RNA Silencing of the Introduced Coat Protein Gene of *Turnip mosaic virus* Confers Broad-Spectrum Resistance in Transgenic *Arabidopsis*

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ABSTRACT

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The coat protein (CP) gene derived from *Turnip mosaic virus* (TuMV) isolate JO was introduced into *Arabidopsis thaliana* and the resulting transgenic progenies were analyzed for resistance to TuMV. Transgenic *Arabidopsis* plants with no detectable transcripts of the introduced CP gene exhibited complete resistance to TuMV. There was no significant correlation between the resistance and the copy number of the transgene. Instead, small interfering RNAs (siRNAs) were detected in these resistant

plants, indicating that the resistance is attributed to RNA silencing. The RNA-mediated resistance was not only inherited over successive generations but also effective against 17 worldwide TuMV isolates with different pathogenicity. Comparative analysis of the CP genes among the 17 TuMV isolates revealed that the 380-nt in the 3' region is highly conserved, suggesting the importance of the 3' conserved region for broad-spectrum resistance. These results indicate that introduction of the TuMV-CP gene into the target Brassicaceae plants followed by selecting transformants that show RNA silencing for the transgenes can be an effective and reliable strategy for developing crucifer crops with a broad spectrum of resistance to TuMV.

Turnip mosaic virus (TuMV), a member of the genus *Potyvirus*, is ranked among the five most damaging plant-pathogenic viruses in the world and infects not only economically important *Brassicaceae* crops such as oil rape, turnip, cabbage, and radish (43) but also a model plant, *Arabidopsis thaliana* (32). TuMV isolates have been classified into four groups based on their phylogenetic relationships and pathogenicity (29,42). Complete genome sequences of several TuMV strains have been determined to reveal the typical potyvirus genome structure (27,28). In addition, Sanchez et al. (32) successfully constructed an infectious cDNA clone that can be used for molecular characterization of TuMV–Brassicaceae plant interaction.

TuMV resistance is one of the major concerns in breeding programs of cruciferous crops. Though several resistance or tolerance loci against TuMV have been identified (34), these loci cannot completely suppress the viral multiplication (20). As for the avirulence factors involved in the virus genome, P3 or the cylindrical inclusion body protein of TuMV was shown to be a determinant for the genotype-specific resistance in the oil rape (*Brassica napus*) and TuMV interaction (20). Recent advances in molecular biology allow the practical use of pathogen-derived genes to develop virus-resistant transgenic plants (4). Various forms of pathogen-derived genes such as coat protein (CP) genes, viral replicase genes, and other viral genome-specific sequences are effective for generating virus-resistant transformants (4,5). Detailed analysis of the transgenic plants has unveiled that most of the examples in pathogen-derived resistance are due to transgene-induced posttranscriptional gene silencing, recently termed

RNA silencing (5). The resistance primarily targets the viral RNA genome and thus is referred to as RNA-mediated resistance (12). RNA silencing is a sequence-specific RNA degradation mechanism that occurs in a broad range of eukaryotic organisms (5,44,46,48). The mechanism involves an initial processing of inducing double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) of 21 to 25 nucleotides (nts), corresponding to both sense and antisense strands of the target gene (15). These siRNAs can act as guide sequences for specific cleavage of homologous RNAs (16,49).

RNA-mediated resistance depends on the sequence similarity between the target transgenes and the invading viral genome (13,26). Jan et al. (19) reported that *Nicotiana benthamiana* transformed with a TuMV-CP gene showed resistance to TuMV infection due to RNA silencing, but the spectrum of the resistance to other TuMV strains with different pathogenicity traits was unknown. Tobacco plants transformed with the RNA-dependent RNA polymerase (RdRp) gene of *Potato virus X* (PVX) showed strain-specific resistance to PVX strains that share more than 88% identity with the RdRp genes (26). In transgenic pea lines expressing the nuclear inclusion b (NIB) gene of *Pea seed-borne mosaic virus* (PSbMV) genome, stable resistance was only expressed when the homology exceeded 89% (21). Similarly, resistance of transgenic *N. benthamiana* expressing the *Groundnut rosette virus* satellite RNA genome was maintained when the sequence identity in the satellite RNA genome was higher than 87% (38). Several other experiments also indicate that similarity in a small target region may be important for viral resistance rather than the overall sequence (2,13,35,37).

