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ORIGINAL ARTICLE

Insights from controlled, comparative experiments highlight the limitations of using BSMV and FoMV for virus-enabled reverse genetics in rice

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Abstract

Virus-enabled reverse genetics (VERG) is a powerful tool for transient gene expression modulation in plants, particularly where stable transformation is challenging. However, the efficacy of VERG varies across species. In this study, we tested two commonly used viral vectors, barley stripe mosaic virus (BSMV) and foxtail mosaic virus (FoMV), for their ability to induce virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX) in rice (*Oryza sativa*). While both vectors successfully altered gene expression in wheat (*Triticum aestivum*), they failed to do so in six rice cultivars from different subspecies and subgroups, despite rigorous optimization of inoculation methods and environmental conditions. The BSMV vector carrying a portion of *phytoene desaturase* (*PDS*) in antisense did not induce the expected photobleaching phenotype, and FoMV-driven GFP expression was absent in rice. These findings contrast with previous reports of successful VERG in other monocots and suggest that intrinsic mechanisms exist in rice that inhibit or reduce the efficacy of VIGS and VOX with these protocols and vectors. Our results highlight the species-specific limitations of VERG and underscore the need for alternative viral systems or novel vector designs for functional genomics research in rice. By sharing our unsuccessful attempts, we aim to prevent redundant efforts and encourage further exploration of VERG in *Oryza* species.

KEYWORDS
gene silencing, *Oryza sativa*, overexpression, VERG, VIGS, VOX

1 | INTRODUCTION

Virus-enabled reverse genetics (VERG) tools are useful for rapidly testing genotype-to-phenotype hypotheses as they transiently change gene expression *in planta*. VERG either induces or reduces gene expression, known as virus-mediated overexpression (VOX) or virus-induced gene silencing (VIGS), respectively. These techniques are particularly useful in plants where stable transformation protocols are either undeveloped or unsuitable, such as for allogamous plants with long generation times and underdeveloped genomic resources (MacGregor, 2020). Accordingly, VERG has successfully been used in a variety of species, such as *Alopecurus myosuroides* Huds. (Mellado-Sánchez et al., 2020; Patterson et al., 2025), *Setaria italica* (L.) Beauv., *Solanum* spp., *Thalictrum thalictroides* (L.) Eames & Boivin, and *Zingiber officinale* Roscoe (reviewed in Dommes et al., 2019). VERG is also well-established in a wide range of agronomically important species

and model plants (Dommes et al., 2019). However, not all VERG protocols are successful or repeatable, even within the same laboratory; for instance, there are conflicting reports on the efficacy of VIGS in *Pennisetum glaucum* (L.) R. Br. (Schoeman, 2011; van Nugteren et al., 2007). Moreover, VERG effectiveness can vary depending on host compatibility and experimental conditions (Dommes et al., 2019).

Among the most commonly used viral vectors for VERG studies, barley stripe mosaic virus (BSMV) and foxtail mosaic virus (FoMV) stand out due to their efficacy, flexibility, and broad host ranges (Dommes et al., 2019; Rössner et al., 2022). BSMV, a tripartite, positive-sense RNA virus from the genus *Hordeivirus*, has been extensively utilised for VIGS applications in monocots, particularly in cereal crops (Lee, Hammond-Kosack, & Kanyuka, 2015). On the other hand, FoMV, a *Potexvirus*, has demonstrated robust systemic infection in multiple monocots and dicots (Kanyuka, 2022), making it a promising alternative to BSMV. FoMV's relatively simple genome organisation and high replication efficiency, alongside a capacity to hold and retain longer inserts than BSMV, contribute to its effectiveness as a VERG tool (Beernink & Whitham, 2023) and a well-characterised vector for overexpression of Green Fluorescent Protein (GFP) is available (Bouton et al., 2018).

Despite the evidenced usefulness of VERG for functional genomics research, there is a distinct lack of data reporting VERG based on BSMV or FoMV in *Oryza* species, even though rice is both experimentally and agronomically important. There is evidence for rice coding sequences having been used for silencing targets in wheat driven by a BSMV-based system (Holzberg et al., 2002), for the heterologous expression of rice genes (e.g., *Hd3a*) in *Panicum miliaceum* L. in a FoMV-based system (Yuan et al., 2020), and more recently, the use of a modified FoMV vector for silencing in *japonica* rice (Huang et al., 2020). Additionally, a few studies (Ding et al., 2006, 2007; Kant et al., 2015; Kant & Dasgupta, 2017; Purkayastha et al., 2010) have reported the use of other viral vectors in rice. Brome mosaic virus (BMV) (Ding et al., 2006) and rice tungro bacilliform virus (Purkayastha et al., 2010) were both validated for VIGS in Asian rice (*Oryza sativa* L.), but the literature building on or effectively using their vectors is scarce. Although *O. sativa* ssp. *japonica* can be reliably transformed, efficient and effective stable transformation of *O. sativa* ssp. *indica* remains challenging (Sulaiman et al., 2019), and we found no data about stable or transient transformation of weedy rice, *O. sativa* f. *spontanea*. Therefore, the aim of this study was to test whether well-established visual reporters of VERG could induce transient gain or loss of function in *O. sativa* subspecies to gain insights into the gene regulation of this complex species.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Seeds from *Nicotiana benthamiana* Domin, wheat (*Triticum aestivum* L. cv. Riband), rice (*O. sativa* ssp. *indica* cv. IR 64, *O. sativa* ssp. *indica* cv. Kasalath, *O. sativa* ssp. *indica* cv. CO-39, *O. sativa* ssp. *japonica* cv. Kitaake, *O. sativa* ssp. *japonica* cv. Koshihikari, *O. sativa* ssp. *japonica* cv. Balilla), and green foxtail (*Setaria viridis* (L.) P. Beauv.) were used. All seeds except for *S. viridis*, which were acquired from Kew's Millenium Seed Bank (Serial Number 31491), were available from Rothamsted Research Seed Stocks. *N. benthamiana* plants were grown according to Lee, Rudd, and Kanyuka (2015). All plants were grown in a high-end DEFRA/HSE-registered controlled environment room set for 26.7/21.1°C in a 16/8 h light/dark regime at 200 µmol m^{-2} s^{-1}. Seeds from wheat, green foxtail, and rice genotypes were surface sterilised with a household bleach solution (50%) for 5 min and rinsed 3 times with distilled water after Lindsey et al. (2017). Seeds were sowed in 20 × 15 cm pots containing wet Rothamsted Standard Compost Mix (75% medium grade (L&P) peat, 12% screened sterilised loam, 3% medium grade vermiculite, 10% grit (5 mm screened, lime free), 3.5 kg m^{-3} Osmocote® Exact Standard 3–4 M, 0.5 kg m^{-3} PG MIX®, 3 kg lime pH 5.5–6.0 and 200 mL m^{-3} Vitax Ultrawet) and covered with perlite. The pots were covered with lids to maintain high humidity. After 5–7 days, seedlings (∼2 cm) were chosen to be transplanted to square 4 cm pots filled with Rothamsted Standard Compost Mix. Propagator lids covered transplanted seedlings for 3 days following transplant. Plants were kept in the same growth room and watered every 2 days.

