Silencing of Multiple Genes in Wheat Using *Barley Stripe Mosaic Virus*

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Virus-induced gene silencing (VIGS) is a powerful reverse genetics tool wherein the expression of a targeted gene is reduced by a viral vector in a sequence homology-dependent manner. A VIGS system using a modified Barley stripe mosaic virus (BSMV) has been developed and successfully employed in the functional characterization of genes in monocots. There are several inherent limitations to the BSMV-VIGS system including silencing variability and silencing transiency. These limitations make it critical to know where and when the targeted gene is silenced for efficient downstream functional analysis. One effective resolution to these limitations is the utilization of a silencing-reporter gene. In wheat the BSMV-VIGS of *phytoene desaturase* (*PDS*) results in a visible photobleaching silencing phenotype. Similarly, BSMV-VIGS of the leaf rust resistance gene, *Lr21* results in a leaf rust resistance-breaking silencing phenotype. Here we used BSMV-VIGS to simultaneously silence *PDS* and *Lr21* to test the utility of concurrently silencing two genes. When both *PDS* and *Lr21* were silenced in the same tissue at the same time, there was no compromise in viral infection efficiency, silencing efficiency, or silencing efficacy. The results discussed here suggest that *PDS* can be used as an endogenous silencing-reporter of a co-targeted gene.

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Introduction

There are two major strategies for functional analysis of genes in plants; gene over-expression and gene under-expression. Gene over-expression approaches rely on the generation of transgenic plants and is an unfavorable strategy in species where gene transformation is a challenge. Gene under-expression approaches such as gene knock-out generation by mutagenesis are valuable in some systems but have limited value in hexaploid wheat (*Triticum aestivum* L.) due to the functional redundancy that results from polyploidy. Further, knock-out approaches cannot be used for analysis of genes that have a lethal knock-out phenotype or embryogenesis related genes. In contrast, a virus-induced gene silencing (VIGS) approach, where a modified viral vector is used to silence genes, requires no transgenesis. Additionally, VIGS assays can knock-down genes temporarily in developed plants allowing analysis of genes that are essential for development. Finally, because VIGS does not knock-out but does knock-down target gene transcript level, this strategy can be used to study genes that would have lethal knock-out phenotypes. In addition, VIGS can target multiple gene homologs at the same time, thus is attractive for functional analysis of genes in polyploids such as wheat.

In VIGS, targeted sequences are silenced by host cell machinery in a sequence-dependent

manner. The viral genome and any endogenous host genes that are sufficiently homologous to the viral sequence are down regulated by the host through cytoplasmic mRNA degradation. It has been demonstrated that virus-induced gene silencing is propagated throughout the plant beyond the infected tissue. Systemic silencing beyond the movement of the viral particles indicates that host cell signaling is initiated in silenced plants (1) though the relationship between trigger and target is not fully understood.

The Hordeivirus, Barley stripe mosaic virus (BSMV) was first shown to induce gene silencing in barley (2, 3) and later in wheat (4). BSMV is a tripartite single-stranded positive sense RNA virus (5) with a host range that includes domesticated and wild monocots (6) as well as some dicots (2). The three BSMV genomes are designated alpha (α), beta (β), and gamma (γ). The genomes are packaged independently into helical particles and each genome is required for viral proliferation. The vector system was developed using recombinant clones of the ND18 stain of BSMV under a T7 bacteriophage promoter (2) allowing for the *in vitro* synthesis of infectious RNA transcripts (2, 3). The BSMV clones maintain the tripartite genome containing only the viral genes necessary for infection and replication and cloning sites in the γ construct.

BSMV-VIGS has been applied to investigate the *Lr21*-mediated leaf rust resistance pathway. The functional analysis of this pathway is important because leaf rust is one of the most economically important wheat diseases worldwide. Leaf rust is caused by the biotrophic fungal pathogen *Puccinia triticina*. Although host genetic resistance is the primary agricultural defense strategy against leaf rust disease, little is known about the molecular mechanisms of resistance. The complexity of the wheat genome and the absence of rust pathogens in model plant systems have been major obstacles in the study of leaf rust resistance. What is known is that the *Lr21* gene confers resistance to leaf rust by inducing a hypertensive response (HR) at the sites of fungal infection. The silencing of *Lr21* compromises leaf rust resistance through the loss of HR leading to fungal sporulation. BSMV-VIGS has been used to study the role of several genes, *Rar1*, *Sgt1*, and *Hsp90* (4), involved in the *Lr21*-mediated leaf rust resistance pathway.

