WHAT MAKES A WEED A WEED? HOW VIRUS-MEDIATED REVERSE GENETICS CAN HELP TO EXPLORE THE GENETICS OF WEEDINESS

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Summary

Reverse genetics investigates what a gene does by testing how the plant responds when the specific gene is changed. These techniques have been in use for decades to assess whether a given gene underpins interesting phenotypes and gain insight into the function of gene networks and families. Weed science has only recently entered the "genomic era" in which genomic and reverse genetics approaches are used to address hypotheses. This review focuses on two reverse genetic techniques used on a variety of plants including agricultural weeds, virus-induced gene silencing (VIGS) and virus-mediated overexpression (VOX), explaining the biology behind them and highlighting how these tools may be used for gene function validation in weed species for which no other transgenic approaches have been developed.

Keywords: Herbicide resistance, reverse genetics, transient transformation, Virus-mediated Reverse Genetics (VMRG), Virus-induced Gene Silencing (VIGS), Virus-mediated Overexpression (VOX), weed molecular biology

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Why Do We Need Reverse Genetics and Genomics in Weeds?

Although weeds in agricultural fields may provide some benefits when the agroecosystem as a whole is considered, it is generally accepted that weeds in fields reduce yields (Oerke, 2006). To achieve high yields, farmers throughout the world rely heavily on agrochemical herbicides to provide very stringent weed control (Pimentel & Burgess, 2014). Since their introduction in the 1940s, herbicides have been key in our ability to control weeds and intensify agriculture. However, there is a steady increase in reports of herbicide resistant weed species appearing in agricultural fields (Heap, 2020). With every new report of an herbicide resistant species, it becomes evident that our over-reliance on herbicides has thwarted our ability to use them effectively (Beckie, 2020). Therefore, to fix the problem and develop sustainable and effective weed management strategies, we must understand how herbicide resistance(s) arose.

We know that weeds can evolve herbicide resistances by changing the protein targeted by the herbicide (target site resistance or TSR) or by avoiding, modifying, or detoxifying the herbicide itself (non-target site resistance or NTSR) (Gaines *et al.* 2020). It is also clear that these two mechanisms are not mutually exclusive, and many populations exhibit both types of resistance (Comont *et al.* 2020).

For several weed species, we have a good molecular-level understanding of TSR. Investigating TSR is straightforward as the thing that is broken, the protein whose function the herbicide is designed to interrupt, is known. The locations of mutated sites within the target proteins, how frequently these occur across populations, and how the resulting changes alter the interaction between the herbicide and target have been identified (reviewed in Gaines *et al.* 2020). These studies give us a molecular-level understanding of why the herbicide no longer inhibits protein function. But TSR mechanisms do not always completely explain how all the weeds in fields are surviving. This highlights the importance of NTSR mechanisms and data suggest NTSR is very widespread (reviewed in Powles & Yu, 2010).

To give future weed management strategies the best chance of working, they must take NTSR into account, particularly because NTSR can confer resistance to herbicides from different modes of action, extending, perhaps, to herbicides not yet even invented. NTSR encompasses all the ways that allow plants to survive herbicides other than changes to the protein target: including uptake, transport, and detoxification of the herbicide molecule. Therefore, to identify the underlying modification, all proteins involved in all these processes must be considered. Researchers have gone about this 'needle-in-a-haystack' search through various routes including comparing the proteomes and/or transcriptomes of herbicide-sensitive with herbicide-resistant plants. This holistic approach, where working systems are compared against broken systems, has worked well to identify potential genes that may underpin NTSR. But these lists are long and it is clear that all herbicide resistant populations do not share a single universal "molecular fingerprint" (Tétard-Jones *et al.* 2018). Therefore, these approaches only reveal correlations between genotypes and phenotypes, but do not establish causation.

