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A sugarcane mosaic virus vector for rapid *in planta* screening of proteins that inhibit the growth of insect herbivores

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Summary

Spodoptera frugiperda (fall armyworm) is a notorious pest that threatens maize production worldwide. Current control measures involve the use of chemical insecticides and transgenic maize expressing *Bacillus thuringiensis* (*Bt*) toxins. Although additional transgenes have confirmed insecticidal activity, limited research has been conducted in maize, at least partially due to the technical difficulty of maize transformation. Here, we describe implementation of a sugarcane mosaic virus (SCMV) vector for rapidly testing the efficacy of both endogenous maize genes and heterologous genes from other organisms for the control of *S. frugiperda* in maize. Four categories of proteins were tested using the SCMV vector: (i) maize defence signalling proteins: peptide elicitors (Pep1 and Pep3) and jasmonate acid conjugating enzymes (JAR1a and JAR1b); (ii) maize defensive proteins: the previously identified ribosome-inactivating protein (RIP2) and maize proteinase inhibitor (MPI), and two proteins with predicted but unconfirmed anti-insect activities, an antimicrobial peptide (AMP) and a lectin (JAC1); (iii) lectins from other plant species: *Allium cepa* agglutinin (ACA) and *Galanthus nivalis* agglutinin (GNA); and (iv) scorpion and spider toxins: peptides from *Urodacus yaschenkoi* (UyCT3 and UyCT5) and *Hadronyche versuta* (Hvt). In most cases, *S. frugiperda* larval growth was reduced by transient SCMV-mediated overexpression of genes encoding these proteins. Additionally, experiments with a subset of the SCMV-expressed genes showed effectiveness against two aphid species, *Rhopalosiphum maidis* (corn leaf aphid) and *Myzus persicae* (green peach aphid). Together, these results demonstrate that SCMV vectors are a rapid screening method for testing the efficacy and insecticidal activity of candidate genes in maize.

Introduction

Maize (*Zea mays*) is one of the world's most important cereal crops, serving not only as a food source for humans and livestock, but also as a raw material for the production of ethanol and other industrial products (Ai and Jane, 2016; Chaudhary *et al*., 2014). The needs of an ever-expanding population will lead to increasing demands on maize production in the coming years. Therefore, maintaining adequate maize yields will require reducing not only the cost of agricultural inputs, but also the negative impacts of biotic and abiotic stresses that limit maize productivity.

More than 100 species of insect pests feed on maize plants in agricultural fields (McMullen *et al*., 2009; Meihls *et al*., 2012). Among these pests, one of the most damaging is *Spodoptera frugiperda* (fall armyworm; Figure 1a), a lepidopteran species that is indigenous to the Americas but recently has become invasive in Africa and Asia (Food and Agriculture Organization of the United Nations, 2018; Goergen *et al*., 2016). By consuming all above-ground plant parts, *S. frugiperda* larvae reduce photosynthetic area, cause developmental delays and decrease yield.

Currently available *S. frugiperda* control methods, both application of chemical insecticides (Togola *et al*., 2018) and transgenic maize producing *Bacillus thuringiensis* (*Bt*) toxins (Huang *et al*., 2014; Tabashnik and Carrière, 2017), are becoming less effective as the insects develop resistance. Therefore, there is a need to screen for additional transgenes that can be used to enhance maize resistance to *S. frugiperda* feeding. Broadly, such approaches can include upregulation of maize defence signalling, overexpression of individual maize defensive proteins and expression of heterologous insecticidal proteins.

Plant elicitor peptides (Peps) trigger anti-herbivore defence responses (Huffaker, 2015; Huffaker *et al*., 2013; Poretsky *et al*., 2020). In maize, *Zm*Pep1 and *Zm*Pep3 up-regulate defences, at least in part by induction of the jasmonic acid (JA) signalling pathway (Huffaker *et al*., 2011, 2013). A key step in the JA pathway is the conjugation of JA with isoleucine by JAR1 (JASMONATE RESISTANT 1) enzymes (Koo and Howe, 2009; Staswick *et al*., 2002) to form JA-isoleucine. Expression of *JAR1a*

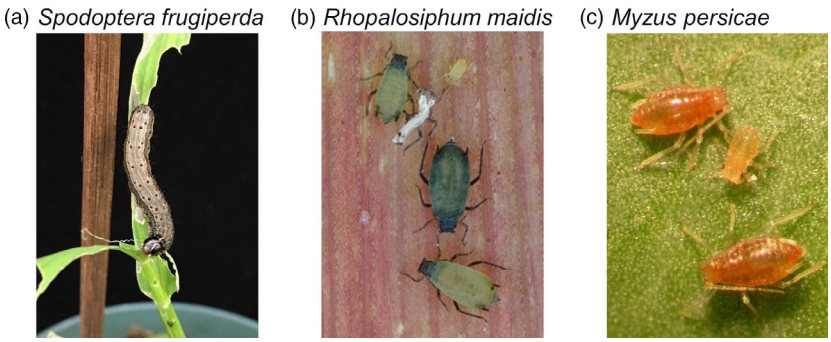

Figure 1 Insect species used in this study. (a) *Spodoptera frugiperda*, fall armyworm, (b) *Rhopalosiphum maidis*, corn leaf aphid and (c) *Myzus persicae*, green peach aphid

and *JAR1b*, two of the five predicted *JAR* genes in maize (Borrego and Kolomiets, 2016), is highly induced by *Spodoptera exigua* (beet armyworm) herbivory (Tzin *et al*., 2017). Thus, these maize genes are good targets for overexpression to enhance resistance against *S. frugiperda*.

Maize and other plants produce ribosome-inactivating proteins (RIPs) that block ribosome function by depurinating a specific adenine residue of the large ribosomal RNA (Bass *et al*., 2004; Zhu *et al*., 2018). These proteins, which are toxic for a variety of insects, including Lepidoptera (Shahidi-Noghabi *et al*., 2009) and Hemiptera (Hamshou *et al*., 2016), have been used previously in transgenic approaches. For instance, the expression of a maize kernel *RIP1* in *Nicotiana tabacum* (tobacco) increased resistance to *Helicoverpa zea* (corn earworm) feeding (Dowd *et al*., 2003). The *RIP2* gene is expressed in all maize tissues except the kernels (Bass *et al*., 2004). *RIP2* expression was induced by *S. frugiperda* herbivory and recombinant RIP2 protein decreased caterpillar growth on artificial diet (Chuang *et al*., 2014a).

Two additional classes of maize proteins with anti-herbivore activity are proteinase inhibitors and antimicrobial peptides (Campos *et al*., 2018; Koiwa *et al*., 1997). Proteinase inhibitors, which are produced by many plant families, impair the growth and survival of insects by disrupting the function of digestive enzymes. Maize proteinase inhibitor (*MPI*) expression was induced by both caterpillar herbivory and JA signalling (Cordero *et al*., 1994; Shivaji *et al*., 2010; Tamayo *et al*., 2000). Heterologous expression of *MPI* in rice increased resistance to *Chilo suppressalis* (striped stem borer) (Vila *et al*., 2005). Cyclotides are macrocyclic insecticidal peptides with the length of about 30 amino acids and a conserved cystine knot motif containing three disulphide bonds (Campos *et al*., 2018; Craik *et al*., 1999; Weidmann and Craik, 2016). Cyclotide Kalata B1 from *Oldenlandia affinis* decreased the growth of *Helicoverpa armigera* (corn earworm) larvae by rupturing epithelial cells in the midgut (Barbeta *et al*., 2008). Cycloviolacins, cyclotides from *Viola odorata*, negatively affected the probing and feeding behaviour of *Myzus persicae* (green peach aphid), suggesting that cycloviolacins limit aphid population growth (Dancewicz *et al*., 2020). Among predicted antimicrobial peptides in maize, a few belong to the cyclotide family (Mulvenna *et al*., 2006; Noonan *et al*., 2017), but their efficacy against insects has not been confirmed.

