

Virus-mediated transient expression techniques enable gene function studies in black-grass

1 Dear Editor,

2 Weeds are arguably among the most economically important groups of plant species. They have
3 major agronomic and environmental impacts and affect food security. For winter-cereal farmers in
4 Western Europe, black-grass (*Alopecurus myosuroides*) is the most problematic weed as it survives
5 chemical control methods (Hicks et al., 2018, Varah et al., 2020) and reproduction occurs within a
6 standard cropping cycle (Moss, 1983, Colbach et al., 2006). Importantly, black-grass also directly
7 reduces yields (Varah et al., 2020). Novel disruptive technologies mitigating herbicide-resistance
8 evolution and enabling better control are therefore urgently required. This could be achieved by
9 gaining a better understanding of black-grass biology and identifying genes underpinning its success
10 as an agricultural weed. However, functional studies have been impeded by the lack of tools for
11 genetic transformation and/or functional genomics in this species. Here we demonstrate the transient
12 expression tools Virus-induced gene silencing (VIGS) and Virus-mediated protein overexpression
13 (VOX) developed for crop monocots can be used in black-grass.

14 Transient expression techniques offer the means to quickly and specifically alter gene expression in a
15 low- to medium-throughput manner even in plant species that are difficult or not yet possible to
16 transform. Those mediated by plant virus-derived vectors, i.e. VIGS and VOX, allow systemic
17 silencing of target genes or protein overexpression throughout young or adult plant tissues. Different
18 viral vectors based on viruses with RNA or DNA genomes have been developed for gene-function
19 studies in monocots (reviewed in Lee et al., 2015, Kant and Dasgupta, 2019). *Barley stripe mosaic*
20 *virus* (BSMV) and *Foxtail mosaic virus* (FoMV) are the most commonly used in wheat and, with
21 variable success, other cereal crops and grass species (Lee et al., 2015, Liu et al., 2016, Bouton et
22 al., 2018, Mei et al., 2019). Therefore, we tested published vectors (Yuan et al., 2011, Lee et al.,
23 2012, Bouton et al., 2018) in black-grass.

24 We used two biotypes that differ in their sensitivity to herbicides (Supplemental Fig. S1): Peldon
25 (multiple herbicide resistant (MHR)) and Rothamsted (sensitive). Under laboratory conditions we
26 infected both biotypes asymptotically with BSMV and FoMV (Fig. 1 and Supplemental Fig. S2),
27 thus enabling loss and gain of function of specific candidate genes in this economically important
28 weed species. BSMV vectors carrying a fragment of black-grass *PHYTOENE DESATURASE* gene
29 (*AmPDS*) in antisense orientation induced leaf photobleaching within 5-11 days post inoculation (dpi)
30 (Fig. 1 and Supplemental Fig. S2). This corresponded with a significant decrease in *AmPDS* mRNA
31 as measured by RT-qPCR (Fig. 1). The photobleaching phenotype was also seen using the published
32 *PDS* fragment from wheat (*asTaPDS*; Lee et al., 2012, Supplemental Fig. S3). When individual tillers
33 were separated, the photobleaching persisted for at least 59 days (Supplemental Fig. S3). Green
34 fluorescence was clearly visible in many but not all leaves of plants inoculated with FoMV:GFP from
35 10-14 dpi onwards when analysed using UV microscopy or a handheld high intensity LED flashlight
36 (Fig. 1 and Supplemental Fig. S2). The presence of the GFP protein was confirmed by Western blot

37 analysis (Fig. 1). None of the individual tillers ($n = 17$) displaying green fluorescence from the
38 FoMV:GFP-treated plants maintained the GFP signal from 12 days onwards after separation and
39 replanting (Supplemental Fig. S4). These data collectively demonstrate that VIGS driven by BSMV
40 and VOX driven by FoMV can be used successfully in black-grass. The stability of BSMV VIGS-
41 induced phenotypes in individual tillers provides an opportunity for clonal analyses. This is particularly
42 important as black-grass is an obligate allogamous species (Sieber and Murray, 1979) with high
43 genetic diversity and low genetic differentiation (Menchari et al., 2007).

