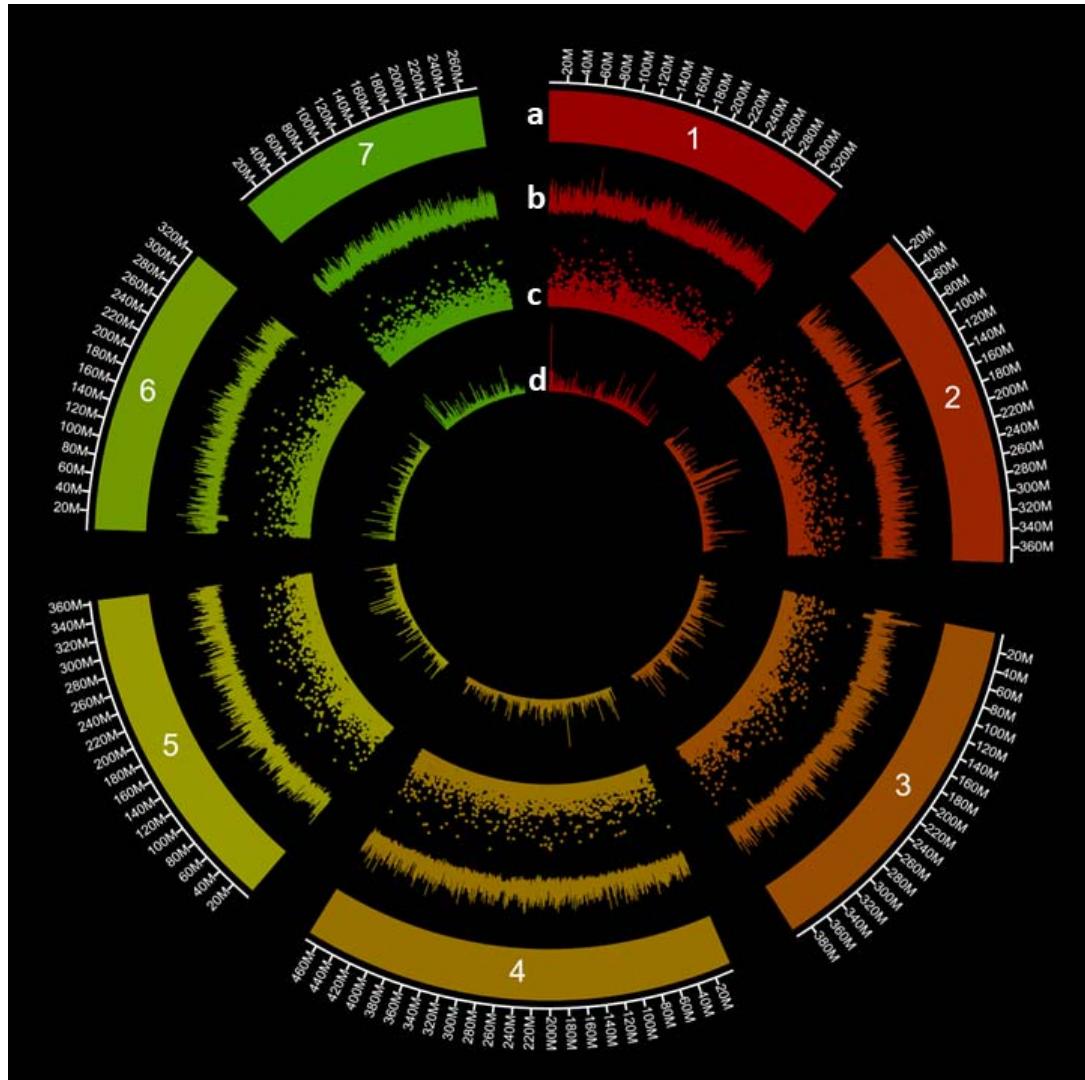
296 using the *data2haplohh* function of the *rehh* package (Gautier & Vitalis, 2012). EHH was
297 calculated from the marker and haplotype information for each of the taxa using the *calc_ehh*
298 function in *rehh*.