If herbicide resistance were a broken machine with a list of potential parts that could be responsible for the problem, we would approach this list in one of two ways: either replace each part to see if it fixes the problem or break the same part in a working machine to see if the problem can be repeated. The

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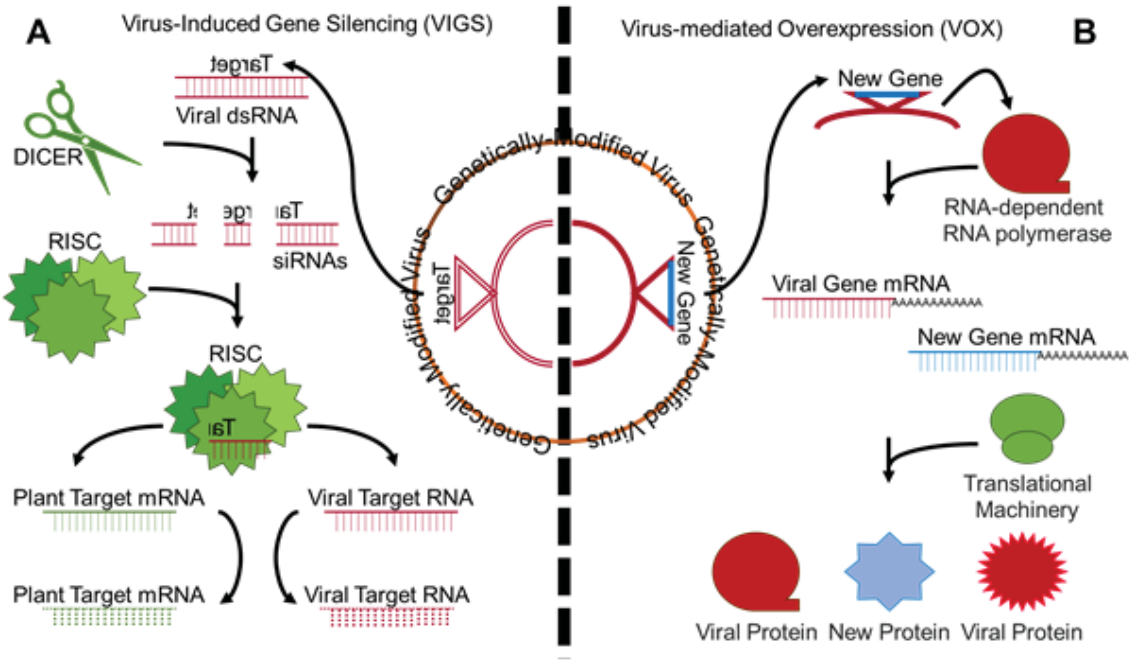

Figure 1. Virus-Induced Gene Silencing (VIGS) and Virus-mediated Overexpression (VOX) are two reverse genetics techniques for changing gene expression. Both use the systemic spread of a genetically-modified virus to ensure the change is manifested and propagated through the plant. For VIGS (A), the virus delivers the modified double stranded RNA (dsRNA) into the cell, which is recognised and all matching copies removed by the plant's RNA silencing pathway (RNAi). In RNAi, the plant ribonuclease DICER cuts the dsRNA into small interfering RNA (siRNA) which are used by the RNA-induced silencing complex (RISC) to find and degrade any matching RNA in the cell, even if it is the endogenous mRNA. VOX (B) uses the fact that during infection and replication, the virus hijacks the plants translational machinery to convert its own RNA into proteins. In VOX, the introduced gene coding sequence is also translated and processed into proteins upon entry, even if it encodes for a heterologous, or new, protein.

molecular biology equivalents of these are to rescue the aberrant phenotype by introducing a wild-type copy of the genetic sequence into the mutant background (as in MacGregor *et al.* 2013) or to isolate or generate a mutation in the gene to determine if it phenocopies the original (as in MacGregor *et al.* 2008). There are many different routes to accomplish these reverse genetic techniques in model or crop species, reviewed elsewhere (Gilchrist & Haughn, 2010; Pereira, 2012). Despite their proven usefulness in model and/or crop species, very few reverse genetics protocols have been applied to the study of agriculturally relevant weed species. This lack of methods to genetically modify weeds has meant that almost no functional validation of genes of interest has been done *in planta* and consequently little data exist to demonstrate that the genes correlated with NTSR cause the resistance phenotype. To continue the analogy, despite having a list of suspected parts, we have not been able to replace parts nor intentionally break others in normally functioning plants. Only by learning about the molecular components underpinning weediness and how they have been altered in resistant plants, will we be able to design weed management strategies that work despite TSR and NTSR and more importantly, do not encourage the development of new resistances in the future.