44 Next, we assessed suitability of VIGS and VOX for evaluating whether a given gene of interest is
45 necessary or sufficient to confer herbicide resistance in black-grass. Previous studies have implicated
46 the glutathione transferase gene *AmGSTF1* in MHR in black-grass and its heterologous expression in
47 *Arabidopsis thaliana* was shown to be sufficient to alter herbicide resistance (Cummins et al., 2013).
48 We used BSMV VIGS to knock-down expression of this gene in the MHR-biotype Peldon and the
49 sensitive-biotype Rothamsted. A BSMV VIGS construct with a portion of *AmGSTF1* coding sequence
50 in antisense orientation was generated and tested. Plants of both biotypes inoculated with
51 BSMV:MCS exhibited the expected phenotypes at 3-4 weeks after herbicide application; compared to
52 unsprayed controls, Peldon plants were unaffected by the herbicide whereas Rothamsted plants were
53 severely affected (Fig. 2). Pre-treatment of Peldon plants with BSMV:asAmGSTF1 significantly
54 decreased plant survival of the application of 1.5x field rate fenoxaprop (Fig. 2). The presence of the
55 recombinant virus in the inoculated plants and knock-down of the expression of the endogenous gene
56 was confirmed by RT-qPCR (Supplemental Fig. S5). These data are direct evidence that *AmGSTF1* is
57 required for MHR in black-grass. We then expressed the *bialaphos resistance (bar)* gene encoding an
58 enzyme that inactivates glufosinate-ammonium herbicides (De Block et al., 1987) from the FoMV
59 vector. Both Peldon and Rothamsted biotypes are susceptible to glufosinate, albeit with different ED₅₀
60 (Supplemental Fig. S1). Testing the inoculated plants by RT-PCR confirmed the presence of FoMV
61 and retention of the full-length *bar* gene coding sequence in the FoMV:bar-inoculated plants
62 (Supplemental Fig. S5). As anticipated, plants inoculated with FoMV:MCS or FoMV:GFP all died
63 within two weeks after application of a lethal dose of glufosinate (Fig. 2). However, both black-grass
64 biotypes pre-treated with FoMV:bar were noticeably less affected by the glufosinate application,
65 remained green and had fresh weights not statistically different from unsprayed plants (Fig. 2).
66 Glufosinate resistance was not stable when FoMV:bar tillers were separated before spraying
67 (Supplemental Fig. S4). With these data we demonstrate that BSMV VIGS and FoMV VOX are
68 suitable for loss- and gain-of-function analyses in black-grass relating to herbicide resistance.

69 Although these techniques can be further improved (Supplemental Fig. S6), the VIGS and VOX
70 techniques established here offer a step change in the type of questions that can now be asked in
71 weed biology. Of main importance will be to apply these techniques to establish a link between
72 specific genes and ability of black-grass to circumvent chemical controls, and thereby to gain a
73 molecular level understanding of what allows black-grass to be such a successful weed.

74 Accession numbers

75 DNA sequences identified for *AmPDS* and *AmGSTF1* in this paper have been deposited to GenBank
76 (<http://www.ncbi.nlm.nih.gov>) with the accession numbers MN936109 and MN936108 (associated
77 with AJ010454.1), respectively.

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83 biotypes. David Comont, Claudia Lowe, and Marie Lamarre are recognized for the data regarding
84 black-grass sensitivity to fenoxaprop and glufosinate. We thank Sergio Cerezo-Medina for providing
85 the *Agrobacterium tumefaciens* strain GV3101 and Caroline Sparks for the plasmid pAL156 carrying
86 the *bar* gene. Our gratitude goes to Helen-L Martin for carrying out the herbicide applications and to
87 Graham Shephard for assistance with photography.

