A Turnip Mosaic Virus Determinant of Systemic Necrosis in *Nicotiana benthamiana* and a Novel Resistance-Breaking Determinant in Chinese Cabbage Identified from Chimeric Infectious Clones

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ABSTRACT

Infectious clones of Korean turnip mosaic virus (TuMV) isolates KIH1 and HJY1 share 88.1% genomic nucleotides and 96.4% polyprotein amino acid identity, and they induce systemic necrosis or mild mosaic, respectively, in *Nicotiana benthamiana*. Chimeric constructs between these isolates exchanged the 5', central, and 3' domains of KIH1 (K) and HJY1 (H), where the order of the letters indicates the origin of these domains. KIH1 and chimeras KHH and KKH induced systemic necrosis, whereas HJY1 and chimeras HHK, HKK, and HKH induced mild symptoms, indicating the determinant of necrosis to be within the 5' 3.9 kb of KIH1; amino acid identities of the included P1, Helper component protease, P3, 6K1, and cylindrical inclusion N-terminal domain were 90.06, 98.91, 93.80, 100, and

100%, respectively. Expression of P1 or P3 from a potato virus X vector yielded symptom differences only between P3 of KIH1 and HJY1, implicating a role for P3 in necrosis in *N. benthamiana*. Chimera KKH infected *Brassica rapa* var. *pekinensis* 'Norang', which was resistant to both KIH1 and HJY1, indicating that two separate TuMV determinants are required to overcome the resistance. Ability of diverse TuMV isolates, chimeras, and recombinants to overcome resistance in breeding lines may allow identification of novel resistance genes.

Keywords: full-length cDNA infectious clones, pathogenicity, phylogenetic analysis

Turnip mosaic virus (TuMV; genus *Potyvirus*, family Potyviridae) infects many plant species, mostly from the family Brassicaceae; the only virus more important in field-grown vegetables worldwide is cucumber mosaic virus (CMV; genus *Cucumovirus*) (Tan et al. 2005; Tomlinson 1987; Walsh and Jenner 2002). TuMV is the most important virus infecting brassicaceous crops worldwide and especially cultivated brassicas in East Asia (Tomimura et al. 2003). Whereas many potyviruses have narrow host ranges, TuMV is known to infect many important crops and weed plants among at least 318 plant species (from 156 genera and 43 families), and it is transmitted nonpersistently by many aphid species (Sánchez et al. 2003).

TuMV has a single-stranded, positive-sense RNA genome of about 9.8 kb encoding a single polyprotein, and flexuous filamentous particles of 700 to 750 nm long (Revers and Garcia 2015; Walsh and Jenner 2002). The genome has a virus-encoded genome-linked protein (VPg) covalently linked to its 5' end and a 3'-polyadenylate tail (Dougherty et al. 1988). The genomic polyprotein is cleaved into

10 mature proteins—P1, Helper component protease (HC-Pro), P3, 6K1, cylindrical inclusion (CI), 6K2, VPg protein, nuclear inclusion protein a protease (NIa-Pro), nuclear inclusion protein b (NIb; RdRp), and the coat protein (CP)—by three virus-encoded proteases (Walsh and Jenner 2002). P1 and HC-Pro each self-cleave at their respective C termini, whereas NIa-Pro is responsible for cleavage at both of its own termini and at all of the remaining cleavage sites in the polyprotein, with differential efficiency (Revers and Garcia 2015). An additional coding region, PIPO, within the sequence of the P3 gene is expressed as a result of polymerase slippage, causing a +2 frameshift (Chung et al. 2008; Olsper et al. 2015; Rodamilans et al. 2015; White 2015).

TuMV isolates of different lineages show differential infectivity on cabbage (*Brassica oleracea* L.) and radish (*Raphanus sativus* L.), largely resulting from variation in the P3 protein, which causes different effects on virus accumulation and viral movement with respect to cabbage and radish (Suehiro et al. 2004). Several resistance genes against TuMV have been identified, such as Tu in lettuce (Robbins et al. 1994); TuRB01, TuRB02, TuRB03, TuRB04, and TuRB05 in *Brassica napus* (Hughes et al. 2003; Jenner et al. 2002a; Walsh et al. 1999); and *retr01* (recessive) and *ConTR01* (dominant) in *Brassica rapa* (Rusholme et al. 2007). The TuMV determinants of avirulence for several *Brassica* resistance genes have been identified. The CI region of TuMV has been reported as the pathogenic determinant for the resistance genes TuRB01 and TuRB01b in the genus *Brassica* (Jenner et al. 2000; Walsh et al. 2002) and the P3 and CI proteins for TuRB04, TuRB03, and TuRB05 (Jenner et al. 2002a; 2003); CI has also been reported as the determinant for the *Rnt1-1* gene in *B. rapa*, which is probably distinct from *TuRBO1b* (Fujiwara et al. 2011). VPg is reported as the determinant for *retr01* and *ConTR01* in *B. rapa*, which are coincident with alleles of *eIF4E* and/or *eIF(iso)4E* (Rusholme

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et al. 2007). In *Arabidopsis thaliana*, systemic necrosis caused by TuMV infection was shown to be a hypersensitive resistance (HR)-like cell death induced by the interaction between P3 and *TuNI* host factor (Kaneko et al. 2004; Kim et al. 2008, 2010).