2.2 | Preparation and cloning of viral vectors

The creation of BSMV:asOsPDS1 and of BSMV:asOsPDS2 followed published procedures (Lee, Rudd, & Kanyuka, 2015) with minor changes detailed below. The *O. sativa phytoene desaturase* (*OsPDS*, LOC_Os03g08570) was aligned with *T. aestivum PDS* (Figure S1) to identify the corresponding regions previously shown to be effective for visual analysis of VIGS efficacy (Lee et al., 2012; Mellado-Sánchez et al., 2020). Two fragments of the *OsPDS* coding sequence were synthesised in the antisense (as) direction. Antisense orientation was used, as this is what is recommended for high-efficiency silencing (Lacomme et al., 2003; Pacak et al., 2010), and more specifically, Killiny (2019) showed that when the antisense orientation was used, expression levels of the *pds* gene were markedly reduced compared to the sense orientation. The sequences covered 185 nucleotides targeting 430–614 bp as asOsPDS1 or 200 nucleotides targeting 900 to 1100 bp as asOsPDS2 (Figure S2). Synthesised dsDNA fragments were cloned into BSMV:RNAγ vector pCa-γbLIC (Yuan et al., 2011) and transformed into *Escherichia coli* JM109. Cloning was confirmed by colony PCR using primers in the viral backbone (2235.F-GATCAA CTGCCAATCGTGAGTA and 2615.R-CCAATTCAGGCATCGTTTTC). Purified plasmid DNA from verified colonies was sequenced using the same primers to confirm cloning orientation and sequence accuracy. A selected positive transformant was transferred to *Agrobacterium tumefaciens* GV3101 following published procedures (Lee, Rudd, & Kanyuka, 2015). Recombinants were selected based on survival of dual selection with kanamycin and gentamycin. Individual colonies were selected, multiplied, and verified by colony PCR with the same primers described above. A selected confirmed transformant colony for each

BSMV:asOsPDS1 and BSMV:asOsPDS2 was stocked in glycerol (15%) and stored at −80°C. BSMV:asTaPDS, BSMV:MCS, BSMV:RNAα (pCaBS-α) and BSMV:RNAβ (pCaBS-β) VIGS constructs (Lee et al., 2012; Yuan et al., 2011) and FoMV:MCS, FoMV:GFP (PV101-GFP), and P19 (pBIN61-P19) VOX constructs (Bouton et al., 2018) were used without further modifications from transformed *A. tumefaciens* strain GV3101 stocked in 15% glycerol at −80°C (Mellado-Sánchez et al., 2020).

2.3 | Preparation of the virus inoculum from *N. benthamiana* for rub inoculation

Detailed protocols using BSMV or FoMV were followed (Mellado-Sánchez et al., 2024). Briefly, glycerol stocks (15% glycerol with 85% bacterial culture at the logarithmic growth stage) of *A. tumefaciens* containing the recombinant vectors were grown in LB broth supplemented with kanamycin (50 μg mL^{-1}) and gentamycin (25 μg mL^{-1}). *A. tumefaciens* cultures were pelleted and resuspended in infiltration buffer [10 mM $MgCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.6, and 150 μM acetosyringone] to a final OD_{600} of 1.5–1.55 for BSMV constructs, 0.6 for FoMV constructs, and 0.3 for P19. BSMV:RNAγ (MCS, asTaPDS, asOsPDS1, and asOsPDS2), BSMV:RNAα, and BSMV:RNAβ were mixed in equal proportions, hereafter referred to as BSMV inoculum. FoMV:GFP and FoMV:MCS were equally mixed with P19, hereafter referred to as FoMV inoculum. BSMV and FoMV inocula were propagated via agroinfiltration in *N. benthamiana*. Infiltrated leaves were harvested 3–5 days for BSMV and 5–7 days for FoMV after infiltration. Three leaves from different *N. benthamiana* plants were weighed into foil packets and instantly frozen in liquid nitrogen before being stored at −80°C.

2.4 | Preparation of the BSMV inoculum from *A. tumefaciens* for injection inoculation, and dipping

A. tumefaciens glycerol stocks were grown and pelleted as described above. For dipping inoculation, pelleted cultures containing BSMV constructs were resuspended in a modified dipping buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, 200 μM acetosyringone, 1% sucrose, and 0.01% Silwet L-77) to a final OD_{600} of 0.6 (Andrieu et al., 2012). For injection inoculation, *A. tumefaciens* cultures were resuspended in a published injection buffer (Kant & Dasgupta, 2017) (10 mM $MgCl_2$, 10 mM MES pH 5.6, 500 μM acetosyringone) and adjusted to a final OD_{600} of 0.8.

2.5 | Inoculation of plants from BSMV or FoMV infiltrated *N. benthamiana*

Rub-inoculation using the abrasive carborundum [Technical, SLR, Extra Fine Powder, ~36 μm (300 Grit), Fisher Chemical cat. 10345170] was performed as published (Lee, Rudd, & Kanyuka, 2015) with minor adjustments. Frozen *N. benthamiana* leaves that had been infiltrated as described above were ground in a 2:1 (w/v) ratio in 10 mM potassium phosphate buffer, pH 7. The sap was used for inoculation by rubbing a leaf 6–10 times. Inoculation by microneedling was performed using a DermaRoller device to wound the leaf once prior to the sap application. The third leaf of 14-day-old wheat plants and 21-day-old green foxtail and rice plants was inoculated.

2.6 | Inoculation of rice from *A. tumefaciens*

For dipping inoculation, the third leaf of 21-day-old rice was wounded by microneedling and dipped in the BSMV:MCS and BSMV:asOsPDS2 *A. tumefaciens* resuspensions for 30 min. For injection inoculation, approximately 0.2 mL of the resuspended BSMV:MCS and BSMV:asOsPDS2 inocula were injected into the meristematic region located at the crown of 14-day-old rice plants using a 1 mL syringe and a 26G needle. After inoculation, rice plants were treated in the same way as those inoculated by rubbing. Only rice cultivars IR64 (*indica*) and Kasalath (*aus*) were used for this experiment.

