Precise Exchange of the Helper-Component Proteinase Cistron Between Soybean mosaic virus and Clover yellow vein virus: Impact on Virus Viability and Host Range Specificity

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ABSTRACT

Soybean mosaic virus and *Clover yellow vein virus* are two definite species of the genus *Potyvirus* within the family *Potyviridae*. Soybean mosaic virus-N (SMV-N) is well adapted to cultivated soybean (*Glycine max*) genotypes and wild soybean (*G. soja*), whereas it remains undetectable in inoculated broad bean (*Vicia faba*). In contrast, clover yellow vein virus No. 30 (CIYVV-No. 30) is capable of systemic infection in broad bean and wild soybean; however, it infects cultivated soybean genotypes only locally. In this study, SMV-N was shown to also infect broad bean locally; hence, broad bean is a host for SMV-N. Based on these observations, it was hypothesized that lack of systemic infection by SMV-N in broad bean and by CIYVV-No. 30 in cultivated soybean is attributable to the incompatibility of multifunctional helper-component

proteinase (HC-Pro) in these hosts. The logic of selecting the HC-Pro cistron as a target is based on its established function in systemic movement and being a relevant factor in host range specificity of potyviruses. To test this hypothesis, chimeras were constructed with precise exchanges of HC-Pro cistrons between SMV-N and CIYVV-No. 30. Upon inoculation, both chimeras were viable in infection, but host range specificity of the recombinant viruses did not differ from those of the parental viruses. These observations suggest that (i) HC-Pro cistrons from SMV-N and CIYVV-No. 30 are functionally compatible in infection despite 55.6 and 48.9% nucleotide and amino acid sequence identity, respectively, and (ii) HC-Pro cistrons from SMV-N and CIYVV-No. 30 are not the determinants of host specificity on cultivated soybean or broad beans, respectively.

The *Potyviridae* represent the largest family of plant RNA viruses and contain eight genera, the largest of which, *Potyvirus*, has >140 definite species as well as a significant number of tentative species (Adams et al. 2012; Gibbs and Ohshima 2010; Wylie et al. 2017). Particles of potyviruses are flexuous filaments containing a single-stranded RNA genome of positive polarity possessing a covalently linked 5'-terminal viral protein (viral protein genome-linked [VPg]) and a 3'-terminal poly(A) tail (Wylie et al. 2017). Expression of a potyviral genome results in the production of a number of multifunctional proteins, including helper-component proteinase (HC-Pro) (Revers and Garcia 2015). In addition, a small open reading frame (i.e., *pipo*) embedded in the P3 cistron encodes a protein in the +2 frame that is involved in virus movement and plays a part in virulence in specific potyviral pathosystems (Atsumi et al. 2016; Choi et al. 2013; Chung et al. 2008; Vijayapalani et al. 2012; Wen and Hajimorad 2010).

A number of potyviruses have a very narrow host range, whereas others are capable of infecting plant species belonging to more than 30 plant families (Adams et al. 2012). The underlying molecular

mechanism(s) of host range and host specificity of plant viruses in general is largely unknown; however, this has been attributed to nonhost resistance to some extent (Hull 2014). Nevertheless, the presence of a specific resistance gene(s) in a host, dominant (*R* gene) or recessive, may also restrict viruses to the site of inoculation via an active or a passive mechanism (Whitham and Hajimorad 2016). It should be noted that in the majority of studies addressing host range specificity, virologists have taken advantage of isolates of the same virus species with a contrasting level of adaptations in order to map the viral determinants (Calvo et al. 2014; Carbonell et al. 2013; Chen et al. 2008; Poulicard et al. 2012; Ryu et al. 1998; Sardaru et al. 2018; Schoelz and Shepherd 1988; Suehiro et al. 2004; Tan et al. 2005; Vassilakos et al. 2015). However, in the absence of isolates of the same virus species with a differential level of pathogenicity on the targeted host, virologists have successfully used two different virus species from the same genera in a few instances (Hilf and Dawson 1993; Mise et al. 1993; Padgett and Beachy 1993; Padgett et al. 1997). For example, the host range determinant of *Tobacco mosaic virus* (TMV) on *N*-genotype tobacco was mapped by taking advantage of two tobamovirus species (Padgett and Beachy 1993; Padgett et al. 1997). It should be noted that TMV-Ob that served as a virulent tobamovirus on *N*-genotype tobacco was subsequently named *Obuda pepper virus* and classified as a distinct species within the genus *Tobamovirus* (Gibbs et al. 2004; Hull 2014). Two features of the TMV/*N*-genotype tobacco pathosystem led to the success of this approach. First, TMV and obuda pepper virus were both capable of differentially infecting host plant species belonging to the same plant genus (i.e., *Nicotiana*). Second, the underlying mechanism restricting TMV to move systemically in this pathosystem was the presence of a single *R* gene (*N* gene) conferring active resistance against the avirulent TMV.

In general, adaptation of plant viruses to a new host species is complex, requiring mutation(s) in multiple viral-encoded proteins involved in replication, cell-to-cell, and long-distance movement

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(Dawson and Hilf 1992). The observation that host jump of viruses is a rare phenomenon provides additional evidence that viruses must overcome many hurdles to overcome host species barriers (Dawson and Hilf 1992; Vassilakos et al. 2015; Webby et al. 2004).