88 Figure Legends

89 **Figure 1:** Virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX) are
90 possible in black-grass. Data are representative of at least three independent replicates. A-C)
91 Phenotypes of black-grass (Peldon) leaves that have been infected with *Barley stripe mosaic virus*
92 (BSMV) carrying either A) an empty multiple cloning site (MCS), or B) the MCS with a portion of
93 *PHYTOENE DESATURASE (PDS)* gene in antisense orientation from black-grass (*asAmPDS*). C)
94 Whole-plant phenotypes of plants from A or B infected with BSMV:MCS or BSMV:asAmPDS as
95 labelled. D-E) Phenotypes of black-grass (Peldon) leaves that have been infected with *Foxtail mosaic*
96 *virus* (FoMV) carrying *GREEN FLUORESCENT PROTEIN (GFP)* gene from Bouton et al. (2018)
97 under either D) bright field microscopy or E) using the GFP3 filter set. F-G) Phenotype of whole black-
98 grass (Peldon) plants that have been infected with FoMV:GFP photographed using a Nikon D90
99 illuminated with F) white light and no filter or G) blue light using a Dual Fluorescent Protein flashlight
100 through a long pass filter. H) RT-qPCR of *PDS* normalised against the *UBIQUITIN (UBQ)* gene in
101 Peldon plants inoculated with BSMV:MCS or BSMV:asAmPDS. Primers used in this study are
102 detailed in Supplemental Table S1. The data are averages and standard errors from five independent
103 biological replicates each. Asterix indicates a significant difference between that treatments using a
104 Student's *t*-test with * indicating $P < 0.05$ and ** $P < 0.01$ compared to the BSMV:MCS treated
105 samples. Supplemental Table S2 reports Student's *t*-test *P* values supporting claims of significance or
106 insignificance of observed results presented in this study. I) Stain free blot showing total protein
107 extracted from Peldon plants inoculated with FoMV:GFP or FoMV:MCS as labelled. Three
108 independent protein extractions per treatment are shown. The size of the bands on the ladder are
109 indicated. J) The Western blot shown in I probed with anti-GFP followed by Anti-Rabbit IgG–
110 Peroxidase antibody and ECL analysed on a CHEMIDOC MP Imaging Instrument using the
111 manufacturers specifications for optimal and automated acquisition.

112 **Figure 2:** VIGS and VOX techniques are applicable for testing hypotheses relating to herbicide
 113 resistance in black-grass. Data are representative of three independent replicates. Averages and
 114 standard errors are shown. Asterisk indicates a significant difference between that treatment and
 115 respective biotype unsprayed using a Student's *t*-test and * indicating $P < 0.05$ and ** $P < 0.01$.
 116 Supplemental Table S2 reports Student's *t*-test *P* values supporting claims of significance or
 117 insignificance of observed results presented in this study. A-B) Altering *AmGSTF1* expression using
 118 *Barley stripe mosaic virus* (BSMV) is sufficient to revert Peldon herbicide resistance to levels
 119 comparable to Rothamsted. A) Phenotypes of Rothamsted and Peldon plants infected with BSMV
 120 with an empty multiple cloning site (MCS), or a 200 bp region of *AmGSTF1* in the antisense
 121 orientation (from 6 to 205 bp after the start codon). Primers used in this study are detailed in
 122 Supplemental Table S1. Photographs were taken 3 weeks after treatment with 1.5x field rate
 123 fenoxaprop. B) Fresh weights of greater than 10 plants per treatment in A taken at 4 weeks after
 124 treatment with 1.5x field rate fenoxaprop and compared to unsprayed plants. C & D) Inoculation with
 125 *Foxtail mosaic virus* (FoMV) carrying the *bar* resistance gene is sufficient to confer resistance to 0.5%
 126 Challenge 60® in Rothamsted or Peldon plants. C) Phenotypes of Rothamsted and Peldon plants
 127 infected with FoMV carrying an empty multiple cloning site (MCS), or the MCS with *GREEN*
 128 *FLUORESCENT PROTEIN* (*GFP*) or *bialaphos resistance* (*bar*) gene treated with 0.5% Challenge.
 129 Photographs were taken 2 weeks after treatment. D) Fresh weights of 9 or more plants per treatment
 130 (only 5 plants in the case of FoMV:bar unsprayed) in Figure C taken at 2 weeks after treatment with
 131 0.5% Challenge and compared to unsprayed plants.

132 Supplemental Data

133 Supplemental Figure S1. Dose-response curves for black-grass biotypes Rothamsted and Peldon to
 134 herbicides A) glufosinate (Challenge 60®) or B) fenoxaprop.

135 Supplemental Figure S2. Virus-induced gene silencing (VIGS) and virus-mediated overexpression
 136 (VOX) are possible in black-grass in the herbicide-sensitive biotype Rothamsted equivalently to the
 137 herbicide-resistant biotype Peldon (Fig. 1).

138 Supplemental Figure S3. Leaf photobleaching correlated to infection with BSMV:asTaPDS or
 139 BSMV:asAmPDS which is stable in individual tillers.

140 Supplemental Figure S4. There is no evidence for stability of the FoMV VOX-induced phenotypes
 141 when individual tillers are separated and rooted.

