The genus *Tobamovirus* includes *Tobacco mosaic virus*, *Tomato mosaic virus* (ToMV) and *Youcai mosaic virus* (this virus is identical to TMV-Cg and, in this report, referred to as TMV-Cg for consistency with our previous publication), as well as many other related viruses (Gibbs, 1999). TMV-Cg is closely related to other crucifer-infecting tobamoviruses and multiplies to higher levels in *Arabidopsis thaliana* than ToMV (Li et al., 1983; Lartey et al., 1996; Yamanaka et al., 1998). The genome of a tobamovirus is a non-segmented positive-strand RNA and encodes 130 and 180 kDa proteins that participate in RNA replication and other functions, a 30 kDa protein which the 130 and 180 kDa replication proteins and the polypeptide of the 130 and 180 kDa replication proteins and the coat protein (CP) (reviewed by Ishikawa & Okada, 2004). Virus multiplication depends not only on virus-coded factors, but also on host-derived factors (reviewed by Buck, 1999; Ahlquist et al., 2003). Quantitative and qualitative changes in these host factors cause a wide range of effects on virus multiplication. Previously, a mutant of *A. thaliana*, YS241 (Ohshima et al., 1998), was isolated and plant lines B1-113 and B1-234 were obtained by back-crossing YS241 plants to the wild-type plants (accession Col-0). The B1-113 line carries defects in both *TOM2A* and *TOM2B*, which encodes a four-pass transmembrane protein (Fig. 1a), and *TOM2B*, which encodes a small basic protein. In B1-113 plants and protoplasts, multiplication of both TMV-Cg and ToMV was significantly reduced (Tsujimoto et al., 2003). It was further demonstrated that (i) the TOM2A protein is co-purified with the 130 and 180 kDa replication proteins from solubilized membranes from ToMV-infected protoplasts (Nishikiori et al., 2006); (ii) TOM2A localizes mainly on vacuolar membranes, with which the 130 and 180 kDa replication proteins and the activity to synthesize tobamovirus-related RNAs are partly associated (Hagiwara et al., 2003); and (iii) TOM2A interacts in yeast with TOM1 (Tsujimoto et al., 2003), another transmembrane protein required for tobamovirus multiplication that interacts with the helicase-like domain polypeptide of the 130 and 180 kDa replication proteins (Yamanaka et al., 2000). Based on these observations, it was proposed that, acting with TOM1, TOM2A plays important roles in the formation and/or maintenance of TMV-Cg replication complexes on membranes. The function of TOM2B has not yet been determined. In YS241 and B1-234 plants, the TOM2A gene is inactivated by deletion, whereas the TOM2B gene is translocated and remains active (Tsujimoto et al., 2003). In these plants, the accumulation of TMV-Cg CP is significantly reduced compared with that in wild-type plants, whereas ToMV CP accumulation is similar to or only slightly lower than that in wild-type plants. Consistent with this observation, an *A. thaliana* mutant in which a T-DNA was inserted in the TOM2A gene and the expression of TOM2A mRNA was not detected (Fig. 1b and c) [tom2a-1 (hereafter, ‘2a’; GABI-Kat 505G01)] showed a similar phenotype to B1-234 (Fig. 2a). One possible explanation...
for the differential effect of the *tom2a* mutation on the multiplication of TMV-Cg and ToMV is that these viruses prefer to utilize different members of the *TOM2A* family. In fact, a BLAST search (http://www.arabidopsis.org/Blast/index.jsp) identified three *TOM2A* homologues in the *A. thaliana* genome (Fig. 1a). Here, the contribution of these homologues to the multiplication of TMV-Cg and ToMV was investigated.
The TOM2A homologues TOM2AH1 (At4g28770), TOM2AH2 (At2g20230) and TOM2AH3 (At2g20740) encode 281, 270 and 221 aa polypeptides, respectively. Like TOM2A, all of them are predicted to be four-pass transmembrane proteins (Fig. 1a). The amino acid sequence identity is 33% between TOM2A and TOM2AH1, 30% between TOM2A and TOM2AH2, 33% between TOM2A and TOM2AH3, 73% between TOM2AH1 and TOM2AH2, 43% between TOM2AH1 and TOM2AH3, and 47% between TOM2AH2 and TOM2AH3. Quantitative real-time RT-PCR analysis revealed that the accumulation levels of TOM2AH1, TOM2AH2 and TOM2AH3 mRNAs in rosette leaves of Col-0 plants 5 weeks after sowing were, respectively, approximately 50, 150 and 110% that of TOM2A mRNA (data not shown). Microarray data (the Bio-Array Resource Database; http://brc.botany.utoronto.ca/efp/cgi-bin/efpWeb. cgi) suggest that mRNAs of TOM2A and its three homologues are ubiquitously expressed (in rosette leaves, cauline leaves, stems, siliques, flowers and roots) and their mRNA accumulation levels are similar in each tissue.

To determine the contribution of the TOM2A homologues to tobamovirus multiplication, *A. thaliana* mutants carrying T-DNA insertions in the TOM2A homologues [tom2ah1-1 (hereafter, ‘h1’; SALK_034692), tom2ah2-1 (‘h2’; SALK_022303) and tom2ah3-1 (‘h3’; GABI-Kat 343E12); Fig. 1b] were utilized. Plant lines that are homozygous for each mutation were established and the specific reduction in accumulation of corresponding mRNAs to undetectable levels was confirmed (Fig. 1c). Plant lines carrying multiple mutations in 2a, h1, h2 and/or h3 were also generated. Among all possible combinations of the mutations, the 2a/h1/h2 triple and the quadruple (2a/h1/h2/h3) mutations led to growth defects, whereas the other single, double and triple mutations had no apparent effect on plant growth (Fig. 1d; data not shown). Seeds of the 2a/h1/h2 triple mutant germinated normally, but the plants started to show growth defects around 3–5 weeks after sowing. The quadruple mutant started to show similar, but more severe, growth defects around 2–3 weeks after sowing (Fig. 1d) and did not produce seeds (data not shown) under the growth conditions described by Fujisaki et al. (2004). These observations suggest that TOM2A and its three homologues play a parallel and essential role in the plant life cycle and that the contribution of TOM2AH3 to plant growth is the least among the TOM2A family members.