Soybean mosaic virus (SMV) and *Clover yellow vein virus* (CIYVV) are both definite *Potyvirus* species members (Wylie et al. 2017). However, SMV has a much narrower host range relative to that of CIYVV (Bos 1972; Hajimorad et al. 2018; Sasaya et al. 1997). Nevertheless, both of these viruses are incapable of systemically infecting a number of plant species within the family of Leguminosae. SMV is well adapted to cultivated soybean (*Glycine max*) as well as wild soybean (*G. soja*), whereas it is undetectable from experimentally inoculated broad bean (*Vicia faba*) (Bos 1972; Cheng et al. 2010; Golnaraghi et al. 2004; Hunst and Tolin 1982; Khatabi et al. 2012; Seo et al. 2009). In contrast, CIYVV is capable of systemic infection in broad bean and wild soybean, whereas infection in inoculated cultivated soybean remains localized (Abe et al. 2019; Bos et al. 1977; Foster and Musgrave 1985; Masuta et al. 2000; Munro 1981; Nakahara et al. 2011; Sasaya et al. 1997). To date, only a single report of natural infection of CIYVV in cultivated soybean has been reported worldwide (Shin et al. 2014). Localized infection of CIYVV in cultivated soybean was attributed to the presence of one major quantitative resistance locus containing recessive gene(s) restricting systemic movement of the virus in mechanically but not graft-inoculated cultivated soybean; however, the viral determinant(s) interacting with this resistance locus remains unknown (Abe et al. 2019). Thus, based on the findings of Abe et al. (2019) and the presence of a naturally occurring CIYVV strain infecting systemically cultivated soybeans (Shin et al. 2014), it appears that the underlying mechanism limiting CIYVV to inoculated soybean leaves does not involve nonhost resistance. In the case of the SMV/broad bean pathosystem, the inoculated plants remain asymptomatic and the virus has not been detected from such plants (Golnaraghi et al. 2004; Hunst and Tolin 1982). Furthermore, to our knowledge, there is no published record of systemic infection of broad bean by a strain of SMV to date. Thus, it is unknown whether the underlying mechanism(s) leading to the lack of detectability of SMV in inoculated broad bean is a consequence of nonhost or host-specific resistance. In a number of pathosystems involving viruses and host genotypes harboring *R* genes (or recessive genes), the phenotype of resistance, which is symptomless, is indistinguishable from nonhost resistance (Adams et al. 1986; Bendahmane et al. 1999; Hajimorad and Hill 2001; Ishibashi et al. 2009).

The aim of this study was to determine whether broad bean is a host or nonhost to SMV and also to determine whether reciprocal exchanges of HC-Pro between SMV strain N (SMV-N) and CIYVV strain No. 30 (CIYVV-No. 30) lead to viable viruses with impacts on host range specificity. HC-Pro is a multifunctional potyviral protein that is involved in many activities, including, but not limited to, cell-to-cell and long-distance movement, pathogenicity, and virulence (Cronin et al. 1995; Hajimorad et al. 2008; Khatabi et al. 2013; Lopez-Moya et al. 2009; Rojas et al. 1997; Sáenz et al. 2002; Valli et al. 2018; Wen et al. 2013). Furthermore, it has been shown that HC-Pro is a relevant potyviral factor in host range specificity (Carbonell et al. 2012; Sáenz et al. 2002). The hypotheses tested were as follows: (i) broad bean is a host to SMV-N; however, SMV-N is incapable of systemic movement in this host; and (ii) multifunctional HC-Pro cistrons from SMV-N and CIYVV-No. 30 play a role in host range specificity and virulence of these viruses in cultivated soybean and broad bean, respectively. Well-characterized molecularly cloned SMV-N and CIYVV-No. 30 (GenBank accessions D00507.2 and NC_003536, respectively), with or without *uidA* gene expressing β -glucuronidase (GUS), were utilized in this study to construct chimeras and to test the above hypotheses (Abe et al. 2019; Masuta et al. 2000; Wang et al. 2006). The results show that HC-Pro cistrons from SMV-N and CIYVV-No. 30 are functionally compatible in infection; however, they are not the

determinants of host specificity on cultivated soybean or broad bean.

For the purposes of this communication, a plant is defined as a host when it supports virus replication at least at the cellular level (Hull 2014). Pathogenicity is defined as the capability of a virus to move systemically in a given host to cause systemic infection in noninoculated leaves regardless of the phenotypes induced (Shaner et al. 1992). Virulence is defined as the ability of a virus to overcome restriction in systemic movement owing to the presence of a known resistance factor in a host (Shaner et al. 1992).

MATERIALS AND METHODS

Viruses, host genotypes, and inoculation. The full-length infectious cDNA clones of SMV-N or CIYVV-No. 30, with or without the *uidA* gene, were used in this study (Abe et al. 2019; Masuta et al. 2000; Wang et al. 2006). SMV-JL1 and SMV-JL3 were originally isolated from infected field-grown soybeans in Jilin province of China. SMV-JL1 was subsequently maintained in soybean, whereas an infectious cDNA clone representing SMV-JL3 was generated (*unpublished data*) and progeny viruses derived from the molecularly cloned SMV-JL3 in soybean were used as an inoculum source in this study. SMV-G7 and SMV-G7d were previously described (Hajimorad et al. 2003). Cultivated soybean (*G. max*) genotypes Williams, Lee68, and recombinant inbred line (RIL) 943 (-3gG2) (Bernard et al. 1991; Hayes et al. 2004); wild soybean (*G. soja*) lines B01167, T106, and B04158 (obtained from the LegumeBase at <https://www.legumebase.brc.miyazaki-u.ac.jp/>); and broad bean (*V. faba* L.) cultivars Windsor, Robin Hood, LiaoN, and SanD were used in this study. Seeds of all broad bean cultivars were purchased from commercial sources. Virus-free seeds of cultivated soybeans were obtained from virus-indexed plants. To establish infection with plasmid DNA, fully expanded primary leaves were biolistically inoculated as described previously (Hajimorad et al. 2003, 2008). To inoculate plants mechanically, progeny viruses in sap extract from biolistically inoculated and systemically infected leaf tissues of soybean Williams or broad bean Windsor in 10 mM of phosphate buffer, pH 7.0, were applied manually to carborundum-dusted fully developed primary leaves of targeted plants. The inoculated plants were kept in a growth chamber at 22°C with a 16-h photoperiod. Histochemical assay of GUS expression in infected tissues was performed as described by Wen and Hajimorad (2010). CIYVV was detected by enzyme-linked immunosorbent assay (ELISA) as described by Abe et al. (2019), whereas detection of SMV coat protein (CP) in infected tissues was done as described by Malapi-Nelson et al. (2009).