142 Supplemental Figure S5. Molecular data demonstrating the viruses are present in the virus-inoculated
 143 plants and alter RNA levels.

144 Supplemental Figure S6: The efficiency of BSMV VIGS and FoMV VOX observed across
 145 experiments.

146 Supplemental Table S1. Student's *t*-test *P* values supporting claims of significance or insignificance of
 147 observed results presented in this study.

148 Supplemental Table S2. Primer sequences used in this study.

149 Supplemental Methods and Author Contributions.

150 Author Contributions

151 DRM conceived the original idea and formulated the research plan. DRM designed the experiments
 152 with input from MM-S and FM. MM-S, FM and DRM performed the experiments. VC and/or DRM
 153 developed the black-grass specific VIGS and VOX constructs. KK provided general guidance and
 154 support regarding BSMV and FoMV biology and VIGS and VOX methodology. DRM wrote the article
 155 with contributions from KK and all the authors. DRM agrees to serve as the author responsible for
 156 contact and ensures communication.

157

158 One-Sentence Summary: Virus-mediated transient expression techniques create loss- and gain-of-
 159 function mutations in black-grass and show causation between specific genotypes and measurable
 160 changes in herbicide resistance.

161

162 Author Names and Affiliations

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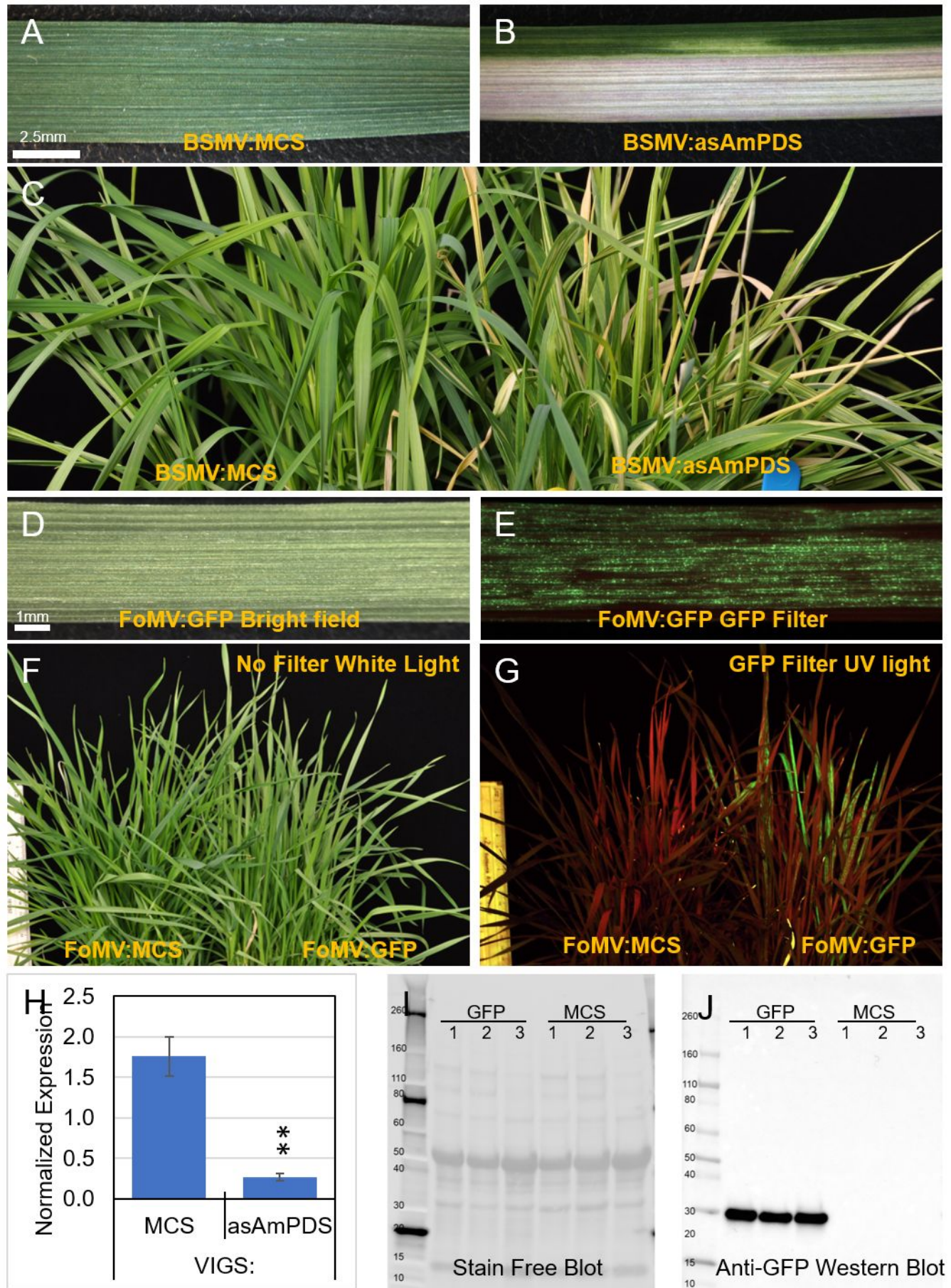