Here, we report that *Arabidopsis thaliana*, in which the full coding region of a TuMV-CP gene was introduced but its expression silenced, showed complete resistance not only to the derivative TuMV isolate but also to 17 different TuMV isolates col-

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lected worldwide. Because the 3' region of the CP genes are highly conserved among the isolates, introduction of the TuMV-CP gene to the target Brassicaceae plants followed by selecting transformants that show RNA silencing for the transgenes can be an effective and reliable strategy for developing crucifer crops resistant to TuMV.

MATERIALS AND METHODS

Viral isolates. A TuMV isolate, TuMV-JO, originally isolated from oil rape, was obtained from the Genetic Resources Center, National Institute of Agrobiological Resources, Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF), No. 01-04035, and used for molecular cloning of the CP gene. Seventeen additional TuMV isolates were selected from each of the four phylogenetic groups (29) and used for determining the resistance spectrum of transgenic *Arabidopsis* plants with the TuMV-CP genes.

Viral inocula were maintained using turnip (*B. campestris*) and *N. benthamiana* plants. TuMV virions were purified from 100 g of infected turnip leaves according to the procedure described by Choi et al. (9) and used for molecular cloning of the CP gene (31). Subsequent production of rabbit polyclonal antiserum against TuMV-CP was performed.

Plants and viral inoculation. *Arabidopsis thaliana* (ecotype Col-0) was grown in a growth chamber under a photoperiod of 12/12 h (light/dark) at 22°C for viral inoculation. Viral inoculum was prepared by grinding 3 g of TuMV-infected turnip leaves with 27 ml of 0.1 M phosphate buffer (pH 7.0). When 10 to 15 leaves of *Arabidopsis thaliana* plants were fully expanded, Carborundum was dusted on the leaf surface followed by rub-inoculation of the inocula on the largest four to five mature leaves per plant with cotton swabs. Inoculated plants were placed in a growth chamber and kept at 25°C with a photoperiod of 12/12 h (light/dark). This condition reproducibly induced severe mosaic and/or stunting symptoms on the inoculated plants. Appearance of the symptoms was monitored daily for at least 4 weeks. Plants not showing any detectable symptoms were assayed for viral multiplication by double-antibody sandwich, enzyme-linked immunosorbent assay (DAS-ELISA) (10).

Cloning of the TuMV-CP gene. After viral RNAs were extracted from the purified virion, the viral genome was reverse transcribed to obtain viral cDNAs using *Avian myeloblastosis virus* reverse transcriptase according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, UK). DNA fragments of 1.3 kbp containing the full coding region of the CP and Nib genes were amplified by polymerase chain reaction (PCR) using EX *Taq* DNA polymerase (Takara, Shiga, Japan) with 0.05 µg of the viral cDNA as the template and a combination of oligo-dT adaptor primer (Takara) and a specific sense primer (5'-CGCGGATCCTGATTGAGTCGTGGGGATAC-3') designed from the highly conserved region within the Nib gene. The reaction mixture was first denatured at 94°C for 4 min and then subjected to 30 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 30 s, and extension at 72°C for 1.5 min. Amplified DNA fragments were separated by 1% agarose gel electrophoresis, and DNA fragments with the expected size were recovered from the gel and purified (31). The purified DNA fragments were then cloned into the pT7Blue vector (Novagen Inc., Darmstadt, Germany) to yield pTuMV. After the DNA sequence of the cloned DNA fragment was determined by cycle sequencing by an automated DNA sequencer (Applied Biosystems, Foster City, CA), the coding region of the CP gene was amplified by PCR under the same conditions described above, using pTuMV as the template. The PCR primers, 5'-CGCGGATCCCAACA-ATGGCAGGTGAGATGCTTGGT-3' (sense) and 5'-ACGAGCTCTCATAACCCCTTAACGCC-3' (antisense), were designed to introduce an ATG start codon and a *Bam*HI restriction site at the 5' end and a *Sac*I site at the 3' end, respectively, as shown by the

underlined letters. Finally, the amplified CP fragments were cloned into the pT7Blue vector to yield pTuCP.

