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ORIGINAL PAPER

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Fine genetic mapping of the *TuNI* locus causing systemic veinal necrosis by turnip mosaic virus infection in *Arabidopsis thaliana*

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Abstract In the pathosystem of turnip mosaic virus (TuMV) and *Arabidopsis thaliana*, two distinct symptoms (mosaic symptom and veinal necrosis) were observed that were dependent upon the combination of the TuMV isolate and the *Arabidopsis* ecotype. The Col-0 ecotype developed mosaic symptoms after infection with the TuMV isolate Azu while the Ler ecotype developed veinal necrosis after infection with the same TuMV isolate. The Ler phenotype is controlled by a single dominant gene *TuNI* (TuMV necrosis inducer) which is located on chromosome 1. The *TuNI* gene was precisely mapped to the ~105 kb interval between the two markers of mXF41 and mRF28 by using several types of DNA polymorphism markers. Within this region, which included largely duplicated sequences, a total of 19 putative genes were predicted and 15 of these were classified into five gene families. The genes belonging to the gene families At1g58480 and At1g58602 may function in response to infection by pathogens. The gene family At1g58480 encodes lipase-like proteins, which might be involved in the induction of defence responses that are mediated by salicylic acid. The gene family At1g58602 encodes the CC-NBS-LRR (CNL) proteins, which are known to function as one of the plant resistance (*R*) proteins against pathogens. In the present study, the possibility that *TuNI* might function as an *R* gene was discussed.

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Introduction

Turnip mosaic virus (TuMV), a member of the genus *Potyvirus*, is one of the most important viruses in the world that infect field-grown vegetables (Tomlinson 1987). The host range of TuMV covers at least 318 species found within over 43 dicot families, including *Brassicaceae*, *Asteraceae*, *Chenopodiaceae*, *Fabaceae* and *Caryophyllaceae* (Walsh and Jenner 2002). TuMV particularly causes damage in horticultural and arable *Brassica* crops in Asia, North America and Europe (Walsh and Jenner 2002). Infection of the plants by TuMV results in various symptoms such as the typical leaf mosaic with stunting (mosaic) and the occurrence of necrosis along leaf veins (veinal necrosis). In most cases, veinal necrosis results in more severe damage than the mosaic symptoms. Although genetic improvement of susceptible crops is the most effective method to control TuMV symptoms, the mechanism for symptom induction in the pathosystem of TuMV and the *Brassica* genus is not fully understood.

In the pathosystems of some members of *Potyvirus* and their host plants, the helper component-proteinase (HC-Pro) and the P3 protein have been identified as the symptom determinants (Gal-On 2000; Saenz et al. 2000; Redondo et al. 2001; Saenz et al. 2001; Jenner et al. 2003; Suehiro et al. 2004). These viral factors are usually associated with the severity of the symptoms displayed by host plants. On the other hand, the NIb replicase in potato virus Y (PVY) was reported to be the factor that elicited veinal necrosis in *Nicotiana tabacum* (Fellers et al. 2002). A single dominant gene, which may be identical to a root knot nematode (RKN) resistance gene, *Rk*, controls the type of symptoms displayed by hosts (either veinal necrosis or mosaic symptoms) (Rufty et al. 1983a). In the pathosystem of PVY and *N. tabacum*, the type of symptoms is determined by the gene-for-gene relationships between the virus and the host plant (Fellers et al. 2002).