Plant lectins, carbohydrate-binding proteins that interact with glycoproteins and glycan structures in insect guts, have antinutritional or insecticidal effects (Macedo *et al*., 2015). For instance, snowdrop lectin (*Galanthus nivalis* agglutinin; GNA), onion lectin (*Allium cepa* agglutinin; ACA) and garlic (*Allium sativum*) leaf lectin reduce nutrient uptake and growth in wide range of insects (Vandenborre *et al*., 2011). Expression of a maize lectin gene, Jacalin 1 (*JAC1*), is induced by JA, an indication that it may provide protection against herbivory (Van Damme *et al*., 2004). There can be additive or even synergistic effects if lectins are co-expressed or fused to scorpion or spider venom peptides. For instance, the insecticidal efficacy of GNA was increased by fusions to Hvt (ω-hexatoxin Hv1a, also called ω-ACTX Hv1a) from *Hadronyche versuta* (Blue Mountains funnel web spider) (Fitches *et al*., 2012), ButaIT (*Buthus tamulus* insect toxin) from *Buthus tamulus*, also known as *Mesobuthus tamulus* (Fitches *et al*., 2010; Wudayagiri *et al*., 2001), AaIT (*Androctonus australis* insect toxin) from *Androctonus australis* (Liu *et al*., 2016) and δ-amaurobitoxin-PIIa from *Pireneitega luctuosus* (Yang *et al*., 2014).

Scorpion and spider venoms, which contain numerous insecticidal toxins (King and Hardy, 2013; Ortiz *et al*., 2015), have been explored as sources of insecticidal peptides. UyCT3 and UyCT5, two antimicrobial peptides that are produced in the venom glands of *Urodacus yaschenkoi* (inland robust scorpion) decrease the fitness of *Acyrthosiphon pisum* (pea aphid) and the density of the primary symbiont, *Buchnera aphidicola*, suggesting that those are promising candidates for the production of insect-resistant transgenic plants (Luna-Ramirez *et al*., 2017). Similarly, Hvt from *H. versuta* is broadly effective against both lepidopteran and hemipteran pests when expressed in transgenic plants (Ullah *et al*., 2015; Javaid *et al*., 2016; Rauf *et al*., 2019). Given the broad insecticidal activity of these spider and scorpion venom proteins, we hypothesized that they would also be effective in reducing *S. frugiperda* growth.

Testing the effectiveness of transgenes for controlling pest insects on maize is limited by the high cost of maize transformation and the often greater than one-year timeline that is required to obtain transgenic maize plants for experiments. Therefore, we are proposing an alternate approach, whereby the efficacy of transgenes that enhance maize pest tolerance is tested by transient expression in maize using a virus vector. Sugarcane mosaic virus (SCMV), a positive-sense single-stranded RNA virus, which has been adapted for efficient transgene expression in maize (Mei *et al*., 2019), is attractive vector for such experiments. Genes of interest are inserted between the SCMV *P1* and *HC-Pro* cistrons in SCMV-CS3, a newly created plasmid vector, maize seedlings are infected by particle bombardment or *Agrobacterium* inoculation, and the pest resistance of the infected plants can be assessed after three weeks. Here, we show that virus-mediated expression of maize defence-regulating proteins, maize insecticidal proteins and exogenous toxins (Table 1) can reduce the growth of insect pests. We demonstrate the insect-controlling properties of not only known proteins but also two maize proteins that were not previously confirmed to have anti-

Table 1 SCMV-expressed transgenes used in this study

Source species	Gene name	GenBank ID	MaizeGDB ID	Description
Zea mays	*Pep1*	XM_008670579	GRMZM2G055447	Peptide elicitor 1
Zea mays	*Pep3*	XM_008670581	GRMZM2G339117	Peptide elicitor 3
Zea mays	*JAR1a*	NM_001174342	GRMZM2G091276	Jasmonate-isoleucine conjugating enzyme
Zea mays	*JAR1b*	NM_001361064	GRMZM2G162413	Jasmonate-isoleucine conjugating enzyme
Zea mays	*RIP2*	NM_001137489	GRMZM2G119705	Ribosome-inactivating protein 2
Zea mays	*JAC1*	NM_001148875	GRMZM2G050412	Jacalin 1, maize lectin
Zea mays	*AMP*	NM_001371023	GRMZM2G032198	Cyclotide antimicrobial peptide
Zea mays	*MPI*	X78988	GRMZM2G028393	Maize proteinase inhibitor
Allium cepa	*ACA*	DQ255944		Onion lectin
Galanthus nivalis	*GNA*	M55556		Snowdrop lectin
Hadronyche versuta	*Hvt*	AJ938032		Spider venom toxin, ω-hexatoxin-Hv1a
Urodacus yaschenkoi	*UyCT3*	JX274241		Scorpion venom toxin, antimicrobial peptide
Urodacus yaschenkoi	*UyCT5*	JX274242		Scorpion venom toxin, antimicrobial peptide

herbivore properties. Furthermore, we show that expression of transgenes using SCMV also is effective in reducing reproduction by two hemipteran pests, *Rhopalosiphum maidis* (corn leaf aphid; Figure 1b) and *M. persicae* (Figure 1c).

Results

SCMV-*GFP* does not affect *S. frugiperda* growth on maize

A previously described SCMV cloning vector (Mei *et al*., 2019) was modified to produce SCMV-CS3 (Figure 2a), and the mEGFP coding sequence (Zacharias *et al*., 2002) was placed in the multiple cloning site to produce SCMV-*GFP*. An empty vector control virus (SCMV-EV) and SCMV-*GFP* were used to inoculate one-week-old maize seedlings. Both SCMV-*GFP* and SCMV-EV caused mosaic symptoms within two weeks after rub-inoculation and continued to spread in newly emerging leaves of the infected plants (Figure 2b). Three weeks post-inoculation, the transcript levels of *GFP* relative to maize endogenous control genes, *Actin* and *EF1-α*, was quantified using quantitative RT-PCR. The *GFP* expression level was significantly higher in plants infected by SCMV-*GFP* than SCMV-EV (Figure 2c). Infected leaves were examined using confocal microscopy, and the green fluorescence signal was only detected in leaves infected with SCMV-*GFP* (Figure 2d). In these short-term experiments, we did not observe a significant reduction in the growth of SCMV-infected maize plants. However, older maize plants, which had been infected for more than four weeks, showed leaf mosaic symptoms associated with the SCMV infection. Neither *S. frugiperda* larval survival (Figure 2e) nor larval growth (Figure 2f) differed significantly between plants infected by SCMV-*GFP* and SCMV-EV. Therefore, SCMV-*GFP* was used as a transgene-expressing virus control treatment in subsequent experiments to test the efficacy of insect growth-inhibiting proteins (Table 1) against *S. frugiperda*, *R. maidis* and *M. persicae* (Figure 1a–c).

