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Author(s)	Fujiwara, Ayaka; Inukai, Tsuyoshi; Kim, Bo Min et al.
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**Combinations of a host resistance gene and the CI gene of *Turnip mosaic virus* differentially regulate symptom expression in *Brassica rapa* cultivars**

Ayaka Fujiwara, Tsuyoshi Inukai, Bo Min Kim and Chikara Masuta

Graduate School of Agriculture, Hokkaido University, 060-8589 Sapporo, Japan

Corresponding author: T. Inukai; E-mail address: [yoshi@abs.agr.hokudai.ac.jp](mailto:yoshi@abs.agr.hokudai.ac.jp)

## Abstract

In the pathosystem of *Brassica rapa* and *Turnip mosaic virus* (TuMV), the type of symptoms expressed by susceptible plants are determined by the gene combinations between the host cultivar and virus strain. In this study, we found that the resistant reaction and symptoms such as systemic lethal necrosis, leaf malformation and mosaic were differentially determined, depending on the combinations of the genotypes for a host locus or two closely linked host loci and the viral CI gene. Systemic necrosis caused by TuMV-UK1 on some *B. rapa* subsp. *pekinensis* cultivars is induced in conjunction with a recessive gene *rnt1-2* (resistance and necrosis to tumv 1-2), which is allelic or closely linked to TuMV resistance gene *Rnt1-1* on chromosome R6. *rnt1-2* is incompletely recessive to *rnt1-3*, which does not cause any necrotic responses. The genotype *rnt1-2/rnt1-3* caused a mild necrosis along leaf veins of severely malformed leaves. A spontaneous mutant TuMV-UK1 (UK1m) with the amino acid substitution (V1827E) in CI broke *Rnt1-1* resistance and altered the systemic necrosis and leaf malformation induced by *rnt1-2*. This single amino acid in the CI protein of UK1 was also associated with severe mosaic and abnormal leaf development, perhaps interacting with a host unknown factor(s). To clarify the relationship of *Rnt1-1* with *TuRB01b*, which was previously reported as a TuMV-UK1 resistance gene on chromosome R6, the *B. rapa* cultivar Tropical Delight carrying *TuRB01b* was inoculated with UK1m or the infectious UK1 clone with the CI V1827E mutation. Because Tropical Delight showed resistance to both mutants, *Rnt1-1* might be different from *TuRB01b*.

## Introduction

To control virus diseases, introduction of resistance genes has been the most useful approach because viruses are difficult to control with chemicals and cultural methods. A number of single, dominant or recessive genes that induce complete resistance to viruses have been identified [1]. One of the issues with using such resistance genes is how to maintain the durability of the resistance because resistance controlled by single major genes is often broken by viral mutation [9]. In some pathosystems in which genetic resources for resistance are limited [e.g. 15], controlling the severity of symptoms may be an effective alternative to minimize losses caused by viruses. The capacity to reduce symptom severity in virus-infected plants is often referred to as tolerance, and several tolerance genes to virus have been reported [e.g. 11, 17]. Genetic control of symptom types is also needed because the damage caused by viral systemic infection varies with the symptoms, e.g., necrosis and mosaic, which are often determined by interactions between host and viral factors [6, 8].

*Turnip mosaic virus* (TuMV), a member of the genus *Potyvirus*, infects a wide range of hosts including *Brassica* crops [13]. In *B. rapa*, the type and severity of symptoms induced by TuMV infection varies in cultivars. Systemic necrosis by TuMV kills infected leaves and/or plants. In Chinese cabbage (*B. rapa* subsp. *pekinensis*), such necrotic tissues are then easily infected by other pathogens such as *Erwinia carotovora* subsp. *carotovora* [12]. Even if a necrotic symptom is mild, the economic damage is serious in plants with necrosis. On the other hand, severe mosaics often accompany leaf malformation and markedly reduce product yield and quality, while mild mosaics are sometimes not evident, depending on the circumstances. Thus, symptom control appears to be effective to decrease losses by TuMV in *B. rapa*, but the mechanisms underlying

symptom induction have not been studied well. There are not many studies on genes controlling symptom expression of TuMV infections in *Brassica* crops. For example, the resistance gene *TuRB01* to TuMV-UK1 cosegregated with a gene inducing systemic necrosis to virulent UK1 mutants in doubled-haploid *B. napus* lines [5]. On the other hand, the TuMV P3 protein was found to be not only an avirulence determinant but also a symptom severity determinant to *B. napus* and *B. juncea* [4]. In *Arabidopsis thaliana*, systemic necrosis caused by TuMV infection was shown to be a hypersensitive reaction (HR)-like cell death induced by the interaction between P3 and a host factor *TuNI* [6, 7, 8].

