Use of virus-induced gene silencing to characterize genes involved in modulating hypersensitive cell death in maize

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Abstract
Plant disease resistance proteins (R-proteins) detect specific pathogen-derived molecules, triggering a defence response often including a rapid localized cell death at the point of pathogen penetration called the hypersensitive response (HR). The maize Rp1-D21 gene encodes a protein that triggers a spontaneous HR causing spots on leaves in the absence of any pathogen. Previously, we used fine mapping and functional analysis in a Nicotiana benthamiana transient expression system to identify and characterize a number of genes associated with variation in Rp1-D21-induced HR. Here we describe a system for characterizing genes mediating HR, using virus-induced gene silencing (VIGS) in a maize line carrying Rp1-D21. We assess the roles of 12 candidate genes. Three of these genes, SGT1, RAR1, and HSP90, are required for HR induced by a number of R-proteins across several plant–pathogen systems. We confirmed that maize HSP90 was required for full Rp1-D21-induced HR. However, suppression of SGT1 expression unexpectedly increased the severity of Rp1-D21-induced HR while suppression of RAR1 expression had no measurable effect. We confirmed the effects on HR of two genes we had previously validated in the N. benthamiana system, hydroxycinnamoyltransferase and caffeoyl CoA O-methyltransferase. We further showed the suppression the expression of two previously uncharacterized, candidate genes, IQI calmodulin binding protein (IQM3) and vacuolar protein sorting protein 37, suppressed Rp1-D21-induced HR. This approach is an efficient way to characterize the roles of genes modulating the hypersensitive defence response and other dominant lesion phenotypes in maize.

Keywords
hypersensitive response, maize, VIGS
1 | INTRODUCTION

The mechanisms underlying programmed cell death (PCD) in plants are complex and include several interacting pathways involved in plant development and disease resistance (Kuriyama and Fukuda, 2002; Williams and Dickman, 2008; Reape et al., 2015; Bruggeman et al., 2015; Dickman et al., 2017). The hypersensitive response (HR) is a specific form of PCD that is characterized by rapid, localized cell death at a point of pathogen infiltration or damage (Wu and Baldwin, 2010; Coll et al., 2011; Balint-Kurti, 2019). It is mediated by dominant resistance (R)-proteins, which are activated in response to the detection of specific pathogen-derived effector proteins (Bent and Mackey, 2007). This activation leads to a so-called effector-triggered immunity (ETI) response that often, though not always (Künstler et al., 2016; Laflamme et al., 2020), includes HR. Other than HR, the ETI response is characterized by several physiological changes, including cell wall strengthening, ion fluxes, changes in phenylpropanoid biosynthesis, and oxidative burst (Kosack and Jones, 1996). Because inappropriate activation of HR is potentially devastating for the host, R-proteins must be tightly controlled so that in the absence of the pathogen they remain in an inactive form that can be rapidly activated when the presence of the cognate pathogen effector is detected (Balint-Kurti, 2019; Wang et al., 2019). Pathogens can also be recognized through detection of conserved microbe-associated molecular patterns (MAMPs) by surface-located receptors called pattern recognition receptors (PRRs). MAMP recognition leads to activation of the MAMP-triggered immunity (MTI) response, which is qualitatively similar to ETI though quantitatively lower, usually not involving an HR (Newman et al., 2013). Recent studies suggest that a functional MTI response is important to enable a full ETI response (Ngou et al., 2020; Yuan et al., 2020).

Most R-proteins possess nucleotide-binding site motifs and leucine-rich repeat domains and are consequently known as NLRs. Within this class of proteins, two major groups exist, defined by whether they possess a coiled-coil (CC) domain or a Toll/interleukin-1 receptor (TIR) domain at their N-terminus. The Rp1 locus in maize is a complex locus encoding multiple tandemly repeated NLR paralogs, several of which confer resistance to specific races of Puccinia sorghi, the causal agent of maize common rust (Hulbert, 1997). Unequal crossover events at this locus are relatively frequent (Hulbert and Bennetzen, 1991). The Rp1-D21 allele is the product of an intragenic recombination event between two Rp1 paralogs, Rp1-D and Rp1-dp2. It encodes a protein that can be spontaneously activated without pathogen recognition and hence causes HR lesions on the leaves of maize plants in the absence of pathogen (Hu et al., 1996; Collins et al., 1999; Smith et al., 2010; Wang et al., 2015b). The severity of the HR conferred by Rp1-D21 is modulated by genetic background as well as by various environmental factors (Hu et al., 1996; Smith et al., 2010; Chaikam et al., 2011; Negeri et al., 2013).

We identified 44 loci associated with variation in the Rp1-D21-induced HR phenotype using genome-wide association (GWA) analysis in the maize nested association mapping (NAM) population (Olukolu et al., 2013). The NAM population is a particularly powerful mapping resource because it is large (5,000 lines), captures a large number of recombination events, and is very densely genotyped (Yu et al., 2008; McMullen et al., 2009). This means that quantitative trait loci (QTLs) can be mapped with very high precision, often to single-gene resolution. We were able to identify candidate causal genes at many of the 44 loci and, in several cases, have verified their effects on HR caused by Rp1-D21 using techniques including transient coexpression in Nicotiana benthamiana, physical association assays including coimmunoprecipitation and bimolecular fluorescence complementation (BiFC) with Rp1-D21 and, in one case, the analysis of a maize line in which the gene was overexpressed due to a nearby transposon insertion (Wang et al., 2015a; Wang and Balint-Kurti, 2016; He et al., 2019a, 2019b). In many cases it is likely that the genes modulating Rp1-D21-mediated HR are also involved in modulating the wild-type defence response. Using the phenotype conferred by Rp1-D21 as a reporter has made the identification of these important components of the defence response more straightforward. One of the main weaknesses of these studies was that the main validating evidence was generated in an ectopic system using agroinfiltration of N. benthamiana because we did not have the ability to easily modulate gene expression in maize.

In many respects maize is an ideal system for genetic research, with abundant publicly available experimental resources (Nannas and Dawe, 2015). The one area where the maize system is less tractable than other plant model genetic systems such as Arabidopsis, rice, and tomato is reverse genetics. With the burgeoning reverse genetics resources for maize genome mapping and analysis, the identification of candidate genes underling traits of interest is becoming easier and more accurate, exacerbating the bottleneck created by the lack of resources for reverse genetics.

Reverse genetics resources are more readily available in several other plant genetic systems. Whereas Arabidopsis, tomato, and rice are relatively easily transformable in any laboratory skilled in tissue culture and molecular biology, maize transformation requires specialized explants and tissue culture techniques, and can only be achieved in certain genetic backgrounds (Yadava et al., 2017). As such, maize transformation is largely limited to a few service laboratories in the public sector, which are necessarily rather costly and can take up to 4–6 months to produce a transgenic line. It should be noted that new technologies promise to improve this situation (Jones et al., 2019), but they are not yet in the public domain. The recent availability of CRISPR/Cas9 technology in maize is revolutionizing the field by allowing us to make targeted genome edits (Svitashev et al., 2016; Liu et al., 2020) but is still limited by a relatively inefficient transformation system.

Populations of sequence-indexed mutant maize lines also exist, the most extensive and available of which is the UniformMu population, which consists of a population of 14,024 stocks in a uniform background of the W22 maize line, each of which carries a number of unique, stabilized transpositions of the Mu transposable element. The transposition sites have been sequenced and together the population carries Mu insertions in or near c.42% of maize genes. However, only about half of these are in the protein coding
sequences (McCarty et al., 2018) meaning that loss-of-function alleles for most maize genes are not available.

In this study, we report the use of a recently developed system for virus-induced gene silencing (VIGS) in maize, based on foxtail mosaic virus (FoMV) (Mei et al., 2016; Mei and Whitham, 2018), to characterize genes associated with Rp1-D21 activity. We developed a quantitative screen for alteration of Rp1-D21-induced HR and used it to characterize the effects of 12 genes. These included the four genes whose effects we had previously validated using the N. benthamiana system (HCT, CCoAOMT, QCR7, and PGH1), other candidates previously identified from GWA analysis (IQM3, VPS37, Pk1b, SL11, LOX9), and several genes previously shown to modulate NLRs in other systems (SGT1, HSP90, RAR1). The FoMV-VIGS system produced robust evidence that validated some of our previous work and further elucidated the mechanisms that control HR in maize.