Arabidopsis thaliana, a weed-like member of the family *Brassicaceae*, is a dicot model plant that possesses many advantages for genetic and host-pathogen interaction studies (Dangl 1993). Since *Arabidopsis* reveals conserved chromosomal synteny to other *Brassica* species (Lan et al. 2000), we consider the pathosystem of TuMV and *Arabidopsis* to serve as a model system for studying the interactions between TuMV and *Brassica* species. Martin-Martin et al. (1999) classified 106 *Arabidopsis* ecotypes into three groups according to the response to the isolate UK1 of TuMV. Four ecotypes classified as group III showed extreme resistance to the isolate UK1. The remaining 102 ecotypes classified as either group I or II were susceptible to the isolate and these ecotypes developed similar symptoms including plant stunting, developmental arrest, leaf mosaic, sawed and curly leaves, and aborted flowers. No susceptible ecotypes showed necrotic lesions. On the other hand, we found that TuMV-infected *Arabidopsis* developed two distinct symptom patterns (mosaic and veinal necrosis) depending on the particular combinations of *Arabidopsis* ecotypes and TuMV isolates. Veinal necrosis was systemically displayed in the infected plants but not concurrent with mosaic symptoms. In order to understand the genetic basis for the development of symptoms in this pathosystem, we identified a 105 kb interval where the genetic locus controlling the type of symptoms induced by TuMV infection resides.

Materials and methods

Plant materials

Seeds from nine *Arabidopsis* ecotypes were purchased from Lehle Seeds (Tucson, Ariz., USA) (Table 1). Bayreuth (Bay-0) seeds were obtained from the Sendai *Arabidopsis* Seed Stock Center (SASSC) (Miyagi University of Education, Japan). For genetic analysis, F_1, F_2 and B_1F_1 plants that were derived from a cross between Columbia (Col-0) and Landsberg erecta (Ler) ecotypes, were produced. Col-0 was used as a recurrent parent in the backcrossing to produce the B_1F_1 plants. One hundred recombinant inbred lines (RILs) derived from the cross between Col-0 and Ler (Lister and Dean 1993) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC) and were used as a mapping population. For the precise mapping of *TuNI*, an additional set of 476 F_2 plants resulting from the cross between C24 and Ler was produced and used to generate additional RILs.

TuMV isolates and inoculation tests

Four TuMV isolates (Azu, TuR1, TuC and C42J) were used for the inoculation tests. TuMV-Azu was isolated from Chinese cabbage (*Brassica rapa* spp. *pekinensis*) in Tochigi prefecture, Japan and has been maintained at the Hokkaido University, Japan for more than 10 years. TuMV-TuR1 and -TuC were derived from the cDNA clones of the original isolates Tu-2R1 and Tu-3, respectively (Suehiro et al. 2004). TuMV isolate Tu-2R1 was isolated from Japanese radish (*Raphanus sativus*) and Tu-3 was from cabbage (*B. oleracea* var. *capitata*) in Tochigi prefecture (Suehiro et al. 2004). TuMV-C42J isolated from turnip (*B. rapa* spp. *rapifera*) in Saga prefecture, Japan, was a kind gift donated by Dr. K. Oshima (Saga University, Japan). Plants were grown in growth rooms under constant light (3,000 lx) and constant temperature (21°C). TuMV isolates were maintained in the susceptible hosts Col-0 or a turnip cultivar Yuki-Hime. Leaves from approximately 2-week-old plants were dusted with carborundum and rub-inoculated with the leaf sap. Infected plants were scored for virus symptoms 2 weeks after inoculation.

Table 1 Comparison of the type of symptoms developed in *Arabidopsis thaliana* ecotypes to infection with TuMV isolates. The abbreviated name for each ecotype is given in parentheses. *M* mosaic, *N* veinal necrosis

Ecotype	Type of symptoms induced by infection of TuMV isolates			
	Azu	TuR1	TuC	C42J
Columbia (Col-0)	M	M	M	M
C24	M	M	M	M
Muhlen (Mh-0)	M	M	M	M
Nossen (No-0)	M	M	M	M
Wassilewskija (WS)	M	M	M	M
Landsberg erecta (Ler)	N	N	M	M
Cape Verde Islands (Cvi-0)	N	N	M	M
Dijon G	N	N	M	M
Niederzenz	N	N	M	M
Bayreuth (Bay-0)	M	N	N	N

Hammer blotting

Hammer blots were prepared as previously described by Takahashi et al. (2001). Two weeks after inoculation the entire plant was pressed onto a filter paper (Advantec No. 2) with a hammer. The tissue print blots were incubated with an anti-TuMV primary antibody and subsequently incubated with a goat anti-rabbit immunoglobulin alkaline phosphatase conjugate (Bio-Rad, Cailif., USA). Color reactions were developed in 15 ml of the substrate solution (100 mM Tris-Cl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) containing 100 µl of 50 mg of nitro blue tetrazolium (NBT) per ml of 70% dimethylformamide and 50 µl of 25 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per ml of dimethylformamide.

