RTM3, Which Controls Long-Distance Movement of Potyviruses, Is a Member of a New Plant Gene Family Encoding a Meprin and TRAF Homology Domain-Containing Protein1[W][OA]

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Restriction of long-distance movement of several potyviruses in Arabidopsis (Arabidopsis thaliana) is controlled by at least three dominant restricted TEV movement (RTM) genes, named RTM1, RTM2, and RTM3. RTM1 encodes a protein belonging to the jacalin family, and RTM2 encodes a protein that has similarities to small heat shock proteins. In this article, we describe the positional cloning of RTM3, which encodes a protein belonging to an undescribed protein family of 29 members that has a meprin and TRAF homology (MATH) domain in its amino-terminal region and a coiled-coil domain at its carboxy-terminal end. Involvement in the RTM resistance system is the first biological function experimentally identified for a member of this new gene family in plants. Our analyses showed that the coiled-coil domain is not only highly conserved between RTM3-homologous MATH-containing proteins but also in proteins lacking a MATH domain. The cluster organization of the RTM3 homologs in the Arabidopsis genome suggests the role of duplication events in shaping the evolutionary history of this gene family, including the possibility of deletion or duplication of one or the other domain. Protein-protein interaction experiments revealed RTM3 self-interaction as well as an RTM1-RTM3 interaction. However, no interaction has been detected involving RTM2 or the potyviral coat protein previously shown to be the determinant necessary to overcome the RTM resistance. Taken together, these observations strongly suggest the RTM proteins might form a multiprotein complex in the resistance mechanism to block the long-distance movement of potyviruses.

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Systemic infection of plants by viruses is the result of compatible interactions between plant and viral factors. These molecular interactions control translation and replication of the viral nucleic acid, assembly of progeny virus particles, and generalized invasion of the host through cell-to-cell and long-distance movements of viral particles or ribonucleoprotein complexes (Carrington and Whitham, 1998; Whitham and Wang, 2004). Plants have developed different mechanisms to resist viruses. Passive resistance generally results from lack of or inappropriate interactions between plant and viral factors, causing a block in one of the viral cycle steps, and is usually controlled by recessive resistance genes (Diaz-Pendon et al., 2004). Active resistance is generally triggered by the recognition of the viruses in plants and can be controlled by at least two types of mechanisms. One well-known mechanism is associated with the hypersensitive response or extreme resistance at initial infection sites and is controlled by dominant resistance genes (R genes) through a gene-for-gene relationship (Soosaar et al., 2005; Maule et al., 2007). The second mechanism concerns the general antiviral defense system of RNA interference, which recognizes and targets the viral nucleic acids (Voinnet, 2005; Maule et al., 2007).
Restriction of long-distance movement of several potyviruses controlled by the dominant restricted TEV movement (RTM) genes in Arabidopsis (Arabidopsis thaliana) does not correspond to any of these known resistance mechanisms (Mahajan et al., 1998; Decrooq et al., 2006). Indeed, in this resistance process, viral replication and cell-to-cell movement in inoculated leaves appear unaffected, the hypersensitive response and systemic acquired resistance are not triggered, and salicylic acid is not involved (Mahajan et al., 1998). First identified as specific to Tobacco etch virus (TEV; Whitham et al., 2000), RTM resistance has recently been shown to be active against two other unrelated potyviruses, Lettuce mosaic virus (LMV) and Plum pox virus (PPV; Decrooq et al., 2006), showing that the RTM genes seem to be associated with a general resistance mechanism against potyviruses that blocks their long-distance movement. Whether the RTM genes are also involved in resistance mechanisms against other viral genera remains to be investigated. Genetic characterization from natural ecotype variation and chemically induced mutants has revealed that at least three dominant genes, named RTM1, RTM2, and RTM3, are involved in the restriction of long-distance movement of potyviruses in the Arabidopsis accession Columbia (Col-0; Mahajan et al., 1998; Whitham et al., 1999). Remarkably, a mutation in any of these three genes is sufficient to completely abolish the restriction of long-distance movement, suggesting that these genes act in an interdependent way to block the generalized invasion of the plant (Whitham et al., 1999). RTM1 encodes a protein belonging to the jacalin family, some members of which are involved in defense against insects and fungi (Chisholm et al., 2000). RTM2 encodes a protein that contains a transmembrane domain and has similarities to small heat shock proteins, although its expression is not heat inducible and does not contribute to thermotolerance (Whitham et al., 2000). Although it is not understood how the RTM proteins restrict long-distance movement of potyviruses, it has been shown that both RTM1 and RTM2 are expressed in phloem-associated tissues, that the corresponding proteins localize to phloem sieve elements (Chisholm et al., 2001), and that the N-terminal end of the potyviral capsid protein (CP) is involved in breaking of the RTM resistance (Decrooq et al., 2009), suggesting a direct or indirect interaction between the potyvirus CP and the RTM factors.