Focus on Virus-mediated Reverse Genetic Techniques

Virus-mediated reverse genetics (VMRG) techniques are tools that rely on plant–virus interactions to transiently alter gene expression. There are many different viruses that have been adapted for VMRG in wide range of plant species (Lange *et al.* 2013). In short, VMRG uses a virus that has been genetically-modified (GM) to either prevent protein production by degrading the plant's messenger RNA (mRNA) through the RNA silencing pathway (RNAi), or to introduce a gene coding sequence of interest into the plant so this protein is made. These two approaches are Virus-Induced Gene Silencing (VIGS) and Virus-mediated Overexpression (VOX) respectively (Figure 1). Both rely on systemic infections of the plant with the GM virus and result in gene expression changes that are usually seen in the new leaves (Blevins *et al.* 2006). VMRG take advantage of the fact that viruses cannot replicate themselves but instead direct the plant's cell machinery to replicate the viral genome and make the protein coat it is packed into so the resulting "virons" can be released to infect another cell. Exactly how they accomplish this depends on the virus type (Hull, 2014). These stages of replication, protein production, and spread, as well as the way that plants fight against this subjugation are key to VMRG.

Although both VIGS and VOX require successful viral infection, the different outcomes rely on different "winners" of the plant-virus competition – VIGS relies on the plant's ability to repress translation of viral genes, while VOX relies on the virus' ability to drive it. Some viruses can induce both VIGS and VOX (Lee *et al.* 2012), so the piece of DNA that is included into the viral genome and the plant's reaction to it determine whether the infection leads to a loss or gain of function (Figure 2). Inserting a full-length coding sequence favours VOX while VIGS occurs when just a portion of the gene is used. Computer programs are available that identify specific regions of mRNA that will be better at inducing VIGS (Lück *et al.* 2019) and inserting these portions in antisense rather

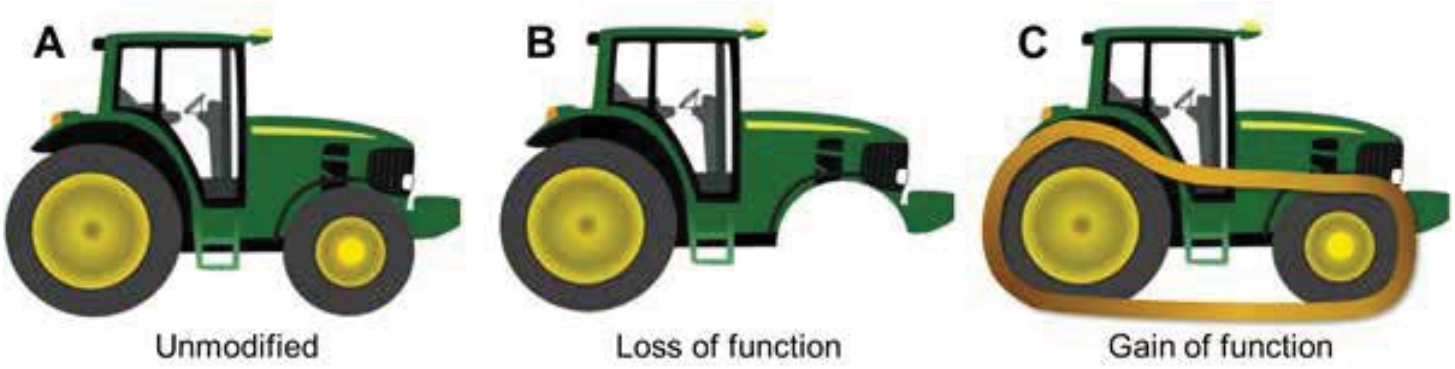

Figure 2. The aim of reverse genetics is to test a specific protein's function. To illustrate this concept, imagine an unmodified and properly functioning tractor (A) as a group of components working together to serve a function. If we hypothesize that the wheels are necessary for proper tractor function, we could design an experiment to test this by observing what happens when we make changes to the wheels. For example, we could use reverse genetics to introduce a 'loss of function' to remove a wheel (B) or a 'gain of function' to introduce a track (C). If the loss of function causes the tractor to malfunction, we have demonstrated the gene encoding for the front wheel is necessary for proper tractor function. On the other hand, if introducing a track gives the tractor better traction, we have demonstrated that adding a gene for a track was sufficient to improve the functionality of the front wheel.

than sense direction results in increased degradation efficiency (Singh *et al.* 2018).