Expression of endogenous maize genes using SCMV enhances *S. frugiperda* resistance

We cloned the 69 bp sequences of the maize *Pep1* and *Pep3* defence elicitors (Huffaker *et al*., 2011, 2013) into pSCMV-CS3 and inoculated seedlings of sweet corn variety Golden Bantam. *Pep1* and *Pep3* expression was confirmed by qRT-PCR three weeks post-inoculation (Figure 3a,b). Relative to SCMV-*GFP*, infection with SCMV-*Pep1* and SCMV-*Pep3* caused increased transcript accumulation of maize proteinase inhibitor (*MPI*; Figure 3c), a JA-induced marker gene with anti-herbivore activity (Chuang *et al*., 2014b). *Pep1* and *Pep3* expression decreased the growth of *S. frugiperda* larvae on Golden Bantam maize by 25% and 51%, respectively, compared to GFP control plants (Figures 3d and S1), but did not affect survival of the larvae (Table S3). Similar results were obtained for *Pep3* using sweet corn (P39) and field corn (B73) inbred lines (Figure 3e,f), showing that the effect on *S. frugiperda* growth is not specific to Golden Bantam.

We targeted previously investigated maize insect resistance genes (*JAR1a*, *JAR1b*, *RIP2* and *MPI*) and two predicted resistance genes (*AMP* and *JAC1*) for overexpression using SCMV. A previously uncharacterized gene (GRMZM2G032198) encoding a maize cyclotide antimicrobial peptides was designated as *AMP*. Expression of both *AMP* and *JAC1* was induced by *S. frugiperda* herbivory (Figure S2), suggesting that these genes are involved in maize insect resistance. The expression levels of *JAR1a*, *JAR1b*, *RIP2*, *JAC1*, *AMP* and *MPI* were significantly higher in plants infected by the corresponding SCMV constructs than in SCMV-*GFP* control plants (Figure 3g–j,m,n). SCMV-*JAR1a* and SCMV-*JAR1b* also significantly increased *MPI* expression (Figure 3k), confirming upregulation of JA-related defence pathways. More importantly, the growth of *S. frugiperda* larvae was up to 60% lower on plants expressing *JAR1a*, *JAR1b*, *RIP2*, *JAC1*, *AMP* and *MPI* than on Golden Bantam control plants infected with SCMV-*GFP* (Figure 3l,o). Although the larval growth rate was reduced in these experiments, larval survival was not significantly affected (Table S3).

Expression of scorpion insecticidal proteins reduces *S. frugiperda* growth

To determine whether SCMV can be used to express heterologous insect resistance genes in maize, we cloned *UyCT3* and *UyCT5*, which encode *U. yaschenkoi* venom toxins, into the SCMV vector. Transgene expression in maize was confirmed by qRT-PCR (Figure 4a,b). Both SCMV-*UyCT3* and SCMV-*UyCT5* reduced *S. frugiperda* weight on P39 and B73 plants compared to SCMV-*GFP* control plants (Figure 4c,d). Relative to control plants, neither SCMV-*UyCT3* nor SCMV-*UyCT5* significantly reduced the larval survival rate (Table S4).

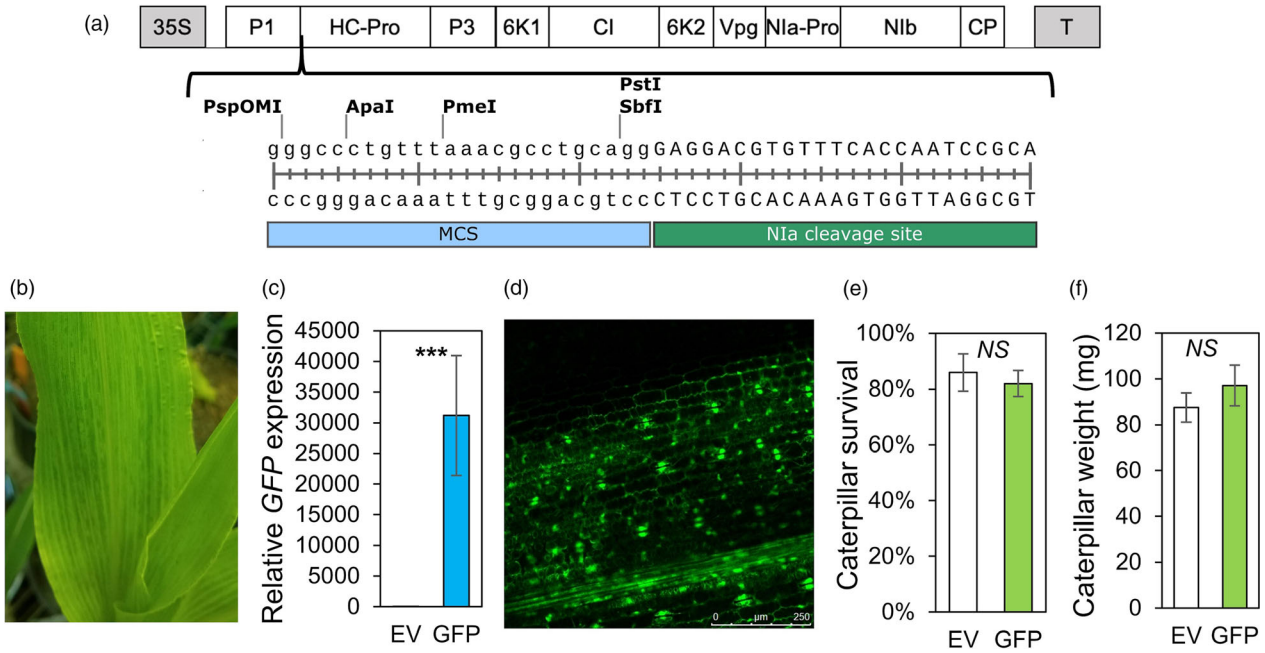

Figure 2 Use of a *sugarcane mosaic virus* (SCMV) to transiently express *GFP* in maize. (a) Schematic diagram of the SCMV-CS3 cloning vector. A multiple cloning site (MCS) was inserted between *P1* and *HC-Pro* genes of SCMV. 35S: cauliflower mosaic virus 35S promoter; T: NOS terminator. (b) Mosaic infection symptoms of SCMV vector in maize three weeks post-inoculation. (c) Transient overexpression of *GFP* in plants infected by SCMV-empty vector (EV) or -*GFP*. Gene expression was determined by qRT-PCR three weeks post-inoculation. Means ± SE of $N = 5$, ***$P < 0.001$, *t*-test. (d) Confocal image of foliar GFP expression. (e,f) *S. frugiperda* survival and larval growth on plants infected by EV or SCMV-*GFP*. Two-day-old caterpillars were fed on infected plants for one week and caterpillar mass was determined. Means ± SE of $N = 10$, EV, empty vector; *NS*, non-significant; MCS, multiple cloning site

Expression of fusion proteins using SCMV has additive effects on *S. frugiperda*

Fusion of spider and scorpion neurotoxins with plant lectins can improve their toxicity (Fitches *et al*., 2012; Liu *et al*., 2016; Rauf *et al*., 2019). We investigated this effect in maize using SCMV constructs. Because SCMV vectors produce a polyprotein precursor from which functional proteins are cleaved by the NIa protease (Mei *et al*., 2019), we generated SCMV constructs with and without an NIa cleavage site between the venom toxin and lectin to determine whether the proteins are more efficacious separately or as fusion proteins. The expression levels of *H. versuta* toxin (*Hvt*) and *A. cepa* agglutinin (*ACA*) genes were significantly higher in plants infected by each corresponding SCMV constructs than SCMV-*GFP* control (Figure 5a,b), and the presence of protease cleavage site between *Hvt* and *ACA* did not affect *Hvt* and *ACA* transcript accumulation. Expression of single proteins did not significantly decrease *S. frugiperda* growth relative to the GFP control (Figure 5c). However, the expression of fusion protein by SCMV-*Hvt-ACA* or two individual proteins of Hvt and ACA by SCMV-*Hvt*-cleavage site-*ACA* reduced the larval growth by 39% and 46%, relative to the GFP control (Figure 5c), respectively, suggesting an additive effect from the expression of the two genes.