2.7 | Visual assessment of VIGS and VOX

Whole plants or leaf segments were photographed using a 48 megapixel camera from iPhone 15 Pro (Apple, Cupertino, CA, USA) and a Velour Vinyl black backdrop (Superior Seamless 234312). For GFP fluorescence, a yellow long-pass (510 nm) filter (Midwest Optical Systems, Palatine, IL, USA) was used in front of the camera, and plants were illuminated with blue light from a Dual Fluorescent Protein flashlight (Nightsea, Lexington, MA, USA).

3 | RESULTS

Individual plants were inoculated and evaluated in biological and technical replicates for each experiment. Riband plants were inoculated in parallel as a positive control for the inoculum and the rubbing process. A total of 286 rice plants from subgroups *indica*, *aus*, and *japonica* were inoculated alongside 105 wheat plants (Table 1). As expected, the efficacy in wheat was high, with an overall efficiency of 77% for FoMV:GFP, whereas the different BSMV:asPDS constructs induced photobleaching in over 91% of the treated plants (Table 1). Unexpectedly, none of the rice plants showed the expected gain or loss of function phenotypes (Table 1). These results are explained further below.

3.1 | VIGS in wheat and rice

BSMV vectors driving an antisense portion of *PDS* are a well-accepted standard for VIGS and have been validated in several different monocot and dicot species under a range of different growth, inoculation, and propagation conditions (Lee et al., 2012; Lee, Rudd, and

TABLE 1 Description of plant materials and number of plants inoculated with each vector and different inoculation methods.

Species	Subgroup	Total number of plants	FoMV: GFP rubbing	FoMV: MCS rubbing	BSMV: asOsPDS1 rubbing	BSMV: asOsPDS2 rubbing	BSMV: asOsPDS2 microneedling	BSMV: asOsPDS2 dipping	BSMV: asOsPDS2 injection	BSMV: MCS rubbing
Triticum aestivum cv. Riband	-	105	48 (37)	4 (0)	15 (12)	22 (19)				4 (0)
Oryza sativa ssp. *indica* cv. IR 64	*Indica*	182	30 (0)	24 (0)	24 (0)	40 (0)	10 (0)	10 (0)	10 (0)	34 (0)
Oryza sativa ssp. *indica* cv. Kasalath	*Aus*	56	6 (0)			10 (0)	10 (0)	10 (0)	10 (0)	10 (0)
Oryza sativa ssp. *indica* cv. CO-39	*Indica*	12	6 (0)			6 (0)				
Oryza sativa ssp. *japonica* cv. Kitaake	*Japonica*	12	6 (0)			6 (0)				
Oryza sativa ssp. *japonica* cv. Koshihikari	*Japonica*	12	6 (0)			6 (0)				
Oryza sativa ssp. *japonica* cv. Balilla	*Japonica*	12	6 (0)			6 (0)				

Note: The number in parenthesis represents plants that showed positive signs of inoculation: Bleaching for BSMV and green fluorescence for FoMV:GFP.

Kanyuka, 2015; Mellado-Sánchez et al., 2020; Patterson et al., 2025; Yuan et al., 2011). Like these previous results, Figure 1a shows that inoculating Riband with either BSMV:asOsPDS1 or BSMV:asOsPDS2 constructs created herein induced photobleaching to a similar degree as inoculation with BSMV:asTaPDS from Lee, Rudd, & Kanyuka, (2015). Further details about the asOsPDS1 and asOsPDS2 sequences and their cloning can be found in the Materials and Methods section. Also, as expected, Riband plants inoculated with empty vector control (BSMV:MCS) remained green (Figure 1a). Systemic bleaching was clearly visible in BSMV:asOsPDS-treated Riband plants from 12 to 35 days after inoculation (Figure 1c). However, parallel inoculation of BSMV:MCS, BSMV:asOsPDS1, BSMV:asOsPDS2, or BSMV:asTaPDS into *indica* rice (IR64) led to a different phenotype (Figure 1b). In IR64, inoculation with any variant of the BSMV vectors led to the development of white spots on the top of the leaf (Figure 1b,d). Importantly, not only was this non-bleaching phenotype similar regardless of which vector was introduced (Figure 1b), similar white spots were also observed in uninoculated rice plants grown in the same room (Figure 1e).

Many different standard "transient expression" protocols that use rice as the target species are available in the literature (Andrieu et al., 2012; Ding et al., 2006, 2007; Huang et al., 2020; Kant et al., 2015; Kant & Dasgupta, 2017; Purkayastha et al., 2010). To rule out that we were not seeing VIGS because the rub inoculation protocol we were using did not sufficiently introduce the virus into the leaf, we tried a variety of inoculation methods (e.g., using microneedles, dipping, or injection) and resuspension buffers (see Materials and Methods for details). None of the alternative inoculation methods induced photobleaching in rice cultivars from the *indica* or *aus* subgroups (Figure 1f). In addition, injection-based inoculation led to mortality in approximately 20% of the treated plants.

3.2 | VOX in wheat, green foxtail, and rice

FoMV driving expression of GFP (PV101-GFP) (Bouton et al., 2018) is a well-established visual reporter which has been proven to be effective in several species, including several different types of monocots (Bouton et al., 2018; Torti et al., 2021; Yuan et al., 2020). As expected, the FoMV:GFP inoculum induced characteristic GFP fluorescence in wheat (Figure 2a). In Riband, fluorescence was visible 7–10 days post rub-inoculation and spread systemically (Figure 2b). Rub inoculation with FoMV:GFP also successfully led to GFP fluorescence in green foxtail, while those treated with FoMV:MCS showed no observable fluorescence or viral infection phenotypes (Figure 2c). Rice plants inoculated in parallel with either FoMV:MCS or FoMV:GFP showed no observable symptoms of viral infection or GFP fluorescence (Figure 2d). This lack of observable phenotype was similar across all the different rice genotypes treated. Plants from all the species were monitored regularly for 5 weeks after inoculation.