In this study, we identified a *B. rapa* gene involved in the induction of necrosis and leaf malformation after infection with TuMV and analyzed the relationship of the host gene to a TuMV resistance gene. Moreover, we found that a single amino acid in the cytoplasmic inclusion protein (CI) gene of TuMV could determine symptom types such as necrosis, leaf malformation and mosaic on *B. rapa*.

## **Materials and methods**

### Plant materials and infectivity tests

Five Chinese cabbage cultivars Aki-masari, Haya-hikari, Yu-shun, Santo-hakusai (ATARIYA, Katori, Japan) and Tropical Delight (SAKATA SEED, Yokohama, Japan), and a turnip (*B. rapa* subsp. *rapa*) cv. Wase-ohkabu (TAKII, Kyoto, Japan) were used in this study. AS9 used in this study is a UK1-resistant S<sub>2</sub> progeny of Aki-masari. SS11 is a UK1-susceptible S<sub>1</sub> progeny of Santo-hakusai. An F<sub>2</sub> population derived from a cross between AS9 and SS11 was used to map the resistance gene to UK1. Plants for infectivity tests were maintained in a greenhouse at 24-26°C with a natural day length.

Plants for mapping were grown in an MLR-350 growth chamber (SANYO, Tokyo) at 21°C with 12-h photoperiod ( $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). The infectious cDNA clone of the TuMV strain UK1 developed by Sánchez et al. (1998) [10] was kindly provided by Dr. F. Ponz. UK1 and the UK1-derived constructs with a point mutation were first used to mechanically inoculate *Nicotiana benthamiana*, and then the infected leaves were used as inoculum. The first pair of true leaves of Chinese cabbage or turnip were dusted with carborundum and rub-inoculated with the inoculum.

Sequencing of a mutant isolate derived from UK1 and site-directed mutagenesis of the infectious cDNA clone UK1

Total RNA was extracted from a non-inoculated upper leaf of an AS9 plant that had systemic necrosis using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). To determine the sequence of the coding region in the UK1 mutant, viral cDNA was synthesized using AMV Reverse Transcriptase XL (Takara, Tsu, Japan) and amplified with KOD-Plus- ver.2 DNA polymerase (Toyobo, Osaka, Japan). The primer sequences used are listed in Table S1. After addition of dATP at the 3' end using ExTaq DNA polymerase (Takara), the amplified fragments were ligated into pCR-XL-TOPO vector (Invitrogen) or pGEM-T vector (Promega, Madison, WI, USA). DNA sequencing was carried out using an automated sequencer (ABI PRIZM 310 Genetic Analyzer) according to the manufacturer's instructions. Point mutations were introduced into the infectious clone of UK1 using the protocol for the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). The infectious constructs UK1-P3m, -CIm and -CPm were first inoculated to *N. benthamiana*, and then the viral progeny of these mutants obtained from the infected leaves were sequenced. After

confirming that the mutations were still maintained and there were no other mutations, they were used as an inoculum.

#### DNA extraction and DNA polymorphism markers

Total DNA was extracted from young leaves by the cetyltrimethylammonium bromide method [6]. The primer sequences for BRMS SSR markers were obtained from the VegMarks database (<http://vegmarks.nivot.affrc.go.jp/VegMarks/jsp/index.jsp>) managed by the National Institute of Vegetable and Tea Science in Japan. Insertion/deletion (indel) PCR markers were developed based on the Chinese cabbage genomic sequences of Scaffold000009 and Scaffold000129 obtained from the *Brassica* Database (BRAD; <http://brassicadb.org/brad/>). The primer sequences are listed in Table S2.