From 1992 to 1994, TuMV was reported to affect radish and Chinese cabbage in the Gangwon alpine area of Korea (Hahm 1995; Hahm et al. 1998). Later, TuMV, CMV, and radish mosaic virus (genus *Comovirus*) were detected in 2014 to 2015 nationwide surveys in radish fields (Chung et al. 2015, 2016). TuMV causes significant economic damage to radish and Chinese cabbage growers in Korea. Isolates of TuMV are also spread throughout much of the subtropical and temperate zones of the world (Gibbs and Ohshima 2010). Although new *Brassica* varieties with TuMV resistance were introduced in 1999 (Walsh et al. 1999), the resistance was soon broken by newly emerging TuMV isolates (Jenner et al. 2002b). The basal *Brassica-Raphanus* (BR) phylogenetic group of TuMV isolates was initially found in Eurasia, and the Asian-BR group mainly from the genus *Raphanus* was largely from Japan (Ohshima et al. 2002). The basal-BR group has been reported from China (Shi et al. 2007; Wang et al. 2009) and also Korea (Gong et al. 2019). The Asian expansion of the basal-BR group may explain the increased prevalence of TuMV in Korean vegetable crops.

To analyze the origins of TuMV in Korea, study pathogenesis determinant(s) in newly collected isolates, and develop tools to screen for TuMV resistance, we have collected isolates during a nationwide field survey of Korean radish crops (Chung et al. 2015, 2016). From these isolates, we have generated full-length complementary DNA (cDNA) clones of 20 isolates of TuMV (Gong et al. 2019; Han et al. 2016). Also, by constructing TuMV chimeric clones between mild and severe isolates, we found that the P3 coding region was the TuMV pathogenicity determinant affecting necrosis in *Nicotiana benthamiana*, although expression of the P3 open reading frame (ORF) from a potato virus X vector failed to induce necrosis. Chimeric constructs also demonstrated that the central region of the polyprotein included an additional virulence determinant (likely the VPg) able to overcome resistance in a commercial cultivar of *B. rapa* with apparent broad-spectrum resistance to TuMV.

MATERIALS AND METHODS

TuMV isolates and sap inoculation. TuMV samples from radish were collected during a nationwide field survey of radish and Chinese cabbage (*B. rapa* subsp. *pekinensis*) from 2014 to 2016 in Korea and selected based on differences in symptom expression in *N. benthamiana*, *R. sativus*, and *B. rapa* subsp. *pekinensis*. Only isolates from radish were utilized in this study. For initial plant inoculations, infected sap was prepared with TuMV-infected radish leaves ground in 1 ml of 1× phosphate buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄, pH 7.4) per 100 mg of leaf and mechanically inoculated to leaves of 2-week-old seedlings of *N. benthamiana* and *R. sativus* ‘Dalgona’ (Kwonnog Seed Co.) dusted with carborundum powder. Later, infectious clones were inoculated to seedlings of Chinese cabbage cultivar Kenshin (HK Seed Co. Ltd.), lines SSD002 and SSD138 (hybrids between Kenshin and cultivar Chiifi; Molecular Genetics Lab, Chungnam National University), cultivar Norang (alchannorang; KS Seed Co. Ltd.), and cultivar Chusarang (CR chusarang; Dongwon Nongsan Seed Co. Ltd.) and to radish cultivars Chunghwa (KS Seed Co. Ltd.) and Iljin (Dongwon Nongsan Seed Co. Ltd.). Chinese cabbage Norang and Chusarang, and radish Chunghwa and Iljin, were selected because they are varieties commonly cultivated in Korea.

Generation of infectious clones of TuMV isolates KIH1 and HJY1 and phylogenetic analysis. The construction of infectious clones of TuMV isolates KIH1 and HJY1 is fully described in a parallel report by Gong et al. (2019). Briefly, total RNA was purified from TuMV-infected *N. benthamiana* inoculated from field-collected radish plants, cDNA produced, and full-length

double-stranded (ds) cDNA produced by PCR amplification using TuMV-specific primers R7_1/3_T7_ApaI_F and R7_3/3_R_XmaI_T(30) (Table 1). The full-length PCR products were digested with *ApaI* and *XmaI*, ligated into similarly digested binary vector pJY1 (Park et al. 2017), and transformed into *Escherichia coli* strain DH5α (Gong et al. 2019).

After identification of infectious clones by agroinfiltration of *N. benthamiana* and sequencing to distinguish clones on sequence differences, isolates KIH1 and HJY1 were selected for more detailed examination based on their severe mosaic and systemic HR-like necrosis (KIH1) or mild mosaic, mottle, and malformation (HJY1) in *N. benthamiana* (Gong et al. 2019).

Nucleotide (nt) and predicted amino acid (aa) sequences were assembled and compared using DNAMAN (Version 5.2.10; Lynnon BioSoft) and Biology Workbench (<http://workbench.sdsc.edu/>). Phylogenetic trees of TuMV genomic sequences were constructed using the maximum likelihood method with 1,000 bootstrap replicates in MEGA version 7.0. Additional TuMV isolate sequences were obtained from the NCBI GenBank (Table 2); potato virus Y (genus *Potyvirus*) genomic RNA and polyprotein sequences were used as outgroups to root trees.

Construction of chimeric TuMV clones. The five chimeric constructs used in this study between TuMV-HJY1 and KIH1 were constructed using restriction enzyme sites unique within the TuMV sequence, including *SalI* (position: in pJY MCS upstream of *ApaI* and at nt 3865), *StuI* (position: nt 6274), and *XmaI* (position: downstream of the poly A) in the viral cDNA infectious clones. Each of the three fragments, 3.9 kb (*SalI-SalI*)/2.4 kb (*SalI-StuI*)/3.5 kb (*StuI-XmaI*), was exchanged between HJY1 and KIH1 using these conserved restriction enzyme sites. Chimeric clones KKH, KHH, HHK, HKH, and HKK are composed of the respective fragments from KIH1 (K) and HJY1 (H).