Plant transformation and in vitro selection. The TuMV-CP gene in pTuCP was digested with *Bam*HI and *Sac*I and inserted into the same restriction site of the binary vector, pBI121 (Clontech Labs, Palo Alto, CA), by replacing the GUS gene located between the 35S *Cauliflower mosaic virus* promoter and nopaline synthase terminator sequences. The resultant plasmid was transferred into *Agrobacterium tumefaciens* C58C1 (24) from *Escherichia coli* DH5α by triparental mating (11) and used for *Agrobacterium*-mediated transformation according to the method of Bechtold et al. (6). pBI121 was used for the vector-control transformation. Transformed T₀ plants were further grown in a growth chamber with a 12/12 h (light/dark) photoperiod at 22°C until self-pollinated seeds of the T₁ generation were fully matured.

For in vitro selection, T₁ generation seeds were surface-sterilized and germinated on Murashige and Skoog media containing 25 and 150 µg of kanamycin and claforan per ml, respectively, to eliminate *Agrobacterium tumefaciens* at 22°C under continuous fluorescent light. No antibiotic was supplemented for raising nontransformed seedlings. After 20 days, when the second true leaves had developed, seedlings were transplanted to clay soil and grown in a growth chamber as described previously.

Molecular characterization of the transgenes. For Southern blot analysis (31), total DNA was isolated from 0.1 mg of non-inoculated remaining leaves (8) after determining the plant response to TuMV infection. Five micrograms of the total DNA was digested with *Bam*HI or *Eco*RV, size-fractionated on a 1.0% agarose gel, and transferred to a nylon membrane (Roche Diagnostics, Indianapolis). Transferred DNAs were then hybridized with digoxigenin (DIG)-labeled TuMV-CP gene-specific probes and detected with autoradiographic film according to the manufacturer's instructions (Roche Diagnostics). For northern and western blot analyses, fully expanded mature leaves (≈150 mg) were harvested from 30-day-old plants before TuMV inoculation and subjected to sample preparation for the analysis. Northern blot analysis was carried out using total RNAs isolated from the leaves (≈100 mg) by the method of Verwoerd et al. (45). Isolated RNAs were size-fractionated on a 1.2% agarose gel containing 0.66 M formaldehyde, transferred to nylon membrane, hybridized with the same probe used for Southern blots, and detected with autoradiographic film as described previously. For western blot analysis, the remaining leaves (≈50 mg) were macerated with 100 µl of 2× Laemmli sample buffer and boiled for 5 min (22). After centrifugation at 3,000 × g, 10 µl of each supernatant was analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membrane, and detected by TuMV-CP rabbit polyclonal antiserum.

Detection of siRNAs. Extraction of low molecular weight RNAs was carried out essentially according to the methods of Papaefthimiou et al. (30). Total RNAs were isolated from 0.2 g of fully expanded, noninfected, mature leaves 30 days after sowing, and low molecular weight RNAs were fractionated using 8 M LiCl. The samples were heat-treated in formamide buffer, loaded onto a 12% polyacrylamide slab gel (12% acrylamide and 0.6% bisacrylamide) containing 7 M urea and TBE buffer (50 mM Tris, 41.5 mM boric acid, and 0.5 mM EDTA), and separated by electrophoresis. Separated RNAs were transferred to nylon membrane with the Trans-Blot SD Cell (Bio-Rad Laboratories, Hercules, CA) and fixed by UV cross-linking. DIG-labeled sense RNA probes were synthesized by transcribing the target regions with T7 RNA polymerase according to the manufacturer's instructions (Roche Diagnostics). As for the templates, linearized pTuCP and pTuCP3' were used for the TuMV-CP full coding region and the 3' conserved region, respectively. The pTuCP3' was constructed by inserting 380 nts at the 3'-terminal region amplified by PCR into the pT7Blue vector (Novagen) using specific sense (5'-TGGAATTCCTCCGATCAAACCG-3') and antisense (5'-ACGAGCT-

CTCATAACCCCTTAACGCC-3') primers under the same conditions with pTuCP construction. Transferred RNAs were hybridized with the TuMV-CP-specific probe and detected with autoradiographic film as described previously.

