Gene expression shapes the patterns of parallel evolution of herbicide resistance in the agricultural weed *Monochoria vaginalis*

Shinji Tanigaki[1], Akira Uchino[2], Shigenori Okawa[3], Chikako Miura[4], Kenshiro Hamamura[5], Mitsuhiro Matsuo[6], Namiko Yoshino[7], Naoya Ueno[8], Yusuke Toyama[9], Naoya Fukumi[10], Eiji Kijima[11], Taro Masuda[12], Yoshiko Shimono[1], Tohru Tominaga[1], Satoshi Iwakami[1*]

[1]Graduate school of agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

[2]Central Region Agricultural Research Center, National Agriculture and Food Research Organization, Tsu 514-2392, Japan

[3]Miyagi Prefectural Furukawa Agricultural Experiment Station, Fukoku 88, Furukawa-Osaki, Osaki 989-6227, Japan

[4]Akita Prefectural Agricultural Experiment Station, 34-1 Yuwaaikawa-azagenpachizawa, Akita 010-1231, Japan

[5]Japan Association for Advancement of Phyto-Regulators (JAPR), 860 Kashiwada-cho, Ushiku 300-1211, Japan

[6]Field Science Center, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-kibanadai-nishi, Miyazaki 889-2192, Japan

[7]Tohoku Agricultural Research Center, National Agriculture and Food Research Organization, 50 Harajukuminami, Arai, Fukushima 960-2156, Japan

[8]Yamanashi Prefectural Agritechnology Center, 1100 Shimoimai, Kai 400-0105, Japan

[9]Shizuoka Prefectural Research Institute of Agriculture and Forestry, 678-1 Tomigaoka, Iwata 438-0803, Japan

[10]Tottori Agricultural Experiment Station, 260 Hashimoto, Tottori 680-1142, Japan

[11]Yamaguchi Prefectural Agriculture and Forestry General Technology Center, 1-1-1 Ouchihikami, Yamaguchi 753-0231, Japan

[12]Faculty of Agriculture, Setsunan University, 45-1 Nagaotoge-cho, Hirakata 573-0101, Japan

*Corresponding Author, E-mail: iwakami.satoshi.2v@kyoto-u.ac.jp, Phone: +81-75-753-6064

Abstract

The evolution of herbicide resistance in weeds is an example of parallel evolution, through which genes encoding herbicide target proteins are repeatedly represented as evolutionary loci. The number of herbicide target-site genes differs among species, and little is known regarding the effects of duplicate gene copies on the evolution of herbicide resistance. We investigated the evolution of herbicide resistance in *Monochoria vaginalis*, which carries five copies of sulfonylurea target-site acetolactate synthase (*ALS*) genes. Suspected resistant populations collected across Japan were investigated for herbicide sensitivity and *ALS* gene sequences, followed by functional characterisation and *ALS* gene expression analysis. We identified over 60 resistant populations, all of which carried resistance-conferring amino acid substitutions exclusively in *MvALS1* or *MvALS3*. All *MvALS4* alleles carried a loss-of-function mutation. Although the enzymatic properties of ALS encoded by these genes were not markedly different, the expression of *MvALS1* and *MvALS3* was prominently higher among all *ALS* genes. The higher expression of *MvALS1* and *MvALS3* is the driving force of the biased representation of genes during the evolution of herbicide resistance in *M. vaginalis*. Our findings highlight that gene expression is a key factor in creating evolutionary hotspots.

Keywords: acetohydroxy acid synthase, acetolactate synthase, convergent evolution, evolutionary constraint, target-site resistance, weed evolution

INTRODUCTION

Agricultural weeds are often subject to artificial lethal stresses employed in an effort to increase crop yield. Such weeding stresses, in turn, act as unintentional selection pressures driving the evolution of weed populations. While multiple evolved traits related to weed adaptation in agricultural ecosystems have been documented, such as flowering phenology, mimicry morphology, seed shattering, seed dormancy, and competitiveness (Fukano, Guo, Uchida, Tachiki, & Cornelissen, 2020; Waselkov, Regenold, Lum, & Olsen, 2020; Wedger & Olsen, 2018; Ye et al., 2019), herbicide resistance remains among the best studied and characterised trait (Powles & Yu, 2010). Evolved herbicide resistance has been documented worldwide in as many as 263 weed species (Heap, 2021), posing a great threat to the current global food security.

While weed resistance has often been studied from an agricultural viewpoint as a threat to food security, it has also attracted the attention of evolutionary biologists as a classic example of an ‘evolution in action’ (Baucom, 2019). An important insight from studies on weed resistance is that nature often mimics itself: certain genes have repeatedly acted as the major components in the evolution of resistance (Martin & Orgogozo, 2013). In particular, resistance to sulfonylurea (SU) herbicides represents the extreme parallelism of molecular evolution. SUs inhibit acetolactate synthase (ALS, or acetohydroxy acid synthase), which catalyses the first committed step in the biosynthesis of branched-chain amino acids (Tranel & Wright, 2002). Due to their low toxicity to animals, high crop selectivity, and high efficacy at low doses, SUs are among the most frequently used herbicides in many countries, including Japan (Hamamura, 2018; Peters & Strek, 2018). Resistance to SUs, particularly in broadleaf

weeds, is caused mainly by a non-synonymous mutation in the *ALS* gene (Yu & Powles, 2014). The amino acid substitution (AAS) caused by this mutation leads to a conformational alteration in the binding site of SUs, resulting in insufficient ALS inhibition. AASs inducing SU resistance have been reported in as many as eight natural weed populations, and most of these mutations were not significantly associated with fitness cost (Yu & Powles, 2014). This nature is considered as the driving force to render *ALS* loci as an evolutionary hotspot for resistance to SU herbicides (Baucom, 2019).

However, little attention has been paid to the effects of duplicated gene copies on the evolution of ALS herbicide resistance. Having undergone whole genome duplications during the long course of evolution (Clark & Donoghue, 2018), many plants possess duplicated copies of *ALS* genes; in addition to polyploid species, even diploid species such as *Senecio vulgaris* and *Zea mays* exemplify this scenario (Delye, Causse, & Michel, 2016; Svitashev et al., 2015). While recent studies have shed some light on the 'dilution effect' (sensu Iwakami, Uchino, Watanabe, Yamasue, & Inamura, 2012) caused by duplicated gene copies (e.g. Deng et al., 2017; Yamato, Sada, & Ikeda, 2013), little data are available on the roles of such duplicated copies in resistance evolution. Few studies have identified cases in which resistance-conferring mutations occur in all *ALS* copies, such as in *Schoenoplectiella juncoides* (Sada & Uchino, 2017), *Descurainia sophia* (Xu, Xu, Li, & Zheng, 2020), *Senecio valgaris* (Delye et al., 2016), and *Echinochloa crus-galli* (Löbmann, Schulte, Runge, Christen, & Petersen, 2021), implying that all these loci act as evolutionary hotspots. Meanwhile, the biased frequency of specific *ALS* loci has also been reported during the evolution of resistance in *Schoenoplectiella mucronatus* (Scarabel, Locascio, Furini, Sattin, & Varotto, 2010),

although only two populations have been investigated. Most studies thus far have investigated only a limited number of populations; therefore, whether there is indeed a bias in the occurrence of resistance-conferring mutations among *ALS* loci remains unclear.

Monochoria vaginalis (syn. *Pontederia vaginalis*) is a noxious allohexaploid (2n = 4x = 52) weed in temperate and tropical Asian paddy fields (Holm, Plucknett, Pancho, & Herberger, 1977). It carries five *ALS* loci, with all genes transcribed at the seedling stage (Iwakami et al., 2020) when plants are exposed to herbicides in agricultural fields. Among the limited number of populations tested to date, mutations known to confer ALS herbicide resistance have been reported exclusively in *MvALS1* or *MvALS3* (e.g. Iwakami et al., 2020; Ohsako & Tominaga, 2007), despite the presence of multiple potential evolutionary hotspots for resistance. In the present study, we confirmed the occurrence of resistance-conferring mutations exclusively in *MvALS1* and *MvALS3* among 68 resistant populations across Japan. Enzyme kinetics and expression analysis of each *ALS* copy and its product unveiled the potential mechanisms that make these two loci the hotspots of resistance evolution.

MATERIALS AND METHODS

M. vaginalis

M. vaginalis was collected from paddy fields across Japan (Figure 1a-b; Table S1). Most of the paddy fields were small, measuring 0.1 to 0.3 ha an average. Plants from a single paddy field are hereafter referred to as a population. The plants were either directly used for resistance diagnosis using the rooting assay or cultivated for the collection of selfed seeds for the following pot assay (Figure 1c).

Resistance diagnosis in *M. vaginalis*

Resistance to SU herbicides was diagnosed using the rooting assay with 45 $\mu g \cdot L^{-1}$ of bensulfuron-methyl, as described by Hamamura et al. (2003). In some populations, the selfed seeds were used for resistance diagnosis using a recommended dose of imazosulfuron [90 g active ingredient (a.i.)$\cdot ha^{-1}$], as described previously (Iwakami et al., 2020). Briefly, germinated seeds were transplanted into autoclaved soil under flooded conditions. When plants reached the three-leaf stage, a commercial formulation of imazosulfuron (75% a.i.) was applied to the surface of standing water. The survival rate of plants was evaluated at 3 weeks post application.