299

300 **RESULTS**

301 **A chromosome-level assembly and annotation for *L. multiflorum***

302 The first step towards elucidating the evolution of herbicide resistance in *L. multiflorum* was to
303 assemble a high-quality reference genome for this species (Fig. 1). We generated 45 \times coverage
304 long-read data from DNA of an herbicide susceptible individual and used proximity ligation (Hi-
305 C) to generate a haploid assembly of the *L. multiflorum* genome (Table S1). The *de novo*
306 assembly of the *L. multiflorum* genome resulted in 297 scaffolds, with $N_{50} = 61$ Mb and $L_{50} = 12$
307 (i.e., the length sum of 12 contigs contributes to at least 50% of the assembly). This initial
308 assembly was improved by ordering scaffolds based on the synteny shared with the closely
309 related species *L. perenne*, resulting in 7 chromosomes containing 2.55 Gb of nuclear content
310 (90% of the genome; $N_{50} = 363$ Mb; Table S1). The genome size obtained *in silico* supports the
311 flow cytometry estimates of 2.72 Gb haploid size. BUSCO analysis indicated that 93% of the
312 single-copy orthologs from the *poales_odb10* dataset were contained in the assembly.
313 Approximately 83% of the DNA content is composed of repetitive elements (Table S2), as
314 expected from a plant species with a large genome size (Schnable et al., 2009). We annotated the
315 *L. multiflorum* genome with 23 Gb of Iso-Seq data from leaf, root, pistil and anther tissue and
316 identified 49,295 protein-coding sequences.



317

318 Fig. 1. Circos plot of the *L. multiflorum* genome and its various features, plotted in 100 kb
319 windows, where peak height represents feature frequency. Tracks represent a) the seven
320 chromosomes, b) repetitive elements, c) small variants (<1000 bp), and d) single-nucleotide
321 polymorphisms.

322

323 In weed management, accurate species identification is crucial, because different species can
324 exhibit distinct responses to efforts to control them. *Lolium multiflorum*, *L. perenne*, and *L.*
325 *rigidum* are often referred to, or treated as, a single species for basic biology and management
326 (Scarabel et al., 2020, Yannicci et al., 2020). Our results confirm these species are closely

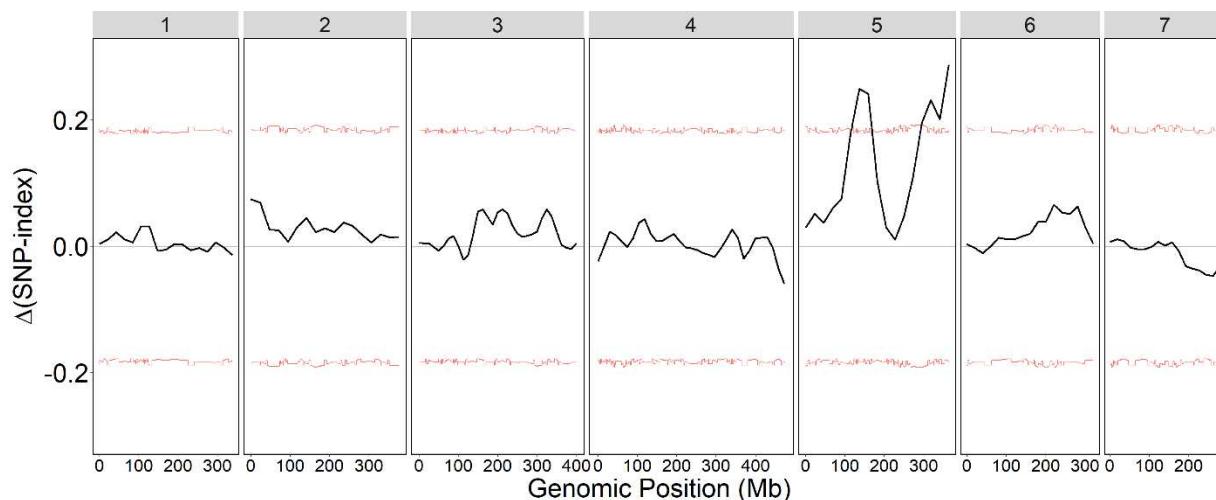
327 related, but they are phylogenetically distinct, confirming morphological and life-history studies
328 performed elsewhere (Fig. S1A) (Bararpour et al., 2017). In addition, based on the synonymous
329 substitution rates (K_s) between homologous gene pairs, we found that *L. multiflorum* appears to
330 have diverged from *L. perenne*, *T. aestivum*, *H. vulgare*, and *B. distachyon* approximately 5.4,
331 26, 27, and 29.4 M years ago (Fig. S1B).

332

333 **Genetic mapping reveals the genetic architecture of resistance**

334 A QTL analysis in an F_2 mapping population identified regions of the genome segregating with
335 the resistance trait (Fig. 2). We identified two regions on chr5 that were significantly associated
336 with paraquat resistance based on the Δ SNP-index exceeding the 95% confidence interval. The
337 intervals of the two detected QTL spanned positions 120,860,887 - 167,802,303, and
338 294,188,538 - 363,361,071 on chr5. This could suggest two separate loci on chr5 are responsible
339 for paraquat resistance, but further analysis is necessary to determine the precise regions
340 involved.