RESULTS

Cloning of CP genes and transformation of *Arabidopsis thaliana*. A DNA fragment of 1.3 kbp was amplified by PCR and cloned into the pT7blue vector to yield pTuMV. Sequence analysis revealed that the cloned DNA fragment shared 96.5 and 94.9% nucleotide sequence identity with those of TuMV isolates from rutabaga (*B. napus* subsp. *napobrassica*) (27) and radish (*Raphanus sativus*) (28), respectively. In addition, translation products of the cloned DNA fragment reacted with the TuMV-CP polyclonal

Fig. 1. Differential responses to Turnip mosaic virus (TuMV) isolate JO in **A**, nontransformed wild-type *Arabidopsis thaliana* (ecotype Col-0) and **B to D**, transformants with the TuMV-coat protein gene. **B, C, and D**, Plants represent resistant, intermediate, and susceptible phenotypes, respectively. **B**, Plant shows no detectable symptoms and abundant mature seeds were obtained later. **C**, Plant developed slight mosaic and stunting symptoms, although the appearance was delayed a few days compared with **D**, the susceptible phenotype. All plants showing resistant and intermediate phenotypes (**B and C**) produced fertile seeds, whereas plants categorized in **A and D** phenotypes were severely stunted with distinct mosaic symptoms in the leaves, resulting in failure of seed formation.

antiserum by western blot analysis when subcloned into an expression vector, pPROEX HTa (Gibco-BRL, Grand Island, NY), and expressed using *E. coli* cells (data not shown). These results confirmed that the 1.3-kbp DNA fragment contained the coding region of the TuMV-CP gene. For plant transformation, a binary vector was constructed by replacing the GUS gene of pBI121 with the TuMV-CP gene to yield pBITuCP. Twenty plants were vacuum infiltrated with pBITuCP, and the T₁ generations seeds were obtained by self-pollination. Upon kanamycin-resistance screening of T₁ generation, 23 regenerated plants were selected and checked for the presence of transgenes by both PCR and Southern blot analyses (data not shown). Each of the T₁ generation plants was numbered consecutively and self-propagated to yield T₂ generation seeds. Progenies of the successive generations were obtained simultaneously for further analyses. In the case of the susceptible lines, seeds were obtained from the remaining uninoculated plants because the inoculated plants used for resistance screening were severely stunted or dead before seeding.

Response of transgenic progenies to TuMV. A TuMV isolate, JO, induced severe mosaic and/or stunting symptoms on both nontransformed wild-type (Fig. 1A) and vector control plants 10 to 14 days after inoculation, whereas transgenic progenies segregated for resistant (R), intermediate, and susceptible (S) phenotypes (Fig. 1B to D). The resistant transformants were completely symptomless and neither viral protein nor viral multiplication was detected by western blot analysis and DAS-ELISA. On the contrary, susceptible transformants (Fig. 1D) were severely affected with distinct mosaic symptoms indistinguishable from that of the nontransformed, wild-type plants (Fig. 1A). In these susceptible plants, TuMV-CP was detected by western blot analysis and DAS-ELISA (data not shown). Transgenic progenies showing the intermediate resistance phenotype developed various levels of mosaic symptoms several days after inoculation, yet later the mosaic symptoms disappeared and the plants grew to produce mature seeds. These transgenic plants were, thus, considered to represent recovery phenotypes (12).

T₂ generation plants from 23 T₁ lines were first screened individually for kanamycin resistance and then 24 seedlings per each line were randomly taken from the resultant regenerants for TuMV inoculation tests. As shown in Table 1, parental T₁ lines, CP4, CP11, CP20, and CP23, yielded resistant T₂ progenies at the rate of more than 50%, whereas the same T₁ parental lines, CP2, CP5,

TABLE 1. Segregation of Turnip mosaic virus (TuMV) resistance over the T₂, T₃, T₄, and T₅ generations of *Arabidopsis thaliana* transformed with the TuMV coat protein (CP) gene

Parental T ₁ lines	Number of T ₂ plants showing R/S ^a		Parental T ₂ plants ^b	Number of T ₃ plants showing R/S		Responses of T ₄ and T ₅ progenies ^c	
	R	S		R	S		
CP4	12	12	CP42	–R1	6	4	Variable
				–R2	2	8	Variable
				–R3	5	5	Variable
				–S1	0	10	All susceptible
CP11	18	6	CP112	–R1	10	0	All resistant
				–R2	8	2	–
				–R3	5	5	–
CP20	12	12	CP202	–R1	10	0	All resistant
				–R2	6	4	–
				–R3	2	8	–
CP23	18	6	CP232	–R1	10	0	All resistant
				–R2	2	8	–
				–R3	7	3	–
CP2	0	24	CP22	–S1	0	10	All susceptible
CP5	0	24	CP52	–S1	0	10	All susceptible
CP8	0	24	CP82	–S1	0	10	All susceptible
CP16	0	24	CP162	–S1	0	10	All susceptible

^a R indicates resistant, no detectable symptom; and S indicates susceptible, severely stunted and/or delayed mosaic symptoms in the entire leaves. Plant responses were determined by inoculating mature leaves of each transgenic plant with cell sap prepared from TuMV-infected turnip leaves followed by incubation in a growth chamber at 25°C with a photoperiod of 12/12 h (light/dark) for 4 weeks.