4 | DISCUSSION

Our well-replicated and highly controlled data show that our protocols and variations thereon for BSMV-driven VIGS or FoMV-driven VOX were ineffective in the different cultivars and subspecies of *O. sativa* we tested. Considerable literature demonstrates these protocols are efficient and effective in a variety of monocots (Bouton et al., 2018; Kanyuka, 2022; Lee et al., 2012; Lee, Hammond-Kosack, & Kanyuka, 2015; Lee, Rudd, & Kanyuka, 2015; Mellado-Sánchez et al., 2020; Patterson et al., 2025; Torti et al., 2021), and here we show they successfully induced VIGS and/or VOX in wheat (Figures 1 and 2) and in green foxtail (Figure 2). Therefore, we do not

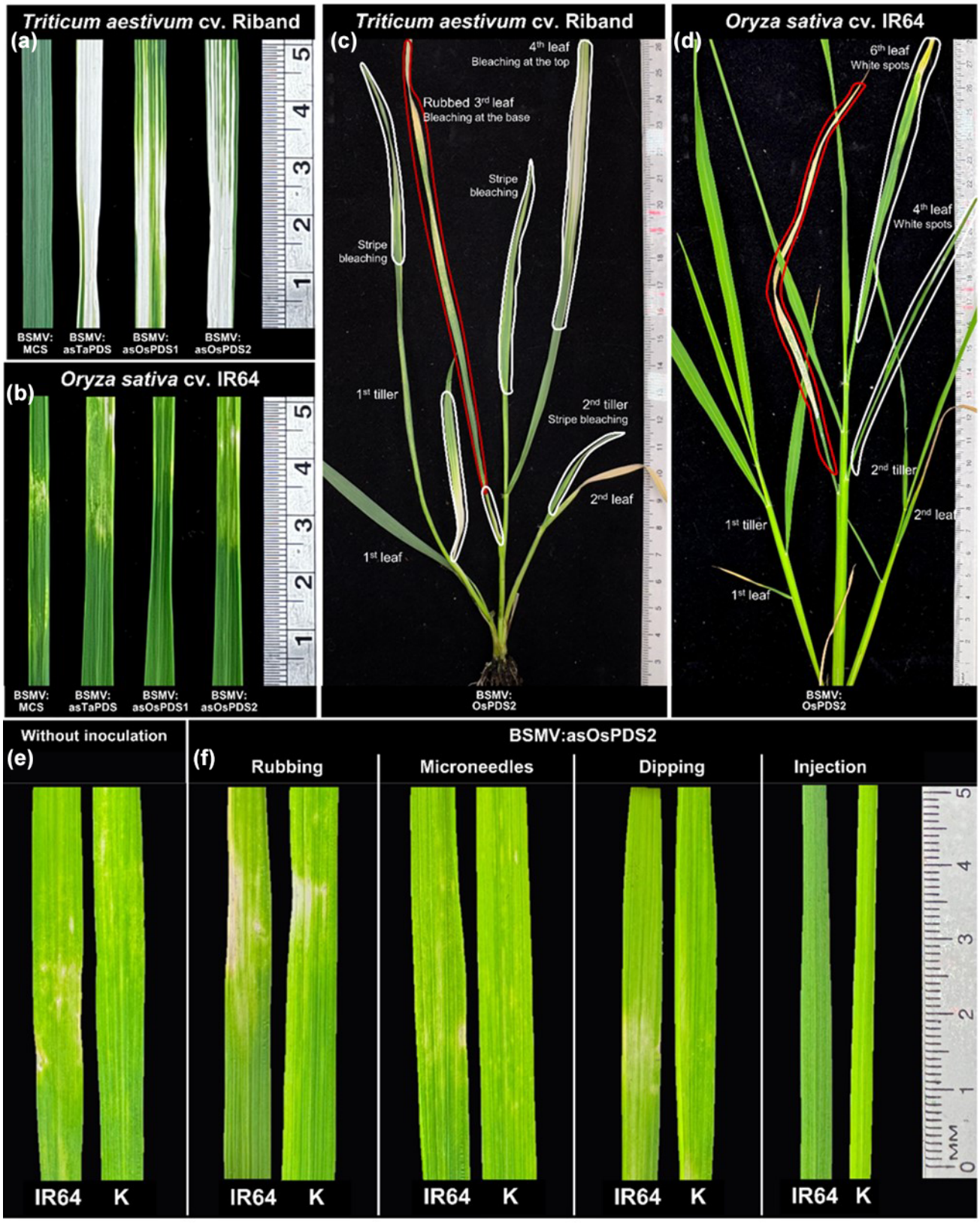

FIGURE 1 Legend on next page.

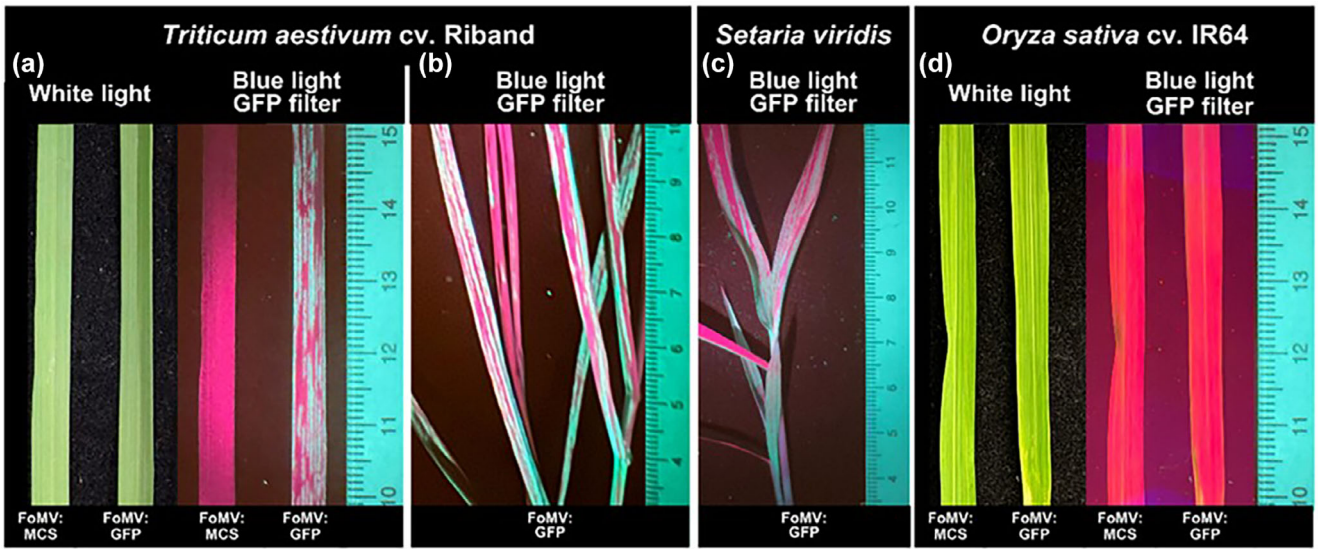

FIGURE 2 Virus-mediated protein overexpression (VOX) using foxtail mosaic virus (FoMV) vector. Phenotypes of wheat (a) and rice (d) leaves that have been inoculated with FoMV carrying a multi cloning site (MCS) or GFP gene under white light or using blue light with a GFP filter set. Photos of the fourth leaf, up next the rub-inoculate leaf with a sap containing grinded leaves of *Nicotiana benthamiana* agroinfiltrated with FoMV:GFP and the abrasive carborundum. Phenotype of whole wheat (b) and *Setaria viridis* (c) plants that have been inoculated with FoMV:GFP showing systemic infection.

feel our lack of phenotype is caused by a failure of the experiment; rather, our data demonstrate that these protocols and vectors are not able to produce a successful VERG response in these cultivars and subspecies of *O. sativa*.