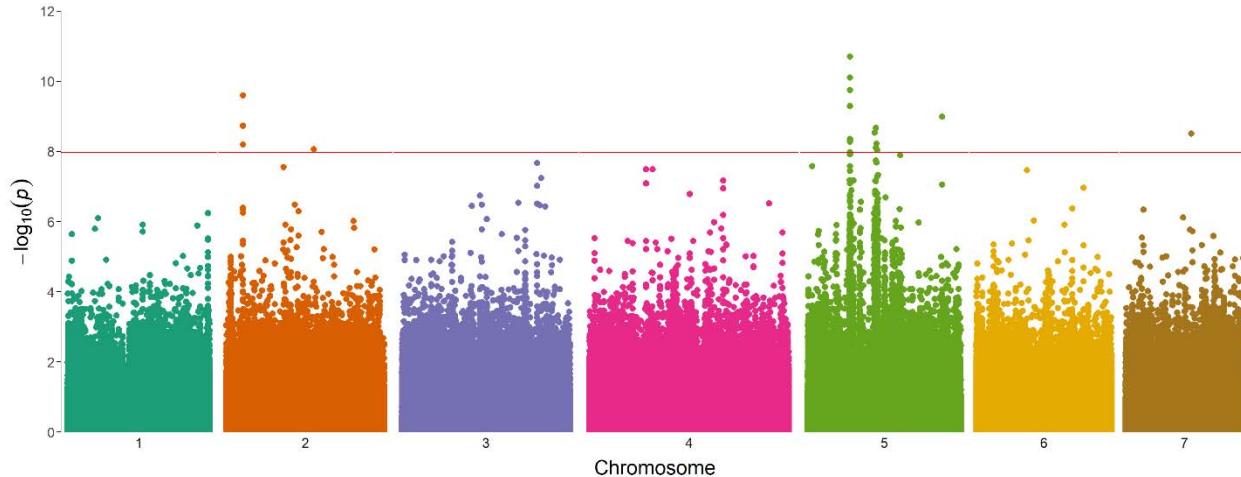
341



342
343 Fig. 2. QTL-seq analysis from an F_2 mapping population between paraquat-resistant and -
344 susceptible *L. multiflorum* individuals. The numbers at the top represent the chromosome
345 number. The red lines represent the 95% bootstrap confidence intervals based on 10,000
346 replicates used for significance testing.

347

348 To further explore the genetic architecture of resistance, we performed a GWAS by resequencing
349 94 *L. multiflorum* individuals to an average 10× coverage, and implemented statistical models in
350 *GAPIT* (Lipka et al., 2012) to identify SNPs and INDELs associated with paraquat resistance.
351 GWAS can take advantage of the genetic variation present across multiple individuals that have
352 a longer history of recombination. Moreover, determining the genomic regions where the QTL
353 and GWAS analyses intersect would provide independent corroboration of potential candidates
354 for resistance. The final GWAS dataset contained 3,385,096 SNPs and 1,359,667 INDELs,
355 which results in an overall marker density of 1.7 variants per 1 kb. In total, we found 22 markers
356 that were significantly associated with the difference between resistant and susceptible plants
357 (Fig. 3). Although five of these markers were statistically significant, they occurred as
358 singletons, with no additional significant markers in those regions. Therefore, we focused only
359 on the remaining 17 markers that localized to three distinct regions of the genome: one on chr2,
360 and two on chr5. The three significant markers found on chr2 spanned a distance of only 289 bp
361 (between positions 40,259,279 – 40,259,568). Of the remaining significant markers, eight of
362 them localized to a 239 kb region on chr5 (between positions 100,183,991 – 100,423,860). The
363 final five markers that were significantly associated with the phenotype also were located on
364 chr5, between positions 159,409,415 – 165,297,925, a distance of 5.89 Mb. Although the first
365 region on chr5 does not directly overlap with the QTL peaks (roughly 20 Mb away), this second
366 region was contained within the first peak on chr5.



367
368 Fig. 3. Genome-wide association of paraquat-resistance in *L. multiflorum*. A total of 4,744,764
369 (n) makers were tested. The red line is the Bonferroni threshold calculated as 0.05/n.