Construction of SMV-N- and CIYVV-No. 30-derived chimeras with precise exchanges of HC-Pro cistron. SMV-N/CIYVV-No. 30 HC-Pro and CIYVV-No. 30/SMV-N HC-Pro chimeras with precise exchanges of HC-Pro cistrons between the two parental viruses (SMV-N and CIYVV-No. 30) were constructed by using fusion-PCR as described by Charlier et al. (2003) and primers as listed in Supplementary Table S1. PrimeSTAR HS DNA polymerase Premix (Takara Bio, Madison, WI) was used in all amplification reactions. The nature of viral constructs with a precise exchange of HC-Pro was confirmed by Sanger sequencing. Details of construction of the chimeras and sequencing are presented in Supplementary Data File S1. Oligonucleotides were used for construction of chimeras as well as sequencing are listed in Supplementary Table S1.

RNA extraction, reverse transcription PCR, Sanger sequencing, and bioinformatics. Total RNA was isolated from systemically infected leaves from biolistically inoculated plants using an RNeasy plant mini kit (Qiagen, Valencia, CA). To verify the stability of the chimeras in planta, the genome of progeny viruses was reverse transcribed using random primer in the presence of SuperScript reverse-transcriptase III as instructed by the manufacturer (Invitrogen, Carlsbad, CA). PCR amplification of the entire HC-Pro cistron was done using oligonucleotide primer

sets outlined in Supplementary Table S1 in the presence of ExTaq polymerase (Takara Bio). The resultant amplicons were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced using specific primers (Supplementary Table S1). Sequencing was done at the University of Tennessee DNA Sequencing Facility and sequences were edited and analyzed using Vector NTI (Invitrogen). Determination of the percent identity of nucleotides and predicated amino acids was performed according to the Jotun Hein method (de Groot et al. 2008).

RESULTS

SMV-N infects broad bean locally but fails to move systemically. To demonstrate whether broad bean serves as a host

to SMV, the attached primary leaves of cultivar Windsor were biologically inoculated with SMV-N GUS. Figure 1A shows that based on expression of GUS, SMV-N GUS caused local infection in the inoculated leaves, albeit to a few foci; however, no GUS expression was observed in adjacent veins at 21 days post-inoculation (DPI). Furthermore, no GUS expression was detected in noninoculated leaves (Fig. 1A). It should be noted that SMV-N GUS was detected in veins of inoculated leaves of both cultivated soybean Williams and wild soybean line B01167 and systemically moved efficiently in both of these hosts (Fig. 1A). SMV-N also systemically infected two other wild soybean lines, T106 and B04158 (*data not shown*), demonstrating that it is well adapted to this host species. To rule out the possibility that the inability of SMV-N GUS to move systemically in broad bean Windsor is a consequence of tagging with *uidA* gene (German-Retana et al. 2000), broad bean cultivar Windsor was also inoculated with SMV-N without the *uidA* gene (GUS). However, the inoculated broad bean cultivar Windsor, similar to those inoculated with SMV-N GUS, also remained asymptomatic and virus was not detectable in noninoculated leaves by ELISA (Table 1). To demonstrate that the lack of systemic infection in Windsor by SMV-N is not cultivar specific, broad bean cultivar Robin Hood was also inoculated; however, no systemic infection occurred (Table 1). To demonstrate that the inability of SMV-N to infect broad bean cultivars is not SMV strain specific, other strains of SMV were also inoculated to broad bean cultivar Windsor as well as Chinese cultivars LiaoN and SanD. Only SMV-JL3 was detected in the upper noninoculated leaves above the inoculated leaves in a single LiaoN plant by ELISA; however, virus was not recovered from these tissues by back-inoculation onto a susceptible soybean (Table 2). Furthermore, SMV-JL3 was not detected in leaves located at the top of this plant by ELISA either. All of the other SMV strains (SMV-G7, SMV-G7d, and SMV-JL1) failed to move systemically in all of the

Fig. 1. β -glucuronidase (GUS) histochemical analysis of **A**, soybean mosaic virus-N (SMV-N) GUS or **B**, clover yellow vein virus-No. 30 (CIYVV-No. 30) GUS infection in biologically inoculated or noninoculated leaves from soybean (*Glycine max*) cultivar Williams, broad bean (*Vicia faba*) cultivar Windsor, or wild soybean (*G. soja*) line B01167. For inoculation, a full-length infectious cDNA clone of each of the viruses harboring GUS was delivered biolistically into fully developed attached primary leaves of approximately 2-week-old seedlings maintained at 22°C. Leaves were analyzed histochemically for GUS expression at 21 days postinoculation. Note that SMV-N GUS is incapable of systemic infection in broad bean Windsor, whereas CIYVV-No. 30 GUS is incapable of systemic movement in soybean cultivar Williams. Scale bar = 2 cm.