Although molecular assays such as quantification of viral loads or transcript profiling might have detected low-level infections or modest changes in target gene expression, detailed characterisation of rice–BSMV or rice–FoMV interactions lies outside the scope of this work. Rather, our focus was on establishing whether our robust and reproducible VERG protocols that efficiently modulate gene expression in other monocots could be applied to *O. sativa*, and in doing so, empowering researchers to reliably and effectively test their reverse genetics hypotheses. In this context, phenotypic assessment provided the most direct and interpretable readout of successful change in gene expression. Accordingly, we focused our efforts on replication, application across different rice cultivars, and assessing different inoculation protocols. Moreover, it is likely that molecular confirmation would have been complicated by the patchy nature of viral infection that occurs in VERG. Although our protocols for BSMV- and FoMV-driven VERG result in specific and systemic changes in the new plant tissue (Bouton et al., 2018; Kanyuka, 2022; Lee et al., 2012; Lee, Hammond-Kosack, & Kanyuka, 2015; Lee, Rudd, & Kanyuka, 2015; Mellado-Sánchez et al., 2020; Patterson et al., 2025; Torti et al., 2021), not all leaves in all plants and not all cells within any given leaf are affected (MacGregor, 2020). Therefore, molecular data could be confounded by the wrong tissue being sampled. Given these constraints and that our goal was to assess the usefulness of variations on the existing VERG protocols, we focused on visual phenotyping as the most robust and interpretable approach in this study.

Although the efficacy of VERG systems is affected by myriad factors such as plant growth temperature, insert orientation, and length (Bouton et al., 2018; Lacomme et al., 2003; Lee, Rudd, & Kanyuka, 2015; Pacak et al., 2010), and there are different variations on the BSMV and FoMV vectors used by the community (described in, e.g., Kant & Dasgupta, 2019) ultimately, the success of VERG is dependent on the appropriate and balanced interaction between the viral vector and the host. Our data gave no indication that an appropriate interaction occurred when any of the rice cultivars we

FIGURE 1 Virus-induced gene silencing (VIGS) using barley stripe mosaic virus (BSMV) vector. Phenotypes of wheat (a) and rice (b) leaves that have been inoculated with BSMV vector carrying a multi-cloning site (MCS) or partial sequences of *phytoene desaturase* (*PDS*) from wheat (asTaPDS) or rice (asOsPDS1 and asOsPDS2). Photos of the fourth leaf (up next to the rub-inoculated leaf). Whole-plant phenotypes from wheat (c) and rice (d) that have been inoculated with BSMV:AsOsPDS2. The use of different methods of inoculation was not sufficient to improve the efficacy of VIGS in *Oryza sativa* ssp. *indica* cv. IR64 from the *indica* subgroup and *O. sativa* ssp. *indica* cv. Kasalath (represented by K) from the *aus* subgroup. No differences were observed between the non-inoculated control (e) and plants inoculated with different methods (f). The named BSMV vector was introduced by rub-inoculation of the third leaf of 21-day-old plants 6–10 times with ground leaves of *Nicotiana benthamiana* agroinfiltrated with BSMV:AsOsPDS2 and abrasive carborundum. Instead of carborundum, a device containing small metallic needles was used to wound the third leaf before the rubbing of BSMV:AsOsPDS2 sap. Inoculation by dipping and injection followed published procedures with adaptations. Photos of the fourth leaf (up next to the inoculated leaf) for rubbing, microneedles, and dipping and of the second leaf for injection.

investigated were inoculated with either tobacco-induced BSMV or FoMV viruses using a variety of methods. One possible explanation for the lack of BSMV- and FoMV-driven VERG phenotypes in rice is that *O. sativa* is known to exhibit multiple defence pathways against viruses (Qin et al., 2019). It is therefore possible that neither BSMV nor FoMV successfully infected rice to the degree required for VERG. Further work would be required to prove this hypothesis, but if true, scientists wanting to do transient functional genomics in rice would need to use alternative viruses as the VERG chassis. Alternative viral systems are available (reviewed in Kant & Dasgupta, 2019) and novel vectors specific to rice could be created. As we recognise the potential usefulness of VERG tools for research in *Oryza* species, we hope that by clearly outlining our failed experimental protocols and combinations, we can help others take alternative routes to deploying VERG protocols for *O. sativa* functional genomics research.

As explained above, successful VERG depends on a balance between the virus and the host that supports successful infection and either gene silencing or heterologous expression of the gene of interest. The first step in this process is infection of the host by the virus. Although rub inoculation is sufficient in wheat and other monocots (Bouton et al., 2018; Ellison et al., 2021; Lee, Hammond-Kosack, & Kanyuka, 2015; Mei et al., 2019; Mellado-Sánchez et al., 2020), we recognise that there are multiple inoculation techniques and buffer formulations commonly used for transient expression in rice (Andrieu et al., 2012; Ding et al., 2006, 2007; Huang et al., 2020; Kant et al., 2015; Kant & Dasgupta, 2017; Purkayastha et al., 2010). We tested many of these, and despite our efforts, none of these protocols induced the expected photobleaching phenotype in either *indica* or *aus* rice genotypes or were more effective than the standard rub inoculation (Mellado-Sánchez et al., 2024). We recognise that other protocols or combinations could have been tried, such as adding vacuum infiltration to the dipping protocols. Zhang et al. (2017) report successful tobacco rattle virus-mediated VIGS in wheat using co-cultivation with agroinfiltration in a novel infiltration solution applied under vacuum (Safitri et al., 2016; Zhang et al., 2017). However, this protocol relies on introducing the *Agrobacteria* carrying the viral vectors into the target plant, whereas our protocol introduces the virus via an inoculum induced in tobacco. Due to our lack of success, researchers looking to do VERG in rice may want to try different protocols or different combinations than what was described herein.