As the presence of a cleavage site between the two protein components did not increase efficacy (Figure 5c), we tested insecticidal activity of fusion proteins without viral protease cleavage sites in subsequent experiments. SCMV constructs were made with a maize defence gene, *RIP2*, and a spider insecticidal protein, *Hvt*, fused to *G. nivalis* agglutinin (*GNA*). We confirmed that each gene was expressed in plants infected by the corresponding construct (Figure 5d,e,g,h). Although the expression of *GNA* alone did not affect *S. frugiperda* larval growth, infection with SCMV-*RIP2* and SCMV-*RIP2-GNA* reduced *S. frugiperda* growth by 28% and 27%, respectively (Figure 5f). In the case of *Hvt* and *GNA*, neither gene by itself significantly reduced caterpillar growth. However, the SCMV-*Hvt-GNA* fusion construct significantly decreased *S. frugiperda* larval growth compared to GFP control protein (Figure 5i). Relative to controls, larval survival was not significantly affected by these SCMV constructs (Table S5). Together, these results indicate that fusion proteins combining lectins and maize defence proteins or venom toxins can improve resistance against *S. frugiperda*.

Expression of maize and scorpion genes enhances resistance to phloem-feeding herbivores

To investigate whether our SCMV constructs also provide protection against phloem-feeding insects, we conducted aphid bioassays using P39 plants infected with a subset of the previously tested constructs: SCMV-*GFP*, SCMV-*Pep3*, SCMV-*RIP2*, SCMV-*UyCT3*, SCMV-*UyCT5*, SCMV-*AMP* and SCMV-*JAC1*. GFP expression did not affect aphid numbers compared to the empty vector control (Figure 6a,b). By contrast, expression of maize defence proteins and scorpion toxins significantly decreased progeny production by both *R. maidis* (Figure 6a,c) and *M. persicae* (Figure 6b,d) compared to SCMV-*GFP* control.

Discussion

Artificial diet assays are commonly employed for testing the oral efficacy of novel insecticidal proteins against insect herbivores (Fitches *et al*., 2012; Panwar *et al*., 2018; Yao *et al*., 2003).

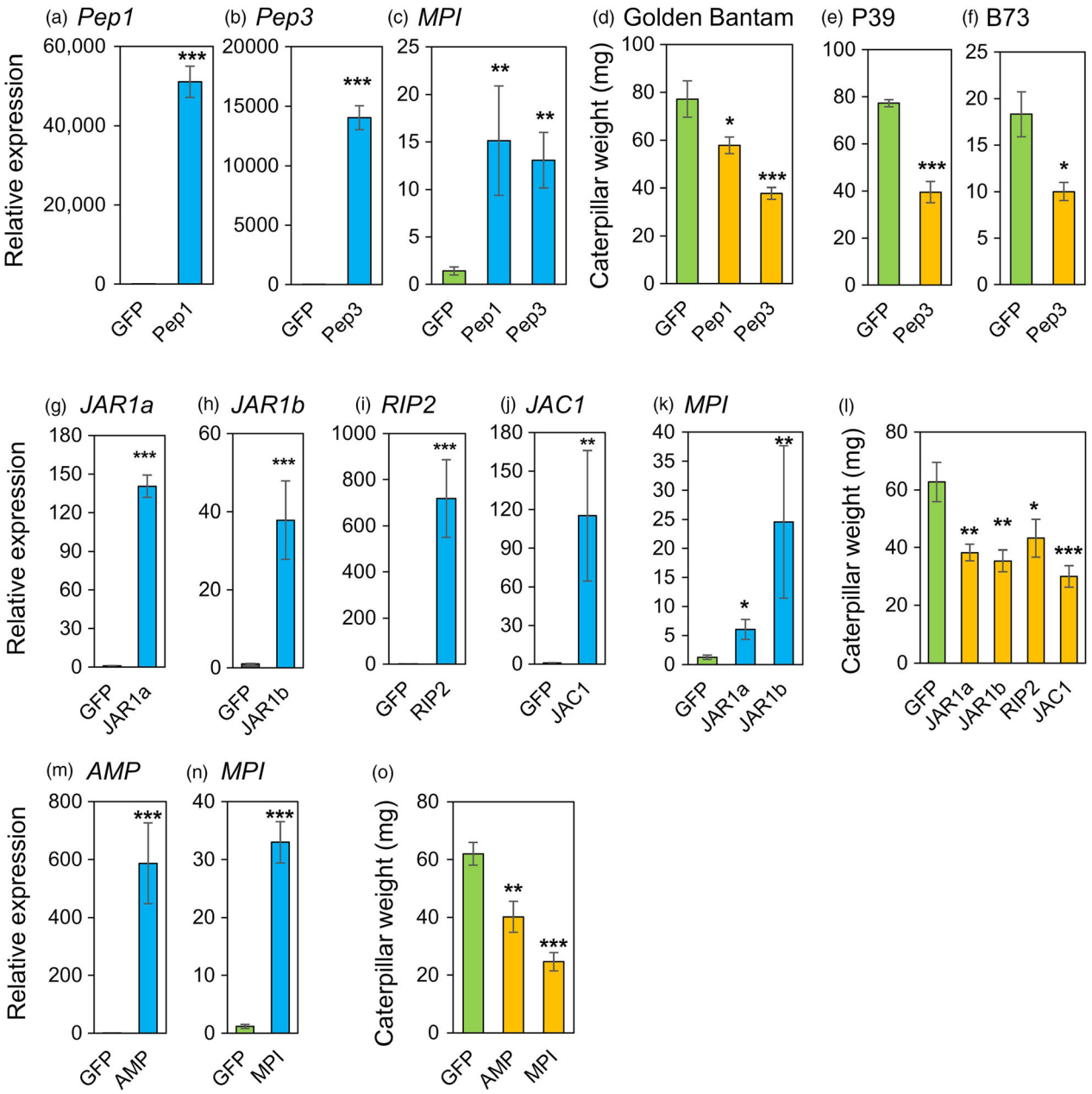

Figure 3 Gene expression and *Spodoptera frugiperda* larval growth on plants expressing maize defence genes. (a,b) Transient overexpression of *Pep1* and *Pep3* in Golden Bantam plants infected by SCMV-*GFP*, -*Pep1* or -*Pep3*. (c) The expression level of *MPI* in plants expressing SCMV-encoded *GFP*, *Pep1* or *Pep3*. Gene expression was determined by qRT-PCR three weeks post-inoculation. (d) Performance of *S. frugiperda* on Golden Bantam plants expressing *GFP*, *Pep1* or *Pep3*. (e,f) Performance of *S. frugiperda* on P39 and B73 plants expressing *GFP* or *Pep3*. (g–j,m,n) Transgene expression in Golden Bantam plants infected with SCMV-*GFP*, -*JAR1a*, -*JAR1b*, -*RIP2*, -*JAC1*, -*AMP* or -*MPI*. (k) Expression level of *MPI* in Golden Bantam plants expressing *GFP*, *JAR1a* or *JAR1b*. (l,o) Performance of *S. frugiperda* on plants expressing *GFP*, *JAR1a*, *JAR1b*, *RIP2*, *JAC1*, *AMP* or *MPI*. Five caterpillars were confined on each infected plant three weeks post-inoculation and average caterpillar weights on each plant were measured one week later. Means ± SE of $N = 5$ for gene expression, $N = 10–12$ for insect bioassays, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ relative to GFP control, Dunnett's test

However, growth inhibition on artificial diet does not always correlate well with the effects that are observed when the same insecticidal proteins are subsequently expressed in transgenic plants (Khan *et al.*, 2020). Both the context of the surrounding plant tissue and the localization of the insecticidal proteins in the plants could affect their toxicity against insect herbivores. Therefore, rather than pre-screening insecticidal proteins by cloning in microbial systems, purification and artificial diet assays, we propose that transient expression using a viral vector such as SCMV will be a more effective approach for rapidly testing the *in planta* efficacy of novel insecticidal proteins.