TABLE 1. Systemic responses of host genotypes to inoculation with soybean mosaic virus-N (SMV-N) or clover yellow vein virus No. 30 (CIYVV-No. 30)^a

Virus	Host genotype (n) ^b			
	<i>Glycine max</i>	<i>Vicia faba</i>		<i>G. soja</i>
	Williams	Windsor	Robin Hood	B01167
SMV-N	19/32	0/14	0/27	10/10
CIYVV-No. 30	0/28	8/8	22/24	13/13

^a Plants were inoculated biolistically or mechanically.
^b Total number of plants systemically infected/total numbers of plants mechanically inoculated on primary leaves with sap extract from infected tissues or biolistically inoculated with infectious cDNA clones. Plants were evaluated for presence of systemic symptoms at 21 days postinoculation. Asymptomatic plants were assayed by enzyme-linked immunosorbent assay.

TABLE 2. Systemic responses of host genotypes to mechanical inoculation with various strains of soybean mosaic virus (SMV)

Virus	Host genotype (n) ^a				
	<i>Glycine max</i>	<i>Vicia faba</i>			
	Williams	Windsor	Robin Hood	LiaoN	SanD
SMV-G7	12/24	0/35	0/30	ND ^b	ND
SMV-G7d	16/30	0/27	0/26	ND	ND
SMV-JL1	4/4	0/8	ND	0/8	0/8
SMV-JL3	4/4	0/8	ND	1/8 ^c	0/8

^a Total number of plants systemically infected/total numbers of plants mechanically inoculated with sap derived from infected tissues. Plants were evaluated for the presence of systemic symptoms at 21 days postinoculation. Asymptomatic plants were assayed by enzyme-linked immunosorbent assay.
^b ND = not done.
^c Asymptomatic infection detected by ELISA as well as reverse transcription PCR; however, SMV was not recovered from the tissues following back-inoculation of sap onto susceptible soybean cultivar Williams.

inoculated broad bean cultivars (Table 2). Collectively, these observations suggest that broad bean is indeed a host to SMV; however, infection of SMV in broad bean is subliminal and the virus is incapable of moving systemically.

The data presented in Figure 1B confirm findings by Abe et al. (2019) showing that cultivated soybean Williams is a host to CIYVV-No. 30 GUS; however, based on the lack of GUS expression in noninoculated leaves, the virus is incapable of moving systemically in this host. On the other hand, CIYVV-No. 30 GUS moved systemically in wild soybean line B01167 and broad bean cultivar Windsor (Fig. 1B). CIYVV-No. 30 was also capable of infecting systemically broad bean cultivar Robin Hood (Table 1).

HC-Pro from SMV-N shares 48.9% amino acid identity with that of CIYVV-No. 30. The SMV-N genome is 9,588 nucleotides long, whereas that of CIYVV-No. 30 is 9,584 nucleotides long (Supplementary Table S2). The encoded polyprotein of SMV-N is 3,066 amino acids long, whereas that of CIYVV-No. 30 is 3,072 amino acids long. Overall, the two genomes shared 55.1 and 46.8% nucleotide and amino acid sequence identity, respectively. Phylogenetic analysis suggests that SMV is distantly related to CIYVV among potyvirus species whose infections have been reported in soybean (Abe et al. 2019). There were significant differences among cistrons of the two viruses at the nucleotide and amino acid levels (Supplementary Table S2). At the amino acid level, P1 and N1b had the lowest and highest percent identities (20.3 and 60.2%, respectively) (Supplementary Table S2). The sequence identity between HC-Pro from SMV-N with that of CIYVV-No. 30 was 48.9% at the amino acid level (Fig. 2; Supplementary Table S2). However, the percent identity at the nucleotide level was slightly higher at 55.6% (Supplementary Fig. S1; Supplementary Table S2). Interestingly, HC-Pro from CIYVV-No. 30 with that of CIYVV-Gm that systemically infects cultivated soybean in South Korea (Shin et al. 2014) (GenBank accession KF975894) differed by four amino acids (Supplementary Fig. S2). It should be noted that the percent identity of HC-Pro between CIYVV-No. 30 and that of CIYVV-Gm at nucleotide and amino acid levels was 94.8 and 99.1%, respectively (Supplementary Table S3).

Precise exchange of HC-Pro cistron between SMV-N and CIYVV-No. 30 resulted in viable chimeric viruses in infection but did not influence host range specificity. To determine whether HC-Pro cistrons from SMV-N and that of CIYVV-No. 30 are functionally compatible in infection and whether they carry the determinant(s) for systemic infection in cultivated soybean

and broad bean, respectively, the two cistrons were precisely switched. As shown in Figure 3A, HC-Pro exchanges did not alter the amino acid sequences located at the C-terminal cleavage site of HC-Pro at the junction with P3 for both recombinant viruses. However, amino acid sequences at the C termini of P1 at the cleavage sites differed between the two viruses. Regardless, this difference had no bearing on the composition of amino acid sequences of HC-Pro from the recombinant viruses; hence, the exchanges of HC-Pro cistrons between SMV-N and CIYVV-No. 30 within the context of the resultant chimeric viruses were precise.