Figure 1: Virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX) are possible in black-grass. Data are representative of at least three independent replicates. A-C) Phenotypes of black-grass (Peldon) leaves that have been infected with *Barley stripe mosaic virus* (BSMV) carrying either A) an empty multiple cloning site (MCS), or B) the MCS with a portion of *PHYTOENE DESATURASE* (*PDS*) gene in antisense orientation from black-grass (*asAmPDS*). C) Whole plant phenotypes of plants from A or B infected with BSMV:MCS or BSMV:*asAmPDS* as labelled. D-E) Phenotypes of black-grass (Peldon) leaves that have been infected with *Foxtail mosaic virus* (FoMV) carrying *GREEN FLUORESCENT PROTEIN* (*GFP*) gene from Bouton et al. (2018) under either D) bright field microscopy or E) using the GFP3 filter set. F-G) Phenotype of whole black-grass (Peldon) plants that have been infected with FoMV:GFP photographed using a Nikon D90 illuminated with F) white light and no filter or G) blue light using a Dual Fluorescent Protein flashlight through a long pass filter. H) qRT-PCR of *PDS* normalised against the *UBIQUITIN* (*UBQ*) gene in Peldon plants inoculated with BSMV:MCS or BSMV:*asAmPDS*. Primers used in this study are detailed in Table S1. The data are averages and standard errors from five independent biological replicates each. Asterix indicates a significant difference between that treatments using a Student's *t*-test with * indicating $P < 0.05$ and ** $P < 0.01$ compared to the BSMV:MCS treated samples. Table S2 reports Student's *t*-test P values supporting claims of significance or insignificance of observed results presented in this study. I) Stain free blot showing total protein extracted from Peldon plants inoculated with FoMV:GFP or FoMV:MCS as labelled. Three independent protein extractions per treatment are shown. The size of the bands on the ladder are indicated. J) The Western blot shown in I probed with anti-GFP followed by Anti-Rabbit IgG–Peroxidase antibody and ECL analysed on a CHEMICON. Downloaded from on April 2, 2020 - Published by www.plantphysiol.org Copyright © 2020 American Society of Plant Biologists. All rights reserved.