Imazosulfuron dose–response analysis in *M. vaginalis*

KST1, KOM1, SMM23, AKT3, NRS2, and SMM13 populations were used in the assay. The dose–response analysis was performed as described by Iwakami et al. (2020), with some modifications. Briefly, five seedlings were transplanted into autoclaved soil in 100 cm^2 plastic pots. The plants were cultivated under flooded conditions. At the two-to-three–leaf stage, the commercial formulation of imazosulfuron was applied to the surface of standing water in individual pots at a rate calculated for the area of each pot (0, 0.09, 0.9, 9, 90, 900, 9,000, and 90,000 g a.i.$\cdot ha^{-1}$). At 3 weeks post application, the shoots were harvested and dry weight was measured.

Sequencing of *ALS* genes in *M. vaginalis*

141 DNA was extracted from the leaves of plants subjected to the herbicide sensitivity assay using
142 the cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). For
143 some old populations, DNA was extracted from dead seeds using the Plant Genomic DNA Kit
144 (Tiangen, Beijing, China). The entire coding sequence of *MvALS1* to *MvALS5* was
145 PCR-amplified as described by Iwakami et al. (2020). The entire coding region of the
146 amplicons was directly sequenced.

147 The nucleotide diversity of each *ALS* gene was calculated using the DnaSP 6 (Rozas
148 et al., 2017). Previously reported full-length coding sequences of three populations (SMM13,
149 SMM23, and TOT1) were also used in the analysis (Iwakami et al., 2020).

150

151 **Expression and *in vitro* assay of ALS**

152 The pGEX6P-1 vector (Cytiva, Tokyo, Japan) was digested with SalI-HF (New England
153 Biolabs, Tokyo, Japan). Alleles of *MvALS1/2/3/5* from the sensitive population (SMM13)
154 were individually ligated at the SalI site of pGEX-6 via SLiCE reaction following the
155 protocol described by Motohashi (2015). Each *ALS* gene was expressed without the estimated
156 chloroplast transit peptide (CTP) region corresponding to 1-86 residues of *A. thaliana* ALS
157 (Chang & Duggleby, 1997; Singh, Schmitt, Lillis, Hand, & Misra, 1991). The primers used
158 for the SLiCE reactions are listed in Table S2. The resultant expression vector was
159 transformed into *Escherichia coli* BL21 (DE3) cells.

160 The transformed cells were inoculated in 200 mL of LB medium containing 100
161 $\mu g \cdot ml^{-1}$ of ampicillin and cultured at 37°C. When OD_{600} reached 0.4-0.7, isopropyl
162 β-D-thiogalactopyranoside was added to the medium at a final concentration of 1 mM, and

the culture was continued at 16°C for 24 h. The cells were harvested by centrifugation at 14,000 ×*g* for 10 min. The cells were resuspended in phosphate-buffered saline with 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 0.5 mM $MgCl_2$ and sonicated for 5 min on ice. GST-fused ALS was batch-purified with glutathione Sepharose 4B (Cytiva) according to the manufacturer' s protocol. ALS was eluted in 400 μL of 50 mM Tris–HCl buffer (pH 8.0) containing 10 mM glutathione, followed by the addition of glycerol at a final concentration of 10% (v/v).

ALS activity was evaluated as described by Chang and Duggleby (1997), with some modifications. The reaction mixture (300 μL) containing 20 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 50 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM thiamine diphosphate, 20 μM FAD, 0.5 ng enzyme, and varying concentrations of sodium pyruvate (0, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 mM) was incubated at 30°C for 60 min, and the reaction was terminated by adding 30 μL of 6N H_2SO_4. The resultant solution was incubated at 60°C for 15 min, followed by the addition of 300 μL creatine (0.5%, w/v) and α-naphthol (0.5%, w/v, in 2.5N NaOH). Absorbance was measured at 525 nm. The data were plotted and curve-fitted to the Michaelis–Menten equation using the drc package (ver. 3.0.1) (Ritz, Baty, Streibig, & Gerhard, 2015).The Michaelis–Menten constant (K_m) and turnover number (k_{cat}) was determined for each ALS. The experiments were conducted in triplicate and repeated twice.

Protein concentration was determined using Bio-Rad Protein Assay Dey Reagent Concentrate (Bio-Rad, CA) with bovine serum albumin as the standard, following manufacturer's instructions.

185 **Targeted mutagenesis of *ALS* genes**

186 Alleles of *MvALS1*/2/3/5 from the SMM13 population and a deleted allele of *MvALS5* from
187 the SMM3 population were cloned into the pCRblunt vector (Thermo Fisher Scientific, Tokyo,
188 Japan). The codons for Pro197 of the *ALS* genes were mutated to carry a resistance-conferring
189 Ser codon. Targeted mutagenesis was performed according to the protocol in QuikChange
190 Site-Directed Mutagenesis System (Stratagene, CA, USA), with some modifications. Briefly,
191 the clones were used as a template for PCR using PrimeSTAR MAX (TaKaRa, Kusatsu,
192 Japan); the primers are listed in Table S2. The PCR products were treated with DpnI-HF
193 (New England Biolabs). The resultant solution was used for *E. coli* DH5α transformation. The
194 success of targeted mutagenesis was confirmed by sequencing the extracted plasmids.
195
196 **Transformation and phenotyping of transgenic *A. thaliana***
197 The Pro197Ser-mutated *ALS* genes were inserted into the pCAMBIA1390 vector under the
198 cauliflower mosaic virus 35S promoter using the In-Fusion DH Cloning Kit (TaKaRa), as
199 described previously (Iwakami et al., 2019). The primers used in this reaction are listed in
200 Table S2. *A. thaliana* transformation was performed using the floral dip method, and the
201 transformants were selected as described previously (Guo et al., 2019).
202 To quantify transgene expression, real-time RT-PCR was performed. Briefly, RNA
203 from 10-day-old T3 homozygous lines was extracted using the Plant Total RNA Mini Kit
204 (Favorgen, Ping-Tung, Taiwan). Contaminated DNA in the extracted RNA was removed using
205 the Turbo DNA-*free* kit (Thermo Fisher Scientific). The resultant RNA was reverse
206 transcribed according to the method described by Iwakami et al. (2019). PCR was run using

207 THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) on QuantStudi 12K Flex
208 Real-Time PCR System (Thermo Fisher Scientific). The primers for transgene expression
209 were designed using the terminator sequence of pCAMBIA1390. *GAPDH* was used as the
210 internal control; primers developed by Czechowski, Stitt, Altmann, Udvardi, and Scheible
211 (2005) were used. Data were analysed using the ΔΔCT method (Schmittgen & Livak, 2008).
212 Imazosulfuron sensitivity of the transformants was evaluated as described
213 previously (Dimaano, Yamaguchi, Fukunishi, Tominaga, & Iwakami, 2020) with some
214 modifications. Seeds were sown on Murashige and Skoog medium (Murashige & Skoog,
215 1962) containing various concentrations of imazosulfuron. Plants were grown for 10 days at
216 22°C. Sensitivity was evaluated based on the emergence of true leaves. The dose that
217 inhibited the growth to 50% (GR_{50}) and 90% (GR_{90}) compared with the control was
218 calculated by fitting the log-logistic curve (two-parameter model) using the drc package (ver.
219 3.0.1) (Ritz et al., 2015).
220
221 **Gene expression analysis in *M. vaginalis***
222 SMM13 and SMM23 were used as representatives of the sensitive and resistant populations,
223 respectively. Germinated seeds were transplanted into autoclaved soils. Six plants from each
224 population were grown in water until the three-leaf stage. Half of the plants were treated with
225 imazosulfuron at a concentration of 90 g a.i.·ha^{-1}. At 24 h post application, the shoots of all
226 plants were snap frozen in liquid nitrogen and stored at -80°C until further use. RNA was
227 extracted using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands), followed by DNase
228 treatment using the Turbo DNA-*free* kit (Thermo Fisher Scientific).

229 To estimate approximate transcript abundance, PCR products generated by the
230 primers designed based on the completely conserved regions among the five copies of *ALS*
231 genes (Table S2) were cloned into the pCRblunt vector (Thermo Fisher Scientific). Over 50
232 clones were sequenced. cDNA was prepared from the RNA of an imazosulfuron-treated
233 sensitive plant. The experiment was performed twice.

234 For RNA-Seq analysis, 12 RNA samples were shipped to GENEWIZ (Kawaguchi,
235 Japan) for stranded RNA-Seq using Illumina HiSeq 4000 (150-bp paired-end reads).
236 Contaminated adaptor sequences were trimmed using 'cutadapt' (ver. 2.5) (Martin, 2011).
237 Low-quality bases were removed using Trimmomatic (ver. 0.38) (Bolger, Lohse, & Usadel,
238 2014) with the following options: 'LEADING:25 TRAILING:25 SLIDINGWINDOW:4:15
239 MINLEN:100'. Reads of a non-treated sensitive plant were used for contig assembly. The
240 assembly was performed using Trinity (ver. 2.4.0) (Grabherr et al., 2011) with a k-mer size of
241 32. The biological function of the contigs was inferred using BLASTX (Blast+ ver. 2.9.0)
242 (Camacho et al., 2009) against rice protein sequences (*Oryza sativa* MSU release 7). Only one
243 contig under the identical gene identifier annotated by Trinity was used, as described by Ono
244 et al. (2015). Briefly, from contigs with hits in the BLAST analysis, those with the highest bit
245 score were selected, and from contigs without hits, the longest ones were selected. Contigs
246 that were annotated as *ALS* genes were replaced with full-length *ALS* genes of *M. vaginalis*
247 (DDBJ accession numbers: LC488950 to LC488953 and LC488956). The reads were mapped
248 using Bowtie2 (ver. 2.3.4.1) (Langmead & Salzberg, 2012) with the following options: '-X
249 900 --very-sensitive --no-discordant --no-mixed --dpad 0 --gbar 99999999'. The concordantly
250 mapped reads were counted using RSEM (ver. 1.3.2) (Li & Dewey, 2011). Multiple

comparisons were performed using the GLM approach with the quasi-likelihood F-test using the edgeR package (ver. 3.28.1.) (Robinson, McCarthy, & Smyth, 2010). Contigs with fold-changes of more or less than 2 and *q*-values less than 0.05 were evaluated as differentially expressed genes (DEGs).