In accordance with our data, previous work showed FoMV was symptomless after mechanical inoculation in rice (Paulsen, 1977). These data align with ours, where no phenotype or clear gain-of-function phenotypes were observed after inoculation with FoMV:MCS or FoMV:GFP (Figure 2). The lack of phenotype in rice contrasts with the clear, systemic fluorescence seen with wheat or green foxtail (Figure 2). Our methods successfully replicated previous publications where GFP was induced in wheat (Bouton et al., 2018; Mei et al., 2016) and green foxtail (Ellison et al., 2021). These FoMV:GFP vectors also successfully induced fluorescence in blackgrass (Mellado-Sánchez et al., 2020) and *N. benthamiana* (Torti et al., 2021), demonstrating they can be applied to a variety of species. As explained above, we did not explore the molecular mechanisms for this lack of visible GFP in rice, and recognise that it could have been due to one or a combination of factors. Even if our protocols are successfully infecting rice with the FoMV:GFP, no functional GFP is being produced under our conditions in these rice cultivars, and therefore, the hypothesis that VOX could be successfully induced using these protocols was not supported. Moreover, we were unable to find any reports showing the use of viral vectors to induce heterologous expression in rice anywhere in the literature.

We recognise there are places in the literature that report BSMV is able to infect rice (Benedito et al., 2004). Figure 1 shows that even if BSMV is successfully infecting rice, the asOsPDS it carries is not leading to a VIGS response. Although we observed a change in the greenness of the rice leaves in the BSMV experiments, a remarkably similar phenotype was seen in the BSMV: MCS-treated plants, which lack the *PDS* sequence, and in the untreated controls (Figure 1b,e,f); these observations indicate that this phenotype is likely a non-specific or environmental response and unlikely to result from either BSMV infection or target-induced VIGS.

Working in wheat or in blackgrass Bouton et al. (2018) and Mellado-Sánchez et al. (2020) respectively demonstrated that not all biotypes have equivalent VERG responses, even if treated with the same vector under equivalent environmental conditions and protocols. We had hoped that by trialling different rice cultivars, we would find an equivalent cultivar-specific response in rice. However, Table 1 shows that none of the plants of O. *sativa* ssp. *indica* cv. IR 64, *O. sativa* ssp. *indica* cv. Kasalath, *O. sativa* ssp. *indica* cv. CO-39, *O. sativa* ssp. *japonica* cv. Kitaake, *O. sativa* ssp. *japonica* cv. Koshihikari, or *O. sativa* ssp. *japonica* cv. Balilla showed the expected VERG phenotypes. These varieties and subspecies are both experimentally and agronomically important. We therefore believe that our lack of VERG is not due to either cultivar- or subspecies-specific issues.

We recognise that others have reported successful VIGS in rice. However, these used vectors based on BMV (Ding et al., 2006) or rice tungro bacilliform virus (RTBV) (Purkayastha et al., 2010). Ding et al., (2006) used two strains of BMV to generate a modified BMV vector and inserted an 86 bp fragment of the *OsPDS* into it. This vector was then used to inoculate *O. sativa* ssp. *indica* cv. IR64 (Ding et al., 2006). However, as no untreated controls were shown, it is not possible to determine whether the reported phenotype was a result of *PDS* silencing or viral symptoms similar to what we observed (Figure 1). Similarly, Purkayastha et al. (2010) modified RTBV and tested it for its ability to silence *PDS*. They showed inoculation by injection—a method we unsuccessfully replicated (Figure 1e and Table 1)—into two cultivars of *indica* rice, which resulted in a weak streaking phenotype (Purkayastha et al., 2010). In contrast to the very clear and robust photobleaching we see in wheat (Figure 1), and that others have reported in other species (Mellado-Sánchez et al., 2020). our data also contradict the report of successful VIGS in *O. sativa* ssp. *japonica* cultivars Tatsuen No. 2, Taikeng No. 9, and Taoyuan No. 3 using a 441 bp sequence of *PDS* from barley that was cloned into a modified FoMV vector (Huang et al., 2020). While their FoMV vector differed from the one we tested, they employed a similar approach by infiltrating

Chenopodium quinoa and rub-inoculating rice seedlings at the 2-leaf stage. However, it was surprising that we were unable to find literature repeating or building on these successes, and we were unable to repeat the successful FoMV-enabled VIGS in rice.

As temperature plays a crucial role in the effectiveness of either VERG method, all our experiments were conducted in high-end, well-monitored, controlled environment rooms using conditions previously demonstrated to be ideal for BSMV and FoMV infection and VIGS and VOX efficacy (Bouton et al., 2018; Fei et al., 2021; Lee, Rudd, & Kanyuka, 2015; Mellado-Sánchez et al., 2020, 2024). The studies reporting successful VIGS in rice utilised standard growth conditions, including constant temperatures ranging from 25 to 28°C or a 25/20°C day/night regime, a 16/8-h light/dark photoperiod, and relative humidity levels exceeding 80% (Ding et al., 2007; Huang et al., 2020; Kant & Dasgupta, 2017). These conditions align with those employed in our study, suggesting that the observed ineffectiveness of the tested VERG protocols is unlikely to be due to environmental factors.

In addition to environmental conditions, it is well known that the efficacy of VIGS depends on the accuracy of the match between target and endogenous RNA (MacGregor, 2020). That said, systematic VIGS was successful in *N. benthamiana* using a wide range of heterologous inserts, indicating that a perfect match to the endogenous copy is not required, providing the viral vector can successfully infect and spread (Senthil-Kumar et al., 2007). Nevertheless, we used two rice-specific BSMV:asOsPDS vectors that targeted two different regions of the *PDS* cDNA. These regions were chosen as they target the same *PDS* regions previously shown to be highly efficacious in wheat (Lee, Rudd, & Kanyuka, 2015) or blackgrass (Mellado-Sánchez et al., 2020). They were also cloned in antisense, as the antisense orientation was shown to be more effective than those cloned in a sense direction (Zhang et al., 2010). Despite the controlled and optimised conditions, and although these newly created vectors efficiently induced photobleaching in wheat (Figure 1a), neither vector caused a characteristic photobleaching phenotype in rice (Figure 1b). We therefore do not believe that the lack of sequence specificity was the cause of our unsuccessful VIGS.