When recombinant viruses were inoculated biolistically onto cultivated soybean Williams, broad bean Windsor, and wild soybean line B01167, both chimeras were viable in infection (Fig. 3B). The viability of HC-Pro chimeric potyviruses is in line with previous publications (Carbonell et al. 2012; Maliogka et al. 2012). However, the pathogenicity of SMV-N/CIYVV-No. 30 HC-Pro and CIYVV-No. 30/SMV-N HC-Pro did not differ from those of the parental viruses (compare Figs. 3B and 4 with Fig. 1A and B and Table 1). The recombinant SMV-N/CIYVV-No. 30 HC-Pro, similar to SMV-N, moved systemically in cultivated soybean Williams as well as wild soybean line B01167, but not in broad bean cultivar Windsor (Fig. 4A). On soybean Williams, similar to wild-type SMV-N, it induced severe mottling of leaves and stunting of the plants whereas on wild soybean line B01167 it caused severe symptoms including leaf necrosis and stunting. On the other hand, the recombinant CIYVV-No. 30/SMV-N HC-Pro, similar to CIYVV-No. 30, moved systemically in broad bean cultivar Windsor and wild soybean line B01167; however, it failed to infect systemically cultivated soybean Williams (Fig. 4B). Its symptoms on broad bean Windsor were more severe than CIYVV-No. 30 and included severe systemic necrosis of leaves (Fig. 4B), tip necrosis, stem necrosis, and eventually death of the infected plants (*data not shown*). However, on wild soybean line B01167, it induced severe systemic mottling of leaves with no necrosis (Fig. 4B). Similar results were obtained when progeny viruses derived from molecularly cloned recombinant viruses were mechanically inoculated onto these plants (Table 3).

HC-Pro from CIYVV-No. 30 did not confer virulence to SMV-N on a recombinant inbred soybean line containing the *Rsv1*-derived resistance factors. L943 (-3gG2) is a soybean RIL derived from a cross between SMV-resistant PI96983 (*Rsv1*) and SMV-susceptible soybean Lee68 (*rsv1*) (Hayes et al. 2004; Wen et al. 2013). Infection by wild-type SMV-N on L943 (-3gG2) is

N (309)	SQNPEAQF	F	RGWKKV	FDKMP	PHVEN	HECTT	DFTNE	QCGELAAAI	SQSI	FPVKKLS	CKQCRQHI	-KHL	SW
No.30 (303)	SA---	GDL	FWKGY	NQTFI	DNRPKDI	GHTCTSD	LNVI	QCGSVMAL	MTLAL	FPCGRI	TCKEC	VEDFHN	QNNR
N (377)	EEYKQ---	FLLAH	MGCHG	PEWET	FQEI	DGMR	----	YVKRVI	ETSTAEN	ASLQTSLEI	VRLT	QNYKSTHM	
No.30 (370)	ERYARTER	FELS	QAMTL	LA---	QTTYPE	FKHSRDI	LQLFRER	LSMENSNAE	ACVETNKA	ITSLTE	----	TPF	
N (439)	LQIQDI	NKAL	MKGPS	SVTQSE	LEQASK	QLLAM	TQWKK	NHMTLT	DEDAL	KVFRNKR	SSKALL	NPSLL	CDNQL
No.30 (433)	NHI	RKI	NEVLL	RYGSL	TNDEV	GVAS	SLLEI	TRYI	RNRTDSI	QRNDLSK	FRNKI	SSKTHI	NLDLMCDNQL
N (509)	DKNGNF	VWGER	GRHS	KRFFA	ANYFEE	VVPSE	GYSKYVI	RKNPN	QGRELAI	GS	LIVPLD	FERARMAL	QGKSV
No.30 (503)	DKNANF	MWGQR	AYHAK	RFLSN	NYFSVI	DPSEGY	DKFI	NRKL	PNGTRELAT	GR	LIVPTN	FESFRD	QMKGT
N (579)	TREPI	TMSCI	SRQDGN	FVYPCC	CVTHDD	GKAFY	SELRS	PTKRHLVI	GTSGDP	KYI	DLPAT	DADRM	MYI
No.30 (573)	DNRPI	GKE	CVSRV	KGAFCY	PCCCTTDDI	GNAI	LSEFK	MPTKY	HLVL	LGQE	AAKYI	ELPSD	SSGMYI
N (649)	GFCYL	NI	FLAML	VNVN	DEAK	KDFTK	MVRDVI	VPR	LKGWPT	MLDVATA	AYMLT	VFHPE	TRNAELPRI
No.30 (643)	GYCHI	NI	FFAML	VNVN	SEDKS	KDFTK	MVRDQI	MPK	LQGWP	TMLDVATA	CWYLT	VWFPD	TLSAELPRI
N (719)	ACQT	TMHVI	DSFGSL	TVGYH	VLKAGT	VNQLI	QFASNDL	QSEM	KFYRVG				
No.30 (713)	KLST	TMHVL	DSYGS	ISTGYH	VLKANI	VSQLI	KFASDDLE	SDL	KFYRVG				

Fig. 2. Alignment of the deduced primary amino acid sequences of helper-component proteinase (HC-Pro) from soybean mosaic virus-N (SMV-N) and clover yellow vein virus No. 30 (CIYVV-No. 30). The amino acids identical between the two viruses are highlighted. The numbering is based on the predicted position of HC-Pro within the polyprotein precursor of SMV-N and CIYVV-No. 30 (GenBank accessions D00507.2 and NC_003536, respectively). N = SMV-N and No.30 = CIYVV-No. 30.