Despite the advantages of BSMV-VIGS, there are limitations of the system including inconsistency of silencing, transiency of silencing, and a lack of a visually discernable silencing phenotype for many genes. The silencing induced by BSMV is not uniform throughout the tissue or from plant to plant. Additionally environmental factors such as temperature and light intensity play a large role in the timing of viral infection and subsequent gene silencing. A visually discernable silencing-reporter gene allows for the identification of silenced tissue. Ideally, silencing-reporter provides a visually identifiable phenotype whereby the silencing of a gene with a non-visually identifiable silencing phenotype can be estimated in terms of time and location. Additionally, a silencing-reporter must express a silencing-phenotype that does not confound the functional analysis of the gene of interest. The concurrent silencing of a reporter gene and candidate gene via VIGS has been demonstrated in dicots (1, 7, 8, 9, 10, 11, 12). Previous concurrent gene silencing studies were primarily qualitative and the effect that silencing of one gene has on the silencing of another gene remains unclear.

To investigate the efficiency and efficacy of concurrent gene silencing via BSMV-VIGS, we simultaneously targeted two genes that independently demonstrate obvious and well-described silencing phenotypes, a reporter gene, *phytoene desaturase* (*PDS*), and the disease resistance gene, *Lr21*. The commonly used endogenous silencing-reporter gene *PDS* was chosen because the silencing of this gene has been well characterized in wheat and has no effect on leaf rust resistance (4). PDS is an enzyme involved in the biosynthesis of carotenoids that protect chlorophyll from photooxidation and the silencing of *PDS* is observed as photobleaching in light-exposed photosynthetic tissue. The leaf rust resistance gene *Lr21* was chosen because the silencing of this gene has also been well characterized in wheat. The silencing of *Lr21* is observed as a leaf rust resistance-breaking phenotype (4). To investigate the concurrent silencing of these two genes we observed the silencing phenotypes and measured the associated target genes transcript levels by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).

The research presented here demonstrates that the BSMV-induced concurrent silencing of the silencing-reporter gene, *PDS* and the leaf rust resistance gene, *Lr21* in hexaploid wheat has efficiencies parallel to that of independent *PDS* and *Lr21* gene silencing.

Materials and Methods

Plant material:

Wheat cultivar Wichita (*lr21*) is susceptible and the near-isogenic line WGRC7 (*Lr21*) is resistant to the leaf rust pathogen *P. triticina*. Wheat seeds were germinated on filter paper at room temperature. At root radical emergence the seedlings were transferred to 98 deep-cone racks containing a soil mixture of 1:1 Sunshine mix: MSU soil mix and transferred to the greenhouse. The plants were maintained at a sixteen hour photoperiod with a daytime temperature of 25-30°C for the duration of growth at the Montana State University Plant Growth Center.

The BSMV vectors:

The BSMV vectors utilized in these experiments were obtained from Dr. Andrew O. Jackson at UC Berkeley (2). The γ vector was reconstructed to include PCR-ready cloning sites following a protocol modified from Holzberg et al. (3). It has been previously demonstrated that BSMV antisense inserts between 120-bp and 400-bp long with high homology to the target gene sequence induce optimal silencing (3, 4). Based on these parameters the same 185-bp *PDS* and 219-bp *Lr21* inserts as described by Scofield (4) were cloned into independent γ vectors.

BSMV inoculation:

Infectious RNA transcripts were synthesized by *in vitro* transcription using T7 RNA polymerase (New England Biolabs, Ipswich, MA) from linearized α, β, and γ plasmids (4). The BSMV inoculum was made by combining an equimolar ratio of α, β, and γ transcripts with excess inoculation buffer containing a wounding agent (FES). The concurrent silencing BSMV inoculum was made by combining the α:β:(γ*PDS*:γ*Lr21*) transcripts in a 2:2:(1+1) ratio with excess FES. The third leaf of each ten day old wheat seedling was inoculated with the synthesized RNA inoculum as described by Scofield (4). The plants were visually assessed for viral infection symptoms six days post-BSMV inoculation and again for virus infection symptoms and photobleaching ten days post-BSMV inoculation.

