Virus-Mediated Transient Expression Techniques Enable Gene Function Studies in Black-Grass[1][OPEN]

Dear Editors,

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

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

We used two biotypes that differ in their sensitivity to herbicides (Supplemental Fig. S1): Peldon (multiple herbicide resistant [MHR]) and Rothamsted (sensitive). Under laboratory conditions, we infected both biotypes asymptomatically with BSMV and FoMV (Fig. 1; Supplemental Fig. S2), thus enabling loss- and gain-of-function of specific candidate genes in this economically important weed species. BSMV vectors carrying a fragment of black-grass *PHYTOENE DESATURASE* gene (*AmPDS*) in antisense orientation induced leaf photobleaching within 5 to 11 d postinoculation (Fig. 1; Supplemental Fig. S2). This corresponded with a significant decrease in *AmPDS* mRNA as measured by reverse transcription quantitative PCR (RT-qPCR; Fig. 1). The photobleaching phenotype was also seen using the published *PDS* fragment from wheat (*asTaPDS*;Supplemental Fig. S3: Lee et al., 2012). When individual tillers were separated, the photobleaching persisted for at least 59 d (Supplemental Fig. S3). Green fluorescence was clearly visible in many but not all leaves of plants inoculated with FoMV:GFP from 10 to 14 d postinoculation onwards when analyzed using ultraviolet microscopy or a handheld high intensity light-emitting diode flashlight (Fig. 1; Supplemental Fig. S2). The presence of the GFP protein was confirmed by western blot analysis (Fig. 1). None of the individual tillers ($n = 17$) displaying green fluorescence from the FoMV:GFP-treated plants maintained the GFP signal from 12 d onwards after separation and replanting (Supplemental Fig. S4). These data collectively demonstrate that VIGS driven by BSMV and VOX driven by FoMV can be used successfully in black-grass. The stability of BSMV VIGS-induced phenotypes in individual tillers provides an opportunity for clonal analyses. This is particularly important, as black-grass is an obligate allogamous species (Sieber and Murray, 1979) with high genetic diversity and low genetic differentiation (Menchari et al., 2007).

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D.R.M. conceived the original idea and formulated the research plan; D.R.M. designed the experiments with input from M.M.-S. and F.M. M.M.-S., F.M., and D.R.M. performed the experiments; V.C. and/or D.R.M. developed the black-grass specific VIGS and VOX constructs; K.K. provided general guidance and support regarding BSMV and FoMV biology and VIGS and VOX methodology; D.R.M. wrote the article with contributions from K.K. and all the authors; D.R.M. agrees to serve as the author responsible for contact and ensures communication.

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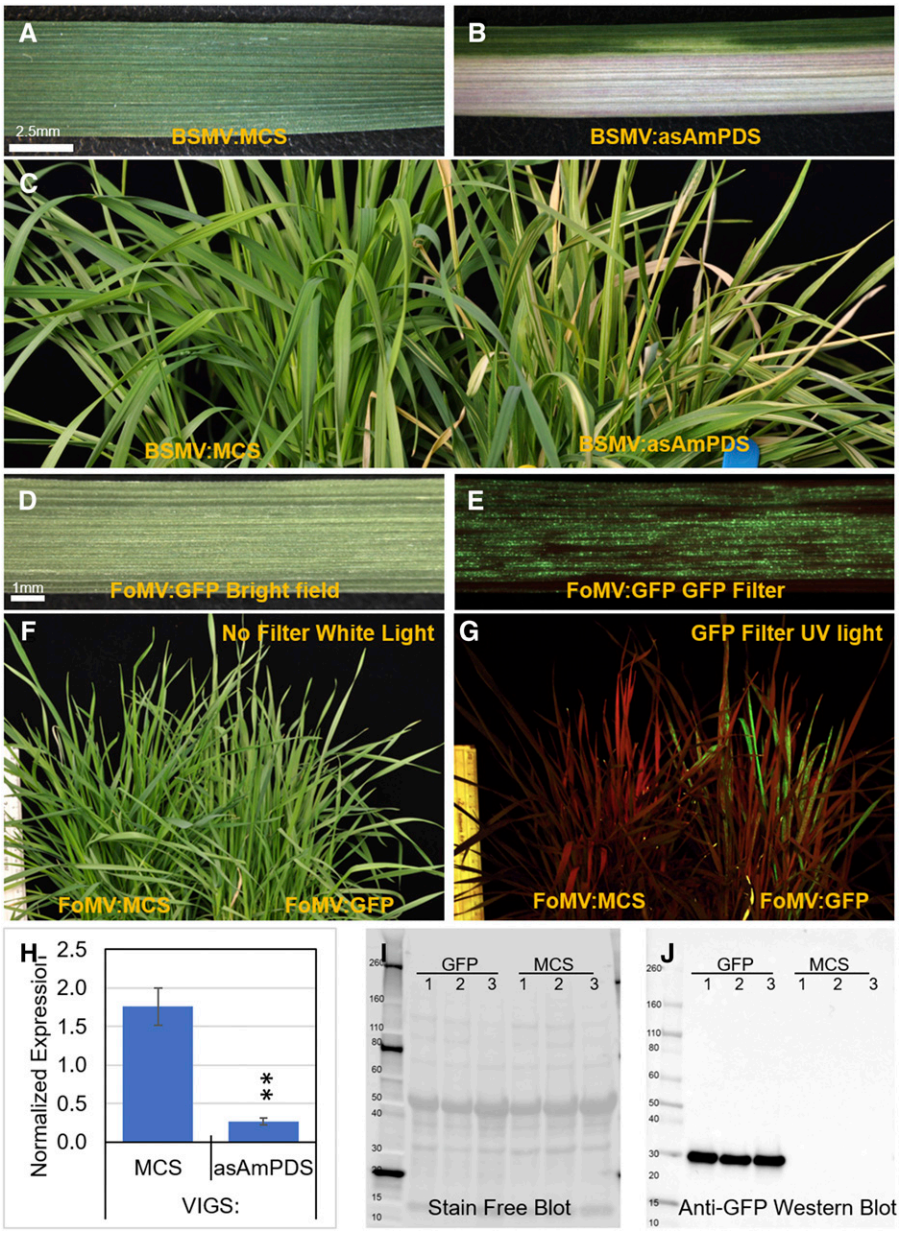