## Results

#### A symptom-inducing factor of *B. rapa* to TuMV-UK1 infection

In *B. rapa* infected with TuMV, distinct symptoms were induced depending on the combination of host and viral genotypes. A Chinese cabbage cv. Yu-shun developed systemic necrotic lesions and veinal necrosis to various levels when infected with TuMV-UK1 (Fig. 1). In Yu-shun, mild mosaic was occasionally associated with necrosis. The necrotic symptom on the Chinese cabbage cv. Haya-hikari was much milder than on Yu-shun. In Haya-hikari, smaller and fewer necrotic lesions developed, and the infected leaves gradually became chlorotic (Fig. 1). Necrosis along the leaf veins of Haya-hikari eventually caused leaf curling. Malformation was also observed in the infected leaves showing only mild mosaic in Haya-hikari. The turnip cultivar Wase-ohkabu developed a typical mosaic to UK1 (Fig. 1). On the other hand, the

Chinese cabbage cv. Aki-masari and the experimental line AS9 derived from Aki-masari were resistant to UK1 (Fig. 1). When UK1 was inoculated to cotyledons of AS9 or Aki-masari, a few necrotic local lesions were observed. Therefore, the resistance of Aki-masari to UK1 appeared to be a HR-type resistance. This resistance was broken by a mutation in UK1, changing to systemic necrosis (Fig. 1). The symptoms on the other three cultivars changed to mild mosaics after such a mutation in UK1. Thus, the symptom type induced by TuMV appeared to depend on the combination of cultivar and virus strain.

To identify the host factors for symptom induction, we made selfed ( $S_1$ ) populations from Yu-shun, Haya-hikari and Aki-masari and investigated their reactions to UK1. While all the  $S_1$  plants derived from Yu-shun developed systemic necrosis, the  $S_1$  plants derived from Haya-hikari and Aki-masari segregated for the reactions to UK1 (Table 1). The Haya-hikari  $S_1$  plants segregated into 34 plants with mosaic and 11 with necrosis. The degree of necrosis in the  $S_1$  plants was severer than that in the parental Haya-hikari and similar to that in Yu-shun.  $S_1$  plants with mild necrosis similar to Haya-hikari were rarely observed. These results suggest that necrosis in Haya-hikari is controlled by a single recessive gene and that the necrosis expressed on the heterozygote could change depending on environmental conditions or genetic background. On the other hand, leaf malformation was observed on 20 of 34  $S_1$  mosaic plants, suggesting that another factor induces leaf malformation in addition to veinal necrosis in Haya-hikari. The Aki-masari  $S_1$  population segregated into 29 resistant and 9 mosaic plants, indicating that Aki-masari carries a single dominant resistance gene to UK1 (Table 1). AS9 was the selected  $S_2$  line carrying this resistance gene at homozygous state. To investigate the allelism between the genes involved in resistance and systemic necrosis, we crossed

Aki-masari and Yu-shun and generated F<sub>1</sub> populations and F<sub>2</sub> families. The F<sub>1</sub> population segregated into 46 resistant plants and 50 symptomatic plants having mild or no necrosis, consistent with the expected ratio of 1:1 (Table 1). In the 12 F<sub>2</sub> families, six families segregated into "resistance" and "lethal necrosis" within each family, while the other six families segregated into "lethal necrosis" and "mosaic or mild necrosis" within each family. The expected segregation ratios in these two groups of families were 3:1 or 1:3, respectively (Table 1). These results indicated that the resistance gene and the necrosis-inducing gene were allelic or closely linked to each other, and the resistance gene was dominant or epistatic to the necrosis-inducing gene. In general, it is difficult to prove allelism between dominant genes or dominant and recessive genes using a classical genetic analysis. For this matter, there are two points of view. One is to treat the genes as separate genes until the allelism is proven. The other is to treat the genes as multiple alleles until the allelism is denied. In order to avoid an excessive increase of the number of gene symbols for disease resistance, the latter option was chosen as for instance in the genetic study of rice blast resistance [3]. Here, we followed these criteria and described the resistance gene in Aki-masari and the necrosis-inducing gene in Yu-shun as multiple alleles *Rnt1-1* and *rnt1-2* at the *Rnt1* (Resistance and necrosis to TuMV) locus for convenience. The recessive allele that originated from Aki-masari and does not induce any necrotic symptom was described as *rnt1-3*. Gene *rnt1-2* was incompletely recessive to *rnt1-3*, and genotype *rnt1-2/rnt1-3* resulted in leaf malformation associated with mild necrosis along leaf veins.