370

371 We further investigated these regions by extracting annotated genes in 2 Mb regions surrounding
372 the marker in each region with the lowest P-value (4 Mb total per region). The focal markers
373 were position 40,259,568 of chr2, and 100,269,722 and 162,257,267 of chr5. A total of 214
374 genes associated with paraquat resistance were annotated near these three regions, of which 89
375 were on chr2, 64 near the first peak on chr5, and 61 near the second peak of chr5 (Table S3).
376 There were several genes with known functions that could be involved with resistance to
377 paraquat. For example, there were genes that had functions related to the response to oxidative
378 stress, those that mediated intracellular transport, and genes known to respond to herbicides.
379 Genes with potential roles in herbicide metabolism were identified, such as cytochrome P450s,
380 as well as a polyamine oxidase gene that regulates polyamine intracellular concentration. Given
381 that paraquat resistance has been previously suggested to be conferred by reduced herbicide
382 movement, genes that encode membrane-bound transporters are of particular interest.
383 Specifically, we identified *SEC31B* (promotes the formation of transport vesicles from the
384 endoplasmic reticulum) on chr2, *Transmembrane 9 superfamily member 1 (TMN1*; involved in
385 cell adhesion and phagocytosis in the secretory pathway), *Lysine histidine transporter-like 8*
386 (*AATL1*; a transmembrane amino acid transporter) and *VATP-P1* (a V-type proton ATPase) in
387 the first region of chr5, and *NPF5.10 (NRT1/PTR FAMILY*, a nitrate or di/tri-peptide

388 transporters), and *DTX10* (a member of the MATE family of proteins) in the second region of
389 chr5.

390

391 **RNAseq identifies genes differentially expressed between susceptible and resistant plants**

392 To complement the results from QTL-seq and GWAS, we performed a differential gene
393 expression experiment in two separate F₃ populations segregating for paraquat resistance. Based
394 on visualization of a multidimensional scaling plot, there was a clear separation between
395 mapping populations. Therefore, analyses were performed separately for each segregating
396 population. A total of 55 genes was differentially expressed in the F₃ population derived from
397 PRHC, and 10 genes from pop60 (Table S4). Of these, only two genes were found to be
398 differentially expressed in both populations. One of these encodes a chloroplastic
399 uncharacterized aarF domain-containing protein kinase, and the other encodes a glutathione
400 synthetase (GSH2). In both cases, the genes were expressed at higher levels in resistant
401 individuals relative to susceptible, with *GSH2* being the most highly expressed gene in the
402 dataset (Table S4). Interestingly, we found a strong over-representation of differentially
403 expressed genes across chr5. Of the 63 differentially expressed genes detected, 32 (51%)
404 occurred on chr5. Among these, there is only one gene that is differentially expressed and found
405 among the annotated genes near the GWAS hits. This gene encodes a protein with sequence
406 similarity to the NPF5.10 protein from *Arabidopsis thaliana* and is expressed at a higher level in
407 resistant plants from the PRHC-derived population.

408

409 **Gene co-expression networks dissect the coordinated expression patterns in response to**
410 **paraquat**

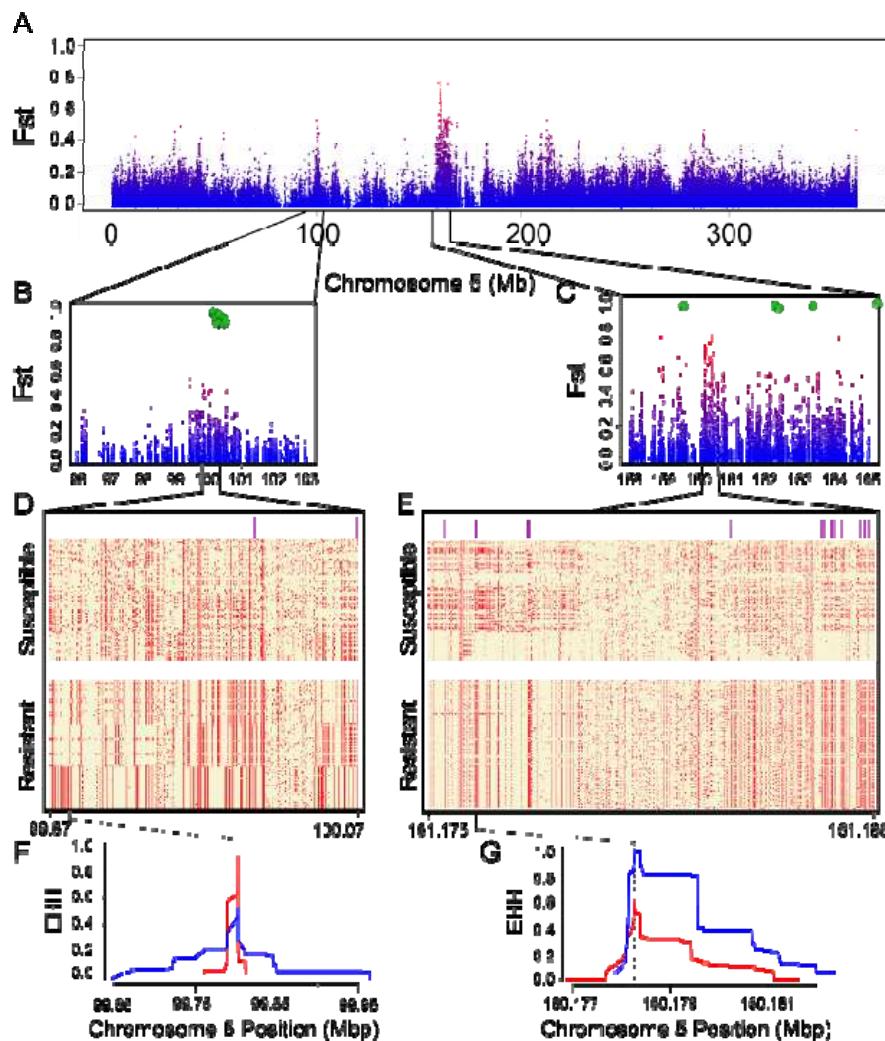
411 The network analysis grouped genes with similar expression patterns into separate modules,
412 resulting in 18 modules identified in the population derived from pop60 and 28 from PRHC (Fig.
413 S2-3). The number of genes contained within the modules ranged from 74 to 3,288 for pop60,
414 and from 38 to 3,286 for PRHC. Similar patterns were observed across the modules that were