In this article, we present the positional cloning of RTM3, which encodes a new type of protein containing a meprin and TRAF homology (MATH) domain, and show that RTM3 directly interacts with RTM1.

RESULTS

RTM3 Corresponds to the At3g58350 Gene

In a previous study, screening of ethyl methanesulfonate-mutagenized M2 Col-0 plants with TEV-bar was used to isolate the TEV-susceptible rtm1, rtm2, and rtm3 mutants. The rtm3 mutant (named A161 in Whitham et al., 1999, and rtm3-1 in this article) was shown to be genetically independent from the rtm1 and rtm2 mutants.

The rtm3-1 mutant, similar to the other rtm mutants, is fully susceptible to systemic infection by some LMV and PPV isolates, whereas the wild-type Col-0 is resistant to these viral isolates (Decrooq et al., 2006). To map the mutation in rtm3-1, F2 populations from a cross between the rtm3-1 line and Ws-2 (which restricts TEV, LMV-AF199, PPV-PS, and PPV-EA to inoculated leaves) were produced. In the largest population, 1,620 F2 plants were inoculated with TEV-GUS and scored for GUS activity in inflorescence tissue at 22 d post inoculation. Two hundred forty-four plants (15%) were TEV-GUS positive. Consistent with previous results (Whitham et al., 1999), the low percentage of susceptible plants suggests that the mutant phenotype was not due to a dominant mutation. However, the 1:5.6 susceptible:resistant ratio deviates substantially from the predicted 1:3 ratio expected if the mutant phenotype was due to a single recessive mutation, which may be due to partial dominance of the rtm3 mutant allele or to hybrid effects resulting from the interaccession cross.

Using a series of molecular markers evenly distributed across the Arabidopsis genome, a preliminary linkage analysis was done with a small population (32 rtm3-1 × Ws-2 F2 plants) exhibiting the TEV-GUS-susceptible phenotype. No association between the susceptibility phenotype and markers proximal to RTM1 or RTM2 was detected, as expected from the independent status of the rtm3 mutation (Whitham et al., 1999). However, an association between the susceptible phenotype and a marker (nga6) derived from near the bottom of chromosome III was detected. Assuming that the TEV-GUS-susceptible plants contained a homozygous, recessive locus that conferred the mutant phenotype, four recombination events in the 64 chromosomes analyzed were detected between this locus and nga6. More detailed genetic mapping was then performed, using a population of up to 244 TEV-GUS-susceptible F2 plants and a set of molecular markers located near nga6. Twenty recombination events among 488 chromosomes were detected between rtm3 and the marker Fus6, which is centromeric to nga6. Twelve recombination events among 330 chromosomes and five among 488 chromosomes were detected with the markers Pur5 and Bgl1a, respectively (centromeric to Fus6), but these recombinants differed from those detected using Fus6. This indicated that rtm3 was located between Bgl1a and Fus6. At this stage, as the F3 progeny from TEV-susceptible F2 plants was also susceptible to LMV-AF199, as expected, the following mapping experiments were done with LMV, for which phenotyping is faster and clearer.

Fine mapping using a new set of 2,962 F2 plants and new polymorphic markers developed specifically
Supplemental Table S1) allowed us to reduce the rtm3 interval between the markers F9D24.40 and F14P22.16, which encompass 22 genes between At3g58200 and At3g58410. Sequencing of all 22 genes in the rtm3-1 mutant identified a single point mutation in the At3g58350 gene when compared with the wild-type Col-0 sequence. This single base mutation was further confirmed by resequencing this gene in both the rtm3-1 line and the wild-type Col-0 sequence. This mutation changes an Asp to a Lys at amino acid position 284 of the corresponding protein. Two T-DNA insertion lines (Salk_017845 [N517845] and Gabi-Kat 801D05) corresponding to this gene were obtained, the T-DNA insertions were validated, and their homozygous state was confirmed (Fig. 1A). In addition, the insertion point of the T-DNA was identified by sequencing in each knockout (KO) line, after nucleotide 251 (from nucleotide 1, which is the A of the ATG) for the KO line Gabi-Kat 801D05 and after nucleotide G of the TGA stop codon for the KO line Salk_017845. Reverse transcription (RT)-PCR analysis of the two KO lines showed that RTM3 expression was no longer detected (Fig. 1B). Both mutant lines and the parent Col-0 were inoculated with LMV-AF199, and inflorescence tissues were analyzed for the presence of virus at 3 weeks post inoculation. Both KO lines were found to be susceptible to LMV, whereas the parental Col-0 line remained resistant as expected (Fig. 1C).