Agrobacterium infiltration and infectivity assay in *N. benthamiana*. All constructed clones were evaluated for pathogenesis using agroinfiltration. *Agrobacterium tumefaciens* strain GV2260 was separately transformed with full-length TuMV cDNA clones, hybrid constructs, or modified PVX constructs; colonies were scraped from fresh plates and diluted to ~optical density (OD)₆₀₀ = 0.6 in infiltration buffer (0.5 M 2-[N-morpholino]ethanesulfonic acid (MES), pH 5.6, and 1 M MgCl₂) containing 150 mM 3',5'-dimethoxy-4'-hydroxyacetophenone (Aldrich). Plants of *N. benthamiana* infiltrated with each of the constructed clones were either maintained in a growth chamber at 22 to 24°C or transferred after 24 h to the growth chamber. *Agrobacterium* infiltration was performed according to the work by Lim et al. (2009).

Overexpression of TuMV P1 or P3 coding regions from a PVX vector. The overexpression vector used in these studies was the infectious clone of pGD-PVX-MCS, which was previously described by Lim et al. (2010). The P1 (1,086-bp), and P3 (1,065-bp) coding regions were separately amplified from isolates TuMV HJY1 (mild) and KIH1 (severe) infectious clones, respectively, adding 5' *BamHI* and 3' *MluI* restriction sites (using primers PVX_TuMV_P1_BamHI_F, PVX_TuMV_P1_MluI_R, PVX_TuMV_P3_BamHI_F, and PVX_TuMV_P3_MluI_R) (Table 1), and they were cloned into *BamHI/MluI*-digested pGD-PVX-MCS, forming PVX(HJY1 P1), PVX(KIH1 P1), PVX(HJY1 P3), and PVX(KIH1 P3) constructs. To evaluate overexpression of P1 and P3 proteins, *A. tumefaciens* GV2260 containing modified PVX constructs was infiltrated to *N. benthamiana* as described by Lim et al. (2009). Note that the P3 ORF expressed includes the full P3N-PIPO sequence and translational slippage site of each respective isolate. Retention of the P1 and P3 genes in the constructs was confirmed by reverse transcription PCR (RT-PCR) using the specific primers listed above. Total protein extracts of healthy plants and plants infected with either wild-type PVX or PVX constructs carrying the P3 genes of HJY1 or KIH1 were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in attempts to detect expression of the P3 protein.

Pathogenicity evaluation of TuMV infectious clones and chimeric constructs in *B. rapa* var. *pekinensis* and *R. sativus* lines. Two TuMV cDNA clones (HJY1 and KIH1) and five chimeric constructs were inoculated to 2-week-old seedlings of *N. benthamiana* by agroinfiltration (strain GV2260) as described (Lim et al. 2009). At 1 day postinoculation (dpi), the inoculated leaves of individual *N. benthamiana* plants were ground with 1 ml of 1× PBS buffer per 100 mg of leaf and mechanically inoculated to leaves of 2-week-old seedlings of

B. rapa subsp. *pekinensis* (lines Kenshin, SSD002, and SSD138; three replicate plants of each line) dusted with carborundum powder. Inoculations of each variant were repeated twice. HJY1 and KIH1 infectious clones and the five chimeric constructs were subsequently mechanically inoculated to *B. rapa* subsp. *pekinensis* ‘Norang’ and ‘Chusarang’ and *R. sativus* ‘Chunghwa’ and ‘Iljin’ from agroinfiltrated tissue of *N. benthamiana* as described above. Symptom expression was evaluated over 3 weeks postinoculation for radish and 4 weeks postinoculation for Chinese cabbage. Infection of

TABLE 1. Primers used for cloning and detection

Primer name	Sequence of primer (5' → 3')	Length, bp
R7_1/3_T7_ApaI_F	GAG GGG CCC TAA TAC GAC TCA CTA TAG GAA AAA TAT AAA AAC TCA ACA CAA CAT ACA CAA AAC G	64
R7_3/3_R_XmaI_T(30)	GAG CCC GGG TTT TTT TTT TTT TTT TTT GTC CCT TGC ATC CTA TCA AAT G	61
TuMV_P1_BamHI_F	GAG GGA TCC ATG GCA GTY ACA TTC G	28
TuMV_P1_MluI_R	GAG ACG CGT TTA RTA RTG CAC GAT CTT RTG	30
TuMV_P3_BamHI_F	GAG GGA TCC ATG GGC ACC AAR TGG GAG	27
TuMV_P3_MluI_R	GAG ACG CGT TTA TTG RTG RAC CAC TGC Y	28
TuMV_CP_F_460	GCC ACG ATA TGG TCT TCA G	19
TuMV_R_460	ATC TGG ATG TGT GCC TCT C	19