To determine the chromosomal location of the *Rnt1* locus, we first used a linkage analysis with the SSR markers released at the VegMarks database and 46 F<sub>2</sub> plants derived from a cross between AS9 carrying *Rnt1-1* and an S<sub>1</sub> line SS11 selected from

Santo-hakusai, which is a Chinese cabbage cultivar susceptible to UK1. The result showed that *Rnt1* was located between the SSR markers BRMS221 and BRMS013 on chromosome R6. For further fine mapping, indel PCR markers were developed based on the scaffold sequences from BRAD containing BRMS221 or BRMS013, and those linkage relationships with *Rnt1* were investigated using 32 susceptible plants among 142 F<sub>2</sub> plants derived from a cross between AS9 and SS11. *Rnt1* was shown to cosegregate with the indel PCR marker 129-center developed on the scaffold000129 sequence (Fig. 2).

Because both *Rnt1-1* and *TuRB01b* (the TuMV resistance gene previously reported [14, 16]) were identified using the same TuMV strain UK1 and mapped on the chromosome R6, and because the avirulence gene in TuMV that corresponded to the two resistance genes was the viral CI gene [15, this study], *Rnt1-1* could be identical to *TuRB01b*. To clarify the relationship between *Rnt1-1* and *TuRB01b*, Tropical Delight, a Chinese cabbage cv. carrying *TuRB01b* [15], was inoculated with UK1m and UK1-CIm that had overcome *Rnt1-1* resistance [see next section]. While AS9 showed systemic necrosis to UK1m and UK1-CIm, Tropical Delight showed resistance to UK1m and UK1-CIm (Fig. 1). Because there were no obvious lesions on the inoculated leaves, the resistance of Tropical Delight seemed to be extreme resistance. These results suggest that *Rnt1-1* might be different from *TuRB01b*. Further fine mapping will be needed to clarify the allelic relationship between the two genes.

#### A symptom-inducing factor of TuMV-UK1

The AS9 resistance to UK1 was broken at very low frequency, and the systemically infected AS9 plants sometimes developed necrosis. Interestingly, Yu-shun, Haya-hikari

and Wase-ohkabu altered their symptoms when inoculated with the sap from TuMV-infected AS9 leaves (Fig. 1). This UK1 mutant (UK1m) was found to induce systemic necrosis in AS9, but Yu-shun and Haya-hikari showed only mild mosaic (Fig. 1). In Haya-hikari, leaf malformation became much milder than that by the original UK1. Wase-ohkabu also showed a milder mosaic. To identify the viral gene(s) responsible for these symptom changes, the genomic sequence of UK1m was amplified by RT-PCR using four pairs of primers (Table S1; Fig. 3), cloned and sequenced. The results showed that UK1m possessed four single base-pair substitutions at position 3518, 3636, 5480, and 9113 in the P3, 6K1, CI and CP genes, respectively (Fig. 3). All mutations except the mutation in 6K1 caused an amino acid change (V1173A, V1827E, and V3038A, respectively). To clarify which mutations are involved in the symptom changes and/or resistance breaking, each mutation was created in the UK1 infectious clone by site-directed mutagenesis. The infectious constructs UK1-P3m, -CI<sub>m</sub> and -CP<sub>m</sub> were first inoculated to *N. benthamiana*, and then the infected leaves were used as an inoculum. The results of the infectivity tests showed that the CI mutation (V1827E) was responsible for all the symptom changes and resistance breaking (Fig. 1; Table 2). While the P3 mutation (V1173A) did not affect any reactions of the cultivars or lines, the mutation in the CP gene (V3038A) only resulted in a slight decrease of mosaic in Wase-ohkabu. These results indicated that the amino acid at position 1827 in the CI protein of UK1 was involved in the induction of both resistance and symptoms (necrosis, mosaic and leaf malformation). The position of this CI mutation (V1827E) differed from those of the resistance-breaking mutations (N1686D, H1857R) previously reported [5].