An allelism test was also conducted by crossing the rtm3-1 line with both KO lines. After checking the heterozygous state of the F1 plants produced from this cross (Fig. 1A) and confirming the RTM3 sequence in each F1 population, 12 F1 plants of both populations were inoculated with LMV-AF199 and accumulated it in inflorescence tissue (Fig. 1C). In contrast, plants from control F1 populations obtained from RTM3 KO lines √ Col-0 or rtm3-1 √ Col-0 crosses were all resistant, confirming that At3g58350 corresponds to RTM3.

RTM3 is 1,511 nucleotides long with 5' and 3' untranslated regions of 126 and 166 nucleotides, respectively, and four introns of 70, 75, 90, and 148 nucleotides. RTM3, therefore, encodes a predicted 301-amino acid protein with a deduced molecular mass of approximately 34,000 D.

**RTM3 Belongs to a New Gene Family**

RTM3 is annotated as a MATH protein (The Arabidopsis Information Resource [www.arabidopsis.org]). Meprins are mammalian tissue-specific metalloendopeptidases of the astacin family implicated in developmental, normal, and pathological processes by hydrolyzing a variety of proteins (Marchand et al., 1995). TRAF proteins were first isolated by their ability fragment of LMV RNA from LMV-inoculated RTM3 KO and F1 line systemic leaves. L, A 100-pb ladder.

![Figure 1. Genotyping and LMV infection phenotyping of the RTM3 KO lines and of the F1 populations produced by crossing each KO line with the mutant rtm3-1 line. KO-g, KO Gabi 801D05; KO-s, KO Salk NS17845; H2O, negative control without genomic DNA; WT, wild type. Numbers 1, 2, 3, and 4 indicate replicates. A, Genotyping of both KO lines and of the F1 populations revealed by PCR on genomic DNA. PCR 1, PCR using the Gabi-Kat-specific primer and the two At3g58350-specific primers (see “Materials and Methods”); PCR 2, PCR using Lba1 and the two Salk-designed-specific primers for NS17845 (see “Materials and Methods”); L, 100-pb ladder. B, RT-PCR amplification of RTM1 and RTM3 transcripts from total RNA from wild-type and RTM3 KO lines. gDNA, Genomic DNA; L, 1-kb ladder. C, RT-PCR of a 277-bp

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to interact with human tumor necrosis factor receptors (Rothe et al., 1994) and share with meprins a conserved region of about 180 residues, named the MATH domain (Sunnerhagen et al., 2002). Using the INTERPRO database (http://www.ebi.ac.uk/interpro), a MATH domain is predicted in RTM3 between amino acid positions 13 and 136. The MATH domain is a fold of seven to eight antiparallel β-sheets found in a variety of proteins among eukaryotes (Zapata et al., 2007). Alignment of the RTM3 MATH domain with those of several human TRAF proteins, notably with TRAF2, for which the crystal structure has been determined (Park et al., 1999), indeed reveals conserved amino acids in the eight TRAF2 β-strands (Supplemental Fig. S1). Interestingly, the three residues of TRAF2 (Arg-393 and Tyr-395 in β3 and Ser-467 in β7) involved in the recognition of the SXE motif in the receptor peptide (Park et al. 1999) are conserved in RTM3.

Protein secondary structure prediction analysis using the Network Protein Sequence Analysis (NPS@) system (Combet et al. 2000) predicts at least six β-strands in the MATH domain of RTM3 (Fig. 2). The β-strands corresponding to TRAF2 β-strands 6 and 7 are not predicted in RTM3, but the same analysis performed on TRAF2 also failed to predict β-strands 6 and 7, which do not appear in the consensus prediction and are only supported by the DSC (for Discrimination of protein Secondary structure Class) method (data not shown).

As shown in Figure 2, the C-terminal domain of RTM3 is predicted to form a long α-helix. PCOIL (Lupas, 1996; Gruber et al., 2005; http://toolkit.tuebingen.mpg.de/pcoils) and MARCOIL (Delorenzi and Speed, 2002; http://www.isrec.isb-sib.ch/webmarcoil/webmarcoilC1.html) predicted the likely presence of a coiled-coil (CC) domain in this region, between positions 245 and 289 (Supplemental Fig. S2). Interestingly, the mutation in rtm3-1 at position 284 lies within this CC domain and corresponds to position d in the heptad repeat, providing direct evi-
idence of the involvement of this domain in RTM resistance.