The biggest drawback to both VIGS and VOX is that unlike stable transformations, where the genetic change is integrated into the plant's genome, neither the level nor uniformity of the change can be guaranteed with VMRG. Despite the many changes to viral genomes that have been made to increase the efficiency of VMRG (some of which are reviewed in Lange *et al.* 2013), VMRG viruses can only alter gene expression if they can infect the host and replicate to a sufficient level. Even then, their effects are limited to an area close to where they are replicating (Himber *et al.* 2003). Because VMRG effects are dependent on the plant cell's ability to fight the virus successfully (VIGS) or unsuccessfully (VOX), the degree to which the change occurs can vary between cells, leaves, tillers, plants, and consequently between experiments. The irregular distribution of the effects of VMRG means these techniques are best suited to visual, binary, or easily quantifiable phenotypes, like changes to herbicide or disease resistance (Lange *et al.* 2013).

Most relevantly to this audience, both VIGS and VOX can be applied to agriculturally relevant weeds and are useful for asking questions about what genes are necessary or sufficient for herbicide resistance. In addition to inducing the widely-accepted visual controls of photobleaching and gain of fluorescence (Figure 3, Mellado-Sánchez *et al.* 2020), VIGS against a gene involved in NTSR (Cummins *et al.* 1997; Tétard-Jones *et al.* 2018) was sufficient to revert the herbicide resistance phenotype of a particular well-known NTSR biotype of black-grass (*Alopecurus myosuroides*) (Mellado-Sánchez *et al.* 2020). Similarly, VOX was used to induce glufosinate resistance via heterologous expression of the *bar* resistance gene (Mellado-Sánchez *et al.* 2020). VIGS approaches have also been used for functional analysis of floral developmental regulators in the basal eudicot species California poppy (*Eschscholzia californica*), common columbine (*Aquilegia vulgaris*), opium poppy (*Papaver somniferum*), and *Thalictrum* species (reviewed in Lange *et al.*, 2013).

Virus-induced Gene Silencing (VIGS) History and Mechanism

Plants are not simply passive victims to viral infection but have developed pathways to fight viral invasions. One of the main molecular antiviral pathways is the RNA silencing or RNAi pathway (Figure 1). This pathway is also essential for regulating endogenous gene expression, forming heterochromatin and controlling transposons, as reviewed elsewhere (e.g. Brodersen & Voinnet, 2006). In short, this process requires two separate plant protein complexes and results in sequence-specific degradation of mRNA. The first protein complex cuts the viral RNA into pieces of 21–23 nucleotides (called short interfering RNA or siRNA) which are used by the second protein complex to find and destroy matching mRNA through complementary base pairing. Any time a match is made, the matching mRNA is degraded so the protein it encoded for cannot be made. As transcript degradation is driven by base pairing between target and siRNA, when plant mRNA is inserted into the viral genome, the endogenous mRNAs are targeted and host gene expression is down regulated (Kumagai *et al.* 1995). To return to the machine analogy (Figure 2), VIGS allows us to take a functioning system and specifically remove a single part to ask whether it is able to cause the expected phenotype. This is an efficient and effective antiviral system for plants because most plant virus species have an RNA genome (Romay & Bragard, 2017). Biologists have effectively co-opted this processto ask questions about genotype-phenotype relationships. As soon as it was identified that plants used RNAi to fight viruses, researchers started to apply this as a technique to investigate gene function (Kumagai *et al.* 1995). One of the first genes targeted this way was *PHYTOENE DESATURASE* (*PDS*, Kumagai *et al.* 1995, Figure 3). It is still routinely used as a positive control for VIGS largely because loss of *PDS* results in photobleaching of the affected area, and white leaves are an easily identifiable phenotype.