Leaf rust inoculation:

To evaluate leaf rust resistance, plants were inoculated with a *P. triticina* isolate PRTUS6 urediospore-oil suspension at the time point of established photobleaching (ten days post-BSMV inoculation). The inoculated plants were immediately transferred to an incubation dew chamber with high humidity and an air temperature of 15°C for eighteen hours. The plants were visually assessed for leaf rust infection type (IT) nine days post-*P. triticina* inoculation as per the standard IT classification (13)(Figure 1). The observed range of leaf rust IT for the control WGRC7 plants was 1 to 1^+. Based on the control plant IT range an observed infection type in WGRC7 higher than 1^+ was considered a resistance-breaking phenotype.

Transcript abundance analysis by qRT-PCR:

To analyze virus-induced changes in transcript abundance leaf tissue samples were collected at point the established photobleaching (ten days post-BSMV inoculation). The tissue samples were collected from the base of the leaf blade displaying photobleaching. Plants that displayed viral infection symptoms and subsequent silencing phenotypes were chosen from each treatment group and used for further analysis. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) and treated with *DNase* I (Invitrogen, Carlsbad, CA) as per the manufacture's protocol. The treated RNA was used as template to synthesize first-strand cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) as per the manufacture's protocol. Transcript abundance was quantified via real time-PCR performed on an iCycler and iQ5 system (Bio-Rad, Hercules, CA) using the SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene specific primers. The gene specific primers used to amplify *Actin* (*ACT*), *PDS*, and *Lr21* were: ACT forward 5'-AAATC-TGGCATCACACTTTCTAC-3', ACT reverse 5'GTCTCAAACATATCTGGG-TCATC-3', amplifying a 127-bp product, PDS forward 5'-A TGTCTTTAGCGTGCAAG-3', PDS reverse 5'-GATGATTTCGGTGTCACT-3', amplifying a 104-bp product, and LR21 forward 5'-GACCGACGAACGAAGATGAC-3', LR21 reverse 5'-AAAATGCAAACCGGG-ACTAA-3', amplifying a 107-bp product. In all cases primers were designed outside of the sequence insert used for silencing to ensure that transcript abundance measurements were based on the endogenous gene copies and not virus vectored transcripts. Transcript abundance was calculated using the ΔΔCt method as described in iCycler manual (Bio-Rad, Hercules, CA), where **fold change = $2^{-\Delta\Delta Ct}$** and **percent transcript abundance = fold change x 100**. The transcript abundance for each group was calculated as the average plus or minus the average difference between the average and the highest and lowest value in that group.

Results and Discussion

Transcript abundance of target genes:

To assess the effects of the mechanical damage induced by the FES inoculation buffer, the oil suspension, and the eighteen hour dew chamber incubation had on the transcript abundance of the target genes, we measured the *PDS* and the *Lr21* transcript levels of twenty individual plants from each treatment group of both Wichita and WGRC7. The native transcript levels of each genotype were measured in plants without any viral inoculation, rust inoculation, or dew chamber incubation. The variation in native transcript levels in all groups was 20-24% in *PDS* and 14-25% in *Lr21* (Table 1). This suggests that only changes in transcript level greater than 25% can be reliably

Table 1. Transcript abundance post-mechanical damage with and without *P. triticina* inoculation

Genotype	Leaf Rust Inoculation	Wounding Buffer Inoculation	Average Percent *PDS* [a]	Average Percent *Lr21* [b]
Lr21	-	-	100 ± 20	100 ± 14
Lr21	-	+	110 ± 18	94 ± 7
Lr21	+	-	159 ± 54	132 ± 8
lr21	-	-	110 ± 25	100 ± 25
lr21	-	+	119 ± 16	98 ± 17
lr21	+	-	93 ± 12	119 ± 17

[a] Average percent *PDS* transcript abundance calculated as $2^{-\Delta\Delta Ct}$ using the average for the no rust, no wounding buffer inoculation control group and treatment group ± standard deviation. [b] Average percent *Lr21* transcript abundance calculated as $2^{-\Delta\Delta Ct}$ using the average for the no rust, no wounding buffer inoculation control group and treatment group ± standard deviation. Each genotype compared with the control group of that genotype listed as the first row in each section.