Using the INTERPRO database, MATH domains are identified in 127 Arabidopsis sequences. Analysis of each sequence allowed the identification of 71 distinct Arabidopsis proteins (Supplemental Table S2). These MATH proteins are classified in four families according to the nomenclature of Zapata et al. (2007). Two proteins belong to the Ubiquitin-Specific Protease 7 family, which includes MATH proteins associated with a ubiquitin protease domain; six proteins belong to the MATHd/Broad complex, Tramtrack, Bric-a-brac (BTB) family, in which MATH proteins are associated to a BTB/Pox virus and zinc finger (POZ) domain; one protein belongs to the MATHd/filament protein family, in which proteins contain four MATH domains associated in the case of this Arabidopsis protein with a Bin-Amphiphysin-Rvs domain; and the 62 remaining proteins including RTM3 belong to the MATHd-only protein family, in which proteins contain one to four MATH domains without any other associated domain (Supplemental Table S2). Most of the MATH genes from this last family are organized in clusters in the Arabidopsis genome (Fig. 3), and RTM3 is included in the largest cluster, which contains 18 of these genes.

Among these 62 MATHd-only proteins, 29 Arabidopsis proteins also contain one or several CC domains (Fig. 4). BLAST analysis using only the CC domain (amino acids 137–301) of RTM3 showed that

Figure 3. Genomic distribution of the RTM3 homologous genes. This map was made using the chromosome map tool from The Arabidopsis Information Resource (http://www.arabidopsis.org/index.jsp). Black arrows indicate genes encoding protein with MATH and CC domains; gray arrows indicate genes encoding protein with only a CC domain. Numbers at the top of the chromosomes indicate the number of each Arabidopsis chromosome.
RTM3 also shares significant homology with 10 proteins that contain only a CC domain (Fig. 4). Surprisingly, the genes corresponding to these 10 proteins are also located in clusters containing genes encoding some of the 29 proteins that contain MATH and CC domains (Fig. 3). In addition, in a given cluster, a high level of identity is found between the MATH and CC domains of the corresponding proteins, suggesting that these clusters result from gene duplication events. For instance, the RTM3 cluster contains 17 genes for which the corresponding protein contains MATH and CC domains (including RTM3), five genes for which the corresponding protein contains only a CC domain, and one gene for which the corresponding protein contains only a MATH domain. Most of the identity among domains in this large cluster is often above 50% (up to 63% amino acid sequence identity with the At3g58360 protein). An alignment of both RTM3 domains with proteins showing more than 50% identity with each domain is presented in Supplemental Figure S3. Interestingly, the amino acid position that is modified in the rtm3-1 mutant is well conserved among these proteins.

Figure 4. List and schematic organization of Arabidopsis RTM3 homologous proteins containing MATH and CC domains. White boxes represent the MATH domain, and gray boxes represent the CC domain.

RTM3 Interacts with Itself and with RTM1

As a first step toward understanding the molecular mechanisms underlying the RTM resistance, protein-protein interaction experiments using the yeast two-hybrid system were performed to test possible interactions between the three RTM proteins and the CPs of LMV and PPV. Indeed, as mutations in the potyvirus CP are sufficient to overcome the RTM resistance (Decroocq et al., 2009), it has been suggested that the CP may interact with the RTM proteins. The three RTM cDNAs and CPs from two virus isolates unable to overcome RTM resistance (LMV-AF199 and PPV-PS) and from virus isolates able to overcome RTM resistance (LMV-AFVAR1 [Decroocq et al., 2009] and PPV-R) were cloned in the yeast two-hybrid vectors. The potential interactions between the RTM proteins and between the CPs and the RTM proteins were then evaluated. These experiments confirmed a previously described self-interaction for RTM1 (Chisholm et al., 2001), although rather weak (only detected on the synthetic dextrose [SD]-Leu-Trp-His medium), and provided evidence for a RTM3-RTM3 self-interaction (Fig. 5A). Interestingly, an interaction was also observed between RTM1 and RTM3, whereas no interaction was detected when RTM1 or RTM3 was tested against empty vectors. No interaction was revealed between RTM2 and RTM1 or RTM3 (Fig. 5A). In the same way, no interaction was revealed between the various CPs and the RTM proteins, whereas as a positive control a self-interaction was detected for the CPs (Fig. 5B).