TABLE 2. Details of turnip mosaic virus isolates used for phylogenetic analysis

Group and accession no.	Isolate name	Host	Country
Orchis group			
AB701690	OM	<i>Orchis militaris</i>	Germany
AB701691	OMA	<i>Orchis militaris</i>	Germany
AB701692	ORM	<i>Orchis morio</i>	Germany
AB701693	OS	<i>Orchis simia</i>	Germany
Basal <i>Brassica-Raphanus</i> group			
AB093601	Cal1	<i>Calendula officinalis</i>	Italy
AB093600	ITA7	<i>Raphanus raphanistrum</i>	Italy
AB701725	ITA8	<i>Abutilon</i> sp.	Italy
AB252125	KWB779J	<i>Brassica rapa</i>	Japan
KX674727	HJY1	<i>Raphanus sativus</i>	Korea
KX674729	KIH1	<i>Raphanus sativus</i>	Korea
Asian <i>Brassica-Raphanus</i> group			
AB252103	CH6	<i>Raphanus sativus</i>	China
AB093627	HRD	<i>Raphanus sativus</i>	China
AB362513	TUR9	<i>Raphanus sativus</i>	Turkey
World <i>Brassica</i> group			
AB701698	BEL1	<i>Rorippa nasturtium-aquaticum</i>	Belgium
AB093611	BZ1	<i>Brassica oleracea</i>	Brazil
DQ648591	CAR37A	<i>Cochlearia armoracia</i>	Poland
AB252107	CZE5	<i>Brassica oleracea</i>	Czech Republic
AB701699	DEU1	Not known	Germany
AB701700	DEU2	<i>Raphanus sativus</i>	Germany
AB701703	DNK3	<i>Brassica rapa</i>	Denmark
AB701708	FRA2	<i>Brassica napus</i>	France
AB701717	GBR83	Wild <i>Brassica oleracea</i>	United Kingdom
AB701719	HUN1	<i>Alliaria petiolata</i>	Hungary
AB701728	POL1	<i>Brassica napus oleifera</i>	Poland
AB701731	POL2	<i>Papaver somniferum</i>	Poland
AB701729	PRT1	<i>Brassica oleracea acephala</i>	Portugal
D10927	Q-Ca	<i>Brassica rapa</i>	Canada
AB093606	RUS1	<i>Armoracia rusticana</i>	Russia
AB093598	AI	<i>Alliaria officinalis</i>	Italy
Basal <i>Brassica</i> group			
AB701697	ASP	<i>Allium</i> sp.	Germany
AB701696	GK1	<i>Matthiola incana</i>	Greece
AB252117	GRC42	Wild <i>Allium</i> sp.	Greece
AB440239	IRNSS5	<i>Sisymbrium loeselii</i>	Iran
AB440238	IRNTra6	<i>Rapistrum rugosum</i>	Iran
AB093602	IS1	<i>Allium ampeloprasum</i>	Israel
AB701720	ITA1A	<i>Brassica ruvo</i>	Italy
AB701721	ITA2	<i>Cheiranthus cheiri</i>	Italy
AB701734	TIGA	<i>Tigridia</i> sp.	Germany
AB701735	TIGD	<i>Tigridia</i> sp.	Germany
Out group			
NC_001616	Potato virus Y	<i>Solanum</i> sp.	France

upper noninoculated leaves was evaluated by PCR at 4 dpi by RT-PCR. Total RNAs were extracted from newly generated leaves of *B. rapa* and *R. sativus* using TRIzol Reagent as above. Isolated total RNAs were used for cDNA synthesis by SuPrimeScript RT Premix (GeNet Bio). To detect TuMV, PCR was performed with Prime taq DNA polymerase (GeNet Bio) and primers TuMV_CP_F_460 and TuMV_CP_R_460 (Table 1). PCR conditions were 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C followed by a final cycle of 5 min at 72°C.

RESULTS

Two infectious clones of TuMV induce differential symptoms in *N. benthamiana*. We previously generated and identified a total of 20 infectious clones of Korean TuMV isolates differing in sequence and pathogenicity in *N. benthamiana*, radish, and/or Chinese cabbage (Fig. 1) (Gong et al. 2019; Han et al. 2016). Here, we selected infectious clones KIH1 and HJY1 for additional experiments to identify the determinants affecting their pathogenicity in *N. benthamiana* and radish. Knowledge of the determinants will aid development of tools to screen for TuMV resistance. TuMV isolates from field-collected radish were initially propagated in *N. benthamiana* to maintain and increase the amount of virus. On the leaves of infected *N. benthamiana*, leaf mosaic and mottle appeared at 5 to 6 dpi, and systemic necrosis was observed at 15 to 20 dpi. Infectious clones were generated in the versatile binary vector pJY (Fig. 2) as described (Gong et al. 2019). These cloning steps allowed us to successfully construct infectious clones suitable for both agroinfiltration and in vitro transcription in a binary vector (Park et al. 2017). Infectious clone KIH1 producing

severe symptoms and systemic HR-like necrosis by 20 dpi (Fig. 1) and a second clone yielding mild mosaic, mottle, and malformation symptoms in *N. benthamiana* (HJY1) were selected for comparison to identify the determinant of symptom severity in this host. Their symptom expression was also examined in radish and Chinese cabbage.

Sequence analysis of isolates HJY1 and KIH1 and phylogenetic tree construction. The full sequences of the selected infectious clones are available as KX674727 (HJY1) and KX674729 (KIH1) (Table 2) (Gong et al. 2019). Pairwise analysis of these two isolates revealed genomic nt and polyprotein aa identities of 88.13 and 96.37%, respectively, with highest nt identities in CI (90.84%), CP (90.74%), and 6K1 (90.38%) and highest aa identities in 6K1 (100%), CI (99.38%), and HC-Pro (98.91%) genes (Table 3). A full polyprotein sequence alignment of HJY1 and KIH1 revealed multiple differential residues (Fig. 1) (Gong et al. 2019).