## **Discussion**

In this study, we demonstrated that the symptom type in the pathosystem of *B. rapa* / TuMV was dependent on the particular combination of host and viral genotypes. One of the host loci involved in differential symptom induction appeared to be the *Rnt1* locus. While *Rnt1-1* induces resistance to UK1 in *B. rapa*, the *rnt1-2* and *rnt1-3* alleles induce systemic necrosis and mosaic to UK1, respectively. Additionally, the heterozygote of *rnt1-2* and *rnt1-3* caused leaf malformation with mild necrosis along leaf veins. This phenotype often fluctuated with environmental factors or genetic background. The allelic relationship between *Rnt1-1* and *rnt1-2* could not be clarified due to limitation in classical genetic analysis. However, because a single amino acid change (V1827E) in the CI gene of UK1 broke resistance by *Rnt1-1* and suppressed systemic necrosis by *rnt1-2*, it is likely that the products of *Rnt1-1* and *rnt1-2* directly or indirectly interact with the same domain of the CI protein. Additionally, *Rnt1-1* itself seemed to cause systemic necrosis to UK1-CIm. These results suggest that *rnt1-2* was one of multiple alleles at the *Rnt1* locus. Although we did not investigate the allelic relationship between the gene inducing mild necrosis in Haya-hikari and the gene *rnt1-2* in this study, Haya-hikari is also likely to possess *rnt1-2* because necrosis was not induced in Haya-hikari and Yu-shun against UK1-CIm unlike UK1.

Because *rnt1-2* causing systemic necrosis was more deleterious than *rnt1-3* for *B. rapa* plants infected by TuMV, *rnt1-2* should be removed in the process of breeding. However, it is difficult to screen the genotype of *rnt1-3/rnt1-3* under viral infection because *B. rapa* plants systemically infected with TuMV are often sterile. Such a trait is more efficiently selected for by using DNA polymorphism markers. In this study, the indel PCR marker 129-center was shown to cosegregate with *Rnt1*. This marker will serve not only as selection marker but also as a milestone for map-based cloning of

### *Rnt1*.

We have not yet elucidated a detailed mechanism for the induction of systemic necrosis after TuMV infection of *B. rapa*. However, we previously reported that the systemic necrosis observed on *Arabidopsis thaliana* inoculated with TuMV was a HR-like cell death, accompanied by defense responses [7]. The *N* gene inducing HR to TMV in *N. tabacum* changed in its response from HR to systemic necrosis after the coding sequence or promoter region in the transgenic plants had been mutated [2], suggesting that the observed systemic necrosis is a form of HR with/without a defense reaction. The difference between resistance associated with HR and systemic necrosis appears to be whether their defense responses succeeded in inhibiting viral replication and movement. Therefore, it is conceivable that the break of the *Rnt1-1* resistance by TuMV with the subsequent induction of systemic necrosis might be mediated by the CI mutation, which enhances the ability of the virus to replicate and move from cell to cell. However, significant differences in the number and size of infection sites between UK1 and UK1-CIm were not observed on the inoculated leaves in Wase-ohkabu (data not shown). Rather, the V1827E mutation in the CI gene seems to affect the interaction between CI and *Rnt1-1*, so the CI gene containing the mutation can no longer interact with the *rnt1-2* gene inducing systemic necrosis.

The CI mutation may also suppress leaf malformation and mosaic. The leaf curling in Haya-hikari with the genotype of *rnt1-2/rnt1-3* resulted from veinal necrosis induced by *rnt1-2*. When mild necrosis is induced along leaf veins in developing leaves, leaf elongation may be partially inhibited at the necrotic veins, resulting in curling of leaves. However, in the S<sub>1</sub> plants derived from Haya-hikari, some plants had severe malformation and mosaics on their leaves, while other plants had either necrosis or mild

mosaic. On the other hand, the CI mutation in UK1 attenuated such symptoms in Haya-hikari. These results indicate that there is also an unknown host factor(s) that controls leaf malformation by interacting with CI.

In this study, we showed that the TuMV symptoms were determined essentially by the host resistance gene locus *Rnt1* and the TuMV gene CI. The *Rnt1* locus regulates not only resistance to TuMV, but also necrosis and/or leaf malformation. Complex interactions of the *Rnt1* locus and the avirulence gene CI result in different symptoms. In addition, the CI protein may interact with different host factor(s) at the same domain. Further analysis on *Rnt1* and CI will be necessary to completely understand the mechanism for differential expression of TuMV symptoms.