Together, these results indicate that neither of the BSMV- nor FoMV-based VERG systems we used provides a reliable platform for transient gene manipulation in the rice cultivars tested. Understanding the precise point at which these VERG interactions failed in rice would require follow-up research; such research should explore whether (1) insufficient viral infection, replication, or systemic movement of active virus through the plant, (2) activation of host antiviral defenses, or (3) an inappropriate RNAi response or failure to produce the heterologous protein was responsible for the lack of observable phenotype.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Andrieu, A., Breitler, J. C., Siré, C., Meynard, D., Gantet, P., & Guiderdoni, E. (2012). An in planta, *Agrobacterium*-mediated transient gene expression method for inducing gene silencing in rice (*Oryza sativa* L.) leaves. *Rice*, *5*, 23. https://doi.org/10.1186/1939-8433-5-23.

Beernink, B. M., & Whitham, S. A. (2023). Foxtail mosaic virus: A tool for gene function analysis in maize and other monocots. *Molecular Plant Pathology*, *24*, 811–822. https://doi.org/10.1111/mpp.13330.

Benedito, V. A., Visser, P. B., Angenent, G. C., & Krens, F. A. (2004). The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genetics and Molecular Research*, *3*, 323–341. https://pubmed.ncbi.nlm.nih.gov/15614725/.

Bouton, C., King, R. C., Chen, H., Azhakanandam, K., Bieri, S., Hammond-Kosack, K. E., & Kanyuka, K. (2018). *Foxtail mosaic virus*: A viral vector for protein expression in Cereals. *Plant Physiology*, *177*, 1352–1367. https://doi.org/10.1104/pp.17.01679.

Ding, X. S., Schneider, W. L., Chaluvadi, S. R., Mian, M. A. R., & Nelson, R. S. (2006). Characterization of a brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. *Molecular Plant-Microbe Interactions*, *19*, 1229–1239. https://doi.org/10.1094/MPMI-19-1229.

Ding, X. S., Srinivasa Rao, C., & Nelson, R. S. (2007). Analysis of gene function in rice through virus-induced gene silencing. In P. C. Ronald (Eds.), *Plant-pathogen interactions*, vol. 354 (pp. 145–160). Humana Press. https://doi.org/10.1385/1-59259-966-4.

Dommes, A. B., Gross, T., Herbert, D. B., Kivivirta, K. I., & Becker, A. (2019). Virus-induced gene silencing: Empowering genetics in non-model organisms. *Journal of Experimental Botany*, *70*, 757–770. https://doi.org/10.1093/jxb/ery411.

Ellison, E. E., Chamness, J. C., & Voytas, D. F. (2021). Viruses as vectors for the delivery of gene-editing reagents. In *Genome editing for precision crop breeding*. Burleigh Dodds Science Publishing. http://dx.doi.org/10.19103/AS.2020.0082.28.

Fei, Y., Pyott, D. E., & Molnar, A. (2021). Temperature modulates mirus-induced transcriptional gene silencing via secondary small RNAs. *New Phytologist*, *232*, 356–371.

Holzberg, S., Brosio, P., Gross, C., & Pogue, G. P. (2002). *Barley stripe mosaic virus*-induced gene silencing in a monocot plant. *The Plant Journal*, *30*, 315–327. https://doi.org/10.1046/j.1365-313X.2002.01291.x.

Huang, Y.-W., Chang, C.-Y., & Hsu, Y.-H. (2020). Virus-induced gene silencing in Poaceae using a *Foxtail mosaic virus* vector. In V. Courdavault & S. Besseau (Eds.), *Virus-induced gene silencing in plants: Methods and protocols* (pp. 15–25). Springer US. https://doi.org/10.1007/978-1-0716-0751-0_2.

Kant, R., & Dasgupta, I. (2017). Phenotyping of VIGS-mediated gene silencing in rice using a vector derived from a DNA virus. *Plant Cell Reports*, *36*, 1159–1170. https://doi.org/10.1007/s00299-017-2156-6.

Kant, R., & Dasgupta, I. (2019). Gene silencing approaches through virus-based vectors: Speeding up functional genomics in monocots. *Plant Molecular Biology*, *100*, 3–18. https://doi.org/10.1007/s11103-019-00854-6.

Kant, R., Sharma, S., & Dasgupta, I. (2015). Virus-induced gene silencing (VIGS) for functional genomics in rice using *Rice tungro bacilliform virus* (RTBV) as a vector. In K. S. Mysore & M. Senthil-Kumar (Eds.), *Plant gene silencing* (pp. 201–217). Springer. https://doi.org/10.1007/978-1-4939-2453-0_15.

Kanyuka, K. (2022). Virus-mediated protein overexpression (VOX) in monocots to identify and functionally characterize fungal effectors. In T. A. Kufer & M. Kaparakis-Liaskos (Eds.), *Effector-triggered immunity: methods and protocols* (pp. 93–112). Springer US. https://doi.org/10.1007/978-1-0716-2449-4_7

Killiny, N. (2019). Shrink the giant: scale down the citrus tree to a model system to investigate the RNA interference efficiency. *Plant Signalling and Behaviour*, *14*(7), 1612681. https://doi.org/10.1080/15592324.2019.1612681

Lacomme, C., Hrubikova, K., & Hein, I. (2003). Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. *Plant Journal*, *34*(4), 543–553. https://doi.org/10.1046/j.1365-313x.2003.01733.x

Lee, W.-S., Hammond-Kosack, K. E., & Kanyuka, K. (2012). *Barley stripe mosaic virus*-mediated tools for investigating gene function in cereal plants and their pathogens: Virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. *Plant Physiology*, *160*, 582–590. https://doi.org/10.1104/pp.112.203489.

Lee, W.-S., Hammond-Kosack, K. E., & Kanyuka, K. (2015). *In planta* transient expression systems for monocots. In K. Azhakanandam, A. Silverstone, H. Daniell, & M. Davey (Eds.), *Recent advancements in gene expression and enabling technologies in crop plants* (pp. 391–422). Springer. https://doi.org/10.1007/978-1-4939-2202-4_14.

Lee, W.-S., Rudd, J. J., & Kanyuka, K. (2015). Virus induced gene silencing (VIGS) for functional analysis of wheat genes involved in *Zymoseptoria tritici* susceptibility and resistance. *Fungal Genetics and Biology*, *79*, 84–88. https://doi.org/10.1016/j.fgb.2015.04.006.

Lindsey, B. E. III, Rivero, L., Calhoun, C. S., Grotewold, E., & Brkljacic, J. (2017). Standardized method for high-throughput sterilization of *Arabidopsis* seeds. *Journal of Visualized Experiments*, *128*, e56587. https://doi.org/10.3791/56587

MacGregor, D. R. (2020). What makes a weed a weed? How virus-mediated reverse genetics can help to explore the genetics of weediness. *Outlooks on Pest Management*, *31*, 224–229. https://www.ingentaconnect.com/content/resinf.