DNA polymorphism markers

Total DNA was extracted from green leaves by the conventional cetyltrimetylammonium bromide (CTAB)

method. For precise mapping of *TuNI*, cleaved amplified polymorphic sequence (CAPS), derived cleaved amplified polymorphic sequence (dCAPS), simple sequence length polymorphism (SSLP), single nucleotide polymorphism (SNP) and insertion/deletion (indel) markers were developed. These sequences were based on the available genome sequences of Col-0 (GenBank accession NC_003070). Table 2 lists the primer sequences, the amplification conditions and the restriction enzymes that were used for the development of CAPS and dCAPS markers. The amplified segments, with or without cleavage by restriction enzymes, were analyzed on a 4% agarose gel. The amplified segment for the SNP marker was directly sequenced using an ABI PRISM 310 genetic analyzer that was operated under standard conditions.

Results

When infected with TuMV-Azu, two *Arabidopsis* ecotypes, Col-0 and Ler, showed different symptoms (Fig. 1a). The Col-0 plants infected with TuMV-Azu showed developmental arrest, leaf mosaic and aborted flowers. However, no systemic necrosis was observed in these plants. On the other hand, systemic veinal necrosis, which eventually led to plant death, was observed in the infected Ler plants. Virus distribution was also different in the two ecotypes (Fig. 1b). In the Col-0 plants, TuMV was detected throughout the entire plant, however in the Ler plants, it was only detected in the vascular tissues (Fig. 1b). These two symptom patterns induced by TuMV-Azu were reproducible and distinct. Moreover, it was evident that the symptoms of all ten ecotypes used in this study were classified as either veinal necrosis (N) or mosaic symptoms (M) when these plants were inoculated with four different TuMV isolates (Azu, TuR1, TuC and C42J) (Table 1). The symptom types appeared to be specifically determined, and dependent upon the combination of the *Arabidopsis* ecotype and the TuMV isolate, thereby suggesting that symptom determinants exist in both the host plant and the virus. The ten *Arabidopsis* ecotypes were classified into three groups (Col-0, Ler and Bay-0) according to their respective response patterns to the four viral isolates.

In order to investigate the inheritance of the symptom determinant, F_1, F_2 and B_1F_1 plants derived from a cross between Col-0 and Ler were inoculated with TuMV-Azu. Since all 17 F_1 plants showed the same N type for Ler, the N type was likely dominant over the M type. In the F_2 and B_1F_1 populations, the observed ratios of N type to M type fitted the theoretical ratios of 3:1 and 1:1, respectively. These data indicated that a single dominant gene conditioned the development of the N type symptom (Table 3). The locus that regulates symptom type was named *TuNI* (TuMV Necrosis Inducer).