A possible limitation of the current study is that, although we have confirmed transgene expression, we have not verified the location and abundance of each individual protein in SCMV-

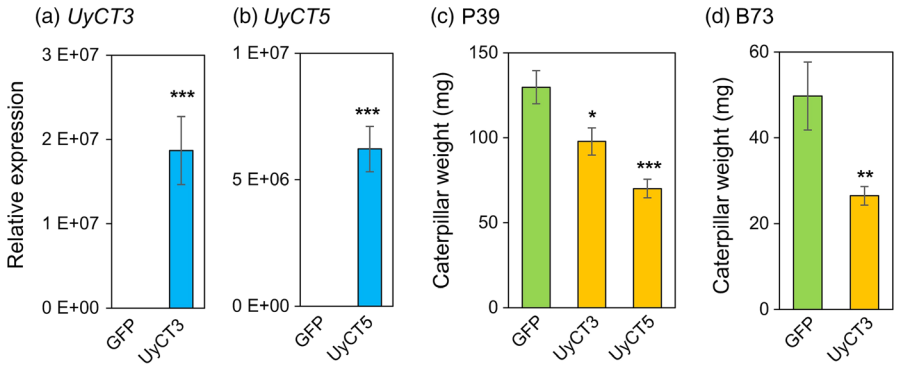

Figure 4 Gene expression and larval growth on P39 plants expressing heterologous insecticidal genes. (a, b) Transient overexpression of *UyCT3* and *UyCT5* in P39 plants infected by SCMV-*GFP*, -*UyCT3* or -*UyCT5*. Gene expression was determined by qRT-PCR three weeks post-inoculation. (c) Performance of *S. frugiperda* on P39 plants expressing *GFP*, *UyCT3* or *UyCT5*. (d) Performance of *S. frugiperda* on B73 plants expressing *GFP* or *UyCT3*. Five caterpillars were confined on infected plants three weeks post-inoculation. Average caterpillar weights on each plant were measured one week later. Means +/- SE of $N = 5$ for gene expression, $N = 7$-14 for insect bioassays, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to GFP control, Dunnett's test

infected maize plants. However, in previous experiments, easily visualized proteins [GFP, GUS (β-glucuronidase) and NLuc (nano luciferase)] were clearly overproduced in maize after gene expression using SCMV vectors (Beernink *et al*., 2021; Mei *et al*., 2019). Production of BAR (bialaphos resistance) using an SCMV vector conferred herbicide resistance to virus-infected plants (Mei *et al*., 2019), indicating that there was successful protein production. Similarly, cloning of genes that encode insecticidal or defence-inducing proteins into SCMV as part of the current study caused reduced *S. frugiperda* caterpillar growth. This is a strong indication that there is not only gene expression but also protein production from these SCMV constructs, and that SCMV is an effective vector for testing the *in planta* effectiveness of proteins that control *S. frugiperda* and other insect herbivores.

Reduced weight gain of *S. frugiperda* in response to SCMV-mediated expression of *Pep1*, *Pep3*, *JAR1a*, *JAR1b*, *RIP2*, *MPI*, *UyCT3* and *UyCT5* as single-gene constructs is consistent with previous reports of these genes providing protection against insect herbivory. Additionally, *AMP* and *JAC1*, two maize genes that are up-regulated in response to *S. frugiperda* feeding (Figure S2), reduced caterpillar weight gain when overexpressed in maize. Although transcriptomic studies have identified numerous maize genes that are up-regulated in response to arthropod feeding (Bui *et al*., 2018; Guo *et al*., 2019; Pan *et al*., 2020; Song *et al*., 2017; Tzin *et al*., 2015, 2017; Wang *et al*., 2017; Yang *et al*., 2020; Zhang *et al*., 2016), the majority of these genes have not been investigated for their roles in plant defence against herbivory. This is at least in part due to the time and cost of creating maize lines that have individual genes overexpressed. Transient expression using SCMV, as we have done for *AMP* and *JAC1*, will accelerate the process of testing the defencing functions of maize genes that are induced in response to herbivory. However, SCMV infection itself changes the abundance of numerous maize proteins (Chen *et al*., 2017). Thus, the possible effects of SCMV infection on the function of insect resistance proteins will need to be considered when selecting candidate genes for SCMV-mediated expression. Moreover, the effects of SCMV-mediated overexpression always should be evaluated with a proper control treatment such as SCMV-GFP and/or SCMV-empty vector.

Although expression of *Hvt*, *ACA* and *GNA* increased resistance in other plant-insect interaction studies (Ullah *et al*., 2015; Liu *et al*., 2016; Vandenborre *et al*., 2011), expression of these single-gene SCMV constructs in maize did not significantly reduce *S. frugiperda* weight gain. Nevertheless, expression of gene fusions, *Hvt-ACA* and *Hvt-GNA*, reduced *S. frugiperda* weight gain, indicating that there are additive effects of the spider venom and the lectin (Figure 5c,i). This is consistent with other studies showing that the toxicity of spider and scorpion toxins was improved by combining with a lectin, which may facilitate the transfer of the venom proteins across the gut lumen (Fitches *et al*., 2002; Javaid *et al*., 2016; Nakasu *et al*., 2014; Rauf *et al*., 2019). In addition to these synergistic effects on larval growth, it is likely that the stacking of multiple toxic proteins with different modes of action in one viral construct will delay the development of resistance in insects (Head *et al*., 2017; Ni *et al*., 2017). In contrast to Hvt-lectin fusions, we did not observe an additive effect when RIP2 was linked to GNA (Figure 5f). This difference may be attributed to the differing origins of the toxin proteins expressed in the SCMV constructs. Whereas spider venoms like Hvt are injected directly into the haemolymph, the maize RIP2 protein would be consumed orally by lepidopteran larvae. As an endogenous maize insecticidal protein, RIP2 may bind to an as yet unknown receptor and thereby enter the midgut cells and/or the insect haemolymph.