The RTM3 form corresponding to the rtm3-1 mutant line (which contains a point mutation in the CC domain) was also evaluated in the two-hybrid system. While this mutant version of RTM3 is still able to self-interact, it is no longer able to interact with RTM1 (Fig. 5C).

To confirm the interactions revealed in yeast, bimolecular fluorescence complementation (BiFC) in planta was used. In this method, two nonfluorescent fragments (YFPN and YFPC) of yellow fluorescent protein (YFP) are fused to two different proteins (Walter et al., 2004). When these two proteins associate with each other, a fluorescent YFP complex is restored. Constructs allowing the expression of each of the RTM proteins in the different BiFC vectors were obtained and combined to bombard onion (Allium cepa) epidermal cells. As shown in Figure 6, the self-interactions for RTM1 and RTM3 as well as the interaction between RTM1 and RTM3 were confirmed in plant cells. We also confirm that there is no interaction between RTM2 and either of the RTM proteins (whatever the vector combinations). As a negative control, no interaction has been revealed between the RTM proteins and the 20S proteasome subunit PAE2 protein.

BiFC experiments were also conducted with RTM3 from the rtm3-1 line. However, in this case, if self-interaction of the mutated RTM3 has been confirmed, we have also observed interaction between RTM1 and the mutated RTM3, which was not observed using the two-hybrid system.

DISCUSSION

In this article, we describe the positional cloning of a third RTM gene, genetically identified after the screening of a Col-0 Arabidopsis mutant library with TEV (Whitham et al., 1999) and involved in the restriction of potyviral long-distance movement. RTM3 belongs to a new protein family that has a MATH domain in its N-terminal region and a CC domain at its C-terminal end. Involvement in the RTM resis-
tance system is the first biological function experimentally identified for a MATH domain-containing protein in plants.

As shown in this study, MATH domain proteins form a large protein family in Arabidopsis, with at least 71 distinct members. The MATHd-only protein type, which includes RTM3, represents the vast majority of the Arabidopsis MATH-containing proteins, with 62 members. This type of MATH protein is found in plants (Medicago, rice [Oryza sativa]), in lower eukaryotes ( Trypanosoma, Plasmodium ), and in lower metazoans such as the nematode Caenorhabditis elegans . In this last species, the MATHd-only protein type also represents the majority of the MATH-containing proteins ( Zapata et al., 2007 ). This MATHd-only protein type is described as lacking any other distinguishable associated domain. However, the analysis reported here shows that RTM3 and a set of RTM3-related proteins are strongly predicted to also contain a CC structure. This CC domain is not only highly conserved between RTM3-homologous MATH-containing proteins but also in proteins lacking a MATH domain. However, as for the MATH-domain proteins, no function has been described until now for CC domain-only proteins. The location in the RTM3 CC domain of the mutation in the rtm3-1 line highlights the role of this domain in the RTM resistance mechanism. The cluster organization of the RTM3 homologs in the Arabidopsis genome, and in particular the large cluster at the bottom of chromosome 3 including RTM3, suggests the role of duplication events in shaping the evolutionary history of this gene family, including the possibility of deletion or duplication of one or the other domain.

The resolved crystal structure of the TRAF2 protein reveals a trimeric self-association of the MATH domain ( Park et al., 1999 ). It has been suggested that the MATH domain may take part in diverse modular arrangements defined by adjacent multimerization domains. Interestingly, TRAF proteins also contain a CC domain, helping to stabilize their multimeric complexes ( Park et al., 1999 ). RTM3 family proteins might form such multimeric structures involving their MATH and CC domains. The protein-protein interaction experiments reported here that demonstrate an RTM3-RTM3 self-interaction are consistent with this hypothesis.

A MATH domain may also interact with other types of proteins. For example, Arabidopsis and rice members of the MATH-BTB family interact with Cullin3 ( CUL3 ) proteins via their BTB domain to form functional E3 ligases targeting specific proteins for ubiquitination ( Weber et al., 2005 ; Gingerich et al., 2007 ). A putative role for the MATH domain of these