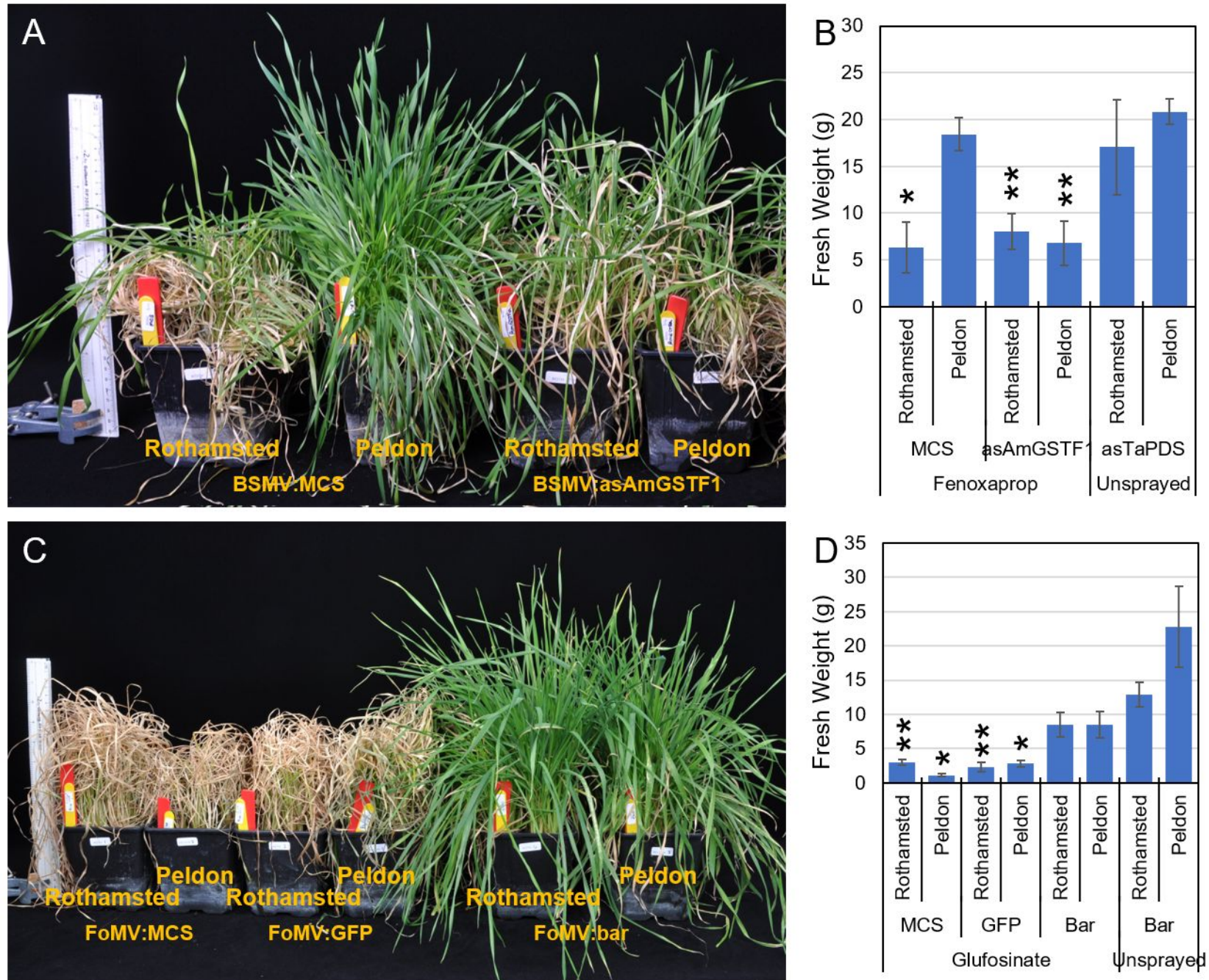


Figure 2: VIGS and VOX techniques are applicable for testing hypotheses relating to herbicide resistance in black-grass. Data are representative of three independent replicates. Averages and standard errors are shown. Asterisk indicates a significant difference between that treatment and respective biotype unsprayed using a Student's *t*-test and * indicating $P < 0.05$ and ** $P < 0.01$. Table S2 reports Student's *t*-test P values supporting claims of significance or insignificance of observed results presented in this study. A-B) Altering *AmGSTF1* expression using *Barley stripe mosaic virus* (BSMV) is sufficient to revert Peldon herbicide resistance to levels comparable to Rothamsted. A) Phenotypes of Rothamsted and Peldon plants infected with BSMV with an empty multiple cloning site (MCS), or a 200 bp region of *AmGSTF1* in the antisense orientation (from 6 to 205 bp after the start codon). Primers used in this study are detailed in Table S1. Photographs were taken 3 weeks after treatment with 1.5x field rate fenoxaprop. B) Fresh weights of greater than 10 plants per treatment in figures A taken at 4 weeks after treatment with 1.5x field rate fenoxaprop and compared to unsprayed plants. C & D) Inoculation with *Foxtail mosaic virus* (FoMV) carrying the *bar* resistance gene is sufficient to confer resistance to 0.5% Challenge 60® in Rothamsted or Peldon plants. C) Phenotypes of Rothamsted and Peldon plants infected with FoMV carrying an empty multiple cloning site (MCS), or the MCS with *GREEN FLUORESCENT PROTEIN* (GFP) or *bialaphos resistance* (*bar*) gene treated with 0.5% Challenge. Photographs were taken 2 weeks after treatment. D) Fresh weights of greater than 10 plants per treatment in the case of FoMV:bar unsprayed) in Figure C taken at 2 weeks after treatment with 0.5% Challenge and compared to unsprayed plants.

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