Mei, Y., Beernink, B. M., Ellison, E. E., Konečná, E., Neelakandan, A. K., Voytas, D. F., & Whitham, S. A. (2019). Protein expression and gene editing in monocots using foxtail mosaic virus vectors. *Plant Direct*, *3*, e00181. https://doi.org/10.1002/pld3.181.

Mei, Y., Zhang, C., Kernodle, B. M., Hill, J. H., & Whitham, S. A. (2016). A Foxtail mosaic virus vector for virus-Induced gene silencing in maize. *Plant Physiology*, *171*, 760–772.

Mellado-Sánchez, M., McDiarmid, F., Cardoso, V., Kanyuka, K., & MacGregor, D. R. (2020). Virus-mediated transient expression techniques enable gene function studies in black-grass. *Plant Physiology*, *183*, 455–459. https://doi.org/10.1104/pp.20.00205.

Mellado-Sánchez, M., Oddy, F., McGroary, P., Turra, G. M., & MacGregor, D. (2024). Infiltration and Rubbing with BSMV or FoMV Vectors for VMRG v1. https://dx.doi.org/10.17504/protocols.io.5jyl8drr6g2w/v1.

Pacak, A., Strozycki, P. M., Barciszewska-Pacak, M., Alejska, M., Lacomme, C., Jarmołowski, A., Szweykowska-Kulińska, Z., & Figlerowicz, M. (2010). The brome mosaic virus-based recombination vector triggers a limited gene silencing response depending on the orientation of the inserted sequence. *Archives of Virology*, *155*(2), 169–179. https://doi.org/10.1007/s00705-009-0556-9

Patterson, E., MacGregor, D. R., Heeney, M. M., Gallagher, J., O'Connor, D., Nuesslein, B., & Bartlett, M. E. (2025). Developmental constraint underlies the replicated evolution of grass awns. *New Phytologist*, *245*, 835–848. https://doi.org/10.1111/nph.20268.

Paulsen, A. Q. (1977). Purification and properties of foxtail mosaic virus. *Phytopathology*, *77*, 1346.

Purkayastha, A., Mathur, S., Verma, V., Sharma, S., & Dasgupta, I. (2010). Virus-induced gene silencing in rice using a vector derived from a DNA virus. *Planta*, *232*, 1531–1540. https://doi.org/10.1007/s00425-010-1273-z.

Qin, J., Wang, C., Wang, L., Zhao, S., & Wu, J. (2019). Defense and counter-defense in rice–virus interactions. *Phytopathology Research*, *1*, 34. https://doi.org/10.1186/s42483-019-0041-7.

Rössner, C., Lotz, D., & Becker, A. (2022). VIGS goes viral: How VIGS transforms our understanding of plant science. *Annual Review of Plant Biology*, *73*, 703–728. https://doi.org/10.1146/annurev-arplant-102820-020542.

Safitri, F. A., Ubaidillah, M., & Kim, K.-M. (2016). Efficiency of transformation mediated by *Agrobacterium tumefaciens* using vacuum infiltration in rice (*Oryza sativa* L.). *Journal of Plant Biotechnology*, *43*(1), 66–75. https://doi.org/10.5010/JPB.2016.43.1.66

Schoeman, I. (2011). Evaluation of two viral vectors for virus-induced gene silencing in pearl millet. Dissertation. Pretoria: University of Pretoria. Available at: https://repository.up.ac.za/handle/2263/31294 [Accessed November 4, 2024].

Senthil-Kumar, M., Hema, R., Anand, A., Kang, L., Udayakumar, M., & Mysore, K. S. (2007). A systematic study to determine the extent of gene silencing in *Nicotiana benthamiana* and other Solanaceae species when heterologous gene sequences are used for virus-induced gene silencing. *New Phytologist*, *176*, 782–791. https://doi.org/10.1111/j.1469-8137.2007.02225.x.

Sulaiman, M., Azman, A. S., & Zaidah, R. (2019). *Agrobacterium*-mediated transformation of rice: Constraints and possible solutions. *Rice Science*, *26*, 133–146. https://doi.org/10.1016/j.rsci.2019.04.001.

Torti, S., Schlesier, R., Thümmler, A., Bartels, D., Römer, P., Koch, B., Werner, S., Panwar, V., Kanyuka, K., von Wirén, N., Jones, J. D. G., Hause, G., Giritch, A., & Gleba, Y. (2021). Transient reprogramming of crop plants for agronomic performance. *Nature Plants*, *7*, 159–171. https://doi.org/10.1038/s41477-021-00851-y.

van Nugteren, I., Hein, I., & Berger, D. K. (2007). Development of a virus-induced gene silencing system in pearl millet (Abstract). *South African Journal of Botany*, *73*, 339. https://doi.org/10.1016/j.sajb.2007.02.201.

Yuan, C., Li, C., Yan, L., Jackson, A. O., Liu, Z., Han, C., Yu, J., & Li, D. (2011). A high throughput *Barley stripe mosaic virus* vector for virus induced gene silencing in monocots and dicots. *PLoS One*, *6*, e26468. https://doi.org/10.1371/journal.pone.0026468.

Yuan, C., Li, H., Qin, C., Zhang, X., Chen, Q., Zhang, P., Xu, X., He, M., Zhang, X., Tör, M., Xue, D., Wang, H., Jackson, S., He, Y., Liu, Y.,

Shi, N., & Hong, Y. (2020). *Foxtail mosaic virus*-induced flowering assays in monocot crops. *Journal of Experimental Botany*, *71*, 3012–3023. https://doi.org/10.1093/jxb/eraa080.

Zhang, C., Bradshaw, J. D., Whitham, S. A., & Hill, J. H. (2010). The development of an efficient multipurpose bean pod mottle virus viral vector set for foreign gene expression and RNA silencing. *Plant Physiology*, *153*, 52–65. https://doi.org/10.1104/pp.109.151639.

Zhang, J., Yu, D., Zhang, Y., Liu, K., Xu, K., Zhang, F., Wang, J., Tan, G., Nie, X., Ji, Q., Zhao, L., & Li, C. (2017). Vacuum and co-cultivation agroinfiltration of (germinated) seeds results in tobacco rattle Virus (TRV) mediated whole-plant virus-induced gene silencing (VIGS) in wheat and maize. *Frontiers in Plant Science*, *8*, 393. https://doi.org/10.3389/fpls.2017.00393.

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