To determine the chromosomal location of *TuNI*, the mapping population, which consisted of 100 recombinant inbreds that were derived from a cross between Col-0 and Ler, was inoculated with TuMV Azu. This mapping population was obtained from the NASC. In this study eight new DNA polymorphism markers were developed for more precise mapping of *TuNI*. The results of the linkage analysis indicated that *TuNI* was located between the two markers mT8L23 and mF23H11 on chromosome 1 (Fig. 2a). For more precise mapping of *TuNI*, 476 F_2 plants from a cross between Col-0 and Ler were developed, and the F_2 plants possessing a recombinant chromosome segment in the interval between the two markers were selected from this population. The selected F_2 plants were subsequently advanced to the F_4 generation and four individual F_4 plants (RIL85, RIL168, RIL224, RIL335) were finally selected for precise mapping of *TuNI*. Comparison of these recombinant genotypes with the six markers in the interval between mT8L23 and mF23H11 showed that the *TuNI* locus was located in the ~105 kb genomic region from mXF41 to mRF28 (Fig. 2b). According to the annotation data of *Arabidopsis* chromosome 1 in GenBank (accession no. NC_003070) and a more detailed report by Meyers et al. (2003), a total of 19 genes are predicted to reside in this genomic region. Most of this region consists of two nearly identical repeats of an approximately 36 kb genomic segment and a 10 kb imperfect repeat. As a result, 15 of the predicted genes actually exist as multiple copies in this region (Kato et al. 1999; Meyers et al. 2003). The 15 putative genes were classified into five gene families (Table 4). The gene families At1g58480, At1g58602 and At1g58684 were predicted to encode a GDSL-motif lipase, the disease resistance protein (CNL) and the 40S ribosomal protein S2, respectively. The two gene families At1g58643 and At1g58766 were predicted to encode hypothetical proteins with unknown functions. The single genes At1g58450, At1g58470, At1g58520 and At1g58460 were predicted to encode a peptidyl-prolyl *cis-trans* isomerase FKBP-type family protein, an RNA binding protein, an early-response to dehydration (ERD) protein and an expressed protein with an unknown function, respectively.

Discussion

In this study, we have shown that the type of symptoms in *Arabidopsis* plants infected by TuMV were determined by the specific interaction between the host plant ecotype and the virus strain. Systemic veinal necrosis was controlled by a single dominant gene, *TuNI*, that was mapped to a 105 kb interval defined by the markers mXF41 and mRF28. This region has been previously described to include the CNL protein gene clusters (Meyers et al. 2003). The CNL protein contains a nucleotide-binding site (NBS), leucine-rich-repeat (LRR) domains and an N-terminal coiled-coil (CC)

Table 2 DNA polymorphism markers developed for mapping of *TuNI*. *CAPS* cleaved amplified polymorphic sequence, *dCAPS* derived cleaved amplified polymorphic sequence, *SSLP* simple sequence length polymorphism, *SNP* single nucleotide polymorphism, *indel* insertion or deletion. A denaturation step at 94°C for 2 min was first carried out before each program. All programs were cycled 40 times followed by a final extension at 72°C for 2 min

Maker	Type of DNA polymorphism	Primer sequence (5′ to 3′)	PCR condition	Restriction enzyme
mT8L23	CAPS	CCATCATCACAAGTCTCGGTTGGC GACCATCTGATACAAGATAAC	94°C (30 s) 50°C (30 s) 72°C (30 s)	*Hae*III
mF16M22	CAPS	GACCGAAATCAAGACATACCGAAACCTCCCTTCATCAACGT	94°C (30 s) 50°C (30 s) 72°C (30 s)	*Psp*1406I
mF19C14	Nested PCR + dCAPS	TCTATAATTGGTTCTGATCGCACGTTCCAGTTCTGCGTGCGTTCTG	94°C (30 s) 50°C (30 s) 72°C (30 s)	*Acc*I
		GCAACTGTTGCTGTCTCTGGTGTCTCAACACTAATTAAGAGTATA	94°C (30 s) 50°C (30 s) 72°C (30 s)	
mXF1	SNP	ATCTGCCTAACTCCTTCCGCCCATGGACATGTCGTCTTAGC	94°C (30 s) 50°C (30 s) 72°C (30 s)	–
mXF41	CAPS	GAGACACTCACTTCATTCTCGGGCTTGACTGTGGACATCAAGC	94°C (30 s) 50°C (30 s) 72°C (30 s)	*Ssp*I
mRF28	indel	GATGCTACCTTGTCCGAATCTCTGAGTTACAAGAGTCCCTCCATC	94°C (30 s) 55°C (30 s) 72°C (1 min)	–
mT30E16	SSLP	CCTAACCACAGCCACGTATGCAAGTTACCCAACACTTAAC	94°C (30 s) 50°C (30 s) 72°C (30 s)	–
mF23H11	CAPS	GTTTACCTTCACCAGCCACTAGTCCATCTGGAGTAACTGAAGAGAGCATC	94°C (30 s) 50°C (30 s) 72°C (30 s)	*Ava*II