The complete nt sequences of HJY1, KIH1, and 37 previously characterized TuMV isolates (Nguyen et al. 2013a, b) (Table 2) from the NCBI were subjected to phylogenetic analysis. All isolates clustered into five previously described groups, including the Orchis group (Nguyen et al. 2013a, b). The pathotypes of TuMV isolates are divided into two types: *Brassica* (can infect *Brassica* spp. but not *Raphanus* spp.) and BR (can infect both *Brassica* and *Raphanus* spp.) (Ohshima et al. 2002). Isolates HJY and KIH belong to the basal-BR group together with isolates from Japan and Italy (Fig. 2) among the isolates compared.

The N-terminal region of the TuMV polyprotein is associated with pathogenicity. To determine which TuMV cistrons were responsible for the severe systemic necrosis in

Fig. 1. A, Symptoms of turnip mosaic virus (TuMV) parental isolates HJY1 and KIH1 at 17 days postinoculation (dpi) on *Nicotiana benthamiana* and 20 dpi on *Brassica rapa* var. *pekinensis* ‘Kenshin’ and breeding lines SD002 and SD138. Healthy plants of the same age are shown as controls. **B,** Cartoon of the TuMV polyprotein showing individual gene products, with amino acid residues differentiating HJY1 and KIH1 indicated above and below the representation of the polyprotein. Lines from the N and C termini of each gene product encompass the residues differentiating HJY1 and KIH1.

Fig. 2. A phylogenetic tree was constructed from the complete nucleotide sequences of the turnip mosaic virus (TuMV) polyprotein by the maximum likelihood method with 1,000 bootstrap replicates; bootstrap values of >50% are indicated at the branches. The genomes of 38 previously characterized TuMV isolates obtained from the NCBI (Table 2 has details) were compared with HJY1 and KIH1 (indicated by arrows). The equivalent sequence of the reference isolate of Potato virus Y was used to root the tree. Isolates are indicated in the tree by access number/isolate designation/country of origin. Both HJY1 and KIH1 fall within the basal-BR subgroup.

N. benthamiana, a series of reciprocal chimeric clones between HJY1 and KIH1 was constructed (Fig. 3). The TuMV genome was divided into three parts using restriction sites conserved between HJY1 and KIH1, and then, five hybrid constructs were generated using the corresponding restriction enzymes (*SalI*, *StuI*, and *XmaI*) (Fig. 3A). Initially, we exchanged the nt 6274 to 3' regions of the two parental isolates containing the C terminus of VPg, all of NIa-Pro, NIb, CP, and 3' untranslated region (containing polyA) (Fig. 3A). Chimeric constructs HHK and KKH were able to infect *N. benthamiana* without altering their pathogenicity compared with the major parental isolates, indicating that the nt 6274 to 3' region of these isolates was not associated with systemic necrosis. Chimera HHK gained the nt 3865 to 6274 region containing most of CI, the complete 6K2, and most of the VPg of KIH1, but the symptoms were not significantly different from those of the major parental isolate, HJY1, indicating that the nt 3865 to 6274 region of isolate KIH1 did not play an important role in the differential symptoms and that the N-terminal region of the polyprotein (5' end to nt 3865; containing all of P1, HC-Pro, P3, 6K1, and the N-terminal domain of CI) includes the determinant responsible for systemic necrosis in *N. benthamiana*. The N-terminal region of the potyvirus polyprotein has been reported previously to include the determinant of TuMV pathogenicity (Jenner et al. 2002a, 2003; Kaneko et al. 2004; Kim et al. 2008, 2010; Suehiro et al. 2004) and other potyviruses (Chu et al. 1997; Dallot et al. 2001; Hjulsgaard et al. 2002; Sáenz et al. 2000; Salvador et al. 2008a, b) in specific hosts. As expected, the chimeric construct HHK caused host symptoms distinct from those of KIH1 (necrosis → mild mosaic), and the reciprocal chimera KKH caused a different response compared with HJY1 (mild mosaic → necrosis) (Fig. 3B). These results confirmed that the viral determinants for necrosis were located in the region including the P1 to CI N terminus. The aa sequences of P1, HC-Pro, P3, P3N-PIPO, 6K1, and the CI N-terminal region of the two isolates had 90.06, 98.91, 93.80, 97.30, 100, and 100% identity (Table 3) (data not shown), respectively. Previous work indicated that HC-Pro was not a determinant of TuMV pathogenicity in *N. benthamiana* (Han et al. 2016); thus, these results suggest that the TuMV P1 and/or P3 gene encode determinant(s) of viral necrosis symptoms in *N. benthamiana*.

To confirm whether this region of the TuMV genome can induce severe symptoms on other susceptible hosts, we also inoculated the TuMV chimeric constructs to *B. rapa* ssp. *pekinensis* lines Kenshin, SSD002, and SSD138. Pathogenicity tests of hybrid constructs in *B. rapa* lines showed that isolate HJY1 and chimeras HHK, KKH, and HHK induced severe symptoms, whereas KIH1 and chimeras KKH and KKH showed mild symptoms (Supplementary Fig. S1) in *B. rapa*. These results indicated that more severe symptoms in *B. rapa* were associated with the N-terminal P1/HC-Pro/P3 region of isolate HJY1, contrary to the results in *N. benthamiana*; however, these results were not representative of all *B. rapa* lines (see below).