restricted to limited foci in the inoculated leaves and the avirulence determinant(s) has been mapped to SMV strain-specific HC-Pro (Wen et al. 2013). On the other hand, infection by CIYVV-No. 30 in L943 (-3gG2), similar to infection in all other cultivated soybean genotypes, is also restricted to limited foci (Abe et al. 2019). It should be noted that inoculation of two SMV-N susceptible soybean cultivars, Lee68 (the susceptible parent of L943 [-3gG2]) and Williams, with the recombinant SMV-N/CIYVV-No. 30 HC-Pro resulted in systemic infection (Fig. 4A; Table 3). Surprisingly, when SMV-N/CIYVV-No. 30 HC-Pro was biolistically inoculated onto unifoliate leaves of L943 (-3gG2), none of the inoculated RIL L943 (-3gG2) showed any sign of systemic infection and the virus was undetectable by ELISA in the noninoculated leaves. Mechanical inoculation of L943 (-3gG2) with progeny viruses derived from bilistically inoculated Williams with molecularly cloned SMV-N/CIYVV-No. 30 HC-Pro also yielded similar results and the virus was undetectable by ELISA in noninoculated leaves of any of 76 mechanically inoculated plants (Table 3). It should be noted that previously it has been shown that precise replacement of HC-Pro from SMV-N with the HC-Pro from SMV-G7 or SMV-G7d, both virulent strains on L943 (-3gG2) resulted in gain of virulence by SMV-N/G7 HC-Pro or SMV-N/G7d HC-Pro on L943 (-3gG2) (Wen et al. 2013). These observations suggest that in L943 (-3gG2) the HC-Pro from CIYVV-No. 30 is functionally equivalent to that of SMV-N HC-Pro because it failed to confer virulence to SMV-N/CIYVV-No. 30 chimera.

DISCUSSION

For a virus to infect a plant systemically, it should have evolved all of the determinants necessary to interact successfully with various host factors to complete critical steps in the infection cycle (Hull 2014). Furthermore, as plants have evolved mechanisms to counter viral invasion, competent viruses have acquired determinants to evade plant defense systems mediated by innate immunity and passive or active mechanisms (Li and Ding 2006; Mandadi and Scholthof 2013; Whitham and Hajimorad 2016). On the other hand, for a plant to serve as a permissive host to a virus following transmission and to allow for systemic invasion, it should lack dominant or recessive resistance genes directed against the invading virus. In addition, such a plant should possess all of the necessary susceptibility factors for compatible interactions with cognate viral-derived elements (Hull 2014; Revers and Nicaise 2014; Sanfaçon

2015). Thus, the inability of a virus to infect a plant and to move systemically could be either a result of the lack of genetic determinants or a consequence of an evolved specific resistance mechanism in the host plant.

One of our aims in this study was to determine whether broad bean is a host to SMV. To date, there has been no published report for isolation of SMV from systemically infected broad bean under field or experimental conditions (Golnaraghi et al. 2004; Hunst and Tolin 1982). The observation that inoculation with SMV-N GUS led to the detection of GUS expression in the inoculated leaves at 21 DPI suggests that broad bean indeed supports the replication of SMV-N locally. It worth noting that previous studies showed that GUS expression in the inoculated leaves following biolistic delivery of SMV-N GUS after 21 DPI is not a consequence of passive transcription and translation of the delivered plasmid and is indicative of virus replication (Abe et al. 2019; Khatabi et al. 2012; Wang et al. 2015; Wen and Hajimorad 2010; Wen et al. 2013; Zhang et al. 2009).

As broad bean supports SMV replication locally, evidenced by expression of GUS, it is a host (Hull 2014). However, because of a lack of determinant(s), SMV is incapable of moving long distance. To identify the determinant(s) via a comparative genomic approach, a well-adapted naturally occurring SMV strain isolated from a systemically infected broad bean is needed. However, because of the absence of such a strain, we instead set out to explore the ability of HC-Pro from CIYVV-No. 30 that systemically infects efficiently various broad bean genotypes (Abe et al. 2019; Bos et al. 1977; Foster and Musgrave 1985; Masuta et al. 2000; Munro 1981; Nakahara et al. 2011; Sasaya et al. 1997). It should be noted that potyviruses do not have a dedicated movement protein and a number of viral-encoded proteins are involved in the movement processes. These include P1, HC-Pro, P3, P3N-PIPO, cytoplasmic inclusion, 6K2, VPg, and CP (Adams et al. 2012; Revers and Garcia 2015; Schaad et al. 1997; Spetz and Valkonen 2004; Vassilakos et al. 2015; Wen and Hajimorad 2010).

In this study, we focused on HC-Pro because of its multifunctional role in the life cycle of potyviruses, its established function in systemic movement, and its involvement in pathogenicity and virulence (Hajimorad et al. 2008, 2011; Kasschau et al. 1997; Khatabi et al. 2013; Rolland et al. 2009; Wen et al. 2013). In addition, it has been suggested that HC-Pro is a relevant factor in host range specificity (Carbonell et al. 2012; Sáenz et al. 2002). To determine whether HC-Pro from CIYVV-No. 30 can replace that of

Fig. 3. A, Schematic representation of genome of parental viruses and derivative recombinant viruses with precise exchanges of helper-component proteinase (HC-Pro). **B**, Systemic responses of soybean (*Glycine max*) cultivar Williams, broad bean (*Vicia faba*) cultivar Windsor, or wild soybean (*G. soja*) line B01167 to biolistic inoculation with a full-length infectious cDNA clone of each of the viruses delivered onto primary leaves. Plants were evaluated at 21 days postinoculation and the absence of viruses in asymptomatic noninoculated leaves was verified by enzyme-linked immunosorbent assay. The identity of progeny viruses as HC-Pro recombinants was verified following reverse transcription PCR and Sanger sequencing of the resultant amplicons. SMV-N = soybean mosaic virus-N, UTR = untranslated region, CI = cytoplasmic inclusion, CP = coat protein, and CIYVV-No. 30 = clover yellow vein virus No. 30.