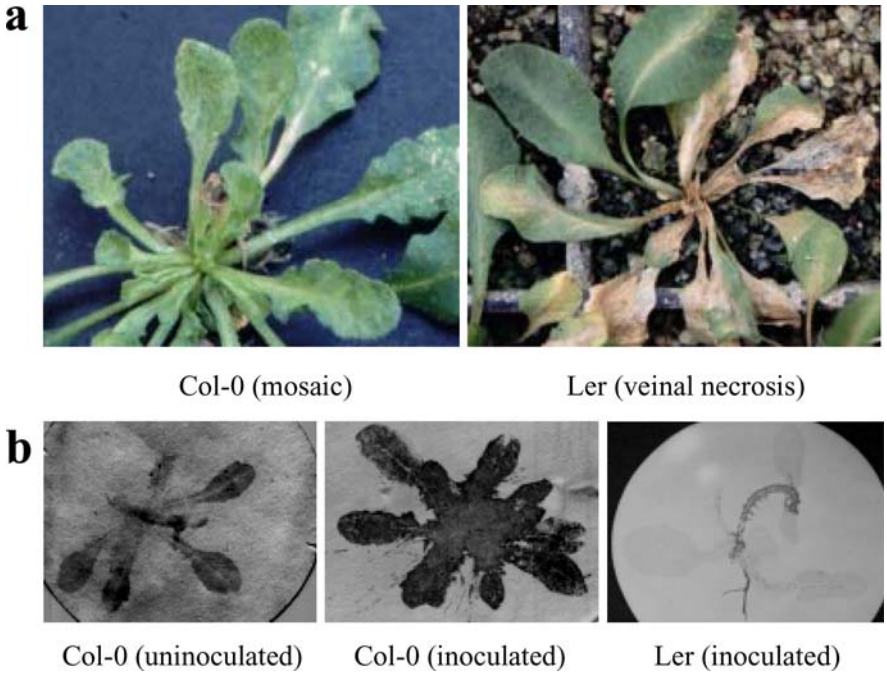

Fig. 1 **a** Symptoms induced by TuMV isolate Azu in *Arabidopsis thaliana* ecotypes Col-0 and Ler 2 weeks after inoculation. A typical mosaic symptom was observed in Col-0, while Ler showed veinal necrosis. **b** Distribution of TuMV in the TuMV-Azu infected plants of Col-0 and Ler 2 weeks after inoculation. TuMV spread into the entire Col-0 plant. However, TuMV was only detected in the vascular tissues in the Ler plant

Table 3 Responses of the F_2 and B_1F_1 populations derived from a cross between Col-0 and Ler ecotypes to TuMV isolate Azu

Cross and parents	Number of plants			Expected ratio (N:M)	Goodness of fit	
	Necrosis (N)	Mosaic (M)	Total		χ^2	Probability
Col-0	0	20	20			
Ler	20	0	20			
Col-0 × Ler F_1	17	0	17			
Col-0 × Ler F_2	52	17	69	3:1	0.005	0.94
(Col-0 × Ler) × Col-0 B_1F_1	10	11	21	1:1	0.048	0.83

motif and is thought to function in pathogen recognition (Dangl and Jones 2001). According to the sequence analysis by Meyers et al. (2003), this region consists of some large duplicated sequences. Specifically, 15 out of 19 potential candidate genes were actually found to exist as multiple copies and have been classified into five putative gene families; including CNL protein gene clusters. In Arabidopsis, at least 51 genes belonging to this CNL group have been identified and were classified into four subgroups based upon the position of introns and sequence conservation (Meyers et al. 2003). All five CNL *R* genes cosegregating with *TuNI* were classified into the CNL-D subgroup. At the present time, we do not have enough evidence to show the relationships between the *R* genes and the necrotic symptoms shown by TuMV-infected *Arabidopsis*. However, transgenic tobacco plants which contain the mutant alleles of *N*, a well known *R* gene against tobacco mosaic virus (TMV) showed incomplete resistance, with systemic necrosis when inoculated with TMV (Dinesh-Kumar and Baker 2000; Dinesh-Kumar et al. 2000). Considering its ability to induce systemic necrosis, it is conceivable that *TuNI* is a candidate for the *R* gene. It is also possible that lipase-like protein genes in the *TuNI* region may be a putative necrosis inducer; lipase-like proteins were found to be required for the induction of defence responses mediated by salicylic acid (SA) (Falk et al. 1999; Jirage et al. 1999; Kumar and Klessig 2003). The *PRLIP* genes encoding lipase-like proteins are tandemly clustered on chromosome 5 in *Arabidopsis* (Jakab et al. 2003), and it has been shown that the expression of some members in this gene family is induced by the resistance-inducer β-aminobutylic acid, SA, methyl jasmonate, ethylene or pathogens (Jakab et al. 2003).