^b R and S represent resistant and susceptible T₂ plants, respectively.

^c — Indicates not present because there are no R plants in the T₂ generation.

CP8, and CP16, yielded only susceptible progenies in the successive generations. The remaining 15 T₁ lines yielded mostly susceptible progenies, although several recovery types were obtained.

Responses to TuMV infection were further examined for the T₃ generation. Resistant and susceptible T₂ plants were randomly selected from each of the eight T₁ lines that generated both resistant and susceptible T₂ progenies (CP4, CP11, CP20, and CP23) and only susceptible progenies (CP2, CP5, CP8, and CP16). In the screening of the T₃ generation, 10 plants were randomly selected from each of the T₃ generation lines through kanamycin-resistance screening and inoculated with TuMV. As shown in Table 1, most of the T₃ generation plants derived from resistant T₂ lines segregated for both resistant and susceptible phenotypes, whereas all of the T₃ progenies from three resistant T₂ lines, CP112-R1, CP202-R1, and CP232-R1, were completely resistant and showed no detectable symptoms even at extremely high inoculum doses. These resistant T₃ plants generated only resistant phenotypes in the successive T₄ and T₅ generations, confirming the stable inheritance of the resistance in these lines. However, CP42-derivative T₂ lines, CP42-R1, CP42-R2, and CP42-R3, segregated for both resistant and susceptible T₃ progenies even though their parental CP42 plants were completely resistant. Successive progenies of these lines were variable for the TuMV resistance. On the contrary, all of the T₃ progenies from susceptible T₂ lines were susceptible to TuMV and did not segregate for resistance in the successive generations.

Response of TuMV-resistant transgenic plants to 17 different TuMV isolates. Seventeen TuMV isolates selected from different phylogenetic groups (29,42) were analyzed for their sequence identity of the CP region with that of JO and inoculated to the T₃ progenies to determine the breadth of resistance. Comparative analysis of the sequence of the TuMV-CP gene among the 17 isolates revealed that 380 nts in the 3'-terminal region showed more than 95.5% identity with that of JO isolate, whereas the identities of the entire CP region ranged from 86.6% (IS1) to 97.0% (2J) (Table 2). When these isolates were independently inoculated to the T₃ progenies derived from two resistant T₂ lines, CP112-R1 and CP232-R1, all of the plants were completely resistant not only to the transgene-derived isolate, JO, but also to all 17 TuMV isolates (Table 2), showing no detectable symptom or

viral protein in the upper leaves even 4 weeks after inoculation. In the case of the wild type, all of the TuMV isolates induced severe mosaic and/or stunting symptoms within 20 days, although the degree of pathogenicity differed slightly among the isolates; Cal1 and CHN12 showed severe and slight stunting symptoms, respectively. These results demonstrate that the TuMV-CP gene confers broad-spectrum resistance of plants to TuMV isolates.

Comparative analysis of the transgene and the mode of expression in terms of TuMV resistance. After determining the plant response to TuMV infection, total DNAs prepared from the infected leaves were used for Southern hybridization. The number of hybridized DNA restriction fragments varied among the progenies regardless of the infection type (Fig. 2A and B). For example, there were resistant lines containing only two transgenes, but also susceptible lines with more than six loci. In northern blot analysis, resistant progenies accumulated no detectable or extremely low levels of the CP transcripts, whereas in the susceptible progenies, distinct hybridization signals with a similar mobility to that expected for the CP RNA (approximately 900 bp) were detected (Fig. 2C). Western blot analysis confirmed the difference in the protein expression level of the transgenes: no detectable hybridization signal was observed in resistant plants versus distinct signals in susceptible plants (Fig. 2D). Similarly, in the successive T₄ and T₅ generations, the resistant progenies accumulated neither transcripts nor translation products of the introduced TuMV-CP gene, whereas all of the susceptible plants transcribed the TuMV-CP gene and produced abundant levels of CP (data not shown). These results demonstrate that TuMV resistance in these resistant lines is expressed during the RNA accumulation stage, most probably the result of RNA silencing.