415 positively correlated with chlorophyll fluorescence (a proxy for paraquat resistance).
416 Specifically, these modules contained co-expressed genes with functions that included
417 transmembrane transport, photosynthesis, glutathione biosynthesis, ABC transporters, and
418 superoxide metabolism (Fig. S2-3). Hub genes included detoxification 21 (*DTX21*) and ABC
419 transporters (*ABCB11*, *ABCA8*). In addition, *psbB* *Photosystem II CP47* and *atpI* ATP synthase
420 subunit α were detected as hub genes in modules associated with photosystem responses and
421 regulation (Fig. S2, module “yellow” and “tan” in pop60 and PRHC, respectively). These results
422 further indicate that membrane-bound transporters are responsive to paraquat application and
423 could be involved in herbicide resistance or to minimize oxidative stress from paraquat
424 application. Of the 60 major hub genes positively correlated with paraquat resistance, 19 (32%)
425 were on chr5. Three annotated genes were found within the QTL interval on chr 5:
426 *LOLMU_00024321* (Protein of unknown function), *LOLMU_00024251* (Probable protein
427 phosphatase 2C 20), and *LOLMU_00027468* (Protein of unknown function).

428

429 **Recent positive selection on resistant haplotypes**

430 By scanning the genome for SNPs with elevated *Fst*, we detected two regions that were highly
431 differentiated between resistant and susceptible individuals (Fig. S4). Importantly, both of these
432 regions occurred on chr5, and they aligned with the two regions on that chromosome identified
433 from the GWAS (Fig. 4). Hereafter, we define these two regions as region 1 and region 2, based
434 on their position on chr5 (region 1: 99.7 Mb, and region 2: 160.1 Mb). Although neither region
435 contained differentially fixed sites (i.e., *Fst* = 1), *Fst* did exceed 0.7 at three sites in region 2,
436 indicating extensive sequence divergence between resistant and susceptible populations.



437

438 Fig. 4. Recent positive selection on resistant haplotypes. A) Fst scan of biallelic SNPs across
439 chromosome 5. The SNPs are color-coded based on their relative values across the entire
440 genome (see Fig. S4), with red indicating higher overall divergence. Panels B and C contain the
441 same information as in panel A, but they are zoomed in on regions 1 and 2. Green hexagons
442 indicate the location of GWAS markers that were significantly associated with the resistance
443 phenotype. D, E) Each row is a haplotype from resistant or susceptible individuals, either for
444 region 1 (D) or region 2 (E), and columns correspond to variant sites. Yellow boxes indicate the
445 presence of the reference allele, while red boxes indicate the alternate allele. Purple lines above
446 the haplotypes denote the sites where Fst between resistant and susceptible populations is greater
447 than 0.4. The range of sites included in each region is reported at the bottom of each panel, in
448 Mb. F, G) The site-specific extended haplotype homozygosity (EHH) statistic for region 1 (F)

449 and region 2 (G), with blue lines corresponding to resistant haplotypes and red lines denoting
450 susceptible haplotypes. The black dotted line indicates the focal SNP included in each analysis
451 (region 1: 99,802,212; region 2: 160,178,236).