Figure 5. Protein-protein interactions between the RTM proteins and the potyviral CP with the yeast two-hybrid system. A, Interactions among the wild-type RTM proteins. The growth of cotransformed yeast was assessed in SD/Leu-Trp (–LT) medium, and then after 3 d, six colonies for each cotransformation experiment were plated on SD/Leu-Trp (as a control), SD/Leu-Trp-His (–LTH), and SD/Leu-Trp-His-Ade (–LTHA) media and were left 4 d at 30°C. C−, negative control; C+, positive control. The clones in pGADT7 are those indicated in the horizontal rows, and those in pGBK7 are indicated in the vertical rows. B, Interactions between the RTM proteins and the PPV CP. For each pair of tested clones, the first is the clone in pGAD-T7 and the second is the clone in pGBK-T7. pGAD-T and pGBK-S3 are the clones supplied in the Clontech kit to be used as positive controls. pGAD is the empty vector pGAD-T7 and pGBK is the empty vector pGBK-T7 used as negative controls. Only the yeast colonies (three colonies for each cotransformation experiment) plated in the –LTHA medium are shown. C, Interactions with the RTM3 mutant protein from the rtm3-1 mutant line. Details are as in B.
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BTB proteins is its possible interaction with substrate protein, as shown in C. elegans for the BTB/POZ-MATH protein CeMel-26, which interacts with CeCUL3 via its BTB/POZ domain and with the substrate protein CeMEI-1 via its MATH domain (Pintard et al., 2003).

As for RTM1, which belongs to the jacalin lectin protein family, and RTM2, which contains a small HSP domain, RTM3 belongs to a large protein family. Members of all three protein families are able to form multimeric structures, such as the tetrameric structure of jacalin (Jeyaprakash et al., 2002), the heterooligomeric structure of small HSPs (van Montfort et al., 2002), or the trimeric structure of TRAF proteins (Park et al., 1999). In our study, in planta self-interaction for RTM3 and RTM1 as well as interaction between RTM1 and RTM3 have been observed, but no such interactions have been shown with RTM2. Taken together, these observations strongly suggest the RTM proteins might form a multiprotein complex.

Our protein-protein interaction experiments do not allow us to conclude about a possible correlation between the inability of the RTM3 form present in the rtm3-1 mutant to interact with RTM1 and its functionality in RTM resistance. Indeed, if no interaction has been shown between RTM1 and the mutated RTM3 in the yeast two-hybrid system, this interaction has been revealed in the BiFC experiments. Such differences between these two approaches were already mentioned in the literature (Ührig et al., 2007).

Regarding the expression of RTM3 in relation with those of RTM1 and RTM2, although not experimentally analyzed in this study, several studies have shown coexpression among these three genes in Arabidopsis. Indeed, it was shown that the three RTM genes are mainly expressed in root, stem, petiole, and some floral organs, in particular the petal, although RTM3 appears to be expressed at lower levels than RTM1 and RTM2 (Supplemental Fig. S4; Schmid et al., 2005; AtGenExpress visualization tool [http://jsp.weigelworld.org/expviz/expviz.jsp]). These tissues and organs are those in which vascular tissues are predominant and in which the RTM1 and RTM2 genes are specifically expressed (Chisholm et al., 2001). Other gene expression studies have also revealed the coexpression of the three RTM genes, which have been shown to be induced together in an in vitro xylem vessel element formation system (Kubo et al., 2005) and to be expressed in phloem-cambium tissues separated from xylem and nonvascular peripheral tissues from root-hypocotyl (Zhao et al., 2005).

Assuming that the RTM proteins may form a multisubunit complex, this complex could interact with the viral particles or with a viral ribonucleoprotein complex involved in virus long-distance movement through the CP N-terminal end. In this case, the RTM factors could sequester this long-distance movement-capable form of the virus, thereby blocking viral movement through the vascular tissue. In the case of potyvirus isolates able to overcome the RTM resistance (Decroocq et al., 2009), mutated positions in the N-terminal end of the CP may not allow the postulated interaction between the RTM complex and the CP to occur, leading to an absence of sequestration and long-distance movement of virus. However, in our study, no interaction has been detected so far between the RTM proteins and the CPs of LMV and PPV, but these negative results do not rule out the possibility that such interactions could occur in plant cells, since a number of artifactual effects may also be responsible for them.

Alternative models are also plausible. The RTM factors, for example, may interact with cellular factors or structures necessary for potyvirus long-distance movement, preventing them from playing their role in this process. In a third model, the RTM factors may induce or activate an antiviral response in the phloem tissue that recognizes and targets the virus movement-competent form through an interaction with the N-terminal end of the CP. Finally, we cannot rule out a catalytic activity of the RTM complex that could, for instance, degrade the viral long-distance movement-capable form.

To gain a better understanding of the mechanisms underlying this original resistance pathway, numerous questions remain to be answered in the future, such as the tissue and the subcellular localization of the block in the viral long-distance movement, the existence of...
an RTM factor-containing multiprotein complex in the phloem and the identity of its other components, if any, and whether virus particles or nucleoprotein complexes interact directly or indirectly with the RTM factors or complexes.