The systemic lethal necrosis induced by *TuNI* apparently causes more severe damage than mosaic symptoms. Although the induction of lethal necrosis by *TuNI* may decrease the fitness of *Arabidopsis* populations, why has such a gene evolved? *TuNI* is actually widely distributed in natural populations of *Arabidopsis* and has even differentiated, giving rise to specificity for TuMV isolates among the different ecotypes. One

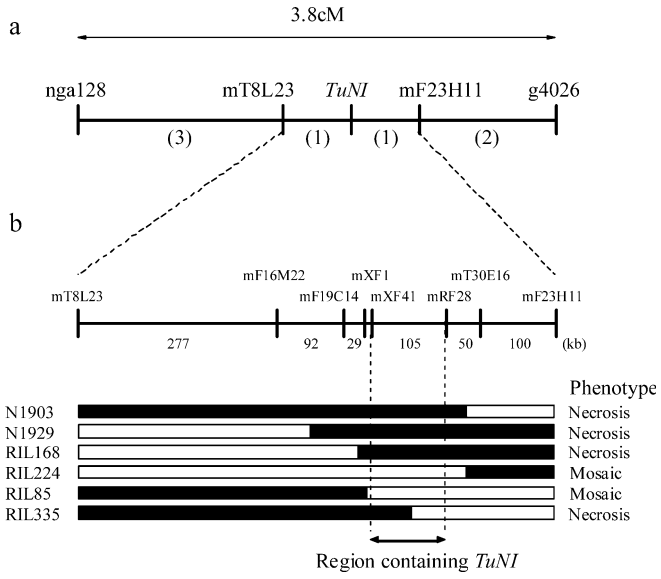

Fig. 2 Genetic and physical mapping of the *TuNI* locus. **a** Linkage relationships between *TuNI* and four DNA polymorphism markers on chromosome 1. The number of recombinants between the markers is indicated by *parentheses*. **b** Graphical genotypes of key recombinants defining the genomic region containing *TuNI* for the DNA polymorphism marker loci. Additional DNA polymorphism markers between mT8L23 and mF23H11 are located on the physical map. The phenotype of each recombinant is indicated on the *right side* of the figure

explanation is that restriction of the virus in veinal tissues followed by plant death could reduce the viral spread through the population more efficiently than mosaic symptoms alone. Since rapid plant death following viral infection could minimize the chance of transmission of the virus by aphids, lethal necrosis could have a positive impact on the host population fitness through a negative impact on the virus. This is likely especially when the *TuNI* allele is dominant in the host population, and the inoculum level is consistently low. Alternatively, it is possible that *TuNI* may possess a pleiotropic effect that is dependent upon different isolates and pathogens. For instance, a systemic veinal necrosis has been reported in tobacco plants that were infected with PVY M^SN^R . This systemic response was found to be controlled by a single gene which was tightly linked to a RKN resistance gene, *Rk*, in tobacco (Rufty et al. 1983a). Since no recombinants were detected between these two traits in a genetic analysis which used more than 15,000 F_2 progeny (Rufty et al. 1983a), it was suggested that these two phenotypes resulted from pleiotropic effects of a single gene. In addition, RKN resistance and development of necrosis by PVY M^SN^R infection showed the same temperature sensitivity and plant age specificity (Rufty et al. 1983b). Another example has been described for soybean *Rsv* genes. In soybean, at least three *Rsv* loci conditioning resistance to soybean mosaic virus (SMV) have been identified and multiple alleles were subsequently differentiated at the *Rsv1* locus (Ma et al. 2003). At this locus, the most dominant alleles were reported to induce either resistance or systemic necrosis to the seven SMV strains while a recessive allele at the *Rsv1* locus showed mosaic symptoms in response to infection with the same SMV strains (Ma et al. 2003). In this pathosystem, both the responses of resistance and systemic necrosis are thought to be triggered by the same gene (Chen et al. 1994). In the TuMV/*Arabidopsis* pathosystem it has been reported that several ecotypes including Bay-0