Our observation of increased defence gene expression (Figure 3c) and reduced *S. frugiperda* weight gain (Figure 3d-f) on plants infected with SCMV-*Pep1* and SCMV-*Pep3* is consistent with experiments showing that pre-treatment of maize plants with Pep1 and Pep3 increases JA levels, defence gene expression and defensive metabolites, leading to reduced growth of *S. exigua* larvae (Huffaker *et al*., 2011, 2013). Under normal circumstances, peptide signalling is initiated when plasma membranes are disrupted and elicitor peptides are released from the cytoplasm into the apoplastic space (Bartels and Boller, 2015). Receptors on neighbouring intact cells recognize these peptides and elicit downstream defence pathways (Lori *et al*., 2015). However, in our experiments, the expression of *MPI* was induced in plants infected by SCMV-*Pep1* and SCMV-*Pep3*, even before initiation of caterpillar feeding (Figure 3c). Pathogen attack can cause plant proteins without secretory signals to be released into

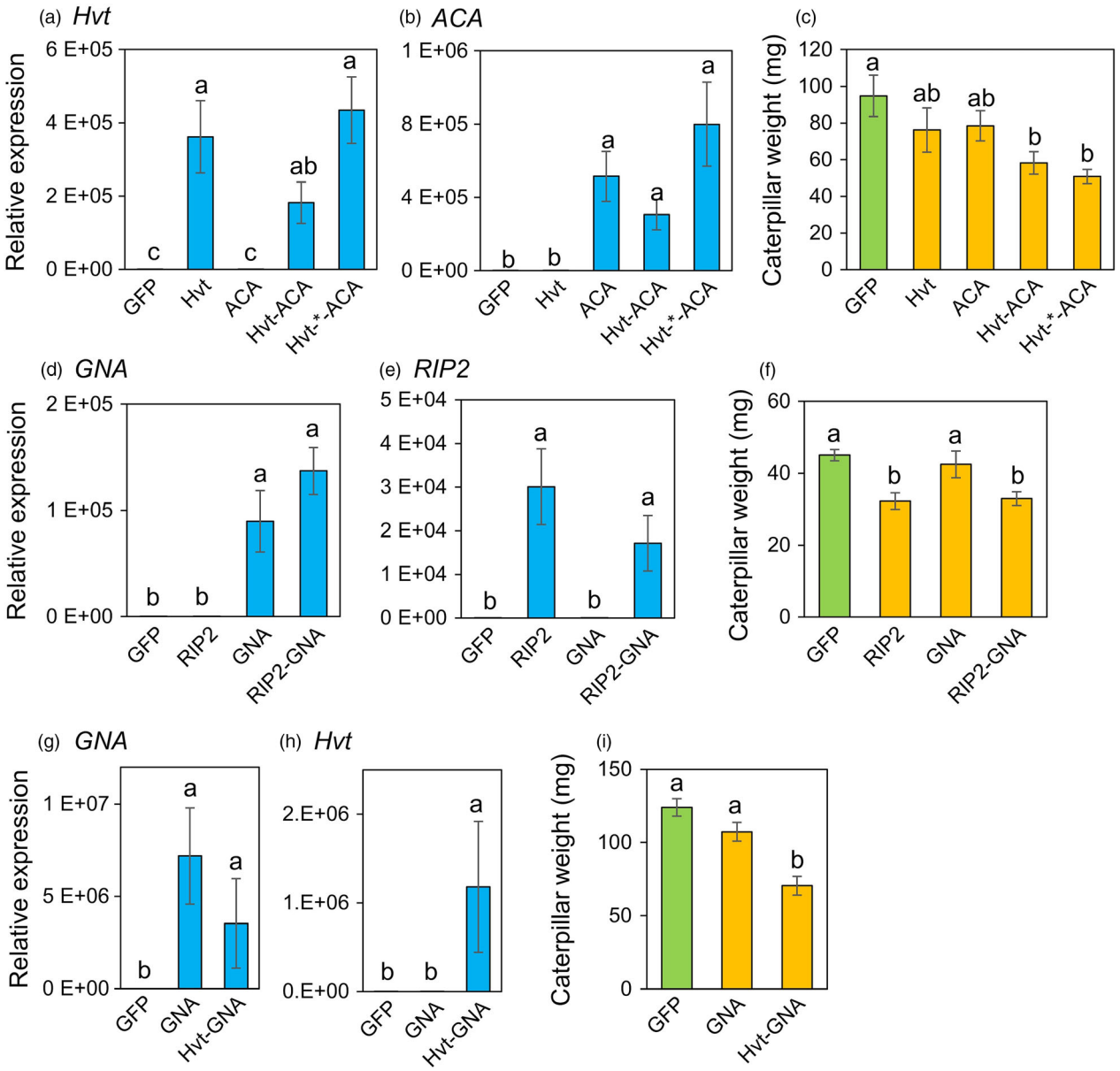

Figure 5 Gene expression and larval growth on plants expressing heterologous insecticidal genes. (a,b) Transient overexpression of *Hvt* and *ACA* in plants infected by SCMV-*GFP*, *-Hvt*, *-ACA*, *-Hvt-ACA* or *-Hvt-*-ACA*. * represents a protease cleavage site. (c) Performance of *Spodoptera frugiperda* on plants expressing *GFP*, *Hvt*, *ACA*, *Hvt-ACA* or *Hvt-*-ACA*. (d,e) Transient overexpression of *GNA* and *RIP2* in plants infected by SCMV-*GFP*, *-RIP2*, *-GNA* or *-RIP2-GNA*. (f) Performance of *S. frugiperda* on plants expressing *GFP*, *RIP2*, *GNA* or *RIP2-GNA*. (g,h) Transient overexpression of *GNA* and *Hvt* in plants infected by SCMV-*GFP*, *-GNA*, *-Hvt* or *-Hvt-GNA*. (i) Performance of *S. frugiperda* on plants expressing *GFP*, *GNA* or *Hvt-GNA*. Gene expression was determined by qRT-PCR three weeks post-inoculation. Five caterpillars were confined on each infected plant three weeks post-inoculation, and average caterpillar weights on each plant were measured one week later. Means ± SE of $N = 5$ for gene expression and $N = 10–12$ for insect bioassays. Different letters indicate significant differences, $P < 0.05$, ANOVA followed by Tukey's HSD test

the apoplast (Agrawal *et al.*, 2010) and is possible that SCMV infection initiated Pep1 and Pep 3 signalling in this manner.

Induced defences, in particular those regulated by the jasmonic acid pathway, typically are turned on after perception of insect herbivory by maize and other plants (Erb and Reymond, 2019; Howe and Jander, 2008). However, initiation of jasmonate-regulated defences takes time and some lepidopteran herbivores may have the ability to suppress jasmonate signalling (Chen *et al.*, 2019a). Therefore, targeted initiation of maize defence responses by expression of regulatory proteins in SCMV may be an approach for increasing pest resistance. Such virus-mediated induction could be deployed in maize fields when there is the specific threat of insect pests such as *S. frugiperda*. However, the negative growth effects that may be associated with longer-term SCMV infection of mature maize plants have not been fully investigated. Future research could be focused on engineering SCMV to be less pathogenic or identifying other maize viruses that lead to asymptomatic infections in mature plants.