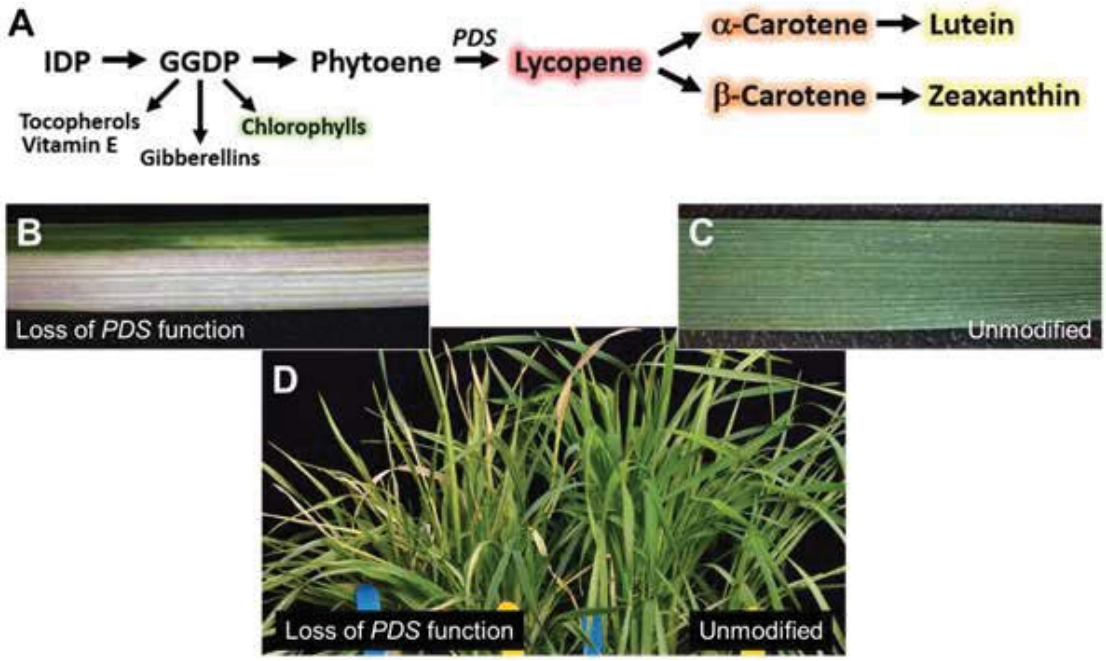

Figure 3. Silencing *PHYTOENE DESATURASE* (*PDS*) is commonly used as a positive control for VIGS experiments. PDS is an essential component of carotenoid biosynthesis, a simplified version of which is shown in A adapted from Guerinot (2000). Carotenoid biosynthesis is responsible for the coloured pigments chlorophyll, lycopene, α-carotene, β-carotene, lutein and zeaxanthin. Abbreviations IDP, isopentenyl-diphosphate; GGDP, geranylgeranyl diphosphate; *PDS, PHYTOENE DESATURASE*. Photobleaching, where the leaves turn white, occurs when PDS function is inhibited. This is shown at the scale of the individual leaf (B) or full plant (D left) compared to an unmodified leaf (C) or plant (D right). Figures B-D adapted from Mellado-Sánchez *et al.* 2020).

Virus-induced Overexpression (VOX) History and Mechanism

To be historically accurate, using viruses to drive protein production in plants came first even though the term VOX is relatively new. While VIGS leads to loss of function, VOX is a way to induce transient gain of function into the plant (Figure 1). Using the machine analogy (Figure 2), VOX allows us to introduce a single part and ask if it alters function. While the GM virus is using the plant's translational machinery to create the proteins it needs to replicate its genome and the outer protein shell that the new genomes go into, it also makes protein from the mRNA that the researchers have included in its genome. For successful VOX, the virus must be present to deliver the mRNA and drive translation, and therefore VOX viruses must be able to carry inserts large enough to make the protein of interest stably. Moreover, as is the case for any protein coding region, any changes in the sequence are likely to create malfunctional or non-functional proteins. Therefore, thesequence must be maintained accurately in its entirety for successful VOX.

One of the most interesting aspects of VOX is that it enables systemic heterologous gene expression. In other words, except for limits to the size of the protein that can be inserted, the protein sequence that is introduced can come from any organism or even be entirely synthetic. Although the introduction of foreign genes into plants is at the very heart of the GMO debate (Blancke *et al.* 2015), having this ability has been key to the study and creation of a plethora of useful phenotypes (Kamthan *et al.* 2016). Like VIGS, VOX is useful for binary and easily visible phenotypes, but unlike VIGS, because the inserted gene can be from another species, VOX can be used to give plants entirely new capabilities. Indeed, using VOX, researchers have been able to make crop plants (Bouton *et al.* 2018; Mei *et al.* 2019) and weeds (Mellado-Sánchez *et al.* 2020) that transiently fluoresce under UV light or survive high levels of the herbicide glufosinate.