associated with any treatment. After mechanical damage induced by inoculation with FES buffer the *PDS* transcript level increased 8% above non-treatment control plus the standard deviation in WGRC7 and 11% above non-treatment control plus the standard deviation in Wichita (Table 1). There was no change in the transcript level of *Lr21* (in WGRC7) or *lr21* (in Wichita) after mechanical damage induced by inoculation with FES buffer (Table 1). In WGRC7, the transcript level of *PDS* increased after *P. triticina* inoculation up to 93% above the non-treatment control plus the standard deviation and the transcript level of *Lr21* increased up to 26% above non-treatment control plus the standard deviation (Table 1). In Wichita, however, there was no change in *PDS* transcript level after *P. triticina* inoculation but the transcript level of *Lr21* increased up to 11% above the non-treatment control plus the standard deviation (Table 1). Increased *PDS* transcript abundance post-fungal inoculation has also been observed by Fofana and co-workers (2007). They found that photosynthesis-related genes were differentially regulated between susceptible and resistant plants by twenty-four hours post-inoculation (14). *P. triticina* spores penetrate wheat leaf stroma and produce primary infection hypha during the first twenty-four post-inoculation (15). It has been hypothesized that the reduction in photosystem II-related components leads to an interruption in the electron transport chain and subsequently to electron leakage and formation of reactive oxygen species (ROS) that are known signals of defense response pathways (14, 16).

We observed a lower density of rust pustules on the photobleached tissue compared to non-photobleached tissue in both resistance-breaking tissues and the susceptible control tissues. The reason for this difference in disease development in photobleached and green tissue is unclear; however it is constantly observed and independent of the action of *Lr21*-mediated resistance.

Efficiency of silencing multiple targets:

To test the efficiency of silencing multiple targets using γ vectors carrying different target inserts, we inoculated seedlings of WGRC7 with BSMV containing only *PDS* (γ*PDS*), or only *Lr21* (γ*Lr21*), or with both *PDS* and *Lr21* (γ*PDS*:γ*Lr21*). At least twenty plants were inoculated in each treatment group. Viral infection symptoms were observed six days post-inoculation indicating successful viral infection. All of our virally inoculated groups demonstrated similar infection efficiencies independent of if the γ vector contained an insert and the identity of the insert (Table 2). The infection efficiency

Table 2. Viral infection and gene silencing efficiencies

Genotype	Vector [a]	Infection Efficiency [b]	*PDS* Silencing Efficiency [c]	*Lr21* Silencing Efficiency [d]
Lr21	γ00	80		
Lr21	γ*PDS*	80	88	
Lr21	γ*Lr21*	81		100
Lr21	γ*PDS*:γ*Lr21*	82	100	100
lr21	γ00	90		
lr21	γ*PDS*	100	100	

Each inoculation was performed with a complete BSMV genome (α, β, and γ). [a] γ vector and gene insert used for each group, γ00 indicates a functional virus with a γ vector containing no target gene insert. [b] Infection efficiency calculated as the **(number of plants with visible viral infection / total number of plants inoculated with BSMV containing that insert(s)) x 100**. [c] *PDS* silencing efficiency calculated as the **(number of plants with visible photobleaching / number of plants with visible viral infection inoculated with BSMV containing that insert(s)) x 100**. [d] *Lr21* silencing efficiency calculated as the **(number of plants with resistance-breaking phenotype / number of plants with visible viral infection inoculated with BSMV containing that insert(s)) x 100**.

using an empty γ vector (γ00), γ*PDS*, γ*Lr21*, or γ*PDS* and γ*Lr21* together was between 80-100% (Table 2). We observed a correlation between viral infection and subsequent gene silencing in all inoculated groups. As expected, if no viral infection symptoms were observed no subsequent gene silencing phenotype was observed. Based on this observation, we know viral infection and replication are essential for the induction of gene silencing. Importantly, however, the observation of viral infection symptoms did not guarantee target gene silencing. When *PDS* alone was targeted with γ*PDS*, 87% of the plants showing viral infection symptoms demonstrated gene silencing observed as photobleaching (Table 2). When *Lr21* alone was targeted with γ*Lr21*, 100% of the plants showing viral infection symptoms demonstrated gene silencing observed as a leaf rust resistance-breaking phenotype (Table 2). When *PDS* and *Lr21* where targeted simultaneously with γ*PDS*:γ*Lr21*, 100% of the plants showing viral infection symptoms demonstrated *PDS* and *Lr21* gene silencing observed as photobleaching and a leaf rust resistance-breaking phenotype (Table 2).