Finally, another intriguing question is whether or not the RTM resistance is also active in other plant species. As put viral CP is not only a determinant necessary to overcome the RTM resistance but also is involved in the viral long-distance movement in numerous hosts (Revers et al., 1999a), the possibility exist that the RTM resistance also might be identified in other plant species.

**Materials and Methods**

**Plant Materials**

Arabidopsis (*Arabidopsis thaliana*) accessions and T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/) or Gabi-Kat (http://www.gabi-kat.de/). Plants were grown under greenhouse conditions (16-h daylength, 18–25°C) and maintained in insect-proof cages after inoculation.

The Arabidopsis A35S8350 T-DNA insertion lines in the Col-0 background (line N517845 [or Salk_017845] and line 801D05 from Gabi-Kat) used in this study were analyzed to verify the right T-DNA insertion sites: genomic (line N517845 [or Salk_017845] and line 801D05 from Gabi-Kat) used in this study were analyzed to verify the right T-DNA insertion sites: genomic

- Virus Inoculation and Detection

LMV-AF199 (Krause-Sakate et al., 2002) and TEV-GUS (Dolja et al., 1992) were used for the inoculation experiments. Inoculation of Arabidopsis plants with both viruses was performed as described by Revers et al. (2003) and Mahajan et al. (1998). ELISA as described by Revers et al. (1997) and RT-PCR using the Nb/P4 primer pair as described by Revers et al. (1999b) were used to detect LMV:GUS activity assays used to detect TEV in inoculated leaves and inflorescence tissues were performed as described by Mahajan et al. (1998).

**rtm3-1 Mapping**

A set of seven markers polymorphic between Col-0 and Wei-2 was generated between the Pst and BglI markers to identify recombination events: four simple sequence repeat markers (TH8101, T30K173, F14P221, F25L231), one single-strand conformation polymorphism marker (F9D24.40), and two cleaved amplified polymorphic sequence-single-strand conformation polymorphism markers (F14P221.16, F9D24.34; Supplemental Table S1).

**Arabidopsis Gene Sequencing**

PCR amplifications of Col-0 or the rtm3-1 line genomic DNA were performed in 50-μl reactions containing 0.5 units of DyNAzyme EXT DNA Polymerase (Finnzymes) and 1 μM of specific primers. Twenty-nucleotide-long primers designed in the 5’ and 3’ untranslated regions of each of the genes in the RTM3-containing interval were used to amplify and sequence the complete coding regions of these genes. The cycling conditions were 35 cycles at 92°C for 30 s, 52°C for 30 s, and 72°C for 2 min after an initial denaturation at 95°C for 3 min using an iCycler thermal cycler (Bio-Rad Laboratories). Automated DNA sequencing of PCR products (two independent PCR products per gene) was performed at Cogenics.

**Sequence Analysis**

The amino acid sequences were aligned using ClustalW (Thompson et al., 1994), which generates and uses a distance dendrogram (Saitou and Nei, 1987) to construct multiple sequence alignments. Secondary structure predictions were produced using DSC (King and Sternberg, 1996), MLRC (for Multivariate Linear Regression Combination; Guermeur et al., 1999), PHD (for profile network method; Rost and Sander, 1993), and Predator (Frishman and Argos, 1996) from NPS@ (Combet et al., 2000; http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html).

**Yeast Two-Hybrid Experiments**

Yeast two-hybrid experiments were carried out with the Matchmaker two-hybrid system (Clontech Laboratories). cDNA synthesis was performed as described above. Appropriate PCR was carried out to amplify each RTM cDNA as well as the CPs from LMV-AF199 (Krause-Sakate et al., 2002), LMV-AFVAR1 (Decroocq et al., 2009), PPV-R (Riechmann et al., 1990), and PPV-PS (Snten et al., 2001) using gene-specific oligonucleotides containing restriction sites in order to clone PCR products in the two-hybrid pGBK17 and pGADT7 vectors. In the case of RTM2, which contains a transmembrane domain usually considered not suitable for two-hybrid experiments, a truncated cDNA excluding the 3’ region corresponding to the transmembrane domain was cloned instead of the full-length sequence. The recombinant plasmids were used to transform yeast AH109 cells using sequential transformation or simultaneous cotransformation protocols according to the instruction manual. The production of the different proteins in yeast was verified by western immunoblotting using anti-hemagglutinin and anti-c-Myc antibodies. Transfomants were plated on SD/Leu-Trp medium and, after 3 d, transferred on SD/Leu-Trp-His and SD/Leu-Trp-His-Ade media. Interactions were confirmed in at least three independent experiments.