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## Figure legends

### Fig. 1

Systemic symptoms on four cultivars of *Brassica rapa* subsp. *pekinensis* and *B. rapa* subsp. *rapa* cv. Wase-ohkabu at 14 dpi with *Turnip mosaic virus* (TuMV) strain UK1, UK1m or UK1-CIm. UK1m was a spontaneous mutant of UK1. An amino acid substitution at position 1827 in CI detected in UK1m was introduced in UK1 to create UK1-CIm. Classification of symptom expression to TuMV in each cultivar is shown in Table 2. In Haya-hikairi systemically infected with UK1, small necrotic lesions and veinal necrosis were observed on the chlorotic leaf. Resistance was only performed in the combinations of AS9/UK1 and Tropical Delight/UK1, UK1m and UK1-CIm. In the rest of combinations of *B. rapa* cultivars and TuMV strains, all plants were systemically infected by TuMV. The systemic infection was confirmed by hammer blotting or ELISA.

### Fig. 2

Linkage relationships between the resistance gene *Rnt1-1* to TuMV-UK1 in Chinese cabbage (*B. rapa* subsp. *pekinensis*) cv. Aki-masari and the SSR markers on linkage group R6. The left map was developed by the National Institute of Vegetable and Tea Science in Japan (<http://vegmarks.nivot.affrc.go.jp/VegMarks/jsp/index.jsp>). Names of SSR markers are to the right of the vertical lines and map distances are to the left.

### Fig. 3

Mutations detected on the genomic sequence in TuMV-UK1. Black and white triangles indicate nonsynonymous and synonymous substitutions, respectively. Primers used for

the RT-PCR are marked by arrows.

**Table 1** Reaction of three Chinese cabbage cultivars and their selfed or crossed populations to *Turnip mosaic virus* (TuMV) strain UK1

Cultivar or cross	Generation	Family <sup>a</sup>	Reaction to TuMV-UK1				Total	Expected ratio	$\chi^2$
			R	NM	nM	M			
Yu-shun			0	18	0	0	18		
Yu-shun	S <sub>1</sub>		0	83	0	0	83		
Haya-hikari			0	0	4	0	4		
Haya-hikari	S <sub>1</sub>		0	11	0	34	45	1:3	0.01 <sup>ns</sup>
Aki-masari			18	0	0	0	18		
Aki-masari	S <sub>1</sub>		29	0	0	9	38	3:1	0.04 <sup>ns</sup>
Aki-masari /Yu-shun	F <sub>1</sub>		46	0	50	0	96	1:1	0.17 <sup>ns</sup>
Aki-masari /Yu-shun	F <sub>2</sub>	R3	10	2	0	0	12	3:1	0.08 <sup>ns</sup>
		R5	5	0	0	0	5	3:1	0.31 <sup>ns</sup>
		R15	12	1	0	0	13	3:1	0.39 <sup>ns</sup>
		R16	8	1	0	0	9	3:1	0.17 <sup>ns</sup>
		R12	74	29	0	0	103	3:1	0.55 <sup>ns</sup>
		R21	80	27	0	0	107	3:1	0.003 <sup>ns</sup>
		S4	0	4	0	13	17	1:3	0.02 <sup>ns</sup>
		S6	0	3	0	15	18	1:3	0.13 <sup>ns</sup>
		S7	0	5	0	17	22	1:3	0.01 <sup>ns</sup>
		S17	0	1	0	6	7	1:3	0.08 <sup>ns</sup>
		S21	0	1	0	5	6	1:3	0.04 <sup>ns</sup>
		S24	0	5	0	13	18	1:3	0.01 <sup>ns</sup>

R: resistance, NM: necrosis with mosaic, nM: mild necrosis with mosaic, M: mosaic

ns: not significant

<sup>a</sup> F<sub>2</sub> families generated from F<sub>1</sub> plants of a cross between Aki-masari and Yu-shun

**Table 2** Type of systemic symptoms induced by the TuMV strain UK1, the mutant derived from UK1 (UK1m) and the UK1 infectious clones after the introduction of single amino acid substitutions in P3, CI and CP (UK1-P3m, -CIm and -CPm), respectively, in three cultivars of *B. rapa* subsp. *pekinensis* and *B. rapa* subsp. *rapa* cv. Wase-ohkabu.