To assess the target gene transcript levels associated with the silenced phenotypes in independent and concurrent silencing events, we performed qRT-PCR as described above. We observed a change in transcript abundance of *PDS* and *Lr21* when plants were infected with the γ00 and accordingly used this as our control group. All transcript levels are reported as a percent change from the transcript abundance observed in plants inoculated with the γ00. In silenced plants targeted with *PDS* alone, *PDS* transcript levels were 12-88% of the *PDS* transcript level in plants inoculated with the γ00 (Table 3). The *PDS* transcript level in γ*PDS*:γ*Lr21* inoculated plants ranged from 14-65% of the *PDS* transcript level inoculated with the γ00 (Table 3). In silenced plants targeted with *Lr21* alone (γ*Lr21*), *Lr21* transcript levels were 52-74% of the *Lr21* transcript level in plants inoculated with the γ00 (Table 3). The *Lr21* transcript level in γ*PDS*:γ*Lr21* silenced plants ranged from 48-86% of the *Lr21* transcript level in the plants infected with γ00. These results suggest that similar silencing levels can be achieved in one and two gene targeting events (Table 3).

Co-silencing of *PDS* with a target gene has been achieved with a single viral vector containing a fused fragment of *PDS* and the target gene (9). This strategy has limitation for BSMV-VIGS

Table 3. Transcript abundance post-BSMV-VIGS

Genotype	Leaf Rust Inoculation	Vector [a]	Average Percent *PDS* [b]	Average Percent *Lr21* [c]
Lr21	+	FES	100 ± 7	100 ± 13
Lr21	+	γ00	106 ± 7	108 ± 4
Lr21	+	γ00	100 ± 7	100 ± 4
Lr21	+	γ*PDS*:γ*Lr21*	35 ± 26	63 ± 19
Lr21	+	γ*Lr21*	105 ± 17	71 ± 14
Lr21	+	γ*PDS*	49 ± 38	112 ± 13

Each inoculation was performed with a complete BSMV genome (α, β, and γ). [a] γ vector and gene insert used for each group, γ00 indicates a functional virus with a γ vector containing no target gene insert, FES indicates inoculation with only the wounding buffer. [b] Average percent *PDS* transcript abundance calculated as $\mathbf{2}^{-\Delta\Delta Ct}$ using the average for the no rust, no wounding buffer inoculation control group and treatment group ± standard deviation. [b] Average percent *Lr21* transcript abundance calculated as $\mathbf{2}^{-\Delta\Delta Ct}$ using the average for the no rust, no wounding buffer inoculation control group and treatment group ± standard deviation.

because the virus tends to lose or partially delete inserts over a certain length. Bruun-Rasmssen and associates showed that BSMV lost a 584-bp *PDS* fragment 14 days post-inoculation and partially deleted an insert of approximately 400-bp (17). Our strategy of using a mixture of two BSMV γ constructs containing single inserts shorter than 400-bp resulted in effective co-silencing of both targets. These results suggest that our strategy is an improvement allowing for increased insert stability.

Localization of silencing of multiple targets:

The location of the photobleaching and leaf rust resistance-breaking silencing phenotypes partially overlapped in silenced plants targeted with γ*PDS*:γ*Lr21* with resistance-breaking observed towards the leaf tip and photobleaching observed towards the leaf base (Figure 1). To investigate the differences in tissue where only one or both of the silencing phenotypes were observed, we measured the transcript abundance of the target genes in these locations. Leaf tissue was collected in one inch increments starting at the base of the leaf blade to yield three tissue collections, each representing the areas where photobleaching, photobleaching and resistance-breaking or resistance-breaking phenotypes were observed. The transcript abundance of the target genes in the three segments was measured by qRT-PCR as described above. Interestingly, both *PDS* and *Lr21* transcript levels were reduced in the tissue segments showing only the resistance-breaking phenotype. It has been observed that the *PDS* gene product is very stable once produced (18) potentially explaining why the reduction of *PDS* transcript abundance is observed earlier than the photobleaching phenotype is observed. This result agrees with our previous observation that resistance-breaking was not coincident with the area of maximum photobleaching (4) when *PDS* and *Lr21* were silenced separately. Previously, we inoculated silenced plants with *P. triticina* nine days post-BSMV inoculation when the first sign of photobleaching was visualized, before the leaf blade displaying photobleaching was fully expanded and therefore less likely to catch the rust spore inoculum (4). In this experiment we inoculated the silenced plants with *P. triticina* when the photobleaching was well established to ensure that the photobleached tissue would catch the rust spore inoculum allowing us to observe the two silencing phenotypes simultaneously. BSMV-VIGS is a transient silencing of a target gene that lasts approximately twenty-four days after viral

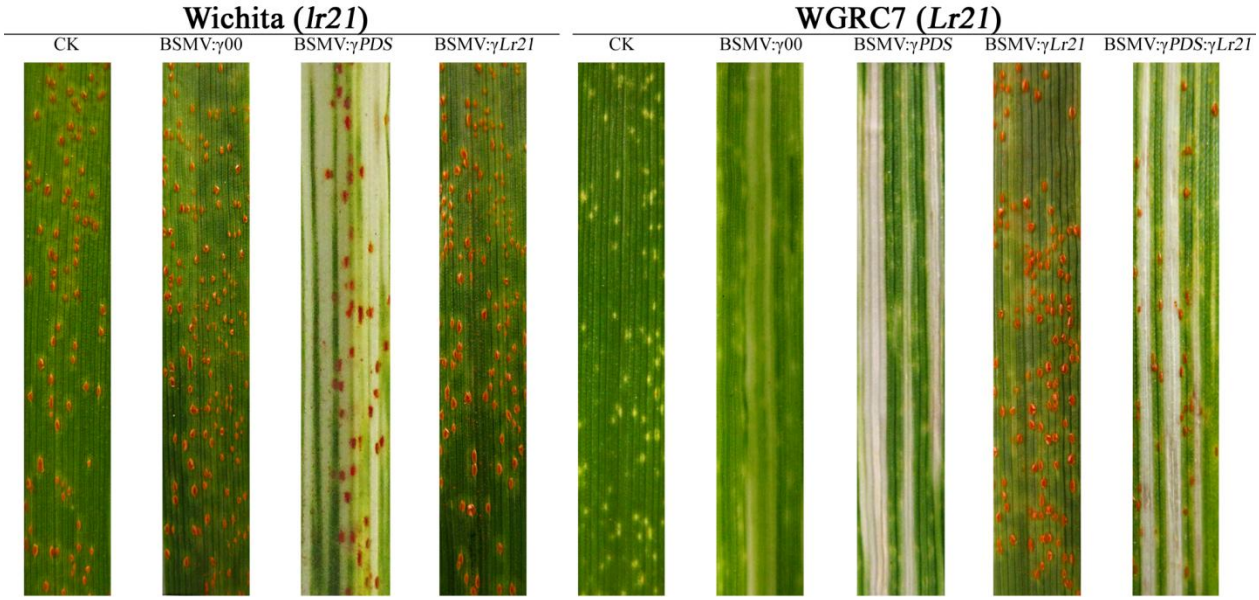

Figure 1. Wheat leaf infection type response to *P. triticina*. Susceptible, Wichita (*lr21*), and resistant, WGRC7 (*Lr21*), plants with no treatment (CK) or treated with BSMV vector containing indicated insert(s) and then challenged with *P. triticina*. Photographs taken nine days post- *P. triticina* inoculation; twenty-one days post-BSMV inoculation.

inoculation. This transiency makes the timing and location of functional challenge crucial for the successful characterization of the target gene. Co-silencing *PDS* with a target gene provides an internal silencing-reporter that is very useful for identifying the time and location of silencing. *PDS* may not be the ideal silencing-reporter for the investigation of target genes that are affected by the *PDS* expression but it may be ideal for use in the study of the *Lr21*-resistance pathway.

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