Table 4 Putative genes co-segregating with *TuNI* in the region between markers mXF41 and mRF28 on chromosome 1. *Arabidopsis* ESTs reported by Kato et al. (1999) or found in GenBank that are identical to the predicted cDNA are shown

Putative gene or gene family	AGI gene code	EST	Note
Single gene			
Peptidyl-prolyl *cis-trans* isomerase FKBP-type family protein	At1g58450		
Expressed protein	At1g58460	BX813897	
RNA binding protein	At1g58470	AB008022	
ERD protein	At1g58520	AB008023	
Gene family			
GDSL-motif lipase	At1g58480		
	At1g58725		
	At1g59030		At1g59030 is identical to At1g58725
Disease resistance protein (CNL class)	At1g58602	AV528757	
	At1g58807	AB028203, AB028222	
	At1g58848	AB028201, AB028202	
	At1g59124	AB028203, AB028222	At1g59124 is identical to At1g58807
	At1g59218	AB028201, AB028202	At1g59218 is identical to At1g58848
Hypothetical protein	At1g58643		
	At1g58936		At1g58936 is identical to At1g58643
	At1g59171		At1g59171 is a fragment of At1g58643
40S ribosomal protein S2	At1g58684	AB008017	
	At1g58983	AB008017	At1g58983 is identical to At1g58684
Hypothetical protein	At1g58766		
	At1g59077		At1g59077 is identical to At1g58766

showed extreme resistance to the isolate UK-1 (Martin-Martin et al. 1999). The Bay-0 resistance was found to be due to an interference with viral cell-to-cell movement (Martin-Martin et al. 1999). Because the inheritance of this resistance in Bay-0 was not known, the resistance gene(s) must be identified to discuss the relationship with *TuNI*.

In the pathosystems of TuMV and the *Brassica* genus, some resistance genes have been reported but they have not yet been isolated (Walsh and Jenner 2002). The viral avirulence (*avr*) genes to *TuRB01* and *TuRB05* is the CI gene (Jenner et al. 2000, 2002), while *TuRB03* and *TuRB04* seem to interact with the P3 gene (Jenner et al. 2002, 2003). When *B. napus* was infected by some virulent TuMV isolates, it is important to note that systemic necrosis, as observed in TuMV-infected *Arabidopsis*, was induced in the lines possessing *TuRB01* or *TuRB03* (Jenner et al. 2000, 2003). Interestingly, we also found that the viral pathogenicity determinant responsible for the *Arabidopsis* symptoms (mosaic or veinal necrosis) occurred within the region containing the P3 and CI genes of TuMV using chimeric constructs between TuMV-TuR1 and -TuC (data not shown). With our current data, we cannot confirm whether the putative *avr* gene to *TuNI* is the P3 or CI gene or both. Future comparative studies of *TuNI* and *TuRB* genes may enable us to understand the relationships between the resistance response and the systemic necrosis against TuMV infection.

In this study, we demonstrated that *TuNI* was located within a region containing the cluster of *R* genes in the *Arabidopsis* genome. However, the multiple repeated sequences within this region complicate our efforts to more precisely map the *TuNI* gene within this region. Complementation tests for *TuNI* which use BAC clones to cover the entire locus are now underway and this will enable us to determine which gene(s) are involved in the induction of systemic veinal necrosis in *Arabidopsis*.

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