452

453 Further inspection of these two regions revealed that the divergence between susceptible and
454 resistant populations was caused primarily by a reduction in diversity and polymorphism in
455 resistant, but not susceptible populations (Fig. 4D-E). Haplotype diversity was 45% lower in
456 resistant individuals than susceptible in region 2 (resistant: $5.4 \times 10^{-4} \pm 2.7 \times 10^{-5}$ S.E.;
457 susceptible: $7.9 \times 10^{-4} \pm 2.9 \times 10^{-5}$ S.E.). Although diversity was similar between susceptible and
458 resistant populations in region 1 (resistant: $2.8 \times 10^{-4} \pm 1.9 \times 10^{-5}$ S.E.; susceptible: $2.9 \times 10^{-5} \pm$
459 1.6×10^{-5} S.E.), the proportion of segregating sites among resistant haplotypes was nearly half
460 that in susceptible individuals in both regions (region 1: resistant = 0.54, susceptible = 0.93;
461 region 2: resistant = 0.61, susceptible = 0.93). These patterns were in stark contrast to the
462 proportion of segregating sites across all chr5, where polymorphism was much more similar
463 between resistant and susceptible populations (resistant = 0.77, susceptible = 0.85). Finally,
464 resistant individuals showed a frequency of the alternate (i.e., non-reference) allele that was
465 nearly twice as high as susceptible individuals in both regions (average frequency among sites
466 for region 1: resistant = 0.22, susceptible = 0.11; region 2: resistant = 0.19, susceptible = 0.10).
467 This difference is even more pronounced when we consider the number of variants where the
468 alternate allele has a frequency greater than 0.5. In region 1, there were 37 sites where the
469 alternate allele was at a frequency > 0.5 in resistant individuals, but only 19 sites in susceptible
470 individuals. In region 2, there were 100 sites in resistant individuals but only 8 among
471 susceptible. The presence of a high frequency of the alternate allele at multiple sites in these
472 regions is consistent with selection increasing the frequency of distinct haplotypes in resistant
473 but not susceptible individuals.

474 We also detected patterns of extended haplotype homozygosity in both regions that were more
475 pronounced in resistant individuals. In region 1, we found a very broad region of homozygosity
476 (roughly 300 kb) in some, but not all, resistant individuals. This is in stark contrast to the pattern
477 in susceptible individuals, where the signal of EHH is quite narrow (Fig. 4). In region 2, the

478 probability of homozygosity was substantially elevated in resistant individuals relative to
479 susceptible, but this region extended only over 5 kb. Thus, even though the patterns are different
480 in each region, they are both consistent with selection driving an increase in the frequency of the
481 alternate allele.

482 Finally, we also asked if the same regions of the genome were differentiated in all three resistant
483 populations relative to the susceptible populations. To do this, we calculated Fst in non-
484 overlapping 25 kb windows for all pairs of resistant and susceptible populations and counted in
485 how many of these pairwise comparisons was each window found in the top 1% of the Fst
486 distribution. We found only two regions in the genome where all nine comparisons between
487 resistant and susceptible populations showed the same window in the top 1% of the distribution,
488 and these corresponded to regions within or directly adjacent to regions 1 and 2 (Fig. S5).
489 Indeed, there was a pileup of windows in these two regions where multiple comparisons showed
490 elevated Fst. No other window across the genome exceeded six comparisons where that window
491 was repeatedly in the top 1% of Fst windows.

492

493 **DISCUSSION**

494 Herbicide resistance is one of the greatest challenges in sustainable crop production, and
495 elucidating the genetic basis of resistance could aid in improving weed management practices.
496 *Lolium multiflorum* is one of the most troublesome weed species in the world, having evolved
497 herbicide resistance in multiple cropping systems and countries. The genomic resources that we
498 generated in this work will be a valuable tool to dissect the genetic bases of herbicide resistance
499 and other traits. Obtaining a contiguous genome is a crucial component of the assembly effort
500 (Lee et al., 2016) that allows researchers to better understand the genomic context in which
501 genes or variants of interest occur. The *L. multiflorum* genome assembled here had 90% of the
502 DNA content placed on 7 chromosomes ($N_{50}=363$ Mb), with 93% completeness, which is
503 comparable to other high-quality assemblies (Cai et al., 2021, Benson et al., 2023).

504 The genetic basis of paraquat resistance was addressed using multiple “-omics” datasets.
505 Implementing multiple approaches has proven necessary to dissect the genetic architecture of

506 plant traits (Du et al., 2018, Jiang et al., 2019), as any one experiment may have shortcomings
507 (Li & Xu, 2022). Overall, our QTL-seq, GWAS, and Fst approaches converged on two regions
508 on chr5 that appeared to be associated with paraquat resistance in *L. multiflorum*. GWAS also
509 identified significant SNPs on chr2 and chr7. Although we used a highly conservative Bonferroni
510 test to correct for multiple testing, these associations may represent false positives due to residual
511 population structure, epistasis, or other indirect effects (Platt et al., 2010, Kaler and Purcell,
512 2019). Furthermore, we found only two regions in the genome where all nine pairwise Fst
513 comparisons between resistant and susceptible populations showed the same window in the top
514 1% of the distribution, and these corresponded to regions within or directly adjacent to regions 1
515 and 2 (Fig. S5).