2 | RESULTS AND DISCUSSION

2.1 | Reduction of Rp1-D21 transcript levels using foxtail mosaic virus-mediated VIGS

The maize Rp1-D21 protein is a chimeric autoactive NLR that triggers spontaneous HR in the absence of a pathogen. A previous genome-wide association study (GWAS) analysis in maize precisely mapped 44 loci associated with variation in Rp1-D21-induced HR and tentatively identified candidate genes underlying these effects (Olukolu et al., 2014). Follow-up studies validated the roles of two of the candidate genes encoding the enzymes HCT and CCoAOMT, which catalyse consecutive steps in the lignin biosynthesis pathway (Wang et al., 2015a; Wang and Balint-Kurti, 2016). The most important aspect of these studies was the observation that specific members of the maize HCT and CCoAOMT gene families (Table 1) suppressed Rp1-D21-induced HR in N. benthamiana transient expression assays. Using similar approaches we subsequently validated the effects on Rp1-D21 activity of two further candidate genes identified in the original GWAS study: a polygalacturonase, PGH1 (He et al., 2019a), and a gene predicted to encode a small protein, QCR7, forming part of the cytochrome b-c1 complex (He et al., 2019b). These studies were performed in an ectopic system because we lacked the ability to conveniently manipulate gene activity in maize.

A maize VIGS system, based on FoMV, was recently shown to be capable of efficiently suppressing the transcript level of specific genes of interest like phytoene desaturase (PDS), which causes photobleaching of leaves (Mei et al., 2016). This system was significantly more effective than the previously reported maize VIGS system based on brome mosaic virus (Ding et al., 2006; Benavente et al., 2012). We used this system for the further characterization of genes associated with the modification of the HR phenotype conferred by Rp1-D21.

### TABLE 1 List of candidate genes associated with variation in the severity of Rp1-D21-induced hypersensitive response (HR)

| Gene ID          | Annotation                              | Silencing level (fold) | Effect on Rp1-D21 | References                      |
|------------------|-----------------------------------------|------------------------|-------------------|---------------------------------|
| Rp1-D21-middle   | Rp1-D21                                 | 3.14                   | Suppress          | Olukolu et al. (2014); Wang et al. (2015) |
| GRMZM2G012631a   | HSP90                                   | 16.83                  | Suppress          | Olukolu et al. (2014)          |
| GRMZM2G017616    | Lipoxigenase9 (LOX9)                    | NS                     | –                 | Olukolu et al. (2014)          |
| GRMZM2G023575    | Modifier of rudimentary protein (VPS37) | 1.63                   | Suppress          | Olukolu et al. (2014)          |
| GRMZM2G061806    | Hydroxycinnamoyl-CoA shikimate/quinate (HCT) | 10.52 | Enhance          | Olukolu et al. (2014); Wang et al. (2015) |
| GRMZM2G099363    | Caffeoyl-CoA O-methyltransferase (CCoAOMT) | 4.16 | Enhance          | Olukolu et al. (2014); Wang and Balint-Kurti (2016) |
| GRMZM2G105019b   | SGT1                                    | 4.61                   | Enhance           | Olukolu et al. (2014); He et al. (2019) |
| GRMZM2G135763    | Polygalacturonase (PGH1)                | 2.46                   | None              | Olukolu et al. (2014); He et al. (2019) |
| GRMZM2G144042    | Protein kinase 1b (Pk1b)                | NS                     | –                 | Olukolu et al. (2014)          |
| GRMZM2G318346    | Cytochrome bd ubiquinol oxidase (QCR7)  | 2.08                   | None              | Olukolu et al. (2014); He et al. (2019) |
| GRMZM2G351387    | Spotted leaf 11/plant U-box 13 (SL11)  | NS                     | –                 | Olukolu et al. (2014)          |
| GRMZM2G439311c   | IQ calmodulin-binding motif family protein (IQM3) | 7.87 | Suppress          | Olukolu et al. (2014)          |
| GRMZM5G868908    | RAR1                                    | 3.93                   | None              | Olukolu et al. (2014)          |

NS, not significant.

aThe PCR product used for virus-induced gene silencing (VIGS) was derived from GRMZM2G012631 but the resulting construct could additionally target four other homologs in the genome.

bThe PCR product used for VIGS was derived from GRMZM2G105019 but the resulting construct could target two homologs in the genome.

cGene annotation is not correct in B73 genome v 4. We designed the primers based on our sequence from amplified PCR product.
First, we used the FoMV VIGS system to silence Rp1-D21 itself in B73:Rp1-D21. A 469-bp fragment corresponding to the leucine-rich repeat (LRR) region of Rp1-D21 was inserted in the antisense orientation at the XbaI and XhoI cloning sites of pFoMV-V (Table 1) to create the pFoMV-Rp1-D21 construct designed to suppress expression of Rp1-D21. Lesions caused by spontaneous HR were visible on the first and second leaves of B73:Rp1-D21 plants c.10 days after germination. Significant reduction of HR in B73:Rp1-D21 plants was observed at 14 days after infection with FoMV-Rp1-D21, compared to B73:Rp1-D21 infected with pFoMV-V (the empty vector control; Figure 1).

Rp1-D21 transcript levels in B73:Rp1-D21 plants infected with FoMV-Rp1-D21 were 2.14-fold lower than control plants infected by FoMV-V (Figure 1b). As well as causing necrotic spots, the presence of Rp1-D21 also causes a general leaf chlorosis. In addition to visual assessment, the severity of the Rp1-D21 phenotype was, therefore, also measured using a soil plant analysis development (SPAD) meter to assess total leaf chlorophyll content as a proxy. The leaves of B73:Rp1-D21 plants infected with pFoMV-Rp1-D21 possessed higher chlorophyll content than those infected with pFoMV-V, confirming our visual assessment that the level of HR had been reduced (Figure 1c).

B73 plants (i.e., plants that did not carry Rp1-D21) infected by pFoMV-Rp1-D21 or pFoMV-V developed typical viral symptoms and did not show any HR symptoms, as expected. Representative pictures of B73 plants infected with pFoMV-Rp1-D21 and every other VIGS construct used in this study are shown in Figure S1. pFoMV-PDS constructs designed to suppress the expression of the PDS gene were also used as a way to test the system. B73:Rp1-D21 plants infected with pFoMV-PDS showed bleached "stripes" typical of the phenotype expected from suppression of PDS expression and PDS transcript levels were reduced by 3.14-fold compared to B73:Rp1-D21 plants infected with pFoMV-V. Chlorophyll content was also slightly lower in B73:Rp1-D21 plants infected with FoMV-PDS compared to those infected with pFoMV-V (Figure S2).

These results demonstrated that the FoMV VIGS system worked well in our hands and could be used as a tool to interrogate the mechanisms controlling the expression of HR induced by Rp1-D21.

**FIGURE 1** Infection of B73:Rp1-D21 by pFoMV-V and pFoMV-Rp1-D21. (a) Image of fourth leaves from inoculated plants 14 days postinoculation (dpi). Black squares outline the area that is shown magnified directly below the main picture. (b) Real-time quantitative reverse transcription PCR analysis of Rp1-D21 expression in B73:Rp1-D21. Significant suppression of Rp1-D21 transcripts is detected in the fourth leaves of plants infected with pFoMV-Rp1-D21 compared with those infected with pFoMV-V. Data normalized using actin as a reference. (c) Soil plant analysis development data from inoculated plants. Significant increase of chlorophyll content is observed in the fourth leaves of B73:Rp1-D21 plants infected with pFoMV-Rp1-D21 compared with those infected with pFoMV-V. Data is derived from at least three biological replications.
Below we describe our findings using the FoMV VIGS system to suppress expression of 12 genes in a B73:Rp1-D21 background. These genes were identified as candidate genes for HR modulation either from our previous work or from the general literature. We first describe the six genes whose suppression led to changes in levels of HR (three up, three down), then the three whose suppression had no measurable effect, and finally three genes whose expression we were unable to suppress with this system.

2.2 Silencing of HCT, CCoAOMT, and Sgt1 enhances Rp1-D21-induced HR

2.2.1 HCT and CCoAOMT

Hydroxycinnamoyltransferase (HCT) and caffeoyl CoA O-methyltransferase (CCoAOMT) (Ye et al., 1994; Hoffmann et al., 2004) are both present as gene families in maize and catalyse adjacent steps in the lignin biosynthetic pathway. We previously demonstrated that members of each gene family (the closely linked HCT genes GRMZM2G061806 and GRMZM2G114918, referred to as HCT1806 and HCT4918, respectively, and the CCoAOMT2 gene GRMZM2G099363) were present at loci associated with modulation of HR conferred by Rp1-D21 and were able to suppress Rp1-D21-induced cell death when coexpressed with Rp1-D21 in N. benthamiana (Wang et al., 2015a; Wang and Balint-Kurti, 2016). We also demonstrated that HCT1806, HCT4918, CCoAOMT2, and Rp1-D21 proteins physically interacted in vitro.