Detection of siRNAs. Occurrence of siRNAs is one of the most important characteristics of RNA silencing and can be a reliable molecular marker that is closely associated with viral resistance in transgenic plants expressing viral genes (15). In the present study, TuMV resistance observed in *Arabidopsis* plants with TuMV-CP genes was predicted to result from RNA silencing. Therefore, occurrence of the siRNAs was determined by northern blot analysis of low molecular weight RNAs prepared from the fully expanded, noninfected, mature leaves of TuMV-resistant T₃ progenies 30 days after sowing. As expected, distinct hybridiza-

TABLE 2. Pathogenicity of different *Turnip mosaic virus* (TuMV) isolates on TuMV-coat protein (CP) transgenic and wild-type *Arabidopsis* plants and the sequence identity of the entire CP and the 3' 380-nt region among the isolates with their relevant characteristics

Designation of TuMV isolates	Pathogenicity on ^a			Identity (%) in ^b		Location isolated	Original host	Phylogenetic group	Source or reference
	CP112-R1 ^c	CP232-R1 ^c	Wild type ^d	CP region	3' 380 nt				
JO	0/10	0/10	5/5	100	100	Japan	<i>Brassica campestris</i>	World-B	This study
2J	0/10	0/10	5/5	97.0	100	Japan	<i>B. pekinensis</i>	World-B	42
59J	0/10	0/10	5/5	90.6	100	Japan	<i>Raphanus sativus</i>	Asian-BR	42
A102/11	0/5	0/5	5/5	88.8	96.6	Italy	<i>Anemone coronaria</i>	Basal-B	42
BZ1	0/7	0/10	5/5	94.0	98.2	Brazil	<i>B. oleracea</i>	World-B	42
Cal1	0/8	0/6	5/5	89.0	97.4	Italy	<i>Calendula officinalis</i>	Basal-BR	42
CHN12	0/6	0/7	5/5	96.8	98.9	China	<i>B. campestris</i>	World-B	20,42
CP845J	0/10	0/10	5/5	88.1	96.3	Japan	<i>C. officinalis</i>	Basal-BR	29
FD21J	0/10	0/10	5/5	90.4	98.7	Japan	<i>R. sativus</i>	Asian-BR	42
HRD	0/10	0/10	5/5	89.4	96.8	China	<i>R. sativus</i>	Asian-BR	42
IS1	0/7	0/7	5/5	86.6	95.5	Israel	<i>Allium ampeloprasum</i>	Basal-B	42
KD32J	0/10	0/10	5/5	90.6	99.7	Japan	<i>R. sativus</i>	Asian-BR	42
KYD81J	0/10	0/10	5/5	87.6	96.3	Japan	<i>R. sativus</i>	Basal-BR	42
PVO104	0/5	0/5	5/5	88.7	97.6	Germany	<i>Lactuca sativa</i>	Basal-BR	42
RUS2	0/9	0/10	5/5	90.8	97.9	Russia	<i>B. napus</i>	World-B	42
SGD311J	0/10	0/10	5/5	89.7	97.9	Japan	<i>R. sativus</i>	Asian-BR	42
TD88J	0/10	0/10	5/5	88.9	97.9	Japan	<i>R. sativus</i>	Basal-BR	42
UK1	0/7	0/7	5/5	96.5	98.4	UK	<i>B. napus</i>	World-B	20,42

^a Results are shown as the number of infected plants/number of inoculated plants.

^b Percentage against that of JO isolate. GenBank accession number for each isolate: JO (AF312909), 2J (AB076504), 59J (AB076485), A102/11 (AB076521), BZ1 (AB076529), Cal1 (AB076518), CHN12 (AY090660), CP845J (AB076513), FD21 (AB076493), HRD (AB076554), IS1 (AB076522), KD32J (AB076500), PVO104 (AB076532), RUS2 (AB076527), SGD311J (AB076509), TD88J (AB076511), and UK1 (AF169561).

^c CP112-R1 and CP232-R1 are resistant T₂ lines of T₃ progenies used for the inoculation experiments and in Table 1.