516 Paraquat resistance in other species, including in the weed *L. rigidum* that is closely related to *L.*
517 *multiflorum* (Yu et al., 2009), is believed to be caused by a single gene with incomplete
518 dominance. By contrast, (Shaaltiel et al., 1988) suggested the possibility of linkage between two
519 genes involved in paraquat resistance. We found two linked loci that appear to be associated with
520 resistance. One explanation for this finding is that there are genes in regions 1 and 2 that work in
521 concert to generate the resistance phenotype. The resistant populations used in this study were
522 collected from fields that have been subjected to recurrent paraquat applications, resulting in a
523 constant selective pressure. However, recombination between these two regions would break up
524 co-adapted genotypes at these loci. Future experiments with larger sample sizes should explore
525 patterns of linkage disequilibrium between these regions to investigate the possibility of epistatic
526 selection maintaining particular genotypic combinations at these loci.

527 Analysis of gene expression patterns produced valuable insights into how plants respond to
528 oxidative stress caused by paraquat. When all time points were analyzed together, we observed
529 an overall higher expression of *GSH2* in resistant plants from both F_3 populations. Closer
530 inspection at each time point provided more information into the temporal dynamics of its
531 expression (Table S4). Notably, we observed that *GSH2* was constitutively expressed at higher
532 levels in the PRHC population (5-fold) prior to herbicide treatment, but this same pattern was not
533 found in the pop60 F_3 population. However, upon paraquat treatment, *GSH2* expression in
534 resistant individuals increased to 17-fold greater than the susceptible individuals in the pop60
535 derived F_3 population. These results are consistent with the different evolutionary histories of

536 pop60 and PRHC (Brunharo and Streisfeld, 2022), which implies that they may have evolved
537 different mechanisms to cope with abiotic stresses. Alternatively, the recent exposure of field
538 populations to paraquat or other herbicides that cause oxidative stress could have induced the
539 constitutive over-expression of oxidative stress-associated genes. By contrast, sublethal oxidative
540 stresses could alter overall gene expression by inducing epigenetic modifications that are trans-
541 generationally stable (Dyer, 2018, Sharma et al., 2022). The WGCNA also found modules with
542 enriched GO terms for hub genes involved in oxidative stress and xenobiotic response that
543 responded to paraquat application.

544 The population genomic analyses revealed molecular signatures that were consistent with the
545 action of recent positive selection in resistant individuals. Fst analysis identified two regions in
546 chr5 that were highly differentiated between resistant and susceptible populations due to a lower
547 diversity and segregating sites in resistant individuals. We also observed a pileup of windows in
548 these regions where multiple comparisons between resistant and susceptible populations showed
549 elevated Fst (Fig. S5). In addition, a greater number of non-reference polymorphisms was found
550 in the resistant individuals compared to susceptible, which resulted in patterns of extended
551 haplotype homozygosity in resistant plants. When combined with the GWAS and QTL analyses,
552 these genomic data suggest that these regions experienced recent positive selection leading to
553 evolution of resistance.

554 In addition to revealing the genetic architecture and evolutionary responses to resistance, our
555 extensive genomic analyses reveal promising candidate genes. Specifically, genes that encode
556 NPF5.10 and DTX10 are of particular interest, as both are contained within the GWAS peaks
557 and are adjacent to region 2 from the Fst analysis. NPF5.10 is also the only gene in these regions
558 that was differentially expressed between resistant and susceptible individuals. It is a member of
559 the NRT1/PTR family, which are nitrate and oligopeptide transporters localized to cellular
560 membranes. It has been suggested that an amino acid substitution in the NPF6.4, a member of
561 the NRT1/PTR family, reduced norspermidine (a polyamine) cell uptake in *Arabidopsis* (Tong et
562 al., 2016). Polyamines are natural compounds responsible for protein synthesis, ion channel
563 modulation and a number of biological processes (Igarashi & Kashiwagi, 2010), and paraquat is
564 known to competitively inhibit polyamine transporters (Hart et al., 1992a, Hart et al., 1992b).
565 (Brunharo & Hanson, 2017) observed that paraquat-resistant *L. multiflorum* pretreated with

566 putrescine (a polyamine) became susceptible upon paraquat application, which suggested that a
567 membrane-bound transporter could be involved in the resistance mechanism.