VIGS constructs pFoMV-HCT and pFoMV-CCoAOMT, designed to silence HCT1806/HCT4918 and CCoAOMT2, respectively, were constructed in pFoMV-V (Table 1). In the case of pFoMV-HCT, the construct was able to silence both HCT1806 and HCT4918 due to their high homology (88%). The primers used for quantitative reverse transcription PCR (RT-qPCR) were also designed to amplify transcripts from both genes. In the case of pFoMV-CCoAOMT the construct was predicted to silence both the CCoAOMT2 gene GRMZM2G099363 and a closely related gene, GRMZM2G127948; however, the primers used for RT-qPCR were also designed to specifically amplify cDNA derived from GRMZM2G099363.

The transcript levels of HCT1806/HCT4918 and CCoAOMT2 in B73:Rp1-D21 plants infected with pFoMV-HCT and pFoMV-CCoAOMT, respectively, were reduced by 10.52- and 4.16-fold compared to plants infected with pFoMV-V 14 days after viral infection (Figure 2b). Stronger HR was observed in B73:Rp1-D21 lines infected with pFoMV-HCT or pFoMV-CCoAOMT compared to B73:Rp1-D21 lines carrying pFoMV-V as determined visually (Figure 2a) and by SPAD analysis (Figure 2c).

The enhancement of HR induced by Rp1-D21 associated with suppression of the HCT and CCoAOMT genes in maize was consistent with our previous findings that their overexpression in N. benthamiana suppressed HR conferred by Rp1-D21 (Wang et al., 2015a; Wang and Balint-Kurti, 2016). These findings suggested that the
FoMV VIGS system was indeed a promising tool to investigate the mechanisms of HR modulation and encouraged us to investigate other genes in a similar way.

2.2.2 | SGT1

The SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1), HEAT SHOCK PROTEIN90 (HSP90), and REQUIRED FOR MLA12 RESISTANCE (RAR1) proteins form a molecular chaperone complex that is an important signalling component of plant immune responses (Shirasu, 2009; Zhang et al., 2010b). This complex interacts with NLRs in numerous systems, stabilizing them and allowing their proper maturation and function (Zhang et al., 2010b). Within this complex, SGT1 plays a central role as it recruits the NLR and also interacts with the E3 ubiquitin ligase subunit Skp1 (Catlett and Kaplan, 2006), part of the Skp1/Cullin/F-box (SCF) ubiquitin ligase complex that targets proteins for degradation (Azevedo et al., 2006; Cheng et al., 2011; Gou et al., 2015). Suppression or mutation of any one of RAR1, SGT1, or HSP90 in many interactions is sufficient to abolish HR and to reduce NLR protein levels (e.g., Hubert et al., 2003; Bieri et al., 2004; Scofield et al., 2005; Azevedo et al., 2006; Kim et al., 2018).

The two maize homologs of SGT1 annotated in maize (B73 genome v. 3) are GRMZM2G105019 and GRMZM2G149704 (here called SGT5019 and SGT9704, respectively). They share a nucleotide identity of 93%. It was not possible to silence only one of these genes specifically due to their high identity across the full length of their coding sequences, so we designed pFoMV-SGT1 to suppress both variants of SGT1. Likewise, the primers used for RT-qPCR were designed to detect the transcripts from both SGT1 homologs. Infection of B73:Rp1-D21 plants with pFoMV-SGT1 reduced total SGT1 transcript levels by 4.61-fold compared to control plants (Figure 2b) and enhanced Rp1-D21-associated HR (Figure 2a,c). We also noted that pFoMV-SGT1 infection of B73 plants often caused the development of lesions that were not seen when B73 was infected with pFoMV-V or with the other constructs used in this study (Figure S1), suggesting that perhaps suppression of SGT1 expression was also causing spontaneous HR in the absence of Rp1-D21.

Suppression of SGT1 expression in most systems studied resulted in lower accumulation of the activated NLR protein and the consequent suppression of resistance and HR (Holt et al., 2005; Cheng et al., 2011). The enhancement of HR associated with suppression of SGT1 expression in maize that we observed was therefore unexpected. However, the role of SGT1 is not completely understood. While it is required for HR induced by NLRs in most studied cases, there are several reports of SGT proteins playing an HR-inhibitory role. In Arabidopsis, inactivation of one of the two functional SGT1 genes led to increased accumulation of the RPS5 NBS-LRR R-protein, suggesting that SGT1 antagonized the effect of RAR1 (Holt et al., 2005), acting to reduce levels of NLR proteins. Similarly, Gou et al. (2015) showed that overexpression of SGT1b suppressed SNC1 protein accumulation, leading to suppression of SNC1-triggered cell death in Arabidopsis. The role of SGT1 may vary depending on the species, the specific member of the gene family in the cases where multiple SGT1 genes are present in the genome, and the particular NLR or its activation state (Azevedo et al., 2006).

Because the effects of SGT5019 and SGT9704 could not be differentiated using VIGS we turned to the N. benthamiana transient expression system. We coexpressed each SGT1 gene with Rp1-D21 in tobacco leaves. Coexpression of SGT5019 but not SGT9704 with Rp1-D21 suppressed Rp1-D21-mediated HR (Figure 3a). Ion leakage conductivity data confirmed that coexpression of Rp1-D21 with SGT5019 significantly reduced ion leakage compared with coexpression of a GUS:GFP control (Figure 3b). This indicates that SGT5019 is involved in regulating Rp1-D21-induced cell death. Based on this result we speculated that SGT5019 might suppress Rp1-D21-induced cell death by reducing Rp1-D21 protein accumulation. To evaluate this possibility, the protein levels of Rp1-D21 and SGT1 were quantified by western blot. Rp1-D21 was detected using antibodies against an HA epitope tag that had been added to the C-terminus of Rp1-D21. SGT1 homologs and β-glucuronidase (GUS) were detected using antibodies that detected their attached green fluorescent protein (GFP) epitope tags. Rp1-D21 was detected at similar levels when coexpressed with GUS:GFP, SGT9704, and SGT5019 (Figure 3c), and the levels of the two SGT1 proteins themselves were comparable. This indicated that there was no evidence that the suppressive effect of SGT5019 on HR induced by Rp1-D21 was due to reduced Rp1-D21 protein levels.

2.3 | Silencing of IQM3, VPS37, and HSP90 suppresses Rp1-D21-induced cell death

2.3.1 | IQM3

GRMZM2G439311 encodes IQ calmodulin-binding (IQM3) protein and is less than 4 kb from a single nucleotide polymorphism (SNP) positioned on chromosome 4 at 188,208,471 bp that was highly associated with variation in Rp1-D21-induced HR (Olukolu et al., 2014). The IQ motif (IQXXXRGXXXR) is a common motif that binds calmodulin, an important calcium sensor that regulates many aspects of the cytoskeleton and mitosis and many other cell functions (Bähler and Rhoads, 2002). Several studies have shown the importance of calmodulin and calmodulin-like proteins in regulating plant defence and HR. CoCaM1 (pepper calmodulin1) overexpression conferred disease resistance by regulating reactive oxygen species accumulation in pepper (Choi et al., 2009). A point mutation in CML24 (Calmodulin 24) gene impaired HR against avirulent Pseudomonas syringae pv. tomato (avrPrt2) in Arabidopsis thaliana and HR was accelerated against avrPrt2 by overexpressing CML43 (Calmodulin 43) (Chiasson et al., 2005; Ma et al., 2008). Based on these studies, we postulated IQM3 may have some role in regulating Rp1-D21 mediated cell death and developed the pFoMV-IQM3 to suppress IQM3 expression in maize.

The transcript abundance of IQM3 was 7.87-fold lower in pFoMV-IQM3-infected plants compared to pFoMV-V-infected plants (Figure 4b). Phenotypic observations and chlorophyll data showed that B73:Rp1-D21 plants infected with pFoMV-IQM3 had less
severe HR lesions than B73:Rp1-D21 plants infected with pFoMV-V (Figure 4a,c). These results suggest that IQM3 silencing suppresses Rp1-D21-induced cell death, and therefore that this protein enhances HR induced by Rp1-D21.

2.3.2 | VPS37

GRMZM2G023575 encodes the vacuolar protein sorting protein 37 (VPS37). It is situated 28.1 kb from a SNP on chromosome 1 at 18,100,677 bp, which is significantly associated with variation in Rp1-D21-induced HR (Olukolu et al., 2014). VPS37 is a component of the ESCRT-I (Endosomal Sorting Complex Required for Transport) complex, which together with the ESCRT-0, -I, -II and -III complexes and the Vps4 complex, as well as other components, enable membrane remodelling and the processing of ubiquitinated proteins to the lysosome (Schmidt and Teis, 2012). In Arabidopsis the ESCRT system and VPS37 in particular have been shown to be important for the endocytic transport of the plant immune receptor FLS2, an LRR-receptor kinase PRR, at the plasma membrane after activation triggered by bacterial flagellin (Spallek et al., 2013). VPS37-1 colocalized with FLS2 at endosome upon flagellin elicitation and vps37-1 mutants compromised vacuolar sorting of FLS2 and flagellin-triggered stomatal closure, and displayed impaired immunity to the bacterial pathogen P. syringae and to the biotrophic oomycete Hyaloperonospora arabidopsidis (Lu et al., 2012). While FLS2 activation and the resulting MTI do not generally lead to an HR, a strong connection has recently been shown between a functional MTI response and a full ETI response, including HR (Ngou et al., 2020; Yuan et al., 2020).