SMV-N successfully in infection, and also complement systemic movement of SMV-N in broad bean, the entire cistron of SMV-N was replaced precisely with that of CIYVV-No. 30. SMV-N/CIYVV-No. 30 HC-Pro was viable in infection; however, it did not move systemically in broad bean. This suggests that the determinant(s) of CIYVV-No. 30 for systemic movement in this host resides elsewhere, or an additional viral-encoded protein is also

needed to complement HC-Pro function in systemic movement. In addition to HC-Pro, other SMV-encoded proteins have a role in mediating movement of potyviruses within the host (Hull 2014). This is further supported by the observation that precise replacement of HC-Pro from CIYVV-No. 30 with that of SMV-N did not abolish systemic movement of this chimera in broad bean. However, CIYVV-No. 30/SMV-N HC-Pro did not move systemically in cultivated soybean. It is worth noting that the role of HC-Pro in the expansion of host range was also studied by Stenger and French (2004), in which HC-Pro from wheat streak mosaic virus (WSMV) was replaced with that of either oat necrotic mottle virus, agropyron mosaic virus, hordeum mosaic virus, tobacco etch virus (TEV), or turnip mosaic virus. However, in all of these exchanges, two amino acids from WSMV HC-Pro at the N terminus were not replaced. Atreya and Pirone (1993) also replaced HC-Pro from tobacco vein mottling virus (TVMV) with that of zucchini yellow mosaic virus (ZYMV); however, the exchange was not complete, as five amino acids located at the N-terminal portion of TVMV HC-Pro were not replaced. Nevertheless, in these experiments, the host range specificity of WSMV- and TVMV-derived chimeras were not altered following partial exchanges of HC-Pro (Atreya and Pirone 1993; Stenger and French, 2004). In the current study, however, we exchanged precisely the entire HC-Pro cistron between SMV-N and CIYVV-No. 30 in a reciprocal manner.

In our study, we did not address the genetic basis of resistance in broad bean that restricts systemic movement of SMV-N. Thus, it remains unknown whether resistance to systemic movement of SMV-N is mediated by a passive or an active mechanism. Interestingly, the symptomless phenotype of resistance against SMV-N in broad bean resembles those mediated by *Rsv1*-derived resistance factors in soybean RIL L943 (-3Gg2) or the *Rsv4* gene in soybean (Khatabi et al. 2012; Wang et al. 2015; Wen et al. 2013). It should be noted that the determinants of SMV interacting with the *Rsv1*-derived resistance factors in soybean RIL L943 (-3Gg2) reside on HC-Pro, whereas those interacting with *Rsv4* reside on P3 (Chowda-Reddy et al. 2011; Khatabi et al. 2012; Wang et al. 2015).

Abe et al. (2019) attributed the inability of CIYVV-No. 30 to move systemically in cultivated soybeans to the presence of a resistance locus containing a recessive gene(s). Interestingly, the phenotype of resistance to CIYVV-No. 30 in cultivated soybean resembles that of SMV-N in broad bean (Abe et al. 2019). However, the determinant(s) of CIYVV-No. 30 interacting with this resistance factor(s) was not determined by Abe et al. (2019). In this study, we ruled out the HC-Pro from CIYVV-No. 30 as the cognate factor interacting with the resistance locus in cultivated soybean. Thus, avirulence determinants of CIYVV-No. 30 reside outside of HC-Pro. It should be noted that the 6K1 and nuclear inclusion protein a-proteinase (NIa-Pro) regions of CIYVV-No. 30 share 100% identity at the nucleotide and amino acid levels with those of CIYVV-Gm, which is a naturally evolved strain capable of systemic infection in cultivated soybeans (Shin et al. 2014; Supplementary Figs. S3 and S4). NIa-Pro as well as 6K1 proteins are both involved in determining the host range of other potyviruses (Chen et al. 2008; Sáenz et al. 2000). Excluding 6K1 and NIa-Pro, there are amino acid

Fig. 4. Systemic responses of soybean (*Glycine max*) cultivar Williams, broad bean (*Vicia faba*) cultivar Windsor, or wild soybean (*G. soja*) line B01167 to biolistic inoculation with **A**, soybean mosaic virus-N (SMV-N)- or **B**, clover yellow vein virus No. 30 (CIYVV-No. 30)-derived helper-component proteinase (HC-Pro) recombinant viruses. For inoculation, a full-length infectious cDNA clone of recombinant HC-Pro viruses was delivered biolistically into fully developed attached primary leaves of approximately 2-week-old seedlings. The inoculated plants were maintained at 22°C until a leaflet from noninoculated leaves was photographed at 21 days postinoculation. The identity of progeny viruses as HC-Pro recombinants was verified following reverse transcription PCR and Sanger sequencing of the resultant amplicons. Absence of viruses in asymptomatic noninoculated leaves was verified by enzyme-linked immunosorbent assay. Scale bar = 2 cm.

TABLE 3. Systemic responses of host genotypes to inoculation with soybean mosaic virus-N (SMV-N)- or clover yellow vein virus No. 30 (CIYVV-No. 30)-derived helper-component proteinase (HC-Pro) chimeras

Virus	Host genotype (n) ^a				
	<i>Glycine max</i>			<i>Vicia faba</i>	<i>G. soja</i>
	Williams	Lee68	L943 (-3Gg2)	Windsor	B01167
SMV-N/CIYVV-No. 30 HC-Pro	8/8 (19/31) ^b	(23/46)	0/6 (0/76)	0/2 (0/17)	8/8
CIYVV-No. 30/SMV-N-HC-Pro	0/8 (0/19)	(ND)	(ND)	8/8 (15/18)	8/8

^a Total number of plants systemically infected/total numbers of plants biolistically inoculated on primary leaves with infectious cDNA clones. Plants were evaluated for presence of systemic symptoms at 21 days postinoculation. Asymptomatic plants were assayed by enzyme-linked immunosorbent assay.