RESULTS

Herbicide resistance of *M. vaginalis* collected across Japan

In this study, we sampled 113 suspected resistant populations across Japan and tested for ALS herbicide sensitivity using the rooting or pot assays (Figure 1). Sixty-six populations (58.4%) were confirmed to be resistant to ALS herbicides. All the resistant populations carried one or two homozygous mutations in the codons of *ALS* genes that are known to confer resistant to the herbicides, namely Pro197, A205, and D376, implying that *M. vaginalis* tend to evolve resistance to ALS inhibitors via target-site resistance (TSR) mechanisms as reported in many non-Poaceae weeds (Yu & Powles, 2014).

Analysis of the codons involved in ALS resistance in the 66 populations tested in this study and 2 previously reported resistant populations (Iwakami et al., 2020) revealed 9 types of resistance-conferring AASs. Mutations at Pro197 dominated the others, as reported in other species under SU selection (Yu & Powles, 2014) (Figure 2a-b). There was no clear association between mutation type (i.e. AASs and genes) and geographical region (Figure 1b; Table S1). Given the predominant self-pollinating nature of *M. vaginalis* and the small population size due to small paddy fields in Japan, most resistant populations have likely evolve resistance independently, as often observed in other self-pollinating species (Kreiner,

Stinchcombe, & Wright, 2018). As relatively rare cases, populations with two independent mutations were also found. AIZ2 and KR1 carried two resistance-conferring mutations, namely Pro197Ser and Asp376Glu, in a single gene *(MvALS1*), while YNZ1 carried Asp376Glu in *MvALS1* and Pro197Ser in *MvALS3*. In addition, mutations that have not been reported in natural weed populations, including Asp376Asn in NDA1 and A205Thr in ANP1, were also detected. Both mutations were confirmed to confer reduced sensitivity to SU in yeast (Bedbrook et al., 1995; Duggleby, Pang, Yu, & Guddat, 2003). Thus, AASs may be the causal mutations for resistance in these weed populations.

Transition was the dominant type of resistance-conferring mutation (Figure 2c), consistent with the results of a previous genome-wide mutation analysis of *A. thaliana* (Weng et al., 2019). The highest frequency of the Pro197Ser transition mutation (CCT→TCT) may be partly attributed to the nature of mutation patterns, although other factors such as high level resistance caused by this mutation (Sada, Ikeda, & Kizawa, 2013) may play a more important role in the observed bias.

Interestingly, the identified resistance-conferring mutations were exclusively found in *MvALS1* or *MvALS3*. To gain insights into this exclusivity, we further analysed the coding sequence of each *ALS* gene in 66 resistant and 30 sensitive populations, along with the previously reported sequences of 2 resistant and 1 sensitive populations (Iwakami et al., 2020) (Table S1). Coding sequences of all *ALS* genes in *M. vaginalis* were homozygous, contrary to the observation in obligate outcrossing weeds such as *Lolium rigidum* and *Papaver rhoeas* (Délye, Boucansaud, Pernin, & Le Corre, 2009; Délye, Pernin, & Scarabel, 2011). This result highlights the predominant self-pollinating nature of *M. vaginalis*. Consistent with previous

295 reports (Iwakami et al., 2020; Ohsako & Tominaga, 2007), a T insertion causing a frameshift
296 was detected in all *MvALS4* alleles investigated in this study (Figure 2a-d). The products
297 encoded by these mutant alleles lacked domains with pivotal roles in ALS activity, indicating
298 that this gene does not encode the functional form of ALS. Thus, the prevalence of
299 frame-shifted alleles in *MvALS4* among *M. vaginalis* populations in Japan explains the
300 absence of resistance-conferring mutations in this gene. While all alleles were pseudogenised
301 in *MvALS4*, variations were observed in *MvALS1* and *MvALS5*; as such a 13 bp deletion was
302 detected in *MvALS1* in 1 population and a 40 bp deletion in *Mv*ALS5 in 12 populations
303 (Figure 2a, TableS1). The reading frame of these alleles also shifted, resulting in premature
304 stop codons (Figure 2d). The putative loss-of-function alleles of *MvALS5* were found in
305 populations sampled from various areas without any geographical association. Contrary to
306 that in *MvALS4*, majority of the alleles were not pseudogenised in *MvALS1* and *MvALS5*.
307 Therefore, allele pseudogenisation cannot solely explain the absence of resistance-conferring
308 mutations in *MvALS1* and *MvALS5*.

309 The analysis of coding sequences revealed that genes with resistance-conferring
310 alleles (*MvALS1* and *MvALS3*) showed a higher nucleotide diversity and greater variation in
311 haplotype number (Figure 2e). This trend held true even when naturally selected
312 resistance-conferring alleles were excluded from the analysis: a greater nucleotide diversity
313 (per site) was observed at three and four positions, irrespective of the resistance selection, in
314 *MvALS1* and *MvALS3*, respectively. Thus, *MvALS1* and *MvALS3* might be more prone to
315 spontaneous mutations, some of which may occur by chance at the resistance-conferring
316 codons. However, the difference in the rate of spontaneous mutations among genes can hardly

317 explain the all-or-nothing resistance-conferring alleles of the *ALS* gene family in *M. vaginalis*.
318 Other more pronounced factors may have played key roles in shaping these biased patterns.
319
320 **Functional characterisation of each ALS**
321 To elucidate the mechanism underlying the biased dependence of *ALS* genes, we next
322 compared the enzymatic functions of the protein encoded by the canonical alleles of each *ALS*
323 gene. ALS activity of the purified GST-tagged protein was assessed (Figure S1). The activity
324 of the recombinant proteins was evaluated based on the conversion of the produced
325 acetolactate to a red insoluble complex. The reaction solutions of the recombinant proteins of
326 MvALS1/2/3/5 developed a red colour, indicating that all isozymes produced acetolactate
327 (Figure 3a). The enzymatic properties of the respective isozymes were similar, although the
328 affinity to the substrate pyruvate (*kcat/Km*) was relatively high in MvALS1.
329 The ALS activity of each isozyme was also compared by ectopic expression in *A.*
330 *thaliana*. We transformed *A. thaliana* with an *ALS* gene carrying the Pro197Ser mutation,
331 which endows extreme resistance to some ALS herbicides such as imazosulfuron (Sada et al.,
332 2013). When overexpressed, all four genes driven by the cauliflower mosaic virus 35S
333 promoter conferred resistance to imazosulfuron (Figure 3b-c). Resistance indices of all
334 transgenic lines were correlated with the transcript level of transgenes (*ALS* genes of *M.*
335 *vaginalis*), and a similar resistance level was observed when the transcripts of the respective
336 *ALS* genes were accumulated at a similar level. Notably, even a small difference in transcript
337 level drastically affected herbicide sensitivity when the transcript levels were within a specific
338 range. For instance, although the transcript levels in three *MvALS1* transgenic lines (lines

339 indicated in pink area in Figure 3b) differed by only 5.1-fold at the maximum, their resistance
340 levels differed by as much as 2,500-fold. Meanwhile, very low or high transcript levels did
341 not affect sensitivity.

342 We also tested the putative loss-of-function allele of *MvALS5*, as it only partially
343 lacks the γ domain—one of the three ALS domains (Figure 2d). All T2 generations in which
344 the allele of *MvALS5* carrying the Pro197Ser mutation was deleted were sensitive to 10 nM
345 imazosulfuron, while at least seven lines carrying the canonical allele of *MvALS5* showed
346 decreased imazosulfuron sensitivity (Figure S2). This result implies that the deleted form of
347 *MvALS5* does not encode functional ALS.

348 Collectively, the results of recombinant protein assay and ectopic expression in *A.*
349 *thaliana* suggest that the activities of the four isozymes were near identical, although a
350 slightly higher substrate specificity was observed in MvALS1 in the recombinant assay.
351 Notably, the recombinant ALS assay is affected by multiple factors, such as the prediction of
352 the CTP cleavage site and presence of a GST tag, which may have influenced the results for
353 MvALS1. Our data suggest that *MvALS2* and *MvALS5* could potentially be involved in the
354 evolution of SU resistance in *M. vaginalis* contrary to the expectation from the nation-wide
355 survey (Figure 2).

356

357 **Transcript levels of *MvALS* genes**

358 Since no significant differences were observed in recombinant protein assays and ectopic
359 expression in *A. thaliana*, we compared the transcript levels of *ALS* genes in *M. vaginalis*
360 seedlings in the presence or absence of imazosulfuron using mRNA-Seq. *De novo* assembly

followed by the curations of *ALS* contigs, we obtained 84,649 contigs with average length of 802.5 bp.