Silencing of VPS37 suppressed Rp1-D21-induced HR in maize (Figure 4a,c). The VPS37 transcript level was reduced by 1.63-fold in plants infected with pFoMV-VPS37 compared to control

![Image](133x741 to 246x756)

**Figure 3** Suppression of Rp1-D21-induced cell death by coexpression of SGT15019 but not SGT9704 in Nicotiana benthamiana. (a) Transient coexpression of SGT5019 or SGT7904 with Rp1-D21 in N. benthamiana. β-glucuronidase (GUS) was used as a negative control. Agrobacterium carrying each construct was diluted to a final concentration of OD_{600} = 1.0. Infiltrated regions are marked with ovals. (b) Ion leakage conductivity was measured at 36 hr after coexpression of SGT5019, SGT7904, or GUS with Rp1-D21. Data represent means ± SD from three independent plants. (*significant difference from Rp1-D21 positive control at p < .01; ns, not significant). (c) Total protein extracted from agroinfiltrated leaves at 30 hr postinoculation (hpi). Leaves infiltrated with empty vector were used as a negative control. Anti-HA was used to detect Rp1-D21 and anti-GFP was used to detect SGT5019, SGT7904, or GUS. Equal loading of protein samples is shown by Ponceau S staining of RuBisCO band. Each experiment was performed with three replications.
plants infected with pFoMV-V (Figure 4b) and pFoMV-VPS37-infected leaves had higher chlorophyll content than those infected by FoMV-V (Figure 4c). This suggests that the ESCRT endosomal sorting complex might be important for controlling the Rp1-D21-mediated defence response.

### 2.3.3 HSP90

HSP90 is a component of the SGT1/HSP90/RAR1 chaperone complex that has been implicated in facilitating NLR-mediated HR in a number of systems and is described above. HSP90 is a protein chaperone that can stabilize NLR proteins or recruit cofactors that allow NLR proteins to function properly in plant defence signalling. In previous studies HSP90-silenced plants exhibited growth reduction and yellowing leaves as well as suppressed NLR-mediated HR and resistance to pathogens (Liu et al., 2004; Zhang et al., 2004; Scofield et al., 2005; Bos et al., 2006; Cakir et al., 2010; Shibata et al., 2011; Kim et al., 2018). We wanted to determine if HSP90 also functions in Rp1-D21-induced HR in maize.

There are five HSP90 homologs in the maize genome (GRMZM2G024668, GRMZM2G012631, GRMZM2G112165, GRMZM2G069651, GRMZM5G833699). Nucleotide identities between them are 82%-93% and pFoMV-HSP90 was designed to knock down all five gene variant transcripts via cross-complementarity to mature mRNA exons. Similarly, the primers used for RT-qPCR were designed to amplify transcripts from all five genes. We observed that cell death was markedly reduced in B73:Rp1-D21 infected with pFoMV-HSP90 (Figure 4a) compared to B73:Rp1-D21 infected with pFoMV-V. The overall transcript levels of the five HSP90 homologs in the silenced plants was 16.83-fold lower than in control plants (Figure 4b). Total chlorophyll content was elevated compared to the plants infected with FoMV-V. In addition, both B73:Rp1-D21 and B73 infected with FoMV-HSP90 displayed yellow stripes and noticeably reduced growth (Figures 4a and S1). Somewhat similar results were reported in N. benthamiana, where suppression of HSP90 expression led to stunting and eventual death (Liu et al., 2004). While we were not able to differentiate the roles of the five HSP90 homologs, these results suggest that HSP90 may play a similar role in controlling HR associated with Rp1-D21 in maize as it does in most of the other systems in which it has been investigated.

### 2.4 Silencing of RAR1, PGH1, and QCR7 do not alter the Rp1-D21-induced HR

#### 2.4.1 RAR1

RAR1 is the third member of the SGT1/HSP90/RAR1 chaperone complex described above. However, RAR1-silenced plants do not always exhibit reduced NLR-mediated resistance (Shibata et al., 2011; Kim et al., 2018). Three RAR1 homologs exist in the maize genome.
(GRMZM5G868908, GRMZM2G156105 and GRMZM5G881347). GRMZM5G868908 and GRMZM2G156105 are 93% identical at the nucleotide level over the whole length of their 587 bp cDNA, while GRMZM5G881347 appears to be an incomplete form of RAR1, with several deletions. pFoMV-RAR1 was designed to silence both GRMZM5G868908 and GRMZM2G156105 but not GRMZM5G881347. Similarly, the primers used for RT-qPCR were designed to amplify transcripts from GRMZM5G868908 and GRMZM2G156105 but not GRMZM5G881347. No phenotypic differences associated with silencing were observed in plants infected with FoMV-RAR1. Rp1-D21-induced cell death was unchanged despite a 3.93-fold decrease in the RAR1 transcripts measured as compared to FoMV-V (Figure 5).

These data do not support a role for RAR1 in the Rp1-D21-induced HR.

2.4.2 ZmPGH1 and ZmQCR7

ZmPGH1 (Zea mays polygalacturonase homolog 1 GRMZM2G135763) was identified from the prior GWAS (Olukolu et al., 2014) as a candidate gene for modulation of Rp1-D21-mediated HR. In subsequent work, He et al. (2019a) showed that coexpression of ZmPGH1 inhibited HR induced by Rp1-D21 and by another autoactive NLR RPM1(D505V) in N. benthamiana. We therefore expected that ZmPGH1 suppression might enhance the Rp1-D21-induced HR. However, reduction of the PGH1 transcript level by 2.46-fold compared to control had no obvious phenotypic effect on Rp1-D21-induced HR in B73:Rp1-D21 (Figure 5).

Similarly to ZmPGH1, ZmQCR7 (GRMZM2G318346, cytochrome b–c1 complex subunit 7) was also identified from the prior GWAS (Olukolu et al., 2014) as a candidate gene for modulation of Rp1-D21-mediated HR. Also similar to ZmPGH1, subsequent analysis showed that coexpression of ZmPGH1 inhibited HR induced by Rp1-D21 and RPM1(D505V) (He et al., 2019b). Silencing of QCR7 by FoMV-QCR7 displayed no obvious effect on the Rp1-D21-induced HR in B73:Rp1-D21, although the QCR7 transcript level was reduced by 2.08-fold compared to control plants (Figure 5).

Based on previous results, we anticipated that suppressing the expression of ZmPGH1 and ZmQCR7 in B73:Rp1-D21 plants would enhance Rp1-D21-induced HR. The fact that we did not observe any such enhancement may be due to several factors. First, the level of gene suppression may not have been sufficient. ZmPGH1 and ZmQCR7 transcript levels were suppressed 2.46- and 2.08-fold, respectively. By contrast, the CCoAOMT2 and HCT genes were suppressed 10.52- and 4.16-fold, respectively. This means that substantial amounts of transcript were still available for translation. Furthermore, relationships between mRNA levels, protein levels, and protein activity are nonlinear and unpredictable (Walley et al., 2016). Both genes are members of broader gene families; however, functional redundancy between family members seems unlikely to be the cause of the lack of effect because assuming these genes are indeed the causal genes underlying the original GWAS association.

**FIGURE 5** Phenotypes of B73:Rp1-D21 plants infected with pFoMV-QCR7, pFoMV-PGH1, and pFoMV-RAR1. (a) Image of the fourth leaves from inoculated plants 14 days postinoculation (dpi). Black squares outline the area that is shown magnified directly below the main picture. (b) Real-time quantitative reverse transcription PCR analysis of QCR7, PGH1, and RAR1 in B73:Rp1-D21. Significant suppression of QCR7, PGH1, and RAR1 transcripts was detected in systemic leaves of plants infected with each virus-induced gene silencing construct relative to pFoMV-V. Values normalized using actin as a reference. (c) Soil plant analysis development data from inoculated plants. No significant difference in chlorophyll content was observed in the fourth leaves of plants that were infected with each virus-induced gene silencing construct relative to pFoMV-V. ns, not significant. Values are derived from at least three biological replications.
(Olukolu et al., 2014), functional redundancy would have also precluded identifying the associations in the first place. It should also be noted that the HR suppression effects of ZmPGH1 and ZmQCR7 in the N. benthamiana transient expression system were substantially weaker than those of the HCT1806, HCT4918, and CCoAOMT2 genes (Wang and Balint-Kurti, 2016; He et al., 2019a, 2019b). By the same token, we might expect that it would be difficult to observe phenotypic effects of suppressing the expression of ZmPGH1 and ZmQCR7 in maize.