HJY1, KIH1, and the five chimeric constructs were also inoculated to radish Chunghwa and Iljin and Chinese cabbage Norang and Chusarang. The constructs had varying ability to infect these varieties and differed in their symptoms in infected plants. Both isolates caused similar mosaic in radish Chunghwa, and both infected Iljin; KIH1 caused significant mosaic in Iljin, whereas HJY1 induced no obvious symptoms. Both isolates infected Chinese cabbage Chusarang with severe symptoms, but neither infected Norang as shown by PCR of upper leaves (Fig. 3B) (data not shown). Similar results were obtained with the chimeric constructs except that chimera KKH was unexpectedly able to infect Chinese cabbage Norang, inducing severe symptoms, whereas Norang was resistant to both parental isolates and the other chimeric constructs (Fig. 3B).

Overexpression of the P1 and P3 ORFs suggests that P3 (and/or P3N-PIPO) is the determinant of symptom severity in *N. benthamiana*. To further identify which of the P1 or P3 proteins was responsible for viral pathogenicity, we modified the pGD-PVX-MCS vector (Lim et al. 2010) to separately

overexpress the P1 and P3 proteins of each of the KIH1 and HJY1 isolates, respectively, in *N. benthamiana* as an added gene. The P1 and P3 genes of each isolate were amplified using P1- or P3-specific primers (Table 1), respectively, and separately introduced into infectious clone PVX-MCS, forming PVX(HJY1 P1), PVX(KIH1 P1), PVX(HJY1 P3), and PVX(KIH1 P3). Each PVX variant was inoculated to *N. benthamiana* by agroinfiltration. WT-PVX induced mild mosaic symptoms in *N. benthamiana*. At 20 dpi, plants inoculated with PVX(HJY1 P1) or PVX(KIH1 P1) showed no significant symptom differences compared with plants infected with WT-PVX (data not shown). However, plants infected with PVX(HJY1 P3) and PVX(KIH1 P3) showed somewhat more severe symptoms than WT-PVX. Comparison of overexpression of the P3 genes of the two different TuMV isolates demonstrated that the KIH1 P3 gene from a severe symptom isolate of TuMV induced more severe symptoms than HJY1 P3 (Supplementary Fig. S2). Maintenance of the P1 and P3 ORFs in the constructs was confirmed by RT-PCR using P1- and P3-specific primers, respectively; however, expression of the proteins could not be directly demonstrated, because no specific antibodies were available, and bands of the appropriate sizes could not be distinguished by SDS-PAGE of total protein extracts (data not shown). Overall, these results suggested that the P3 cistron is associated with the intensity of pathogenicity in *N. benthamiana*. However, the more severe symptoms induced by PVX(KIH1 P3) did not include necrosis similar to that induced by TuMV clone KIH1 (Fig. 1).

Additional analysis of differences in the P3 sequence. The TuMV P3 protein has previously been reported as a determinant of the ability to systemically infect radish (Suehiro et al. 2004). We, therefore, examined the P3 aa sequence in more detail. Because P3 also includes the PIPO reading frame expressed as a P3N-PIPO fusion (Chung et al. 2008), we also examined the differences in the predicted P3N-PIPO sequences of isolates KIH1 and HJY1 used to create the chimeric constructs from which the P3 region was associated with systemic necrosis in *N. benthamiana*. There were only three differential residues in the P3N region (S47, N119, and S126) and three in PIPO (D168, G215, and I217) in HJY1, all substitutions of residues with similar properties (Fig. 4).

We then examined the transcriptional slippage site and PIPO ORF of isolates HJY1 and KIH1 in comparison with those of the TuMV reference sequence (NC_002509), which is derived from TuMV-UK1 (a B host-type isolate) (Ohshima et al. 2002; Tomimura et al. 2003). Analysis of the transcriptional slippage site and surrounding nt sequences revealed that mild isolate HJY1 and reference isolate UK1 shared the same sequence at the transcriptional slippage site, GG(A)₆, whereas severe isolate KIH1 had AG(A)₆, with no downstream differences for a minimum of 8 nt (UK1) or 10 nt (HJY1) (Supplementary Fig. S3).

DISCUSSION

In this study, we generated infectious clones of two isolates collected as part of a nationwide survey of radish fields (Chung et al.

2016; Gong et al. 2019). Isolate HJY1 showed mild mosaic and stunting equivalent to the previously characterized R007 (Han et al. 2016), and isolate KIH1 induced severe symptoms and systemic HR-like symptoms in *N. benthamiana*. Our examination of chimeric constructs between mild symptom isolate HJY1 and necrosis-inducing KIH1 confirms the localization of a host-specific infection phenotype and symptomatology to the P3 ORF (Suehiro et al. 2004), and it extends these host-specific effects to induction of systemic necrosis in *N. benthamiana*. There were no differences between HJY1 and KIH1 in the nt sequence immediately downstream of the shifty hexanucleotide, which has been shown to affect the expression level of P3N-PIPO (Olspert et al. 2016), but the possible influence of the G > A change in KIH1 immediately upstream of the conserved G(A)₆ is not known; however, multiple other Korean TuMV isolates inducing severe mosaic and systemic necrosis in *N. benthamiana* share the AGAAAAA sequence observed in KIH1, and three other isolates inducing mild symptoms similar to those of HJY1 (Gong et al. 2019) have the canonical GGAAAAA sequence. It is worth noting that, in the context of the related P1N-PISPO slippage site of sweet potato feathery mottle virus as a secondary G(A)₆ site downstream of the green fluorescent protein (GFP) sequence in TuMV-GFP (an infectious clone of TuMV-UK1 with the GFP coding sequence inserted between P1 and HC-Pro), mutation of GG(A)₆ to AG(A)₆ increased the level of slippage (from 2.09 ± 0.42 to 3.23 ± 0.32%), but these constructs also differed in the sequences immediately upstream and downstream of the shifty G(A)₆ sequences (Olspert et al. 2016) and are, therefore, not directly comparable.