^b Numbers shown in parentheses indicate the total number of systemically infected plants/total number of plants inoculated mechanically with progeny viruses in sap extract derived from systemically infected leaves of biolistically inoculated Williams or Windsor.

differences between all of the other encoded proteins of CIYVV-No. 30 compared with those of CIYVV-Gm, with the percent amino acid sequence identities varying from 94.1 to 99.8% (Supplementary Table S3). The P1 cistron is the most variable region between the two viruses, with 92.4 and 94.1% sequence identity at the nucleotide and amino acid levels, respectively (Supplementary Table S3). In general, the P1 cistron is the most variable region among potyviruses, which has led to assumptions that it is a key factor in host adaptation and evolution (Adams et al. 2005; Valli et al. 2007). However, experimental evidence in support of this hypothesis is limited (Salvador et al. 2008b; Shan et al. 2018). In the search for host range determinants of SMV-N and CIYVV-No. 30, future research will focus on the role of the P1 cistron.

The observation that some potyvirus members (e.g., bean yellow mosaic virus [BYMV]) have an extensive natural host range encompassing both dicots and monocots, whereas others (e.g., SMV) have a very narrow host range is intriguing (Bos 1972; Hajimorad et al. 2018; Kehoe et al. 2014). SMV and BYMV both have a single-stranded RNA genome and are naturally transmitted by seed as well as in a nonpersistent manner by aphids. Thus, it is unlikely that replication strategy and mode of transmission has much of influence on the generation of variation and consequently evolution toward host specialization. Recombination has been reported for both of these viruses, which has the potential to generate genome variation and possibly lead to host adaptation (Gagarinova et al. 2008; Kehoe et al. 2014; Wylie and Jones 2009). Thus, it seems that the determinants of host range specificity of potyviruses are complex. In addition to HC-Pro, other exchanges of genomic parts among potyviruses included P1 between plum pox virus (PPV) and TVMV (Salvador et al. 2008b), CP between PPV and ZYMV (Tóbiás et al. 2001), and CP between ZYMV and watermelon mosaic virus (Ullah et al. 2003). None of these exchanges led to the expansion of host ranges of the experimentally generated recombinant viruses. However, replacement of the PPV P1 cistron with that of TVMV resulted in a loss of infectivity of the chimera in the PPV-specific host, highlighting the relevance of P1 in host adaptation (Salvador et al. 2008b). Regardless, the results from these experiments revealed the importance of specific potyviral encoded proteins in host adaptation and requirements for the involvement of multiple viral-encoded proteins in determining the host range (Calvo et al. 2014; Carbonell et al. 2012; Dallot et al. 2001; Rajamaki and Valkonen 1999; Sáenz et al. 2000; Salvador et al. 2008a; Shan et al. 2018; Vassilakos et al. 2015). The involvement of multiple viral proteins in host adaptation points to multiple and overlapping functions that each of the viral-encoded proteins have in the potyviral infection cycle (Kasschau and Carrington 2001; Rojas et al. 1997). Even to overcome resistance mediated by dominant resistance loci, multiple potyviral encoded proteins are involved in some instances (Chu et al. 1997; Eggenberger et al. 2008; Hajimorad et al. 2008, 2011; Jenner et al. 2002; Krause-Sakate et al. 2005; Wen et al. 2013). These observations suggest that a plant virus must overcome many barriers in order to infect a host systemically, which requires multiple changes within viral genomes and the proteins involved (Dawson and Hilf 1992).

The finding that HC-Pro from CIYVV-No. 30, similar to that of SMV-N, did not confer virulence to the recombinant SMV-N/CIYVV-No. 30 HC-Pro on RIL L943 (-3gG2) was unexpected. This recombinant virus systemically infected Lee 68, which is the susceptible parental soybean genotype of RIL L943 (-3gG2) (Table 3). It should be noted that SMV-N causes only local infection in RIL L943 (-3gG2), which contains five genes (*IeG30*, *5gG3*, *IeG15*, *6gG9*, and *IgG4*) derived from the complex multigenic *Rsv1* locus of the parental SMV-resistant soybean genotype PI96983 (Hayes et al. 2004; Wen et al. 2013). However, it remains unknown whether lack of function of HC-Pro from CIYVV-No. 30 within the context of SMV-N/CIYVV-No. 30 HC-Pro in RIL L943 (-3gG2) is a consequence of recognition by any of these *Rsv1*-derived resistance

genes. It should be pointed out that recognition of different potyvirus species by resistance genes located within a single host locus is not unusual. The restricted TEV movement (RTM)-mediated resistance identified originally in *Arabidopsis thaliana* against TEV also recognizes other potyviruses, including PPV and lettuce mosaic virus (Decroocq et al. 2009). Interestingly, two recessive alleles, *bc-u* and *bc-3*, in common bean (*Phaseolus vulgaris*) when present together in a single host confer resistance to multiple strains as well as multiple potyviruses (Feng et al. 2015). In common bean, two recessive genes, *bc-1* and *bc-2*, also confer resistance to bean common mosaic virus and bean common mosaic necrosis virus by preventing systemic spread (Feng et al. 2017, 2018). Recessive resistance genes against CIYVV in *A. thaliana* or soybean have already been identified or mapped (Abe et al. 2019; Sato et al. 2005). The possibility that HC-Pro from CIYVV-No. 30 may be recognized by one of the *Rsv1*-derived genes in soybean warrants additional study because it could provide a new source for *R* genes against CIYVV.

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