2.5 | VIGS constructs fail to silence Pk1b, LOX9, and SL11 targets in vivo

Not all of the candidate genes chosen for this work could be silenced using the constructs we produced. GRMZM2G144042 encodes a protein kinase 1b (Pk1b) and was identified as a candidate gene based on its proximity (816 bp) to a GWAS hit on chromosome 5 at 2,282,955 bp associated with Rp1-D21-induced HR (Olukolu et al., 2014). Pk1b belongs to the receptor serine/threonine kinase (STK) family and is involved in a signaling cascade. STKs are thought to relay signals derived from LRR-containing receptors to activate downstream defence responses (Sessa and Martin, 2000; Afzal et al., 2008).

GRMZM2G017616 encodes a lipoygenase 9 (LOX9). Included in its sequence is a SNP (chromosome 1, 16,578,218 bp) that was highly associated with variation in Rp1-D21-induced HR in our previous GWAS (Olukolu et al., 2014). LOX9 belongs to the 13-lipoxygenases family and was found to have a role in defence against southern leaf blight (Bipolaris maydis) in maize (Christensen et al., 2015). It also has a role in jasmonic acid biosynthetic pathways (Porta and Rocha-Sosa, 2002; Woldemariam et al., 2018). Considering these potential roles in the defence response, we inferred that GRMZM2G017616 might have a role in regulating Rp1-D21-induced HR.

GRMZM2G351387 encodes a homolog of spotted leaf 11 (SL11)/plant Ubox 13 (PUB13) protein. It is located on chromosome 3, 11.5 kb from a SNP at 126,183,689 bp that was associated with variation in Rp1-D21-induced HR (Olukolu et al., 2014). Rice SL11 displays E3 ubiquitin ligase activity and was found to negatively regulate plant defence and cell death (Zeng et al., 2004). The Arabidopsis ortholog of SL11, called PUB13, was also involved in negative regulation of plant innate immunity by direct ubiquitination of flagellin receptor FLS2 (Lu et al., 2011) and chitin receptor LYK5 (Liao et al., 2017). T-DNA insertions in Arabidopsis PUB13 resulted in enhanced cell death, salicylic acid accumulation, and resistance to biotrophic pathogens (Li et al., 2012). It was therefore targeted as a promising candidate gene for regulation of Rp1-D21 activity.

The levels of the transcripts of each of these genes, Pk1b, LOX9, and SL11, were unaffected in B73:Rp1-D21 plants infected with the VIGS constructs designed to suppress their expression (Figure S3b). As might be expected, there were also no differences in the severity of the HR caused by Rp1-D21 in any of the B73:Rp1-D21 infected by these constructs (Figure S3a). To further test the role of Pk1b in regulating Rp1-D21-induced HR, we also employed transient expression in N. benthamiana. Coexpression of Pk1b with Rp1-D21 did not significantly affect Rp1-D21-induced cell death (Figure S4).

Because these genes were not silenced, we are unable to draw any conclusions about their roles in mediating Rp1-D21-induced cell death. There are several plausible reasons for ineffective silencing. The most common is the instability of the silencing vector leading to the loss of the target gene-specific insert (Scofield and Nelson, 2009). As described in the materials and methods, we checked each infected plant for retention of the sequence inserted in pFoMV-V and tried to only select plants for further analysis where we could determine that the insert was retained. The only construct that consistently lost its insert was pFoMV-Pk1b, so for this construct at least we have a simple explanation for the failure to suppress expression. In the other two cases, constructs designed to target other portions of the gene might be more effective. It is hard to predict which regions of the gene to target to achieve the most efficient suppression of expression (Yeom et al., 2012). When selecting the fragment for silencing, our overriding consideration was specificity and size (about 500 bp), as discussed by Liu and Page (2008). Software such as siRNA-Finder may offer some more sophisticated methods of identifying target sequences for RNAi applications (Lück et al., 2019). Using a VIGS system based on the bean pod mottle virus, Zhang et al. (2010a) noted that targeting the 3′ end of open reading frames seemed to be more efficient than targeting the 5′ ends. In our case SL11 and LOX9 were targeted at the 3′ end but were not silenced (Figure S3). Liu et al. (2016) used inverted repeats to achieve efficient gene silencing in monocots with an independent FoMV-based VIGS system.

3 | CONCLUSIONS

Here we have attempted to use the FoMV VIGS system to characterize the roles of 12 genes in modulating the HR induced by the Rp1-D21 autoactive R-protein in maize. In six cases we have observed an effect and we can therefore assign a role to the encoded protein. In six other cases we did not observe an effect and in these cases the conclusions are less obvious. Even in the cases where the expression of the targeted genes was suppressed, we still cannot definitively conclude that the encoded proteins play no role in regulating HR because neither the relationship between RNA and protein levels nor the relationship between protein levels and expected phenotype are clear. This highlights a weakness of the VIGS approach: although the name of the technique implies otherwise, VIGS does not ever completely silence gene expression. Therefore, the role of a gene cannot be definitively ruled out using VIGS even if no phenotypic change is observed. In this study, of our 12 constructs, three provided no detectable suppression of mRNA levels of the targeted genes while the other nine gave various levels of suppression ranging from 1.63- to 16.83-fold. The reasons for this variability are unclear but are probably due to the secondary structure of the mRNAs and the stability of the specific inserts in the FoMV vector. Other disadvantages of VIGS include the fact that it may be hard to differentiate the effects
of genes with very similar sequences and that it is transient and can only be used in immature maize plants.

Nevertheless, we were able to use VIGS to efficiently assess the roles of several candidate genes associated with modulation of HR. In four cases we examined the roles of genes that we had previously validated in a N. benthamiana ectopic transient expression system. In two of these cases, the HCT and CCoAOMT genes, the phenotypes we observed in maize conformed to our expectations. In two others, the PGH1 and the QCR7 genes, we did not observe the expected effect. While, as discussed above, we cannot draw definitive conclusions in these later cases, it is likely that the lack of effect observed for PGH1 and QCR7 may be due to a combination of lower gene suppression (2.46- and 2.08-fold, respectively) compared to HCT and CCoAOMT (10.52- and 4.16-fold) and the fact that their effect on HR is more subtle in any case, as demonstrated in the N. benthamiana system (He et al., 2019a, 2019b). In addition, we were able to implicate two previously uncharacterized genes, VPS37 and IQM3, in the modulation of Rp1-D21-induced HR.

We also examined the role of the HSP90/SGT1/RAR1 chaperone complex. Scofield et al. (2005) used the barley stripe mosaic virus (BSMV) VIGS system to show that silencing homologs of each of these three genes in wheat compromised resistance to leaf rust conferred by the Lr21 NLR resistance gene. As discussed above, several other studies have also demonstrated similar effects of these genes on NLR-mediated resistance and HR in other studies. In our experiments we found that suppressing expression of HSP90 homologs suppressed Rp1-D21-induced HR as expected but suppressing RAR1 expression had no effect on HR while suppressing SGT1 expression actually enhanced the HR phenotype. Further work will be required to deconvolute these effects. Interpretation is complicated by the fact that each of these genes are members of small gene families in maize. In each case our constructs were designed to suppress expression of all functional homologs of each gene, five HSP90 homologs, two SGT1 homologs, and two RAR1 homologs. With SGT1 we were able to use the N. benthamiana system to show that the one of the two SGT1 homologs, SGT5019, was probably important for modulating Rp1-D21 activity.

In conclusion, this study demonstrates the feasibility of performing high-throughput functional genomics in maize using FoMV VIGS. Using this system, we can produce robust evidence to validate GWAS predictions in the endogenous maize system rather than using a proxy heterologous N. benthamiana expression system as we had previously. Additionally, this approach could be easily adapted for the characterization of the numerous other lesion phenotypes identified in maize (Johal, 2007).

4 | MATERIALS AND METHODS

4.1 | Plant materials and virus infection procedures

The B73:Rp1-D21 line, the commonly used B73 line into which the Rp1-D21 gene has been introgressed through multiple backcrosses, was used for all experiments. B73:Rp1-D21 is maintained by crossing a B73:Rp1-D21 plant heterozygous for Rp1-D21 to B73, and therefore it segregates at a 1:1 ratio for the presence/absence of the Rp1-D21 mutant allele. When “B73:Rp1-D21 plants” are referred to in this study, it always refers to the segregants that are heterozygous for Rp1-D21 and display the spontaneous lesion phenotype.