The transcriptome of herbicide-treated sensitive plants formed a distinct cluster (Figure 4a), indicating that herbicide treatment triggered massive reprogramming of gene expression within 24 h in these plants. Statistical analysis revealed 12,845 differentially expressed contigs between the treated and untreated sensitive populations (Figure S3). In contrast, gene expression in the resistant population remained almost unchanged following herbicide treatment, which was expected based on a previous report that herbicide treatment only slightly suppressed growth in this resistant population (Iwakami et al., 2020). Moreover, the difference between the untreated sensitive and resistant populations was minor, with only 528 differentially expressed contigs (Figure S3).

The transcription of *ALS* genes was almost stable among all populations (Figure 4b). The only exception was *MvALS2*, which was slightly downregulated (2.36-fold, $q<0.01$) in herbicide-treated sensitive plants. As the differences among the populations were small, we averaged the transcripts per kilobase million (TPM) data of 12 libraries and examined the transcript level of each *ALS* gene. The TPM value of *MvALS1* and *MvALS3* ranked 2,487 and 3,062, respectively, and these values were much higher than the TPM values of *MvALS2*, *MvALS5*, and *MvALS4* (Figure 4c-d). Moreover, respectively 45.3% and 36.9% of the reads mapped to the *ALS* genes were from *MvALS1* and *MvALS3*, followed by 9.6% from *MvALS2*. These findings were consistent with the results of gene cloning (Figure S4). Therefore, the two genes carrying resistance-conferring mutations (*MvALS1* and *MvALS3*) in natural *M. vaginalis* populations were among the most highly expressed *ALS* genes.

383

384 **Roles of *MvALS1* and *MvALS3* in resistance evolution**

385 We compared the roles of *MvALS1* and *MvALS3* in the resistant phenotype of *M. vaginalis*.
386 Imazosulfuron sensitivity of populations carrying the same mutation in *MvALS1* or *MvALS3*
387 was compared. Three populations with the Pro197Ser mutation showed similar GR_{50} and
388 GR_{90} values, irrespective of the mutated genes (Figure 5). Moreover, similar GR_{50} and GR_{90}
389 values were observed in another set of populations carrying the Asp376Glu mutation.
390 Therefore, the mutated alleles of *MvALS1* or *MvALS3* equally contribute to resistance in *M.*
391 *vaginalis*.

392

393 **DISCUSSION**

394 Here, we studied the molecular mechanisms underlying the evolution of herbicide resistance
395 in 68 resistant populations of *M. vaginalis* in Japan. Resistance-conferring mutant alleles were
396 exclusively identified in two of the five *ALS* genes, namely *MvALS1* and *MvALS3*.
397 Sequencing of the coding regions of all *ALS* loci revealed the prevalence of loss-of-function
398 alleles in *MvALS4*, implying that *MvALS4* does not meet the criteria of a resistance gene.
399 Although the enzymatic properties of the isozymes MvALS1/2/3/5 were similar, the transcript
400 levels of genes encoding these isozymes were markedly different. The transcript levels of two
401 genes (*MvALS1* and *MvALS3*) carrying the resistance-conferring mutations were higher in
402 resistant *M. vaginalis* seedlings. Taken together, these findings suggests that the variable
403 transcript levels of the five *ALS* genes shape the biased pattern of resistance-conferring alleles
404 in resistant populations of *M. vaginalis* in Japan.

Functional characterisation of *MvALS1/2/3/5* revealed no marked differences in their functions, indicating that *MvALS2* and *MvALS5*, which did not carry resistance-conferring mutations, are the 'potential' hotspots for resistance evolution, similar to *MvALS1* and *MvALS3*. However, resistance-conferring mutations in *ALS* genes that exhibit low expression would not confer herbicide resistance in *M. vaginalis* in agricultural fields, as evidenced in transformed *A. thaliana* lines with low *MvALS* expression (Figure 3b). Assuming that the transcript abundance of *ALS* genes is proportional to the amount of ALS protein, MvALS2 and MvALS5 accounted for <10% of the ALS pool, whereas MvALS1 and MvALS3 accounted for nearly 40%. This result indicates that over 90% ALS would be inhibited by herbicides if a resistance-conferring mutation is present in *MvALS2* or *MvALS5*; this proportion would further decrease in the first generation of plants carrying a spontaneous mutation due to the heterozygous status, and such plants would likely not survive herbicide stress in agricultural fields. In addition to relative abundance, absolute transcript abundance may also play important roles. We observed that *MvALS1* and *MvALS3* were close to the first inflexion point on the sigmoid curve of the transcription ranking plot, while the other three genes were in the middle or lower parts of the curve (Figure 4c). Therefore, *MvALS1* and *MvALS3* may be among the transcriptionally active genes in *M. vaginalis*. Interestingly, three *ALS* genes in hexaploid wheat, each of which confers resistance when Pro197 is substituted (see below), were also placed close to the first inflexion point (Figure S5a). These observations, together with the mRNA–GR_{50} relationships in transformed *A. thaliana* (Figure 3b), suggest that a certain degree of transcript abundance may be required for protein expression. Further comparative studies in other species with multiple copies of *ALS* genes

might provide some insights into the transcript levels of *ALS* genes required for resistance expression.

Targeted mutagenesis can be employed to validate whether transcript levels shape the pattern of resistance-conferring mutations in the *ALS* gene family. Recent advances in genome editing tools have enabled the induction of particular mutations in the genes of interest. Zhang et al. (2019) successfully induced mutations at the same codon in each of the three *ALS* genes in hexaploid wheat and observed similar decreases in the SU sensitivity of mutant plants. According to the database (Ramírez-González et al., 2018), the transcript levels of the three *ALS* genes in hexaploid wheat did not differ significantly (Figure S5). Establishment of a transformation system for *M. vaginalis* can enable the above manipulations, which will further our understanding of resistance evolution in this weed.

Meanwhile, we cannot rule out the role of other factors in the biased representation of mutated genes inducing resistance. Firstly, given the observed higher sequence variations in *MvALS1* and *MvALS3* (Figure 2e), the spontaneous mutation rate might be higher for these genes, leading to their overrepresentation. According to the classic evolutionary theory, the spontaneous mutation rate is random across a genome, and the apparent bias is the consequence of fitness costs. However, a recent sequencing study revealed that cytogenetic features influenced the mutation rate itself to a certain degree (Monroe et al., 2020). Thus, the spontaneous mutation rate may drive the biased representation of genes. Secondly, multi-layered DNA repair systems (Kimura & Sakaguchi, 2006) may also play a role in the bias. These repair mechanisms are affected by the flanking sequences, local DNA structure, and gene expression, among other factors, and do not act uniformly throughout the genome.

449 The local DNA environment of each *ALS* locus may have affected the manifestation of
450 spontaneous DNA mutations. Thirdly, our gene expression results must be interpreted with
451 caution, as we analysed only two populations. There may be natural variations in *ALS* gene
452 expression among *M. vaginalis* populations. However, similar herbicide responses observed
453 among populations carrying the same mutations in this study (Figure 5) may alternatively
454 indicate relatively conserved gene expression among *M. vaginalis* populations in Japan.
455 Large-scale analyses, such as population transcriptomics, combined with comprehensive
456 investigations of differential mutation rates in the *ALS* gene family would provide a more
457 holistic view of evolutionary patterns in *M. vaginalis*.

458 Notably, resistance-conferring mutations were more frequent in *MvALS1* than in
459 *MvALS3* (Figure 2a). This may be because a mutation in *MvALS1* may confer higher
460 resistance than that in *MvALS3*, which is beneficial to plants, particularly those carrying
461 mutations conferring low resistance, such as Pro197Leu (e.g. Sada et al., 2013), in the
462 heterozygous status. However, comparison of plants carrying the identical mutations in
463 different genes did not reveal significant differences in herbicide sensitivity (Figure 5), which
464 is in agreement with an independent study (K. Ohta, Y. Fujino & Y. Sada, unpublished).
465 Furthermore, in transformed *A. thaliana*, *MvALS1* and *MvALS3* conferred a similar level of
466 resistance when expressed equally (Figure 3b). Thus, although higher substrate affinity was
467 detected for MvALS1 in our recombinant enzyme assay (Figure 3a), other factors (e.g. the
468 bias of material sampling) were likely responsible for the bias between *MvALS1* and *MvALS3*.

469 The present country-scale study identified TSR mutations that have rarely been
470 reported in the natural weed population. Substitutions of Ala205 and Asp376 have frequently

been reported in diverse species, but Ala205Thr and Asp376Asn have not been reported (Tranel, Wright, & Heap, 2021). Moreover, double resistance-conferring mutations in a single gene or a single mutation each in two genes have rarely been reported. These mutations are particularly rare in predominantly self-pollinating species, probably because a single mutation often confers sufficient resistance to field-applied herbicides. Regarding the discovery of rare mutations, we speculate that the transition of herbicide use in Japan may have played an important role. In Japan, the introduction of SU herbicides, such as bensulfuron-methyl and imazosulfuron, in the late 1980s led to the explosion of resistance evolution from the mid-1990s to the early 2000s (Peterson, Collavo, Ovejero, Shivrain, & Walsh, 2018). Mutations at the Pro197 codon was inferred to be the major mechanism of evolved resistance in weeds during this SU selection period (Yu & Powles, 2014). Indeed, the Pro197 mutation was the most frequently reported in *Schoenoplectiella juncoides* and *Lindernia* spp. (Uchino et al., 2007; Uchino & Watanabe, 2002) as well as in *M. vaginalis* in this study (Figure 2b). For the management of these resistant weeds, ALS herbicides belonging to different chemical classes such as penoxsulam and pyrimisulfan or recently commercialised SU herbicides such as propyrisulfuron and metazosulfuron, all of which are effective against resistant plants with mutations at the Pro197 residue, have been used. Transition to these new ALS herbicides may have driven the evolution of the above rarely reported mutations, specifically double mutants carrying Asp376Glu, which confers resistance to ALS herbicides of all chemical classes (Tranel et al., 2021). The sensitivity of these new mutations to various ALS herbicides should be studied for predicting the dynamics of resistance mechanisms in *M. vaginalis*.