Figure 1. VIGS and VOX are possible in black-grass. Data are representative of at least three independent replicates. A and B, Phenotypes of black-grass (Peldon) leaves that have been infected with BSMV carrying either an empty multiple cloning site (MCS; A) or the MCS with a portion of *PHYTOENE DESATURASE* (*PDS*) gene (B) in antisense orientation from black-grass (as*AmPDS*). C, Whole-plant phenotypes of plants from A or B infected with BSMV:MCS or BSMV:asAmPDS as labeled. D and E, Phenotypes of black-grass (Peldon) leaves that have been infected with FoMV carrying *GFP* gene from Bouton et al. (2018) under either bright-field microscopy (D) or using the GFP3 filter set (E). F and G, Phenotype of whole black-grass (Peldon) plants that have been infected with FoMV:GFP photographed using a Nikon D90 illuminated with white light and no filter (F) or blue light using a dual fluorescent protein flashlight through a long-pass filter (G). H, RT-qPCR of *PDS* normalized against the *UBIQUITIN* gene in Peldon plants inoculated with BSMV:MCS or BSMV:asAmPDS. Primers used in this study are detailed in Supplemental Table S1. The data are averages and SEs from five independent biological replicates each. Asterisks indicate a significant difference between that treatments using a Student's *t* test with $^{**}P < 0.01$ compared to the BSMV:MCS-treated samples. Supplemental 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 labeled. 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 analyzed on a CHEMIDOC MP imaging instrument using the manufacturers' specifications for optimal and automated acquisition. For further details of methods used see the Supplemental Methods.

Next, we assessed suitability of VIGS and VOX for evaluating whether a given gene of interest is necessary or sufficient to confer herbicide resistance in black-grass. Previous studies have implicated the glutathione transferase gene *AmGSTF1* in MHR in black-grass, and its heterologous expression in Arabidopsis (*Arabidopsis thaliana*) was shown to be sufficient to alter herbicide resistance (Cummins et al., 2013). We used BSMV VIGS to knock down expression of this gene in the MHR biotype Peldon and the sensitive biotype Rothamsted. A BSMV VIGS construct with a portion of *AmGSTF1* coding sequence in antisense orientation was generated and tested. Plants of both biotypes inoculated with BSMV:MCS exhibited the expected phenotypes at 3 to 4 weeks after herbicide application; compared to unsprayed controls, Peldon plants were unaffected by the herbicide, whereas Rothamsted plants were severely affected (Fig. 2). Pretreatment of Peldon plants with BSMV:asAmGSTF1 significantly decreased plant survival of the application of 1.5× field rate fenoxaprop (Fig. 2). The presence of the recombinant virus in the inoculated plants and knockdown of the expression of the endogenous gene was confirmed by RT-qPCR (Supplemental Fig. S5). These data are direct evidence that *AmGSTF1* is required for MHR in black-grass. We then expressed the *bialaphos resistance* (*bar*) gene encoding an enzyme that inactivates glufosinate-ammonium herbicides (Block et al., 1987) from the FoMV vector. Both Peldon and Rothamsted biotypes are susceptible to glufosinate, albeit with different percentages of plants that die at a given dose of herbicide (Supplemental Fig. S1). Testing the inoculated plants by RT-PCR confirmed the presence of FoMV and retention of the full-length *bar* gene coding sequence in the FoMV:bar-inoculated plants (Supplemental Fig. S5). As anticipated, plants inoculated with FoMV:MCS or