Currently available insect-resistant transgenic maize varieties, in particular those expressing *Cry*, *Cyt* or *Vip* genes from *B.*

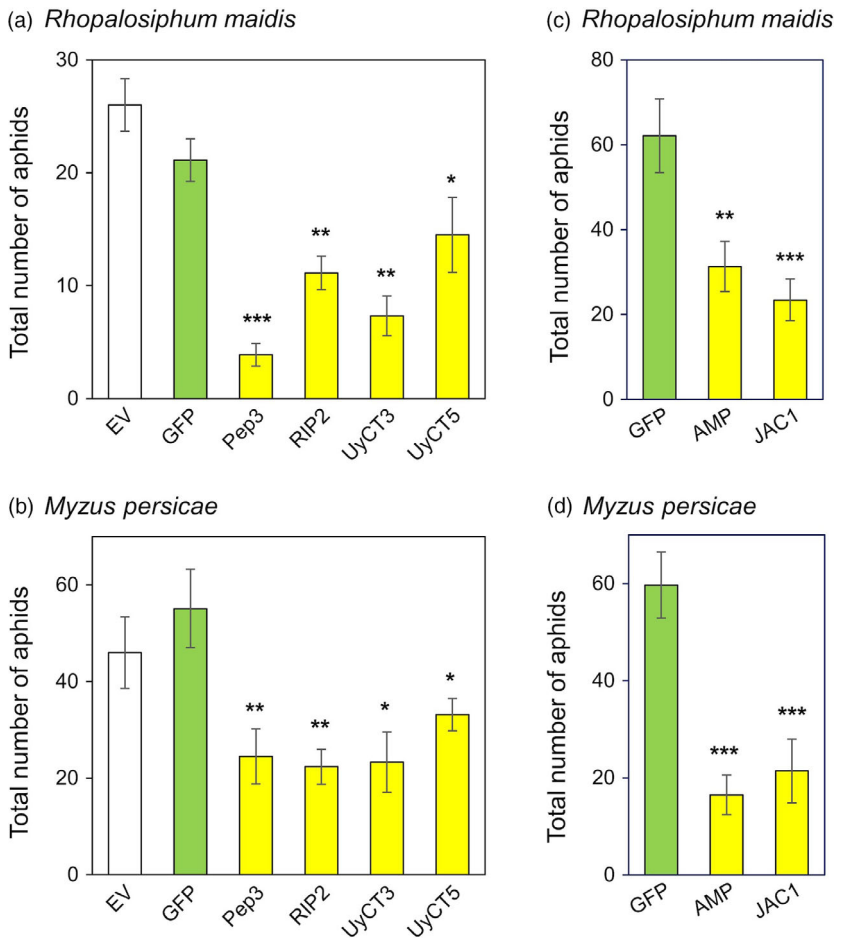

Figure 6 Total number of aphids on P39 plants expressing maize defence genes and scorpion toxin genes. (a,c) *Rhopalosiphum maidis* (b,d) *Myzus persicae*. Eight *R. maidis* adults or ten *M. persicae* adults were confined on plants infected by SCMV-EV, *-GFP*, *-Pep3*, *-RIP2*, *-UyCT3*, *UyCT5*, *AMP or JAC1* three weeks post-inoculation. Surviving aphids and their progeny were counted one week later. Means ± SE of $N = 8$–10, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ relative to GFP control, Dunnett's test

thuringiensis (Bravo *et al*., 2011; Chakroun *et al*., 2016), are not effective against phloem-feeding insects such as *R. maidis* (corn leaf aphid; Figure 1b) or *M. persicae* (green peach aphid; Figure 1c). Although the toxicity of *Bt* toxins can be increased by incorporating peptide sequences that bind to aphid guts (Chougule *et al*., 2013), this has not yet resulted in a commercially viable *Bt* toxin directed at hemipteran herbivores. Thus, there is an interest in identifying additional insecticidal proteins that can be expressed in the plant phloem to enhance aphid resistance. Our demonstration that transient expression of both endogenous maize proteins and insecticidal proteins from other species can reduce reproduction of *R. maidis* and *M. persicae* (Figure 6) suggests that SCMV-mediated overexpression can be used to screen proteins for their effectiveness against hemipteran pests of maize.

Whereas overexpression of endogenous maize proteins is likely to be considered low risk in a food crop, other constructs that we have tested would have difficulty gaining regulatory approval for commercialization. Some of the tested spider venom proteins have insect-specific toxicity (Fletcher *et al*., 1997) and have been approved by United States Environmental Protection Agency (Bilgo *et al*., 2017). However, further studies are required to investigate whether they would have negative effects on vertebrates when incorporated into transgenic food crops. Additional consumer-related concerns include dietary restrictions in some segments of the population. For instance, maize expressing an arthropod protein might not be considered suitable for a vegetarian diet.

Our results demonstrate the utility of SCMV-mediated overexpression for screening the efficacy of proteins that reduce insect growth on maize plants. Virus-mediated transient expression assays included genes encoding maize regulatory proteins, endogenous maize defensive proteins and non-maize insecticidal proteins. The main advantage of the SCMV overexpression system is a timeline that makes it possible to test the effectiveness of single- and multi-gene constructs in actual maize plants in less than two months. Although the main focus of our efforts was a lepidopteran herbivore, *S. frugiperda*, we also showed efficacy of SCMV constructs against two aphid species, *R. maidis* and *M. persicae*, suggesting that the SCMV-mediated transient expression approach will be broadly useful for experiments with both chewing and piercing/sucking herbivores of maize. Proteins that inhibit insect growth after transient gene expression using SCMV infection of maize are good candidates for further experiments to determine their effectiveness in stable transgenic maize plants.

Materials and methods

Plants and insects

Maize (*Zea mays*) plants, sweet corn variety Golden Bantam (West Coast Seeds, British Columbia, Canada) and inbred lines P39 and B73, were grown in a maize mix [0.16 m^3 Metro-Mix 360 (Scotts, Marysville, OH, USA), 0.45 kg finely ground lime, 0.45 kg Peters Unimix (Griffin Greenhouse Supplies, Auburn, NY, USA), 68 kg Turface MVP (Banfield-Baker Corp., Horseheads, NY, USA), 23 kg coarse quartz sand and 0.018 m^3 pasteurized field soil]. All

plants, including those used for SCMV propagation and insect bioassays, were maintained in a growth chamber at 23 °C with a 16:8 h light:dark cycle. Unless specified otherwise, Golden Bantam maize was used for the described experiments.

Eggs of *S. frugiperda* were purchased from Benzon Research (Carlisle, PA, USA) and maintained on an artificial diet (Fall Armyworm Diet, Southland Products Inc, Lake Village, AR, USA) in an incubator at 28 °C. A colony of a genome-sequenced *R. maidis* lineage (Chen *et al*., 2019b) was maintained on maize (Golden Bantam or P39), and a colony of a previously described tobacco-adapted strain of *M. persicae* (Ramsey *et al*., 2007) was maintained on *Nicotiana tabacum* (tobacco) at 23 °C under 16:8 h light:dark cycle. Both *R. maidis* and *M. persicae* were originally collected in USDA-ARS greenhouses by Stewart Gray (Robert W. Holley Center for Agriculture & Health, Ithaca, NY, USA).

Cloning of candidate genes into *Sugarcane mosaic virus* for protein expression

The pSCMV-CS3 expression vector used in this work was derived from pSCMV-CS2 (Mei *et al*., 2019), which was modified to contain the CS3 restriction sites in the multiple cloning site between the *P1* and *HC-Pro* cistrons (Figure 2a). The modified pSCMV-CS2 genome plus the flanking 35S promoter and NOS terminator was amplified with SuperFi polymerase (Thermo Fisher Scientific, Waltham, MA) using primers DCPacI 1380 F and DCPacI 1380 R (Table S1). The pCAMBIA1380 backbone (www.cambia.org) was amplified with SuperFi polymerase using primers 1380F and 1380R (Table S1). The two PCR fragments were subsequently assembled into pCAMBIA1380-SCMV-CS3, hereafter referred to as pSCMV-CS3, by Gibson Assembly (New England Biolabs, Ipswich, MA). pSCMV-GFP was created by amplifying the mEGFP coding sequence (Zacharias *et al*., 2002) using SuperFi polymerase with the GFP-PspOMI and GFP-SbfI primers (Table S1). The resulting amplicon was digested with *Psp*OMI and *Sbf*I, gel purified and ligated into similarly digested pSCMV-CS3.