The presence or absence of variation in loss-of-function mutations within an *ALS*

493 gene have not been reported in natural weed populations. While the loss-of-function
494 mutations in *ALS* genes are considered lethal in species with a single copy of that *ALS* gene,
495 loss-of-function alleles of a single *ALS* gene could be induced by targeted mutagenesis in a
496 species with multiple copies of *ALS* genes (Butler, Atkins, Voytas, & Douches, 2015; Veillet
497 et al., 2019). High expression of either *MvALS1* or *MvALS3* would be sufficient to produce
498 the minimum amount of branched-chain amino acids required in *M. vaginalis*. Meanwhile, it
499 would be interesting to compare the fitness of individuals with or without loss-of-function
500 mutations, particularly in plants with loss-of-function mutations in *MvALS1*, which was
501 revealed to be highly expressed in the present study.

502 In conclusion, comprehensive analysis of *ALS* gene sequences in Japanese
503 populations of *M. vaginalis* revealed that variable transcript levels of these genes are
504 associated with the biased patterns of mutations for the evolution of herbicide resistance. This
505 study provides a novel insight that gene expression quantitatively provides evolutionary
506 competency to potential hotspots across genome. In other words, gene expression level can
507 act as a genomic constraint for the parallel evolution of herbicide resistance. Of note, the
508 Japanese populations of *M. vaginalis* may only reflect a particular genetic group of this
509 globally distributed weed (mainly in eastern and southern Asia), considering that Japan is a
510 small island country. Thus, further large-scale studies of the global populations would provide
511 a more comprehensive understanding of the evolutionary strategies of herbicide resistance in
512 *M. vaginalis*, which may not be limited to the two hotspots (*MvALS1* and *MvALS3*) detected
513 in this study. In the era of genomics, it is anticipated that numerous genomes with more
514 complex DNA structures, such as polyploid genomes, will be compiled, which will inevitably

515 impose more attention to the roles of redundant copies during the course of evolution. The
516 perspective and framework of this study are applicable to research on TSR evolution in
517 agricultural weeds as well as broader studies focusing on the process of bias for genetic loci in
518 parallel evolution.
519

520 **ACKNOWLEDGEMENTS**

521 We thank Nihon Nohyaku for providing seeds of *Monochoria vaginalis*, and members of the
522 weed science lab in Kyoto University for the cooperation to collect *M. vaginalis*, Yuri
523 Nakamura for technical assistance. Computations were partially performed on the NIG
524 supercomputer at ROIS National Institute of Genetics. This work was partly supported by
525 Japan Association for Advancement of Phyto-Regulators (JAPR) grant no. 2017-4, 2018-4 (to
526 A.U. and S.I.), 2019-2 (to S.O. and S.I.).
527

528 **AUTHOR CONTRIBUTIONS**

529 Project Conception, S.I.; Experimental Design, S.T., T.M., and S.I.; Performance of
530 Experiments, S.T., A.U., S.O., S.I.; Bioinformatics Analysis, S.T. and S.I.; Material Collection
531 and/or Maintenance, S.T., A.U., S.O., C.M., K.H., M.M., N.Y., N.U., Y.T., N.F., E.K., Y.S.,
532 T.T., and S.I.; Visualization, S.T. and S.I.; Writing, S.I.; Supervision, T.T., S.I.
533

534 **DATA ACCESSIBILITY:**

535 mRNA-Seq data have been deposited in the DDBJ Sequence Read Archive (DRA) database
536 with BioProject ID PRJDB11326 (BioSample: SSUB017551).
537

REFERENCES

Baucom, R. S. (2019). Evolutionary and ecological insights from herbicide-resistant weeds: what have we learned about plant adaptation, and what is left to uncover? *New Phytologist*, *223*, 68-82.

Bedbrook, J. R., Chaleff, R. S., Falco, S. C., Mazur, B. J., Somerville, C. R., & Yadav, N. S. (1995).

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*, 2114-2120.

Butler, N. M., Atkins, P. A., Voytas, D. F., & Douches, D. S. (2015). Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system. *PLoS One*, *10*, e0144591.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. *BMC Bioinformatics*, *10*, 421.

Chang, A. K., & Duggleby, R. G. (1997). Expression, purification and characterization of *Arabidopsis thaliana* acetohydroxyacid synthase. *Biochemical Journal*, *327*, 161-169.

Clark, J. W., & Donoghue, P. C. J. (2018). Whole-genome duplication and plant macroevolution. *Trends in Plant Science*, *23*, 933-945.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., & Scheible, W. R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant Physiology*, *139*, 5-17.

Délye, C., Boucansaud, K., Pernin, F., & Le Corre, V. (2009). Variation in the gene encoding acetolactate-synthase in *Lolium* species and proactive detection of mutant, herbicide-resistant alleles. *Weed Research, 49*, 326-336.

Delye, C., Causse, R., & Michel, S. (2016). Genetic basis, evolutionary origin and spread of resistance to herbicides inhibiting acetolactate synthase in common groundsel (*Senecio vulgaris*). *Pest Management Science, 72*, 89-102.

Délye, C., Pernin, F., & Scarabel, L. (2011). Evolution and diversity of the mechanisms endowing resistance to herbicides inhibiting acetolactate-synthase (ALS) in corn poppy (*Papaver rhoeas* L.). *Plant Science*, *180*, 333-342.

Deng, W., Yang, Q., Zhang, Y., Jiao, H., Mei, Y., Li, X., & Zheng, M. (2017). Cross-resistance patterns to acetolactate synthase (ALS)-inhibiting herbicides of flixweed (*Descurainia sophia* L.) conferred by different combinations of ALS isozymes with a Pro-197-Thr mutation or a novel Trp-574-Leu mutation. *Pesticide Biochemistry and Physiology,*

136, 41-45.

Dimaano, N. G., Yamaguchi, T., Fukunishi, K., Tominaga, T., & Iwakami, S. (2020). Functional characterization of cytochrome P450 CYP81A subfamily to disclose the pattern of cross-resistance in *Echinochloa phyllopogon*. *Plant Molecular Biology, 102*, 403-416.

Duggleby, R. G., Pang, S. S., Yu, H., & Guddat, L. W. (2003). Systematic characterization of mutations in yeast acetohydroxyacid synthase. Interpretation of herbicide-resistance data. *European Journal of Biochemistry, 270*, 2895-2904.

Fukano, Y., Guo, W., Uchida, K., Tachiki, Y., & Cornelissen, H. (2020). Contemporary adaptive divergence of plant competitive traits in urban and rural populations and its implication for weed management. *Journal of Ecology, 108*, 2521-2530.

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., . . . Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology, 29*, 644-652.

Guo, F., Iwakami, S., Yamaguchi, T., Uchino, A., Sunohara, Y., & Matsumoto, H. (2019). Role of CYP81A cytochrome P450s in clomazone metabolism in *Echinochloa phyllopogon*. *Plant Science, 283*, 321-328.

Hamamura, K. (2018). Development of herbicides for paddy rice in Japan. *Weed Biology and Management, 18*, 75-91.

Hamamura, K., Muraoka, T., Hashimoto, J., Tsuruya, A., Takahashi, H., Takeshita, T., & Noritake, K. (2003). Identification of sulfonylurea-resistant biotypes of paddy field weeds using a novel method based on their rooting responses. *Weed Biology and Management, 3*, 242-246.

Heap, I. (2021). The international survey of herbicide resistant weeds. Online. Internet. Thursday, February 11, 2021. Available www.weedscience.com.

Holm, L. G., Plucknett, D. L., Pancho, J. V., & Herberger, J. P. (1977). *The world's worst weeds. Distribution and biology*. Honolulu: University of Hawaii.

Iwakami, S., Kamidate, Y., Yamaguchi, T., Ishizaka, M., Endo, M., Suda, H., . . . Matsumoto, H. (2019). CYP81A P450s are involved in concomitant cross-resistance to acetolactate synthase and acetyl-CoA carboxylase herbicides in *Echinochloa phyllopogon*. *New Phytologist, 221*, 2112-2122.

Iwakami, S., Tanigaki, S., Uchino, A., Ozawa, Y., Tominaga, T., & Wang, G. X. (2020). Characterization of the acetolactate synthase gene family in sensitive and resistant

biotypes of two tetraploid *Monochoria* weeds, *M. vaginalis* and *M. korsakowii*. *Pesticide Biochemistry and Physiology, 165*, 104506.

Iwakami, S., Uchino, A., Watanabe, H., Yamasue, Y., & Inamura, T. (2012). Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase in *Echinochloa phyllopogon*, a polyploid weed species. *Pest Management Science, 68*, 1098-1106.

Kimura, S., & Sakaguchi, K. (2006). DNA Repair in Plants. *Chemical Reviews, 106*, 753-766.

Kreiner, J. M., Stinchcombe, J. R., & Wright, S. I. (2018). Population genomics of herbicide resistance: adaptation via evolutionary rescue. *Annual Review of Plant Biology, 69*, 611-635.

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods, 9*, 357-359.