The chimeric constructs between HJY1 and KIH1 differ in only three substitutions in the PIPO region and three in P3N of the predicted P3N-PIPO protein (Fig. 4), and we speculate that the observed symptom differences are more likely because of the 22 differences in the P3 protein (Fig. 1) rather than P3N-PIPO. This will be further tested by reciprocal mutagenesis of the differential PIPO residues between HJY1 and KIH1 or coexpression of separate P3 and P3N-PIPO constructs in *N. benthamiana* from the PVX vector. However, both isolates induced severe symptoms in Chinese cabbage Chusarang, and neither infected Norang, whereas both infected radish Chunghwa and Iljin, with KIH1 causing more prominent symptoms (Fig. 3). The failure of expression of P3 of either KIH1 or HJY1 from the PVX vector to induce the necrosis seen in *N. benthamiana* with infection of KIH1 suggests a possible necessity for interaction with either P3N-PIPO or another TuMV product or higher expression levels to induce necrosis. However, we note that, as we expressed the P3 ORF from PVX modified only by addition of start and stop codons and not as a GFP fusion or with addition of an epitope tag, we were unable to directly demonstrate expression of the P3 protein, because we had no P3-specific antibody, and we were unable to distinguish a band of the anticipated sizes of either P3 or P3N-PIPO from the background of *N. benthamiana* proteins by SDS-PAGE of extracts of plants infected with PVX carrying either the HJY1 or KIH1 P3 genes.

TABLE 3. Comparison of untranslated regions and gene nucleotide and amino acid sequence identities between mild turnip mosaic virus (TuMV) HJY1 and severe TuMV KIH1 isolates^a

	P1		HC-Pro		P3		P3N-PIPO		PIPO		6K1	
5' UTR; nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
95.35	87.48	90.06	87.41	98.91	87.23	93.80	89.69	97.30	96.05	94.92	90.38	100
	CI		6K2		Nla-VPg		Nla-Pro		Nlb		CP	
3' UTR; nt	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
95.75	90.84	99.38	83.65	94.34	86.28	94.27	83.40	96.71	86.33	96.52	90.74	97.22

^a HC-Pro, helper component protease; aa, amino acid; CI, cylindrical inclusion; CP, coat protein; Nla-Pro, nuclear inclusion protein a protease; Nla-VPg, nuclear inclusion protein a virus-encoded genome-linked protein; Nlb, nuclear inclusion protein b; nt, nucleotide; and UTR, untranslated region.

The P3 residues 153 and 312 identified by Jenner et al. (2002a, 2003) as associated with overcoming resistance conferred by *TuRBO3* and *TuRBO4* are identical (153I and 312F) in both HJY1 and KIH1 (data not shown). This suggests that the resistance of Norang is not owing to the presence of either *TuRBO3* or *TuRBO4* and that these residues are not related to the observed symptom differences in other hosts.

The ability of chimera KKH but neither parental isolate or any other chimeric construct to infect *B. rapa* ‘Norang’ suggests that two different viral determinants are important in the observed interactions in the three hosts examined here; it has previously been noted in the TuMV/*B. rapa* pathosystem that symptom type is dependent on the particular virus isolates and plant genotypes examined (Fujiwara et al. 2011). It has also been demonstrated that

Fig. 3. A, Scheme for generation of chimeric isolates by exchange of restriction fragments between full-length infectious clones HJY1 and KIH1. Positions of *SalI*, *ApaI*, *Sall*, and *XmaI* are indicated. Symptoms in *Nicotiana benthamiana* (M indicates mild mosaic, and N indicates necrosis) and reverse transcription PCR (RT-PCR) detection of turnip mosaic virus (TuMV) infection from newly expanding upper leaves of radish cultivars Chungghwa and Iljin and Chinese cabbage cultivars Norang and Chusarang (+ indicates TuMV infection, and - indicates that no TuMV was detected) at 4 days postinoculation (d.p.i.) are indicated to the right of cartoon representing each construct. **B**, Symptoms of healthy and TuMV-inoculated plants at 10 or 20 dpi (*N. benthamiana*) or 20 dpi for radish and Chinese cabbage cultivars as indicated on the left of the panel. Note that symptoms in *N. benthamiana* segregated with the origin of the N-terminal portion of the polyprotein and that only chimera KKH and not the parental isolate or any other chimera was able to infect Chinese cabbage cultivar Norang. Other details are in the text.

different TuMV resistance genes interact with different viral determinants; thus, the P3 protein is the determinant interacting with *TuRBO3* (Jenner et al. 2003) and *TuRBO4* (Jenner et al. 2002a), and CI is the determinant for *TuRBO5* (Jenner et al. 2002a), *TuRBO1/TuRBO1b* (Jenner et al. 2000; Walsh et al. 2002), and *Rnt1-1* in Chinese cabbage, a gene probably differentiated from *TuRBO1b* (Fujiwara et al. 2011). The interaction of different potyviral proteins with different hosts is not limited to TuMV; the plum pox virus (PPV) region containing the C-terminal portion of the P3 gene and the 6K1 gene was shown to be associated with symptom phenotype in *N. clelandii*, *Pisum sativum* (Sáenz et al. 2000), and plum but not peach (Dallot et al. 2001), whereas additional determinants of host specificity for *N. clelandii* and peach were identified in the N-terminal region of the PPV CP (Salvador et al. 2008b). This also suggests that Norang has two independent resistance genes or that two (or three) TuMV proteins must have favorable interactions to overcome one (presumably dominant) resistance gene. This has previously been demonstrated in the case of the tobacco etch virus interaction of P3 and NIa-VPg with tabasco peppers to cause wilting (Chu et al. 1997). Similarly, both the HC-Pro and P3 proteins of compatible soybean mosaic virus isolates are required to overcome the dominant *Rsv1* resistance in soybean (Eggenberger et al. 2008). It is, therefore, likely that the determinants affecting infectivity and symptom production in radish and Chinese cabbage are in a different genomic region than that affecting symptom severity in *N. benthamiana*, which our experiments indicate to be in either the P3 or P3N-PIPO protein. Infection of *B. rapa* 'Norang' by chimeric construct KKH suggests either the CI or VPg protein as an additional determinant. Comparison with the case of tobacco etch virus in tabasco pepper (Chu et al. 1997) suggests the combination of P3 and VPg, one each from the 5' and central regions of the chimeric KKH genome, although this must be considered speculative in the absence of experimental evidence.