All VIGS experiments used a standardized growth and screening timeline (Figure S5). All VIGS experiments used at least three individual plants for each treatment and were repeated three times. Seeds were treated with 1% vol/vol H2O2 for 2 min and incubated in water containing 2 mg/ml calcium sulphate to enhance germination. Seeds were germinated using water-imbibed germination paper for 6 days in a growth chamber set to 22/18°C using a 16:8 hr photoperiod. Methods for biolistic inoculation of FoMV DNA constructs were derived from a prior publication with amendment (Mei and Whitham, 2018). Among 6-day-old seedlings grown in germination paper, those possessing an emerged third leaf (V1 stage) or any symptoms of damping off were discarded. Only healthy seedlings possessing an extended cotyledon and first true leaf were used for biolistics. Biolistics were carried out using a Biolistic PDS-1000/HE Particle Delivery System located in the North Carolina State University (NCSU) Phytotron. Bombarded seedlings were sown in 6-inch pots containing standard mix (33% Sunshine Redi-Earth Pro Growing Mix [Canadian Sphagnum] peat moss 50%-65%, vermiculite, dolomitic lime, 0.0001% silicon dioxide, and 66% pea gravel) and grown for an additional 2 weeks using a 12:12 hr 26/22°C day/night cycle with a 2 hr night interruption for c.2 weeks at the NCSU phytotron prior to sample collection. Twenty days after seed germination, the fourth leaves of plants were removed and used to collect SPAD measurements, sampled for RNA isolation, and electronically scanned to collect information about visual phenotypes.

The plants used for data collection and downstream analysis underwent a selection process to distinguish genetic background and successful viral infection. Bombarded B73:Rp1-D21 plants used for this work were screened for the presence of Rp1-D21 based on the appearance of HR lesions on cotyledons 10 days after germination. Bombarded plants were additionally screened based on the visible presence of FoMV infection (a slightly discolored mosaic pattern that was most notable at the leaf tip) and marked between 13 and 15 days after germination. This was done to distinguish plants that were infected from those that failed to infect after bombardment. This had to be done at 13-15 days because later in development Rp1-D21-associated lesions tended to mask the visible symptoms of FoMV infection.

N. benthamiana plants were grown under artificial lighting in a growth chamber at 23°C for a 16 hr light:8 hr dark photoperiod. Transient assays were performed using 4-week-old N. benthamiana plants.

4.2 | Construction of FoMV VIGS vectors

VIGS inserts were amplified using B73 cDNA as a template. Total RNA was isolated from maize leaf using Trizol reagent (Invitrogen)
according to the manufacturer’s protocol. cDNA was synthesized from a total RNA template (3 µg) using RevertAid reverse transcriptase (Thermo Scientific). This cDNA was used for amplification of the maize candidate genes shown in Table 1. PCR was performed using each primer pair listed in Table S1, and the product was digested with XbaI and XhoI for insertion in the antisense orientation into pFoMV-V to generate the candidate gene-silencing constructs. Digested products were purified using a silica column (DNA Clean & Concentrator-5; Zymo Research) and eluted in 6 µl of elution buffer. Purified inserts were ligated with pFoMV-V digested by the same enzymes using T4 DNA ligase. Ligated product was transformed into Escherichia coli DH5α by a heat shock method. Resulting clones were sequenced by the Genomic Sciences Laboratory laboratory at NCSU.

4.3 Measurement of SPAD

SPAD measurements were taken using a Minolta chlorophyll meter (Agriculture Solutions LLC). The leaf-clip sensor was attached to the fourth leaf, and three readings per leaf were measured. A minimum of 18 readings across six leaves were recorded for each treatment. The total chlorophyll content (µg/cm²) was calculated using the following formula (Cerovic et al., 2012):

\[
\text{Total chlorophyll content} = \frac{(99 \times \text{mean SPAD data})}{(144 - \text{mean SPAD data})}
\]

The statistical significance between treatments was determined using one-way analysis of variance (ANOVA) at \( p < .05 \).

4.4 RNA isolation and RT-qPCR

RNA from infected maize was extracted from the lower half of the fourth leaf used for SPAD measurements. One hundred milligrams of leaf tissue samples was collected using a 3 mm biopsy punch. RNA was isolated using TRIzol (Invitrogen) and cDNA was synthesized using a Revert Aid First Strand cDNA synthesis Kit (Thermo Scientific). RNA material from plants presenting a visible FoMV infection were then screened for the presence of VIGS inserts using primer pair FM-5840F and FM-6138R (Table S2). With the exception of pFoMV-Pk1b infections, only samples displaying the correctly amplified insert were further analysed by RT-qPCR. Real-time qPCR was conducted using iTaq Universal SYBR Green Supermix (Bio-Rad) and StepOne Plus (Applied Biosystems). The primers used are provided in Table S1. Fold change was calculated using \( 2^{\Delta\Delta C_t} \) (Livak and Schmittgen, 2001). The statistical significance between treatments was determined using one-way ANOVA at \( p < .05 \). RNA sampling and RT-qPCR were performed for at least six individual plants per control or experimental group. Two technical replicates were used for each of three biological replicates.

4.5 Construction of clones for transient studies in \( N. benthamiana \)

The purified PCR products of SGT5019, SGT9704, and Pk1b amplified from maize B73 cDNA were cloned into pCR8/GW/TOPO TA vector following the manufacturer’s protocol (Thermo Fisher Scientific). The resulting ligation mixtures were transformed into \( E. coli \) and positive clones were identified by colony PCR. Positive clones were then subcloned into pGWB641 featuring a recombinant C terminal EYFP tag (Nakamura et al., 2010) using LR clonase II (Thermo Fisher Scientific). Positive clones were then transformed into Agrobacterium tumefaciens GV3101.

4.6 Transient expression in \( N. benthamiana \)

\( A. tumefaciens \) GV3101 (pMP90) carrying recombinant clones was grown at 28°C overnight in 50 ml of lysogeny broth (LB) with appropriate antibiotics. Cultures were centrifuged at 3,400 \( \times \) g for 30 min at 4°C to obtain a pellet. The resultant pellet was resuspended in infiltration buffer and diluted to an optical density of 600 nm (OD\(_{600}\) = 0.6. GV3101 (pMP90) carrying a helper plasmid expressing TBSV p19 (helper plasmid) at OD\(_{600}\) = 0.3 was added to each pellet suspension. The resultant mixture was used for transient expression in \( N. benthamiana \) as previously performed (Wang et al., 2015a).

4.7 Ion leakage assays

Five leaf discs (1.5 cm diameter) were collected from at least three leaves on three separate \( N. benthamiana \) plants for each treatment and incubated in 5 ml of sterilized distilled water at 100 rpm for 3 hr at room temperature. The initial conductivity (C1) was measured using a conductivity meter (model 4403; Markson Science, Inc.). Samples were boiled at 95°C for 15 min and final conductivity was measured (C2). Ion leakage was obtained by calculating the C1:C2 ratio. Statistical analysis was performed using one-way ANOVA at \( p < .05 \).

4.8 Western blotting

At least three leaf discs (1.2 mm diameter) were collected from the area infiltrated with \( A. tumefaciens \) in each \( N. benthamiana \) leaf at 30 hr postinfiltration (hpi) for protein expression analysis. Total protein was isolated using 300 µl of protein extraction buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 10 mM dithiothreitol, 40 mM MgCl₂, and 1 x plant protein protease inhibitor mixture; Sigma-Aldrich). Protein sample preparation and SDS polyacrylamide electrophoresis was carried out as previously
described (Wang et al., 2015b). Protein gels were transferred to nitrocellulose and incubated with anti-HA antibody conjugated with horseradish peroxidase (HP; Roche) at 1:350 dilution. GFP was detected using a 1:6,000 dilution of anti-GFP (monoclonal, mouse; Abcam) followed by incubation with 1:15,000 of goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) (Sigma). The HRP signal was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and detected by chemiluminescence.