Li, B., & Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics, 12*, 323.

Löbmann, A., Schulte, M., Runge, F., Christen, O., & Petersen, J. (2021). Occurrence, resistance factors and cross-resistance patterns to herbicides inhibiting acetolactate synthase (ALS) of *Echinochloa crus-galli* (L.) Pal. Beauv. in Central Europe. *Journal of Plant Diseases and Protection*. Retrieved from doi:10.1007/s41348-021-00434-1

Martin, A., & Orgogozo, V. (2013). The loci of repeated evolution: a catalog of genetic hotspots of phenotypic variation. *Evolution, 67*, 1235-1250.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal, 17*.

Monroe, J. G., Srikant, T., Carbonell-Bejerano, P., Exposito-Alonso, M., Weng, M.-L., Rutter, M. T., . . . Weigel, D. (2020). Mutation bias shapes gene evolution in *Arabidopsis thaliana*. *bioRxiv*. Retrieved from doi:https://doi.org/10.1101/2020.06.17.156752

Motohashi, K. (2015). A simple and efficient seamless DNA cloning method using SLiCE from *Escherichia coli* laboratory strains and its application to SLiP site-directed mutagenesis. *BMC Biotechnology, 15*, 47.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum, 15*, 473-497.

Murray, M., & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research, 8*, 4321-4326.

Ohsako, T., & Tominaga, T. (2007). Nucleotide substitutions in the acetolactate synthase

637 genes of sulfonylurea-resistant biotypes of *Monochoria vaginalis* (Pontederiaceae).
638 *Genes & Genetic Systems, 82*, 207-215.
639 Ono, H., Ishii, K., Kozaki, T., Ogiwara, I., Kanekatsu, M., & Yamada, T. (2015). Removal of
640 redundant contigs from *de novo* RNA-Seq assemblies via homology search improves
641 accurate detection of differentially expressed genes. *BMC Genomics, 16*, 1031.
642 Peters, B., & Strek, H. J. (2018). Herbicide discovery in light of rapidly spreading resistance
643 and ever-increasing regulatory hurdles. *Pest Management Science, 74*, 2211-2215.
644 Peterson, M. A., Collavo, A., Ovejero, R., Shivrain, V., & Walsh, M. J. (2018). The challenge
645 of herbicide resistance around the world: a current summary. *Pest Management*
646 *Science, 74*, 2246-2259.
647 Powles, S. B., & Yu, Q. (2010). Evolution in action: plants resistant to herbicides. *Annual*
648 *Review of Plant Biology, 61*, 317-347.
649 Ramírez-González, R., Borrill, P., Lang, D., Harrington, S., Brinton, J., Venturini, L., . . .
650 Pasha, A. (2018). The transcriptional landscape of polyploid wheat. *Science, 361*.
651 Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *PLoS*
652 *One, 10*, e0146021.
653 Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2010). edgeR: a Bioconductor package
654 for differential expression analysis of digital gene expression data. *Bioinformatics, 26*,
655 139-140.
656 Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P.,
657 Ramos-Onsins, S. E., & Sanchez-Gracia, A. (2017). DnaSP 6: DNA Sequence
658 Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution, 34*,
659 3299-3302.
660 Sada, Y., Ikeda, H., & Kizawa, S. (2013). Resistance levels of sulfonylurea‐resistant
661 *Schoenoplectus juncoides* (Roxb.) Palla with various Pro197 mutations in acetolactate
662 synthase to imazosulfuron, bensulfuron‐methyl, metsulfuron‐methyl and
663 imazaquin‐ammonium. *Weed Biology and Management, 13*, 53-61.
664 Sada, Y., & Uchino, A. (2017). Biology and mechanisms of sulfonylurea resistance in
665 *Schoenoplectiella juncoides*, a noxious sedge in the rice paddy fields of Japan. *Weed*
666 *Biology and Management, 17*, 125-135.
667 Scarabel, L., Locascio, A., Furini, A., Sattin, M., & Varotto, S. (2010). Characterisation of
668 ALS genes in the polyploid species *Schoenoplectus mucronatus* and implications for
669 resistance management. *Pest Management Science, 66*, 337-344.

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nature Protocols, 3*, 1101-1108.

Singh, B., Schmitt, G., Lillis, M., Hand, J. M., & Misra, R. (1991). Overexpression of acetohydroxyacid synthase from *Arabidopsis* as an inducible fusion protein in *Escherichia coli*: production of polyclonal antibodies, and immunological characterization of the enzyme. *Plant Physiology, 97*, 657-662.

Svitashev, S., Young, J. K., Schwartz, C., Gao, H., Falco, S. C., & Cigan, A. M. (2015). Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiology, 169*, 931-945.

Tranel, P. J., & Wright, T. R. (2002). Resistance of weeds to ALS-inhibiting herbicides: what have we learned? *Weed Science, 50*, 700-712.

Tranel, P. J., Wright, T. R., & Heap, I. M. (2021). Muations in herbicide-resistant weeds to ALS inhibitors. Retrieved accessed 11 May 2019 http://www.weedscience.com

Uchino, A., Ogata, S., Kohara, H., Yoshida, S., Yoshioka, T., & Watanabe, H. (2007). Molecular basis of diverse responses to acetolactate synthase-inhibiting herbicides in sulfonylurea-resistant biotypes of *Schoenoplectus juncoides*. *Weed Biology and Management, 7*, 89-96.

Uchino, A., & Watanabe, H. (2002). Mutations in the acetolactate synthase genes of sulfonylurea-resistant biotypes of *Lindernia* spp. *Weed Biology and Management, 2*, 104-109.

Veillet, F., Perrot, L., Chauvin, L., Kermarrec, M. P., Guyon-Debast, A., Chauvin, J. E., . . . Mazier, M. (2019). Transgene-free genome editing in tomato and potato plants using agrobacterium-mediated delivery of a CRISPR/Cas9 cytidine base editor. *International Journal of Molecular Sciences, 20*.

Waselkov, K. E., Regenold, N. D., Lum, R. C., & Olsen, K. M. (2020). Agricultural adaptation in the native North American weed waterhemp, *Amaranthus tuberculatus* (Amaranthaceae). *PLoS One, 15*, e0238861.

Wedger, M. J., & Olsen, K. M. (2018). Evolving insights on weedy rice. *Ecological Genetics and Genomics, 7-8*, 23-26.

Weng, M. L., Becker, C., Hildebrandt, J., Neumann, M., Rutter, M. T., Shaw, R. G., . . . Fenster, C. B. (2019). Fine-grained analysis of spontaneous mutation spectrum and frequency in *Arabidopsis thaliana*. *Genetics, 211*, 703-714.

Xu, Y., Xu, L., Li, X., & Zheng, M. (2020). Investigation of resistant level to

703 tribenuron-methyl, diversity and regional difference of the resistant mutations on
704 acetolactate synthase (ALS) isozymes in *Descurainia sophia* L. from China. *Pesticide*
705 *Biochemistry and Physiology*, 104653.
706 Yamato, S., Sada, Y., & Ikeda, H. (2013). Characterization of acetolactate synthase from
707 sulfonylurea herbicide-resistant *Schoenoplectus juncoides*. *Weed Biol Manag*, *13*,
708 104-113.
709 Ye, C. Y., Tang, W., Wu, D., Jia, L., Qiu, J., Chen, M., . . . Fan, L. (2019). Genomic evidence
710 of human selection on Vavilovian mimicry. *Nature Ecology & Evolution*, *3*,
711 1474-1482.
712 Yu, Q., & Powles, S. B. (2014). Resistance to AHAS inhibitor herbicides: current
713 understanding. *Pest Management Science*, *70*, 1340-1350.
714 Zhang, R., Liu, J., Chai, Z., Chen, S., Bai, Y., Zong, Y., . . . Gao, C. (2019). Generation of
715 herbicide tolerance traits and a new selectable marker in wheat using base editing.
716 *Nature Plants*, *5*, 480-485.

717

718

719 **Figure legends**

720 **Figure 1. *Monochoria vaginalis* studied in this work**

721 (a) Infestation of *M. vaginalis* in paddy fields in Japan. (b) Collection sites of *M. vaginalis*.
722 *MvALS1*, populations carrying resistance-conferring mutations in *MvALS1*; *MvALS3*,
723 populations carrying resistance-conferring mutations in *MvALS3*; *MvALS1&3*, populations
724 carrying resistance-conferring mutations in *MvALS1* and *MvALS3*; N.D., populations with no
725 resistance-conferring mutations in *ALS* genes. (c) Methods for resistance diagnosis. In rooting
726 assay, roots were cut at 1 cm from the base of shoots and placed in bensulfuron-methyl
727 solution for 7-10 days. Plants with emerging roots were diagnosed as resistant. In the pot
728 assay, seeds were germinated in a tube, followed by transplantation to pots filled with
729 autoclaved soil. The recommended dose of imazosulfuron (90 g a.i.·ha^{-1}) was applied at the
730 three-leaf stage. Plant survival was evaluated at 3 weeks post application.