^d Nontransformed *Arabidopsis thaliana* ecotype Col-0.

tion signals that correspond to the 21- to 25-nt RNAs, homologous to the CP transcripts, were detected in resistant transformants, whereas no such signal was observed in susceptible transformants (Fig. 3A). When northern blot analysis was carried out using sense 380-nt RNA probes prepared from the 3'-terminal region that is highly conserved among the TuMV isolates, a positive hybridization signal of 21 to 25 nts was detected in the resistant plant but not in the susceptible and nontransformed wild-type plants (Fig. 3B), confirming that the conserved 3'-terminal region of the TuMV-CP gene was actually degraded into siRNAs. These results therefore indicate that RNA silencing of TuMV-CP transgene confers the TuMV resistance of transgenic *Arabidopsis* sp. and that the 3' conserved region in the TuMV-CP gene could be a target for sequence-specific RNA degradation.

DISCUSSION

Numerous examples of pathogen-derived resistance have been reported for a wide range of plant virus combinations. In most of the pathogen-derived resistances, resistance occurs generally at the posttranscriptional stage in which RNA silencing has been in-

duced (4). The mechanism of RNA silencing involves an initial processing of inducing dsRNA into siRNAs of 21 to 25 nts, corresponding to both the sense and antisense strands of the target gene (15). These siRNAs associate with several kinds of proteins and make an RNA-induced silencing complex that serves as a guide to select the target RNAs and precedes further degradation of the homologous RNAs (16,17,49). In this study, we have demonstrated that transgenic *Arabidopsis* plants with no detectable transcripts of the introduced TuMV-CP genes exhibited complete resistance to TuMV. In these resistant transformants, siRNAs characteristic to RNA silencing were detected, whereas the susceptible transgenic plants transcribed the mRNAs and accumulated abundant levels of CP (Figs. 2 and 3). These results indicate that the TuMV resistance observed in the resistant transgenic *Arabidopsis* plants is attributed to RNA silencing. Jan et al. (19) also obtained TuMV-resistant *N. benthamiana* transgenic for the CP gene of a TuMV strain isolated from garden balsam (*Impatiens balsamina*) and confirmed that resistance was expressed at the posttranscriptional level. Interestingly, the TuMV-resistant *Arabidopsis* plants obtained in the present study were apparently not affected by a potyviral suppressor, the helper component proteinase (HC-Pro) of TuMV, even though HC-Pro is considered to interfere with RNA silencing by blocking the maintenance of RNA silencing in tissues where silencing has already been established (1,7). This might be because the RNA silencing state in the TuMV-resistant transgenic plants is of such a nature that dsRNA intermediates produced during the replication of the inoculated TuMV were efficiently degraded (40), resulting in the failure of successful replication and thus translation of the HC-Pro gene. The results of our study are consistent with previous observations that the potyviral resistance of the transgenic plants carrying the sense CP gene sequence could not be overcome by the transgene-derived virus (3,12,14,19,37).

RNA-mediated virus resistance resulting from RNA silencing has been reported to be associated with multiple copies of the transgenes (14). Many of the resistant transgenic plants obtained in this study also contained multiple copies of the transgene. However, comparative analysis of the resistant and susceptible progenies by Southern blot revealed no convincing correlation be-

Fig. 2. A and B, Southern, C, northern, and D, western blot analyses of the T₂ generation of *Arabidopsis thaliana* (ecotype Col-0) transformed with *Turnip mosaic virus* (TuMV) coat protein (CP) gene. Lanes 1 to 4 and 5 to 8 show the results of resistant and susceptible phenotypes, respectively. Each lane represents a series of the analyses for the same T₂ plant as follows: lane 1, TuMV-CP4; lane 2, CP11; lane 3, CP20; lane 4, CP23; lane 5, CP2; lane 6, CP5; lane 7, CP8; and lane 8, CP16. In the Southern blot analyses, isolated DNAs were digested with A, *Bam*HI or B, *Eco*RV separated by 1% agarose gel electrophoresis and blotted onto a nylon membrane followed by hybridization using a TuMV-CP gene-specific probe labeled with digoxigenin. C, For northern blot analyses, total RNAs were denatured and resolved by electrophoresis with 1% agarose gel containing 18% formaldehyde. RNAs were then transferred onto a nylon membrane and hybridized with the same probe used in the Southern blot analysis. The lower panel shows the loading level of each RNA sample by ethidium bromide staining. D, In the western blots, protein samples were boiled with 2× Laemmli sample buffer, separated with 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted onto a polyvinylidene difluoride membrane, and detected with rabbit antiserum raised against TuMV.