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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
Afzal, A.J., Wood, A.J. and Lightfoot, D.A. (2008) Plant receptor-like serine threonine kinases: roles in signaling and plant defense. Molecular Plant-Microbe Interactions, 21, 507–517.
Azevedo, C., Betsuyaku, S., Peart, J., Takahashi, A., Noël, L., Sadanandom, A. et al. (2006) Role of SGT1 in resistance protein accumulation in plant immunity. The EMBO Journal, 25, 2007–2016.
Bähler, M. and Rhoads, A. (2002) Calmodulin signaling via the IQ motif. FEBS Letters, 513, 107–113.
Balint-Kurti, P. (2019) The plant hypersensitive response: concepts, control and consequences. Molecular Plant Pathology, 20, 1163–1178.
Benavente, L.M., Ding, X.S., Redinbaugh, M.G., Nelson, R.S. and Balint-Kurti, P. (2012) Virus-induced gene silencing in diverse maize lines using the brome mosaic virus-based silencing vector. Maydica, 57, 206–214.
Bent, A.F. and Mackey, D. (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annual Review of Phytopathology, 45, 399–436.
Bleri, S., Mauch, S., Shen, Q.-H., Peart, J., Devoto, A., Casais, C. et al. (2004) RAR1 positively controls steady state levels of barley MLA resistance proteins and enables sufficient MLA6 accumulation for effective resistance. The Plant Cell, 16, 3480–3495.
Bohjan, D., Johann, S., Johann, S., and Balint-Kurti, P. (2019a) A maize polygalacturonase function as a suppressor of programmed cell death in plants. BMC Plant Biology, 19, 310.
Bruggeman, Q., Raynaud, C., Benhamed, M. and Delarue, M. (2015) To die or not to die? Lessons from lesion mimic mutants. Frontiers in Plant Science, 6, 24.
Cakir, C., Gillespie, M.A. and Scofield, S.R. (2010) Rapid determination of gene function by virus-induced gene silencing in wheat and barley. Crop Science, 50, 577–584.
Catlett, M.G. and Kaplan, K.B. (2006) Sgtp1 is a unique co-chaperone that acts as a client adaptor to link Hsp90 to Skp1p. Journal of Biological Chemistry, 281, 33739–33748.
Cerovic, Z.G., Masdoumier, G., Ghzolen, N.B. and Latouche, G. (2012) A new optical leaf-clip meter for simultaneous non-destructive assessment of leaf chlorophyll and epidermal flavonoids. Physiologia Plantarum, 146, 251–260.
Chaiakon, V., Negeri, A., Dhawan, R., Puchaka, B., Ji, J., Chintamanani, S. et al. (2011) Use of mutant-assisted gene identification and characterization (MAGIC) to identify novel genetic loci that modify the maize hypersensitive response. Theoretical and Applied Genetics, 123, 895–997.
Cheng, Y.T., Li, Y., Huang, S., Huang, Y., Dong, X., Zhang, Y. et al. (2011) Stability of plant immune-receptor resistance proteins is controlled by SKP1-CULLIN1-F-box (SCF)-mediated protein degradation. Proceedings of the National Academy of Sciences of the United States of America, 108, 14694–14699.
Chiasson, D., Ekengren, S.K., Martin, G.B., Dobney, S.L. and Snedden, W.A. (2005) Calmodulin-like proteins from Arabidopsis and tomato are involved in host defense against Pseudomonas syringae pv. tomato. Plant Molecular Biology, 58, 887–897.
Choi, H.W., Lee, D.H. and Hwang, B.K. (2009) The pepper calmodulin gene CaCaM1 is involved in reactive oxygen species and nicotin oxide generation required for cell death and the defense response. Molecular Plant-Microbe Interactions, 22, 1389–1400.
Christensena, S.A., Huffaker, A., Kaplan, F., Sims, J., Ziemen, S., Doehlemm, G. et al. (2015) Maize death acids, 9-lipoxygenase-derived cyclopentene (a) ones, display activity as cytotoxic phytoalexins and transcriptional mediators. Proceedings of the National Academy of Sciences of the United States of America, 112, 11407–11412.
Coll, N., Epplle, P. and Dangl, J. (2011) Programmed cell death in the plant immune system. Cell Death & Differentiation, 18, 1247–1256.
Collins, N., Drake, J., Ayliffe, M., Sun, Q., Ellis, J., Hulbert, S. et al. (1999) Molecular characterization of the maize Rp1-D rust resistance haplotype and its mutants. The Plant Cell, 11, 1365–1376.
Dickman, M., Williams, B., Li, Y., De Figueiredo, P. and Wolpert, F. (2017) Reassessing apoptosis in plants. Nature Plants, 3, 773–779.
Ding, X.S., Schneider, W.L., Chaluvadi, S.R., Mian, M.A. and 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.
Gou, M., Zhang, Z., Zhang, N., Huang, Q., Monaghan, J., Yang, H. et al. (2015) Opposing effects on two phases of defense responses from concerted actions of HEAT SHOCK COGNATE70 and BONZAI1 in Arabidopsis. Plant Physiology, 169, 2304–2323.
He, Y., Karre, S., Johal, G.S., Christensen, S.A. and Balint-Kurti, P. (2019a) A maize polygalacturonase functions as a suppressor of programmed cell death in plants. BMC Plant Biology, 19, 310.
He, Y., Kim, S.-B. and Balint-Kurti, P. (2019b) A maize cytochrome b1 complex subunit protein ZmQRCC7 controls variation in the hypersensitive response. Planta, 249, 1477–1485.
Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C. et al. (2004) Silencing of hydroxycinnamoyl-coenzyme A reductase causes changes in seed growth and agronomic traits of Arabidopsis thaliana. Crop Science, 44, 1985–1994.
Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C. et al. (2004) Silencing of hydroxycinnamoyl-coenzyme A reductase causes changes in seed growth and agronomic traits of Arabidopsis thaliana. Crop Science, 44, 1985–1994.
shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. The Plant Cell, 16, 1446–1465.

Holt, B.F., Belkhadir, Y. and Dangl, J.L. (2005) Antagonistic control of disease resistance protein stability in the plant immune system. Science, 309, 929–932.

Hu, G., Richter, T.E., Hulbert, S.H. and Pryor, T. (1996) Disease lesion mimicry caused by mutations in the rust resistance gene rp1. The Plant Cell, 8, 1367–1376.

Hubert, D.-A., Tornero, P., Belkhadir, Y., Krishna, P., Takahashi, A., Shirasu, K. et al. (2003) Cytosolic HSP90 associates with and modulates the Arabidopsis RPM1 disease resistance protein. The EMBO Journal, 22(21), 5679–5689.

Hulbert, S.H. (1997) Structure and evolution of the rp1 complex conferring rust resistance in maize. Annual Review of Phytopathology, 35, 293–310.

Hulbert, S.H. and Bennetzen, J.L. (1991) Recombination at the Rp1 locus of maize. Molecular and General Genetics, 226, 377–382.

Johal, G.S. (2007) Disease lesion mimic mutants of maize. In APSNet Features. St. Paul, MN: American Phytopathological Society. https://doi.org/10.1094/APSNet-2007-0707

Jones, T., Lowe, K., Hoerster, G., Anand, A., Wu, E., Wang, N.et al. (2019) Maize transformation using the morphogenic genes baby Boom and Wuschel2. In: Kumar, S., Barone, P. and Smith, M. (Eds.), Transgenic Plants: Methods and Protocols. New York, NY: Springer, pp. 81–93.

Kim, S.-B., Lee, H.-Y., Choi, E.-H., Park, E., Kim, J.-H., Moon, K.-B. et al. (2018) The coiled-coil and leucine-rich repeat domain of the potyvirus resistance protein Pvr4 has a distinct role in signaling and pathogen recognition. Molecular Plant-Microbe Interactions, 31, 906–913.

Kosack, H. and Jones, J. (1996) Resistance gene-dependent plant defense response. The Plant Cell, 7, 1773–1786.

Künstler, A., Bacsó, R., Gullner, G., Hafez, Y.M. and Király, L. (2016) Calmodulin-like protein. Journal of Biological Chemistry, 291, 1367–1372.

Kuriyama, H. and Fukuda, H. (2002) Developmental programmed cell death in plants. Current Opinion in Plant Biology, 5, 568–573.

Lafamme, B., Dillon, M.M., Martel, A., Almeida, R.N.D., Desveaux, D. and Martel, A. (2006) Plant lipoxygenases. Physiological and Molecular Plant Biology, 159, 239–250.

Liao, D., Cao, Y., Sun, X., Espinoza, C., Nguyen, C.T., Liang, Y. et al. (2017) Arabidopsis E3 ubiquitin ligase PLANT U-BOX13 (PUB13) regulates chitin receptor LYSIN MOTIF RECEPTOR KINASES (LYK 5) protein abundance. New Phytologist, 214, 1646–1656.

Liu, E. and Page, J.E. (2008) Optimized cDNA libraries for virus-induced gene silencing (VIGS) using tobacco rattle virus. Plant Methods, 4, 5.

Liu, H., Jian, L., Xu, J., Zhang, Q., Zhang, M., Jin, M. et al. (2020) High-throughput CRISPR/Cas9 mutagenesis streamlines trait gene identification in maize. The Plant Cell, 32, 1397–1413.

Liu, N., Xie, K., Jia, Q., Zhao, J., Chen, T., Li, H. et al. (2016) Foxtail mosaic virus-induced gene silencing in monocot plants. Plant Physiology, 171, 1801–1807.

Liu, Y., Burch-Smith, T., Schiff, M., Feng, S. and Dinesh-Kumar, S.P. (2004) Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins Sgt1 and Rar1 to modulate an innate immune response in plants. Journal of Biological Chemistry, 279, 2101–2108.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCT method. Methods, 25, 402–408.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J. et al. (2011) Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. Science, 332, 1439–1442.