731

732 **Figure 2. *ALS* genes of *Monochoria vaginalis***

733 (a) Status of *ALS* genes in 68 resistant populations analysed in this study. YNZ1 carries
734 mutations both in *MvALS1* and *MvALS3*. AIZ2 and KR1 carry two independent mutations in
735 *MvALS1*. F.S., frameshift mutation. (b) Patterns of resistance-conferring mutations. (c)
736 Patterns of DNA conversions of resistance-conferring mutations. (d) Schematic representation
737 of frameshifted alleles found in *MvALS1*, *MvASL4*, and *MvALS5*. *MvALS4* alleles encoding
738 the canonical form of ALS were not detected in the present study. Triangles represent a
739 premature stop codon. (e) Nucleotide variations in the five *ALS* genes. The coding sequences
740 of 68 resistant and 31 sensitive populations were analysed. The plot shows nucleotide

741 diversity calculated at each nucleotide position. Positions causing an amino acid replacement
742 that confers resistance are indicated in red. Nucleotide diversity of the entire genes (π) and
743 haplotype numbers were calculated.
744
745 **Figure 3. Enzymatic functions of ALS encoded by each gene of *Monochoria vaginalis***
746 (a) Enzymatic properties of recombinant ALS. (b) Relationship of transcript level and
747 imazosulfuron sensitivity in transgenic *Arabidopsis thaliana* expressing each *ALS* gene. The
748 site-directly mutated allele of each gene (Pro197Ser) was transformed into *A. thaliana*. (c)
749 Response of transgenic *A. thaliana* to 10 nM imazosulfuron. The plants correspond to the
750 lines indicated by circles in Figure 3b.
751
752 **Figure 4. Expression of *ALS* genes in *Monochoria vaginalis***
753 (a) Transcriptome of sensitive (S) and resistant (R) *M. vaginalis* with or without herbicide
754 imazosulfuruon (H) application. Transcriptome of the R plants was almost unchanged, while
755 that of the S plants was drastically affected. (b) Transcripts per kilobase million (TPM) of
756 each *ALS* gene. *q<0.05. (c) Expression rank in *M. vaginalis* transcriptome. Data represent
757 the average of 12 mRNA-Seq samples. (d) Relative transcript abundance of *ALS* genes in *M.*
758 *vaginalis*.
759
760 **Figure 5. Herbicide sensitivity of *Monochoria vaginalis* carrying a mutation in *MvALS1***
761 **and *MvALS3***
762 (a) *M. vaginalis* 3 weeks post imazosulfuron application. (b) Dose response to imazosulfuron.

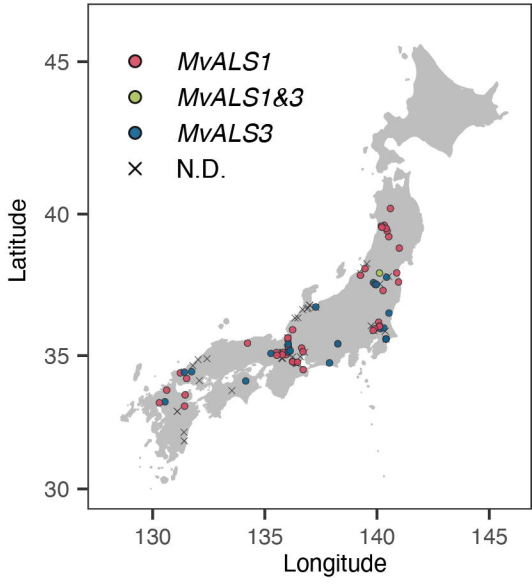
763 Bars represent standard error (n=3). (c) Doses that inhibited the growth to 50% (GR_{50}) and

764 90% (GR_{90}).

765

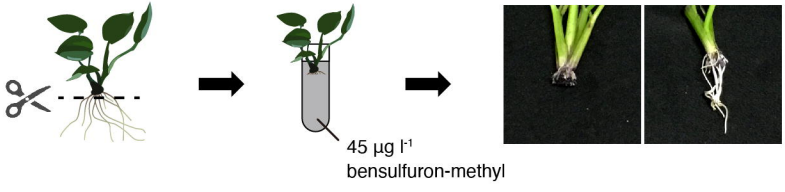
(a)

(b)



(c)

1. Rooting assay

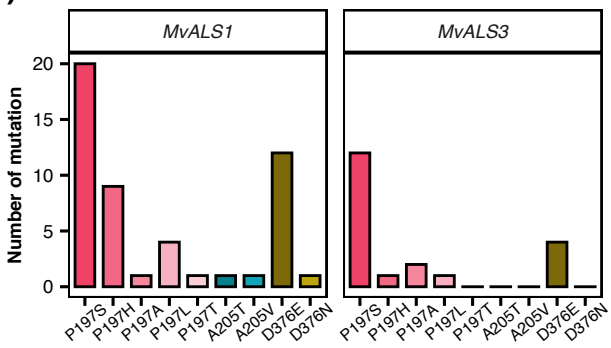


2. Pot assay

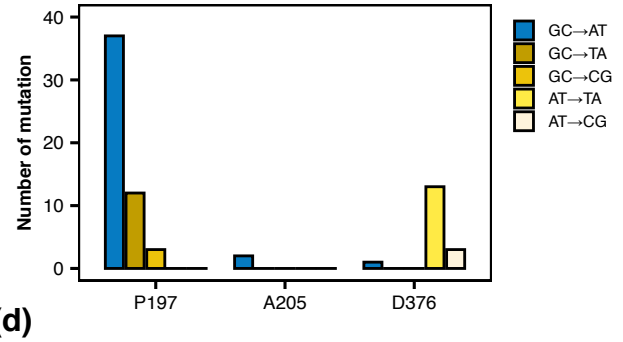
(a)

Population	*MvALS1*	*MvALS2*	*MvALS3*	*MvALS4*	*MvALS5*
YOK1	D376E	–	–	F.S.	–
DSN1	P197H	–	–	F.S.	–
DSN2	P197S	–	–	F.S.	–
DSN3	D376E	–	–	F.S.	–
ODT1	P197S	–	–	F.S.	–
AKT1	P197S	–	–	F.S.	–
AKT2	P197S	–	–	F.S.	–
AKT3	D376E	–	–	F.S.	–
KUR1	P197H	–	–	F.S.	–
KUR2	P197A	–	–	F.S.	–
MSM1	P197S	–	–	F.S.	–
TKU1	P197L	–	–	F.S.	F.S.
TKU2	D376E	–	–	F.S.	–
TKU3	P197S	–	–	F.S.	–
TKU4	P197S	–	–	F.S.	–
TKU5	P197H	–	–	F.S.	–
NDA1	D376N	–	–	F.S.	F.S.
ETZ1	P197H	–	–	F.S.	–
KTK1	P197T	–	–	F.S.	–
TRG1	D376E	–	–	F.S.	F.S.
TRG3	D376E	–	–	F.S.	F.S.
NRS1	P197S	–	–	F.S.	–
ANP1	A205T	–	–	F.S.	–
AIS1	P197H	–	–	F.S.	F.S.
KST1	P197S	–	–	F.S.	–
TKS1	P197H	–	–	F.S.	–
TKS4	P197H	–	–	F.S.	–
TKS8	P197H	–	–	F.S.	–
TNB1	D376E	–	–	F.S.	–
KU1	D376E	–	–	F.S.	–
KMO1	P197S	–	–	F.S.	–
ISE1	P197S	–	–	F.S.	–
IGA1	A205V	–	–	F.S.	–
IGA2	P197L	–	–	F.S.	F.S.
IGA3	P197S	–	–	F.S.	–
TSU3	P197S	–	–	F.S.	–
TOT1	P197L	–	–	F.S.	–
YMG2	P197S	–	–	F.S.	–
FUK1	P197S	–	–	F.S.	–
TKE1	P197S	–	–	F.S.	–
TKD1	P197H	–	–	F.S.	–
SGA1	D376E	–	–	F.S.	–
AIZ2	P197S, D376E	–	–	F.S.	F.S.
KR1	P197S, D376E	–	–	F.S.	–
MRK2	P197L	–	–	F.S.	F.S.
AGN1	P197S	–	–	F.S.	–
NGT1	P197S	–	–	F.S.	–
YNZ1	D376E	–	P197S	F.S.	–
KTG1	–	–	P197S	F.S.	–
TYM2	–	–	P197A	F.S.	–
USK1	–	–	P197S	F.S.	–
HIT1	–	–	P197S	F.S.	–
MIU1	–	–	P197S	F.S.	–
SMM23	–	–	P197S	F.S.	–
SMM3	–	–	P197S	F.S.	F.S.
SMM4	–	–	P197S	F.S.	–
NRS2	–	–	D376E	F.S.	–
IWT1	–	–	D376E	F.S.	–
TKS6	–	–	P197H	F.S.	–
NTN1	–	–	P197L	F.S.	–
MIM1	–	–	P197S	F.S.	–
KRM1	–	–	P197S	F.S.	–
FK1	–	–	D376E	F.S.	–
AIZ1	–	–	P197T	F.S.	–
OMH1	–	–	P197S	F.S.	–
OMH2	–	–	P197S	F.S.	–
YMG1	–	–	D376E	F.S.	–
HG1	F.S.	–	P197A	F.S.	–

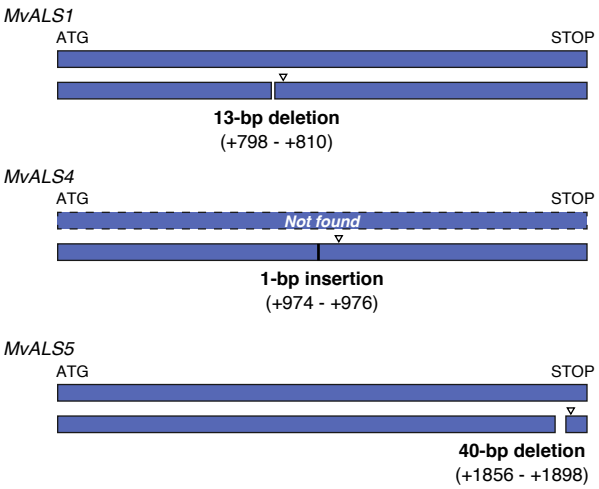
(b)