Interestingly, a mutation in the CI protein of TuMV-UK1, resulting in a V1827E substitution, broke the resistance conferred by *Rnt1-1* in Chinese cabbage (Fujiwara et al. 2011); however, both of the radish isolates and the chimeras examined here have V1827 and also share N1686 and H1857 as for wild-type TuMV-UK1 (data not shown) rather than substitutions N1686D and H1857R previously reported from other resistance-breaking mutants (Jenner et al. 2000). Only four residues differentiate the CI of KIH1 and HJY1, of which all substitutions have similar properties (D1306N, T1586A, I1659V, and T1848A) (Fig. 1), whereas 6K2 has 3 of 53 differential residues (F1902L, S1915M, and L1917I) (Fig. 1), of which residues at two positions fall into different classes.

It is noteworthy that VPg of many potyviruses is known to interact with either eIF4E or eIF(iso)4E, and they are known as recessive resistance genes in many hosts (Le Gall et al. 2011; Moury et al.

2014). Indeed, broad-spectrum resistance to TuMV in Chinese cabbage has been associated with genes *retr01* and *ConTR01*, with *ConTR01* being coincident with one of three loci of *eIF4E* and possibly one locus of *eIF(iso)4E* in the *B. rapa* genome and *retr01* possibly coincident with a locus of *eIF(iso)4E* (Rusholme et al. 2007). In addition, overexpression of eIF(iso)4E variants in transgenic *B. rapa* has been shown to confer broad-spectrum resistance to TuMV (Kim et al. 2014). VPg is thus an attractive candidate for the TuMV determinant of infectivity on Chinese cabbage Norang. However, the source of resistance in Norang is unknown, and although it is possible that combination of the VPg N-terminal portion from KIH1 with the C-terminal portion from HJY1 in chimera KKH (Fig. 3) may yield a VPg variant able to overcome a potential *eIF4E*- or *eIF(iso)4E*-mediated resistance, this remains to be determined. For comparison, a maximum of three aa residues in the central and C-terminal domains of tobacco etch virus VPg determines the infectivity in *Capsicum* species (Perez et al. 2012), and most of the VPg residues that differ between KIH1 and HJY1 are in the central and C-terminal domains (Figs. 1 and 3). Substitution of only the chimeric VPg of KKH into both KIH1 and HJY1 would potentially determine whether the chimeric VPg is sufficient to overcome the resistance of Norang, although the failure of HKH to infect this cultivar suggests otherwise.

The expansion of basal-BR isolates into China (Shi et al. 2007; Wang et al. 2009) and Korea (Gong et al. 2019), possibly with pathogenicity determinants distinct from those of previously prevalent strains, has several implications. The emerging basal-BR isolates may have increased pathogenicity, and they may be able to overcome the resistance present in cultivars selected before the regional emergence of basal-BR strains. However, identification of new TuMV strains and recombinants also offers opportunities for selection and pyramiding of new resistance genes to provide more effective and longer-lasting resistance to TuMV.

In summary, we have tested two contrasting TuMV isolates, and our results suggest the importance of TuMV P3 and/or P3N-PIPO for induction of systemic necrosis in *N. benthamiana*. In addition, we have observed that the symptoms in Chinese cabbage and radish were not correlated with those in *N. benthamiana* and that symptoms in Chinese cabbage Norang require at least one additional TuMV determinant, which may be the VPg. These results form the basis for additional examination of specific virus-host interactions affecting symptom severity and resistance, expanding our knowledge of an important potyvirus and its host interactions. In addition, our experiments with chimeric constructs derived from HJY1 and KIH1 demonstrate the potential for natural recombinants to break currently available broad-spectrum resistance deployed in commercial cultivars and breeding lines. The differential host responses to isolates KIH1, HJY1, and the chimeric constructs and particularly, the ability of chimera KKH to overcome

Fig. 4. Predicted amino acid sequence of P3N-PIPO of the necrosis-inducing turnip mosaic virus (TuMV) isolate KIH1 and mild isolate HJY1 used to generate chimeric constructs. The P3N portion of the sequence is shown in black font, with green highlighting the sequence differences in HJY1; the PIPO portion of the sequence is shown in blue font, with yellow highlighting the three differential residues in HJY1. The blue highlighting indicates the location of the HC-Pro cleavage site immediately upstream of the P3 sequence. Consensus key: asterisks indicate conserved residue, colons indicate conservation of strong groups, and periods indicate conservation of weak groups.

the resistance of Chinese cabbage Norang suggest that these variants may be valuable for screening of germplasm to identify new sources of resistance to TuMV.

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