Lu, Y.-J., Schornack, S., Spallek, T., Geldner, N., Chory, J., Schellmann, S. et al. (2012) Patterns of plant subcellular responses to successful oomycete infections reveal differences in host cell reprogramming and endocytic trafficking. Cellular Microbiology, 14, 682–697.

Lück, S., Kreszies, T., Strickert, M., Schweizer, P., Kuhlmann, M. and Douchkov, D. (2019) siRNA-Finder (si-Fi) software for RNAi-target design and off-target prediction. Frontiers in Plant Science, 10, 1023.

Ma, W., Smigel, A., Tsai, Y.-C., Braam, J. and Berkowitz, G.A. (2008) Innate immunity signaling: cytosolic Ca2+ elevation is linked to downstream nitric oxide generation through the action of calmodulin or a calmodulin-like protein. Plant Physiology, 148, 818–828.

McCarty, D.R., Liu, P. and Koch, K.E. (2018) The UniformMu resource: construction, applications, and opportunities. In: Bennetzen., J. Flint-Garcia, S., Hirsch, C & Tuberosa, R. (eds.), The Maize Genome. Cham, Switzerland: Springer, pp. 131–142.

McMullen, M.D., Kresovich, S., Villeda, H.S., Bradbury, P., Li, H., Sun, Q. et al. (2009) Genetic properties of the maize nested association mapping population. Science, 325, 737–740.

Mei, Y. and Whitham, S.A. (2018) Virus-induced gene silencing in maize with a Foxtail mosaic virus vector. In: Lagrimini, L. (ed.), Maize Methods in Molecular Biology (Vol. 1676). New York, NY: Humana Press, pp. 129–139.

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

Nakamura, S., Mano, S., Tanaka, Y., Ohnishi, M., Nakamori, C., Araki, M. et al. (2010) Gateway binary vectors with the bialaphos resistance gene, as a selection marker for plant transformation. Bioscience, Biotechnology, and Biochemistry, 74, 1315–1319.

Nannas, N.J. and Dawe, R.K. (2015) Genetic and genomic toolbox of Zea mays. Genetics, 199, 655–669.

Negeri, A., Wang, G.-F., Benavente, L., Kibiti, C.M., Chaiikam, V., Johal, G. et al. (2013) Characterization of temperature and light effects on the defense response phenotypes associated with the maize Rp1-D21 autoinoculative resistance gene. BMC Plant Biology, 13, 106.

Newman, M.-A., Sundelin, T., Nielsen, J.T. and Erbs, G. (2013) MAMP (microbe-associated molecular pattern) triggered immunity in plants. Frontiers in Plant Science, 4, 139.

Ngou, B.P.M., Ahn, H.-K., Ding, P. and Jones, J.D. (2020) Mutual potentiation of plant immunity by cell-surface and intracellular receptors. bioRxiv [Preprint]. https://doi.org/10.1101.2020.04.10.034173

Olukolu, B.A., Negeri, A., Dhawan, R., Venkata, B.P., Sharma, P., Garg, A. et al. (2013) A connected set of genes associated with programmed cell death implicated in controlling the hypersensitive response in maize. Genetics, 193, 609–620.

Olukolu, B.A., Wang, G.-F., Vontimitta, V., Venkata, B.P., Marla, S., Ji, J. et al. (2014) A genome-wide association study of the maize hypersensitive defense response identifies genes that cluster in related pathways. PLoS Genetics, 10, e1004562.

Porta, H. and Rocha-Sosa, M. (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiology, 130, 15–21.

Reape, T.J., Brogan, N.P. and McCabe, P.F. (2016) Foxtail mosaic virus vector. In: Lagrimini, L. (ed.), Maize Methods in Molecular Biology (Vol. 1676). New York, NY: Humana Press, pp. 129–139.
Shibata, Y., Kawakita, K. and Takemoto, D. (2011) SGT1 and HSP90 are essential for age-related non-host resistance of *Nicotiana benthamiana* against the oomycete pathogen *Phytophthora infestans*. *Physiological and Molecular Plant Pathology*, 75, 120–128.

Shirasu, K. (2009) The HSP90-SGT1 chaperone complex for NLR immune sensors. *Annual Review of Plant Biology*, 60, 139–164.

Smith, S.M., Steinau, M., Trick, H.N. and Hulbert, S.H. (2010) Recombinant Rp1 genes confer necrotic or nonspecific resistance phenotypes. *Molecular Genetics and Genomics*, 283, 591–602.

Spallek, T., Beck, M., Khaled, S.B., Salomon, S., Bourdais, G., Schellmann, S. et al. (2013) ESCRT-I mediates FLS2 endosomal sorting and plant immunity. *PLoS Genetics*, 9, e1004035.

Svitasev, S., Schwartz, C., Lenderts, B., Young, J.K. and Cigan, A.M. (2016) Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nature Communications*, 7, 13274.

Walley, J.W., Sartor, R.C., Shen, Z., Schmitz, R.J., Wu, K.J., Urich, M.A. et al. (2016) Integration of omic networks in a developmental atlas of maize. *Science*, 353, 814–818.

Wang, G.-F. and Balint-Kurti, P.J. (2016) Maize homologs of CCoAOMT and HCT, two key enzymes in lignin biosynthesis, form complexes with the NLR Rp1 protein to modulate the defense response. *Plant Physiology*, 171, 2166–2177.

Wang, G.-F., He, Y., Strauch, R., Olukolu, B.A., Nielsen, D., Li, X. et al. (2015a) Maize homologs of hydroxycinnamoyltransferase, a key enzyme in lignin biosynthesis, bind the nucleotide binding leucine-rich repeat Rp1 proteins to modulate the defense response. *Plant Physiology*, 169, 2230–2243.

Wang, G.-F., Ji, J., Ei-Kasmi, F., Dangl, J.L., Johal, G. and Balint-Kurti, P.J. (2015b) Molecular and functional analyses of a maize autoactive NB-LRR protein identify precise structural requirements for activity. *PLoS Pathogens*, 11, e1004674.

Wang, J., Hu, M., Wang, J., Qi, J., Han, Z., Wang, G. et al. (2019) Reconstitution and structure of a plant NLR resistosome conferring immunity. *Science*, 364, eaav5870.

Williams, B. and Dickman, M. (2008) Plant programmed cell death: can’t live with it; can’t live without it. *Molecular Plant Pathology*, 9, 531–544.

Woldemariam, M.G., Ahern, K., Jander, G. and Tzin, V. (2018) A role for 9-lipoxygenases in maize defense against insect herbivory. *Plant Signaling & Behavior*, 13, 4709–4723.

Wu, J. and Baldwin, I.T. (2010) New insights into plant responses to the attack from insect herbivores. *Annual Review of Genetics*, 44, 1–24.

Yadava, P., Abhishek, A., Singh, R., Singh, I., Kaul, T., Pattanayak, A. et al. (2017) Advances in maize transformation technologies and development of transgenic maize. *Frontiers in Plant Science*, 7, 1949.

Ye, Z.-H., Kneusel, R.E., Matern, U. and Varner, J.E. (1994) An alternative methylation pathway in lignin biosynthesis in *Zinnia*. *The Plant Cell*, 6, 1427–1439.

Yoom, S.-I., Seo, E., Oh, S.-K., Kim, K.W. and Choi, D. (2012) A common plant cell-wall protein HyPRP1 has dual roles as a positive regulator of cell death and a negative regulator of basal defense against pathogens. *The Plant Journal*, 69, 755–768.

Yu, J., Holland, J.B., McMullen, M. and Buckler, E.S. (2008) Genetic design and statistical power of nested association mapping in maize. *Genetics*, 178, 539–551.

Yuan, M., Jiang, Z., Bi, G., Nomura, K., Liu, M., He, S.Y. et al. (2020) Pattern-recognition receptors are required for NLR-mediated plant immunity. *bioRxiv* [Preprint]. https://doi.org/10.1101/2020.04.10.031294

Zeng, L.-R., Qu, S., Bordeos, A., Yang, C., Baraoidan, M., Yan, H. et al. (2004) Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. *The Plant Cell*, 16, 2795–2808.

Zhang, C., Bradshaw, J.D., Whitham, S.A. and Hill, J.H. (2010a) 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.

Zhang, M., Kadota, Y., Prodromou, C., Shirasu, K. and Pearl, L.H. (2010b) Structural basis for assembly of Hsp90-Sgt1-CHORD protein complexes: implications for chaperoning of NLR innate immunity receptors. *Molecular Cell*, 39, 269–281.

Zhang, Y., Dorey, S., Swiderski, M. and Jones, J.D. (2004) Expression of *RPS4* in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90. *The Plant Journal*, 40, 213–224.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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