Potential Applications and Implications of VMRG for Weed Science

The use of genetic modification and transgenic approaches have underpinned many of the advances used by modern day science. They have and will continue to provide solutions to our agricultural problems – by applying them to weeds as well as crops, we can better "know the enemy" and progress in the battle against weeds can be made more effectively and efficiently.

In the short term and within the confines of the laboratory, using reverse genetic tools on weed species will enable us to explore gene function *in planta*. The loss of function induced by VIGS allows researchers to ask if a specific component is necessary, while gain-of-function through VOX asks if it is sufficient (Figure 2). It is possible to make resistant plants sensitive, and sensitive plants resistant (Mellado-Sánchez *et al.* 2020). In black-grass, the VIGS-induced phenotypic change persists when individual tillers are separated and replanted (Mellado-Sánchez *et al.* 2020). This ability makes possible the potential for clonal analyses, which is essential for molecular biology and physiological experiments with monocot weeds. Moreover, like many weeds black-grass must be cross-pollinated (Sieber & Murray, 1979) and because of this, it exhibits high genetic diversity and low genetic differentiation

(Menchari *et al.* 2007); therefore separating out transiently transformed tillers is a unique opportunity to compare like genotypes directly to like.

The key advantages of VMRG are that they change the plant's behaviour within a matter of weeks using young plants grown in controlled laboratory conditions. VMRG therefore uncouples weed science experiments from agricultural fields and quickly leads to whole-plant phenotypes in response to the induced change. Together with the information generated from an increasing number of weed genomes that are becoming available (as described by Ravet *et al.* 2018) weed science will finally have a full parts list as well as the means to query function of those individual components.

In the longer term, one can imagine different scenarios where field-based applications of VMRG would be desirable; however, most will require a myriad of complex technical and regulatory issues to be addressed first. For instance, a population of black-grass with high levels of NTSR could be killed with a field-rate application of fenoxaprop after VIGS was used to knockout a key gene involved in the metabolic degradation of the herbicide (Mellado-Sánchez *et al.* 2020). To accomplish this, single plants were inoculated manually under strictly controlled laboratory conditions using a GM virus that is known to infect wheat and barley (Lee *et al.* 2012; Bouton *et al.* 2018), that had been adapted to reduce expression of a specific enzyme (Cummins *et al.* 1997; Tétard-Jones *et al.* 2018), which is part of a large family of genes that grass crops rely on to survive the herbicide application (Nakka *et al.* 2019). Therefore, it is impractical to think that this breakthrough will lead quickly to changes in how black-grass is treated in the field. Instead, reverse genetics experiments provide functional validation of the specific gene involved; they demonstrate that removing this critical component leads to a measurable reversion of the resistance phenotype or alternatively that providing another is enough to generate a new, more desirable phenotype. Reverse genetics experiments will therefore inform the development of diagnostics, new herbicides, and management strategies that are used to assess and fight herbicide resistance in the future.

Summary and Outlook

Weeds are among the most economically important groups of plant species. They, and the side-effects of our efforts to control them, result in major agronomic and environmental impacts. Although considerable progress has been made in understanding weeds from an ecological and/or agronomic perspective, relatively little progress in weed molecular biology has been made. Molecular biology provides us with the tools to investigate the regulation of specific genes and/or proteins in response to given stimuli and to determine how those proteins alter growth and development so the plant survives and reproduces successfully. The understanding gained from decades of molecular biology, and specifically reverse genetics, has been used to design crops that are able to survive abiotic and biotic stresses better. It is now time to adapt those tools to gain a molecular-level understanding of what is allowing the weeds to keep up. Tools like VMRG can specifically alter gene expression in species not typically seen inside of a molecular laboratory because they only require infection with the GM virus and a response to it. With these techniques we can undertake cause-and-effect studies to determine what impact a change in expression has on a given measurable phenotype at a whole-plant scale. Moreover, VMRG can do this within short times and sufficiently high through-puts to have real impact on today's problems. These tools can be applied to any phenotype that can be measured accurately, is not dependent on every cell or every leaf being altered, and where the change can be induced at the right developmental stage. Molecular techniques allow us to understand weed evolution in response to anthropogenic selection by agricultural weed management, and by understanding these processes, weaknesses will be identified that can be exploited in tomorrow's weed management practices. As annotated genomes provide a complete blueprint of the proteins that a weed can make, molecular techniques like VMRG give us the ability to alter proteins of interest and therefore determine what function it performs in giving weeds their weediness.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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