**BiFC Experiments**

RTM1 and RTM3 cDNAs were amplified and cloned using Gateway technology (Invitrogen) in BiFC vectors (a gift of Dr. Tatsuyoshi Nakagawa, Shimane University) that were constructed from pUGW2 and pUGW0 vectors (Nakagawa et al., 2007). Appropriate combinations of recombinant BiFC vectors were cobambedared using 1.6-μm gold particles and the biotic particle delivery system (Bio-Rad PDS-1000/He; Bio-Rad Laboratories) into onion (*Allium cepa*) epidermal cell layers. After incubation at 25°C for 18 h in darkness, the epidermal cell layers were viewed with a Leica TCS SP2 confocal microscope fitted with a 20× or a 40× water-immersion objective. For imaging the expression of YFP constructs, excitation lines of an argon ion laser of 514 nm were used. Image analysis was carried out with Leica LCS and Adobe Photoshop 6.0. Interactions were confirmed in at least two independent experiments.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Amino acid sequence alignment of the MATH domain of RTM3, TRAF1, TRAF2, TRAF3, and TRAF5.

**Supplemental Figure S2.** CC structure prediction in RTM3.

**Supplemental Figure S3.** Alignment of RTM3 with homologous proteins sharing more than 50% identity with either the MATH or the CC domain.

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Supplemental Figure S4. Comparison of the expression patterns of the RTM genes.

Supplemental Table S1. Molecular markers developed for the fine mapping of RTM3.

Supplemental Table S2. Arabidopsis genes encoding MATH domain-containing protein.

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LITERATURE CITED

Carrington JC, Whitham SA (1998) Viral invasion and host defense: strategies and counter-strategies. Curr Opin Plant Biol 1: 336–341

Chisholm ST, Mahajan SK, Whitham SA, Yamamoto ML, Carrington JC (2000) Cloning of the Arabidopsis RTM1 gene which controls restriction of long-distance movement of tobacco etch virus. Proc Natl Acad Sci USA 97: 489–494

Chisholm ST, Farra MA, Anderberg RJ, Carrington JC (2001) Arabidopsis RTM1 and RTM2 genes function in phloem to restrict long-distance movement of tobacco etch virus. Plant Physiol 127: 1667–1675

Combet C, Blanchet C, Geourjon C, Deleage G (2000) NPS@: Network Protein Sequence Analysis. Trends Biochem Sci 25: 147–150

Decrooq V, Salvador B, Sicard O, Glasa M, Cosson P, Svanella-Dumas L, Revers F, Garcia JA, Candresse T (2009) The determinant of potyvirus ability to overcome the RTM resistance of Arabidopsis thaliana maps to the N-terminal region of the coat protein. Mol Plant Microbe Interact 22: 1302–1311

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Decrooq V, Sicard O, Alamillo JM, Lansac M, Eyquard JP, Garcia JA, Candresse T, Le Gall O, Revers F (2006) Multiple resistance traits control plum pox virus infection in Arabidopsis thaliana. Mol Plant Microbe Interact 19: 541–549

Delorenzi M, Speed T (2002) An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. Bioinformatics 18: 617–625

Diaz-Pendon JA, Trunerig V, Nieto C, Garcia-Mas J, Benhadame A, Aranda MA (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233

Dolja VV, McBride HJ, Carrington JC (2004) Advances in understanding recessive resistance to plant viruses. Mol Plant Pathol 5: 223–233
Walter M, Chaban C, Schütze K, Batistic O, Weckermann K, Näke C, Blazevic D, Grefen C, Schumacher K, Oechting C, et al. (2004) Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40: 428–438
Weber H, Bernhardt A, Dieterle M, Hano P, Mutlu A, Estelle M, Genschik P, Hellmann H. (2005) Arabidopsis AtCUL3a and AtCUL3b form complexes with members of the BTB/POZ-MATH protein family. Plant Physiol 137: 83–93
Whitham S, Wang Y. (2004) Roles for host factors in plant viral pathogenicity. Curr Opin Plant Biol 7: 1–7
Whitham SA, Anderberg RJ, Chisholm ST, Carrington JC. (2000) Arabidopsis RTM2 gene is necessary for specific restriction of tobacco etch virus and encodes an unusual small heat shock-like protein. Plant Cell 12: 569–582
Zhao C, Craig JC, Petzold HE, Dickerman AW, Beers EP. (2005) The xylem and phloem transcriptomes from secondary tissues of the Arabidopsis root-hypocotyl. Plant Physiol 138: 803–818