Fig. 3. Detection of *Turnip mosaic virus* (TuMV) coat protein (CP) gene-specific small interfering RNAs (siRNAs) in transgenic *Arabidopsis thaliana* (ecotype Col-0) carrying the TuMV-CP gene by northern blots using probes prepared from the A, full coding region and B, 3' 380-nt sequence of the TuMV-CP gene, respectively. A, Lanes 1 to 4 and 5 to 8 show the results of resistant and susceptible phenotypes, respectively. Each lane corresponds to the same T₂ line as follows: lane 1, TuMV-CP4; lane 2, CP11; lane 3, CP20; lane 4, CP23; lane 5, CP2; lane 6, CP5; lane 7, CP8; lane 8, CP16; and M, size marker. B, Lanes 1 and 2 represent the results of the T₃ progenies from resistant (CP112-R1) and susceptible (CP52S-1) T₂ lines, respectively. Lane 3 shows the reaction of the nontransgenic wild-type plant. The sizes of the marker DNA oligomers (20 and 30 nt, respectively) are presented on the left.

tween the resistance and the copy number of the introduced CP gene; most of the resistant lines contained two to four transgene loci, whereas some of the susceptible lines contained more than six transgene loci. The same phenomena have been reported by the analysis of *N. benthamiana* transformed with the sense or antisense protease gene of *Potato virus Y* (47). Alternatively, Waterhouse et al. (48) pointed out the importance of the generation of dsRNAs from the transgenes, and Smith et al. (36) and Tenllado et al. (39) have shown that transgene constructs capable of expressing dsRNAs or single-stranded self-complementary RNAs effectively induce RNA silencing. Although, in this study, we have not determined how and where the TuMV-CP gene is integrated within the *Arabidopsis* genome, stable inheritance of the resistance over successive generations suggests that the transgenes have been homozygously integrated (2). Thus, resistance screening over three generations would be a minimal requirement for raising transgenic lines stably resistant to TuMV.

The spectrum of the RNA-mediated virus resistance is generally restricted to viral strains with greater than $\approx 90\%$ sequence homology to the introduced transgenes (3,21,23,25,41), though, in some cases, the viral resistance of transgenic plants is overcome by the different potyviral strains or isolates that share more than 96% sequence identity (23). In the transgenic papaya and *Papaya ringspot virus* (PRSV) combination, Bau et al. (3) obtained a transformant immune to the heterologous PRSV strains that share 89.7 to 92.5% nucleotide identities in the CP region. Jones et al. (21) determined the breadth of PSbMV resistance in transgenic pea lines with the NIB gene of a PSbMV isolate and concluded that 89% of the sequence homology among the isolates was borderline for resistance. However, our transgenic *Arabidopsis* plants with the CP gene of one TuMV isolate, JO, showed complete resistance to the other 17 representative TuMV isolates, even though the least overall sequence homology of the CP region to isolate JO is 86.6%. These 17 isolates have distinct differences in their pathogenicity and infect over five different plant genera other than Brassicaceae (29). However, a region, representing 380 nts of the 3'-terminal region of the CP gene, is highly conserved at greater than 95.5%. In the case of transgenic papaya with the CP gene of PRSV, Bau et al. (3) reported that 100 nts in the 3' region including the 3' noncoding region of the CP gene was crucial for the resistance. In *N. benthamiana* transformed with the CP gene of the *Tamarillo mosaic virus*, the presence of 135 nts in the 3' part of the transcribed transgenes was sufficient to induce RNA silencing (2). In the current study, we detected siRNAs derived from 380 nts in the 3' conserved region in TuMV-resistant transgenic *Arabidopsis* plants. In addition, we have obtained TuMV-resistant *N. benthamiana* plants by transforming only with the above 3'-terminal fragment (data not shown). These results suggest that the sequence-specific degradation of the 3' conserved region may confer the broad spectrum of TuMV resistance in transgenic *Arabidopsis* lines. Thus, introduction of the TuMV-CP gene, specifically containing the highly conserved 3' region, into the target Brassicaceae plants followed by selecting transformants that show RNA silencing for the transgenes will be an effective and reliable strategy for developing crucifer crops with broad-spectrum resistance to TuMV. However, the possibility of breakdown of the CP-mediated resistance cannot be excluded if the CP transgene silencing was suppressed by following infection with heterologous viruses (33), since crops are frequently infected with multiple viruses simultaneously in the fields. Therefore, introduction of a chimeric transgene, in which heterologous virus genome sequences are fused to each other, would be a promising method to engineer broad resistance (18).

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