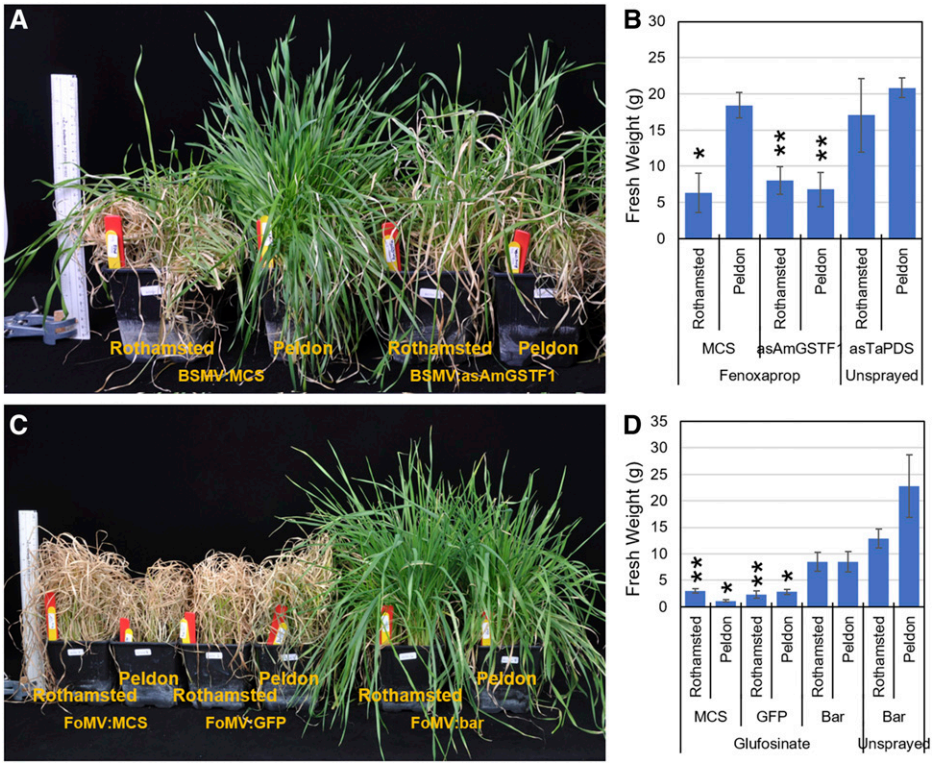

Figure 2. VIGS and VOX techniques are applicable for testing hypotheses relating to herbicide resistance in black-grass. A and B, Altering *AmGSTF1* expression using BSMV is sufficient to revert Peldon herbicide resistance to levels comparable to Rothamsted. A, Phenotypes of Rothamsted and Peldon plants 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). Photographs were taken 3 weeks after treatment with 1.5× field rate fenoxaprop. B, Fresh weights of greater than 10 plants per treatment in A taken at 4 weeks after treatment with 1.5× field rate fenoxaprop and compared to unsprayed plants. C and D, Inoculation with FoMV carrying the *bialaphos resistance* (*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 *GFP* or *bar* gene treated with 0.5% challenge. Photographs were taken 2 weeks after treatment. D, Fresh weights of nine or more plants per treatment (only five plants in the case of FoMV:bar unsprayed) in C taken at 2 weeks after treatment with 0.5% challenge and compared to unsprayed plants. Data are representative of three independent replicates. Averages and SEs are shown. Asterisks indicate a significant difference using a Student's *t* test with $*P < 0.05$ and $**P < 0.01$ between the sample indicated and the unsprayed FoMV:bar treated control for that biotype. Supplemental Table S2 reports Student's *t* test *P* values supporting claims of significance or insignificance of observed results presented in this study.

FoMV:GFP all died within 2 weeks after application of a lethal dose of glufosinate (Fig. 2). However, both black-grass biotypes pretreated with FoMV:bar were noticeably less affected by the glufosinate application, remained green, and had fresh weights not statistically different from unsprayed plants (Fig. 2). Glufosinate resistance was not stable when FoMV:bar tillers were separated before spraying (Supplemental Fig. S4). With these data, we demonstrate that BSMV VIGS and FoMV VOX are suitable for loss- and gain-of-function analyses in black-grass relating to herbicide resistance.

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

Accession Numbers

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

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Dose-response curves for black-grass biotypes Rothamsted and Peldon to herbicides glufosinate (challenge 60) or fenoxaprop.

Supplemental Figure S2. VIGS and VOX are possible in black-grass in the herbicide-sensitive biotype Rothamsted equivalently to the herbicide-resistant biotype Peldon (Fig. 1).

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

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

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

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

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

Supplemental Table S2. Primer sequences used in this study.

Supplemental Methods. Further details of methods used in the article.

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