Genes encoding maize defence regulators and protein toxins were amplified from the B73 cDNA template with gene-specific primers containing restriction sites at the 5′ end for cloning into the SCMV vector. PCR products were gel purified, digested with the corresponding restriction enzymes and cloned into the SCMV vector (Figure 2a). The *UyCT3* and *UyCT5* genes (codon optimized for maize), as well as the maize *Pep1* and *Pep3* genes, were synthesized by Genscript Biotech (Piscataway, NJ, USA) and cloned into the SCMV vector. To generate SCMV with *Hvt*, *ACA*, *GNA*, *RIP2*, *Hvt-ACA*, *Hvt*-ACA*, *RIP2-GNA* and *Hvt-GNA*, primers listed in Table S1 and DNA synthesized by Genewiz (South Plainfield, NJ, USA) were used as template and the resulting PCR products were gel purified and cloned into PspOMI-SbfI cut SCMV by following the NEBuilder HiFi DNA Assembly method (NEB, Ipswich, MA, USA). Primers, restriction sites and cloning methods used in this study are listed in Table S1.

Inoculation of maize with SCMV constructs

SCMV constructs were delivered into maize plants by particle bombardment using a Biolistic PDS-1000/He system (Bio-Rad, Hercules, CA, USA) as described previously (Mei and Whitham, 2018). Five μg of the plasmid DNA was coated on 3 mg 1.0 μm diameter gold particles, and the coated gold particles were distributed onto five microcarriers and allowed to air dry. Plants were placed in the dark 12 h before the particle bombardment. Two leaves of one-week-old plants, randomly oriented so that the adaxial or abaxial surface was targeted, were bombarded with using 1100 psi rupture discs at a distance of 6 cm (between stopping screen and leaves; Figure S3). Successfully infected plants were obtained with a frequency of 5% to 40%, in particle bombardment experiments.

Agroinoculation was used to initiate maize infections with the SCMV constructs containing *Hvt*, *ACA* and *UyCT3*, as described previously (Beernink *et al*., 2021). The constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101, and an *A. tumefaciens* suspension with optical density at 600 nm (OD_{600}) = 1.0 in infiltration buffer (200 μM acetosyringone, 10 mM MES, pH 5.6 and 10 mM $MgCl_2$) was injected above the coleoptile node of one-week-old plants. Successfully infected plants were obtained with a frequency of 1% to 5%, in agroinoculation experiments.

Due to the relatively low infection rate with both particle bombardment and agroinoculation, SCMV constructs in maize were further propagated and used to infect experimental plants by rub-inoculation. Leaf sap of SCMV-infected plants was prepared by grinding 0.5 g leaf tissue in 5 mL of 50 mM pH 7.0 potassium phosphate buffer. One-week-old maize plants were dusted with 600-mesh carborundum and mechanically inoculated by rubbing leaf sap from virus-infected maize plants on two leaves. Successfully infected plants were obtained with a frequency of 50% to 80%, in rub-inoculation experiments.

Confocal microscopy

Three weeks post-inoculation, leaf samples were collected from the seventh or eighth leaves of maize plants infected with SCMV-*GFP*. The samples were observed at an excitation of 488 nm. The emitted fluorescence signal was monitored from 505 to 545 nm using a SP5 Leica Confocal Microscope in the Plant Cell Imaging Center of Boyce Thompson Institute. A scan of fluorescence across a range of wavelengths (lambda scan) was used to confirm that the observed signal was derived from GFP rather than endogenous maize fluorescence.

Insect bioassays

To determine the effect of defensive proteins on the growth of *S. frugiperda*, SCMV-infected plants, three weeks post-inoculation, were used for caterpillar bioassays. Five two-day-old caterpillars were placed on each plant and enclosed using perforated plastic bags (13 × 61 cm, https://www.clearbags.com). The fresh mass of surviving caterpillars was measured one week later. The average mass of caterpillars from each plant was used as a biological replicate when conducting comparisons between maize plants infected with different SCMV constructs. For aphid bioassays, 8 ten-day-old apterous adult *R. maidis* or 10 ten-day-old apterous adult *M. persicae* were placed on each virus-infected plant 15–18 days after SCMV inoculation and enclosed using perforated plastic bags. The total numbers of aphids on each plant were counted one week later.

RNA extraction, cDNA synthesis, RT-PCR and quantitative real-time PCR (qRT-PCR)

Three weeks post-inoculation and prior to the insect bioassays, leaf tissue was collected from the seventh or eighth leaves of infected plants, flash-frozen in liquid nitrogen and stored at −80 °C. RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). One microgram of RNA was used

to synthesize first-strand cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random primers. To verify the expression of insect resistance genes cloned into the SCMV vector, qRT-PCR was conducted with gene-specific primers (Table S1). For the inserts of less than 75 bp (*Pep1*, *Pep3*, *UyCT3* and *UyCT5*), one primer was designed to bind to the insert and another primer was designed to bind to the region flanking the cloning site, with the amplification products ranging in size from 100 to 150 bp. The reactions consisted of 5.0 μL of the PowerUp SYBR Green PCR master mix (Applied Biosystems), 0.6 μL primer mix (300 nM for the final concentration of each primer) and 2 μL of cDNA (1:10 dilution with nuclease-free H_2O) in 10 μL total volume. Template-free reactions were included as negative controls. The PCR amplification was performed on QuantStudio 6 Flex Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) with the following conditions: 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer specificity was confirmed by melting curve analysis. Mean cycle threshold values of duplicates of each sample were normalized using two reference genes, *Actin* and *EF1-α*. Relative gene expression values were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All statistical analyses were conducted using R (R Core Team, 2017). Data for gene expression and larval mass of *S. frugiperda* larvae, and aphid fecundity were compared using analysis of variance (ANOVA) followed by Tukey's test, Dunnett's tests relative to the GFP control, or *t*-tests. Gene expression data were log2 transformed before the statistical analysis to meet the assumptions of ANOVA but untransformed data are presented in the figures. Survival of *S. frugiperda* larvae was analysed using non-parametric Kruskal–Wallis tests. Raw data underlying the bar graphs are presented in Tables S2-S7.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

SHC, GJ, SPDK and SAW: designed experiments. SHC, RRL, BC and UN: cloned genes and constructed virus vectors. SHC and MB: conducted insect bioassays. SHC and GJ: analysed data. SHC, MB and GJ: involved in manuscript writing. SAW, SPDK and GJ: obtained funding and other essential resources. All authors approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Representative pictures of *Spodoptera frugiperda*caterpillars raised on P39 maize plants infected with SCMV.
Figure S2 Gene expression of (a) *AMP*and (b) *JAC1*in P39 maize plants damaged by *Spodoptera frugiperda*caterpillars.
Figure S2 One-week-old P39 maize plants were placed in the vacuum chamber of a Biolistic PDS-1000/He delivery system for inoculation with SCMV constructs.

Table S1 List of primers and restriction sites.
Table S2 Raw data for the graphs in Figure 2.
Table S3 Raw data for the graphs in Figure 3.
Table S4 Raw data for the graphs in Figure 4.
Table S5 Raw data for the graphs in Figure 5.
Table S6 Raw data for the graphs in Figure 6.
Table S6 Raw data for the graphs in Figure S3.