TuMV strain and mutant	Reaction or symptom type to TuMV			
	AS9	Yu-shun <sup>a</sup>	Haya-hikari <sup>a</sup>	Wase-ohkabu <sup>a</sup>
UK1	R	NM	nM	M+++
UK1m	NM	M+	M+	M+
UK1-P3m	R	NM	nM	M+++
UK1-CIm	NM	M+	M+	M+
UK1-CPm	R	NM	nM	M++

R: resistance, NM: necrosis with mosaic, nM: mild necrosis with mosaic, M: mosaic

<sup>a</sup> Number of plus signs indicates the severity of the mosaic

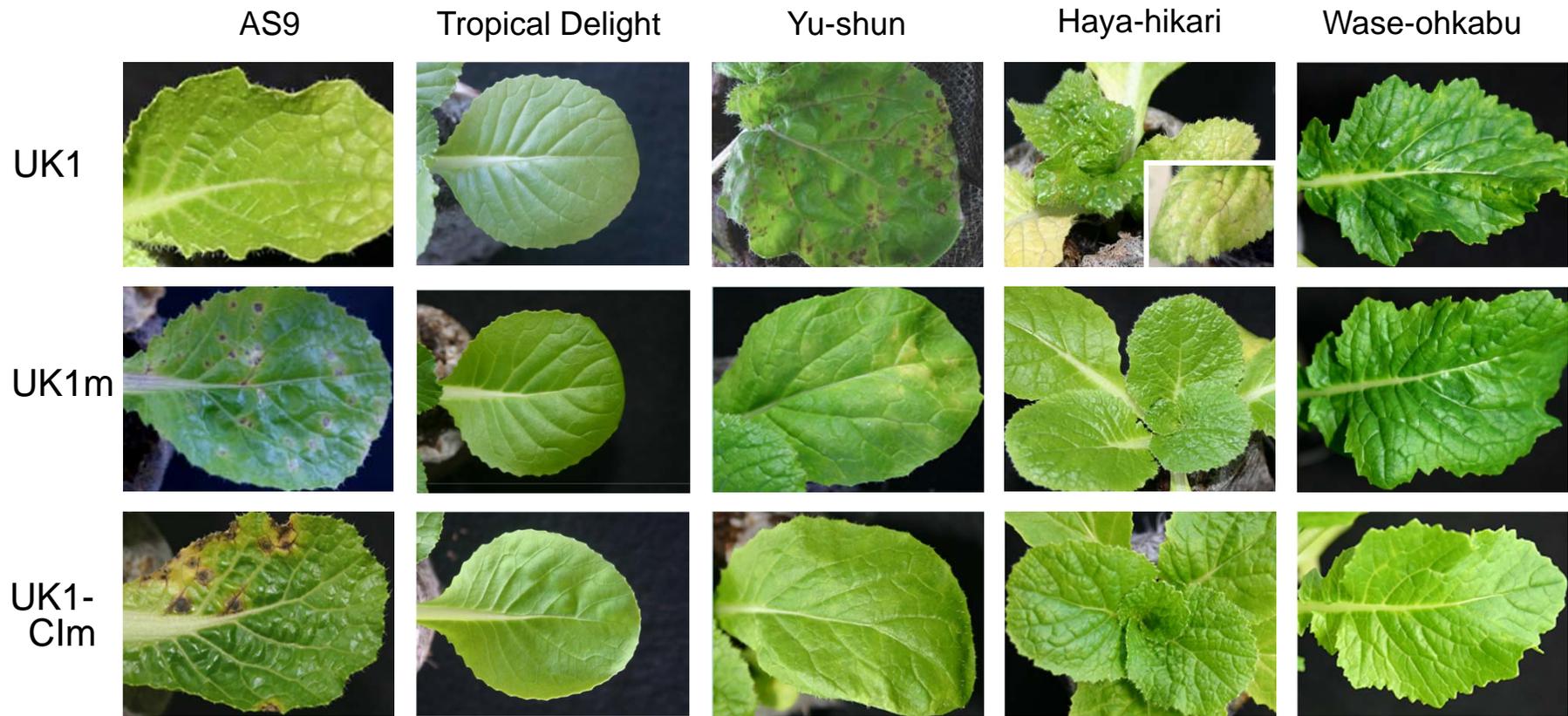


Fig. 1

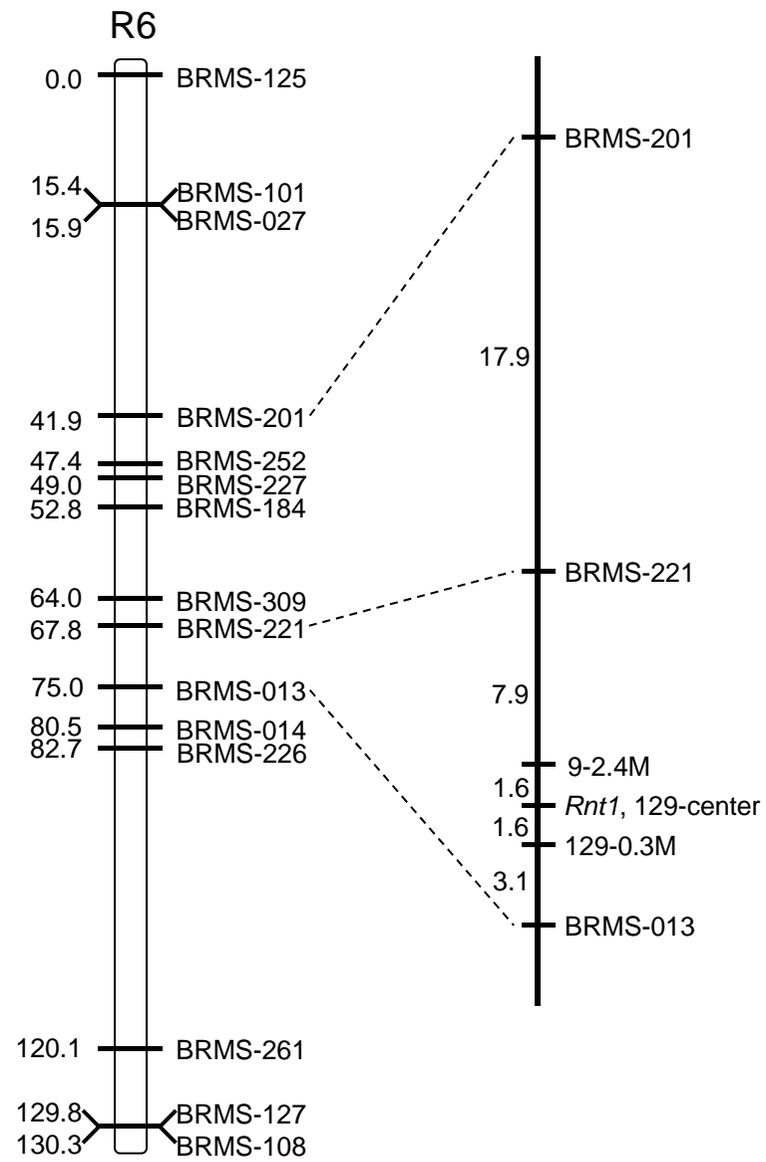


Fig. 2

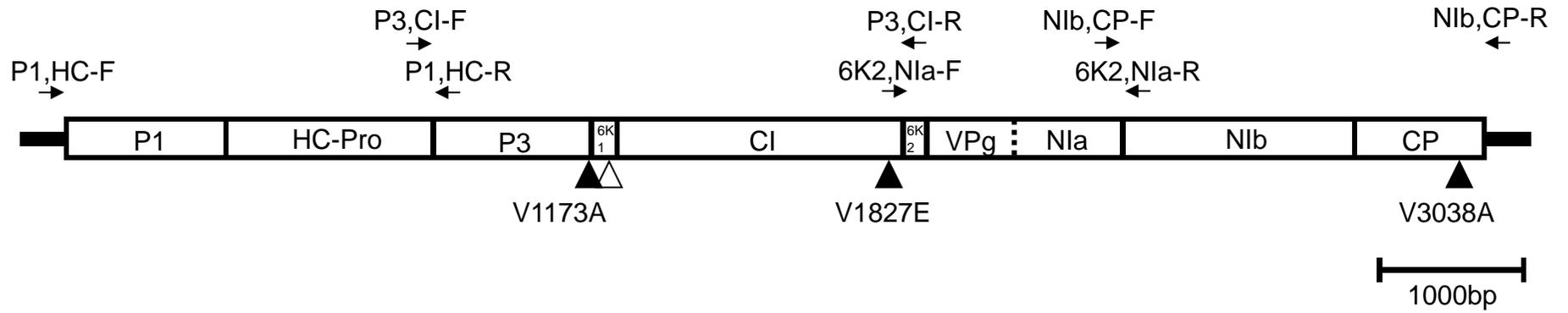


Fig. 3