568 DTX10 is a member of the MATE family (Multidrug And Toxic compound Extrusion) that is
569 found in prokaryotes and eukaryotes. These transporters are localized to the tonoplast (Zhang et
570 al., 2017), Golgi apparatus (Seo et al., 2012), and plasma membrane (Rogers and Guerinot,
571 2002). A total of 58 MATE proteins have been annotated in *Arabidopsis* (Hvorup et al., 2003)
572 and 53 in rice (Tiwari et al., 2014), and they have been shown to transport cationic compounds
573 across cellular and organellar membranes (Kusakizako et al., 2020). Most importantly, an amino
574 acid substitution in the DTX6 has previously been shown to confer paraquat resistance in an
575 experimentally-derived *Arabidopsis* accession (Lv et al., 2021a). DTX6 is localized in the
576 endomembrane trafficking system and is responsible for increased vacuolar sequestration and
577 cellular efflux of paraquat (Lv et al., 2021a).

578 In this work, we elucidated the genetic architecture of paraquat resistance in *L. multiflorum*, and
579 we identified promising candidate genes for future functional validation studies. Understanding
580 the genetic basis of herbicide resistance is crucial to improve the sustainability of cropping
581 systems. A key component of weed management is to limit dispersal of herbicide resistant
582 individuals at multiple levels of the production operation, such as in the field, warehouses, or
583 shipping containers during international trade. This could be accomplished with the development
584 quick resistance identification assays using genetic markers (Brusa et al., 2021), and the
585 development of lateral flow assays that target the genetic variants (Baerwald et al., 2020). The
586 genomic resources generated in this work will be valuable for a wide range of plant scientists.
587 *Lolium multiflorum* is not only an agricultural weed, but domesticated varieties are cultivated as
588 a cover crop and for forage, and the assembled genome could be used by breeders for trait
589 improvement.

590

591 **ACKNOWLEDGEMENTS**

592 Funding for this project was provided by the College of Agricultural Sciences at The
593 Pennsylvania State University and Oregon State University.

594

595 **COMPETING INTERESTS**

596 The authors declare that they have no competing interests.

597

598 **AUTHOR CONTRIBUTION**

599 CAB conceived and designed the study, collected data, assembled and annotated the genome,
600 performed QTL, GWAS, RNA-seq, and drafted and revised the manuscript. AWS performed Fst
601 scans and haplotype networks, and revised the manuscript. LKB performed the WGCNA, and
602 revised the manuscript. MAS performed Fst and haplotype networks, and drafted and revised the
603 manuscript.

604

605 **SUPPORTING INFORMATION**

606 Fig. S1. Evolutionary relationships between *L. multiflorum* and other species in the Poaceae
607 family. A) Phylogenetic tree of *L. multiflorum*, *L. perenne*, *Brachypodium distachyon*, *Triticum*
608 *aestivum*, *Hordeum vulgare*, *Setaria viridis*, *Echinochloa crus-galli*, and *Oryza sativa*. B)
609 Distribution of the synonymous substitution rate (K_s) between *L. multiflorum* and closely related
610 species.

611 Fig. S2. Weighted gene co-expression network analysis of the F_3 paraquat-resistant pop60
612 population. (A) Heat map of module-trait relationships shows a correlation from more negative
613 (blue) to more positive (red) for each module, which are given names with different colors. Each
614 column indicates a comparison between time points or individuals. (B) Hierarchical cluster trees
615 show the co-expression modules identified by WGCNA.

616

617 Fig. S3. Weighted gene co-expression network analysis of the F₃ paraquat-resistant PRHC
618 population. (A) Heat map of module-trait relationships shows relationship from more negative
619 (blue) to more positive (red) of each module color. Each column indicates a comparison between
620 time points or individuals. (B) Hierarchical cluster trees show the co-expression modules
621 identified by WGCNA.

622 Fig. S4. Genome scan of Fst at all bi-allelic SNPs between paraquat-resistant and -susceptible
623 populations of *L. multiflorum*.

624 Fig. S5. The number of pairwise comparisons between resistant and susceptible populations
625 where Fst (calculated in 25 kb windows) was found in the top 1% of the distribution across
626 windows.

627 Table S1. Summary of *L. multiflorum* assembly and annotation.

628 Table S2. Repetitive element content in the *L. multiflorum* genome.

629 Table S3. Genomic regions identified in GWAS and number of annotated genes.

630

631 **DATA AVAILABILITY**

632 Raw sequencing data is available at the NCBI Sequence Read Archive under BioProject
633 PRJNA1046158. The genome and annotation files will be available at the Weed Genomics
634 Consortium at <https://weedpedia.weedgenomics.org/> upon publication.

635

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