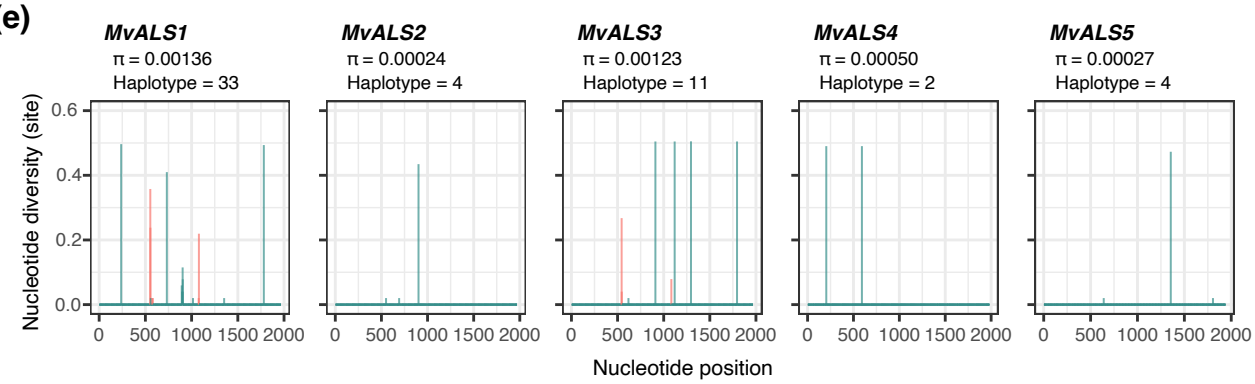
(c)



(d)



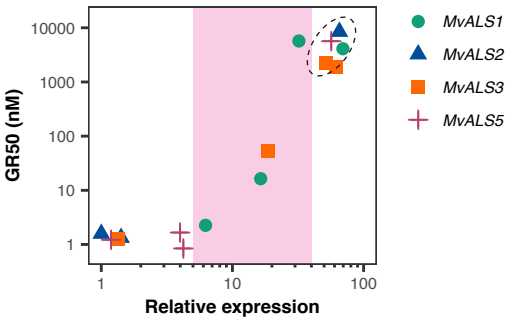
(e)



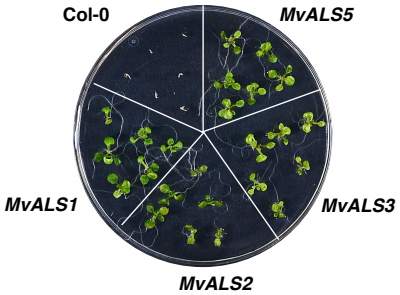
(a)

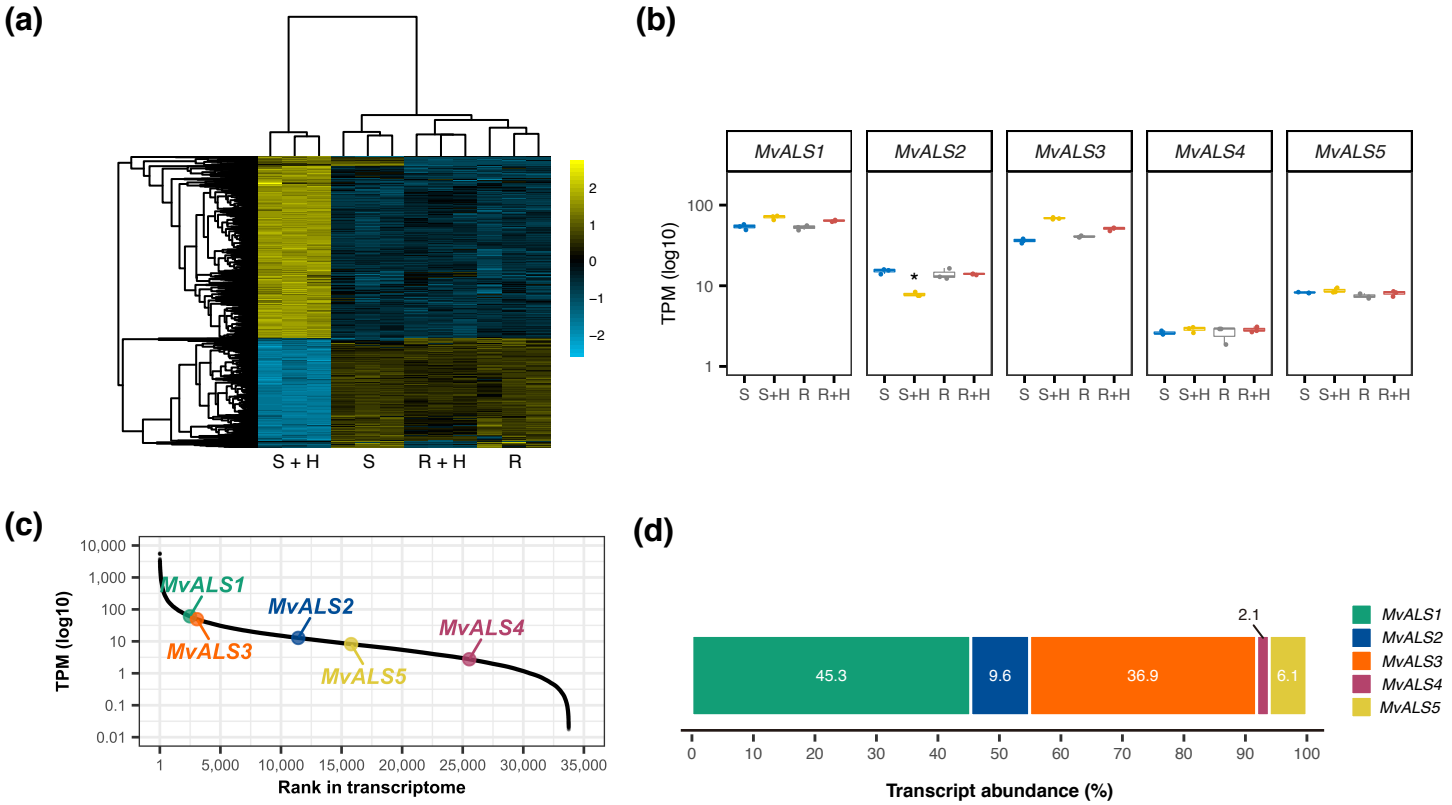
Gene	Reaction	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($min^{-1}mM^{-1}$)
(control)		–	–	–
MvALS1		0.95 ± 0.06	8.53 ± 1.51	0.111 ± 0.021
MvALS2		0.83 ± 0.07	13.13 ± 3.03	0.063 ± 0.016
MvALS3		0.78 ± 0.05	17.25 ± 2.42	0.045 ± 0.007
MvALS5		0.81 ± 0.04	14.51 ± 1.70	0.056 ± 0.007

(b)

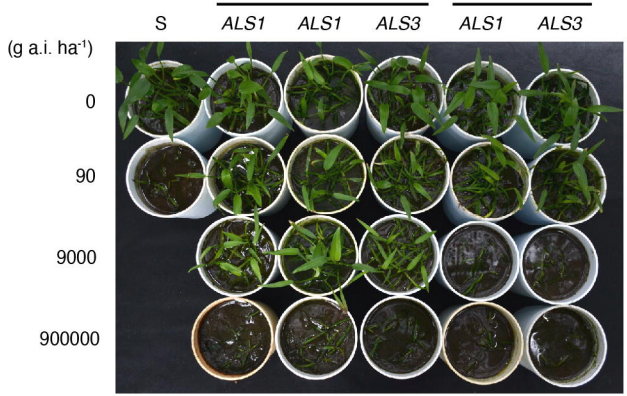


(c)

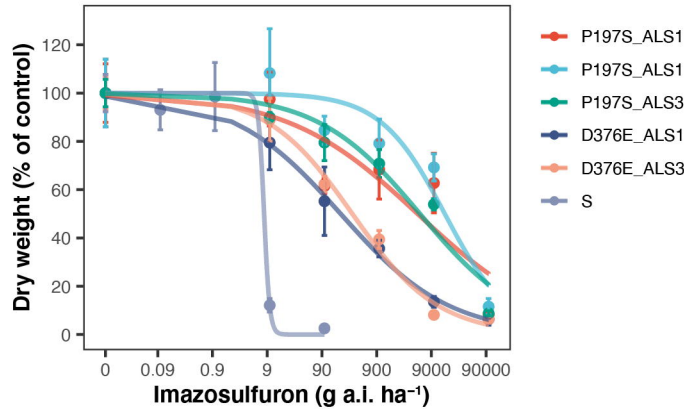




(a)



(b)



(c)

Population	Mutation	Gene	GR_{50}		GR_{90}	
			g a.i. ha^{-1}	R/S	g a.i. ha^{-1}	R/S
KST1	P197S	*MvALS1*	15799	2000	308232	30219
KOM1	P197S	*MvALS1*	5029.9	637	211830	20768
SMM23	P197S	*MvALS3*	5553.9	703	630419	61806
AKT3	D376E	*MvALS1*	197.5	25	29757	2917
NRS2	D376E	*MvALS3*	323	41	17118	1678
SMM13	-	-	7.9	1	10.2	1