To investigate the contribution of the three TOM2A homologues to tobamovirus multiplication, plants carrying h1, h2 and/or h3 mutations were established. In these plants, including the h1/h2/h3 triple mutant, TMV-Cg CP accumulated to wild-type levels (Fig. 2a, b; data not shown). To more sensitively detect the contribution of the TOM2A homologues to TMV-Cg multiplication, TMV-Cg CP accumulation in the normally growing triple mutants 2a/h1/h3 and 2a/h2/h3 was compared with that in the 2a single mutant (Fig. 2b). Quantification of the CP bands in
Fig. 2(b) by the Image J program (http://rsb.info.nih.gov/ij/) suggested that accumulation levels of TMV-Cg CP in the 2a/h1/h3 and 2a/h2/h3 triple mutant leaves were 60 and 40 %, respectively, of that in the 2a single mutant leaves 14 days after infection [the difference between 2a/h1/h3 or 2a/h2/h3 and 2a was significant according to the Student’s t-test (P<0.05)].

It has been demonstrated that the tom2a mutation inhibits TMV-Cg multiplication in protoplasts (Tsujimoto et al., 2003). TMV-Cg multiplication in 2a/h1/h3, 2a/h2/h3 and h1/h2/h3 triple and 2a single mutant protoplasts was then examined. Consistent with our previous report (Tsujimoto et al., 2003), the 2a single mutation led to low level accumulation of TMV-Cg CP and additional h1/h3 and h2/h3 mutations in the 2a background further reduced CP accumulation levels (Fig. 3a). Quantification of the CP bands in Fig. 3(a) showed that the accumulation levels of TMV-Cg CP in 2a/h1/h3 and 2a/h2/h3 triple mutant protoplasts were 6 and 15 %, respectively, of that in 2a single mutant protoplasts [difference between 2a/h1/h3 or 2a/h2/h3 and 2a was significant according to the Student’s t-test (P<0.05)]. The reduced accumulation of TMV-Cg CP in the triple mutant protoplasts was associated with parallel decreases in the accumulation of genomic and CP subgenomic RNAs of TMV-Cg (Fig. 3b). In h1/h2/h3 triple mutant protoplasts, TMV-Cg-related molecules accumulated as in wild-type protoplasts. These results indicate that the three TOM2A homologues contribute to TMV-Cg multiplication, but their contribution is much less than that of TOM2A.

ToMV multiplication was examined in plants carrying single or multiple mutations in TOM2A and its homologues. In the normally growing mutant plants, ToMV CP accumulated to levels that were close to that in wild-type plants (Fig. 2a, b). Furthermore, in 2a single and 2a/h1/h3, 2a/h2/h3 and h1/h2/h3 triple mutant protoplasts, ToMV CP, genomic RNA and subgenomic RNA accumulated to wild-type levels (Fig. 3a, b). These results indicate that,
unlike TMV-Cg, ToMV multiplication does not strongly depend on any particular member of the TOM2A family. In the mutant plants showing serious growth defects (2a/h1/h2 and 2a/h1/h2/h3), CP accumulation of either TMV-Cg or ToMV was not detected (data not shown). However, this result does not necessarily indicate that TOM2A and its homologues are absolutely required for TMV-Cg and ToMV multiplication because the inhibition of virus multiplication might be due to an indirect effect caused by abnormal physiological conditions of the plants.

Cucumber mosaic virus (CMV), which belongs to a different taxonomic group than tobamoviruses and which is known to be insensitive to the *tom2a* mutation (Fig. 2b; Ohshima et al., 1998), also multiplied to wild-type levels in 2a/h1/h3, 2a/h2/h3 and h1/h2/h3 triple mutant plants and protoplasts (Figs 2b, 3a, b), indicating that the observed restriction of TMV-Cg multiplication is due to a specific effect.

In this study, it has been shown that at least some of the TOM2A homologues function to support TMV-Cg multiplication, but their contribution is much less than that of TOM2A. With regard to the respective contribution of each TOM2A homologue, a slight decrease in TMV-Cg CP accumulation was observed at an early time point of infection (4 days after inoculation) in 2a/h1 and 2a/h2 double mutants, but not in the 2a/h3 mutant, compared with that in 2a single mutant plants (data not shown). Therefore, the contribution to TMV-Cg multiplication is likely to be TOM2A>>TOM2AH1, TOM2AH2>>TOM2AH3. This conclusion highlights the strong dependence of TMV-Cg multiplication on TOM2A.

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**Fig. 3.** Effects of mutations in TOM2A family members on virus multiplication in protoplasts. *Arabidopsis* protoplasts (0.5–1.0×10^6) of the indicated genotypes were prepared from fully expanded leaves and inoculated with TMV-Cg, ToMV or CMV virion RNAs (3 μg each), according to the protocol obtained from J. Sheen’s laboratory (http://genetics.mgh.harvard.edu/sheenweb/main_page.html). Consistent results were obtained in three independent experiments and a typical dataset is indicated here. (a) CP accumulation. Inoculated protoplasts were harvested at 20 h post-inoculation (h p.i.). Viral CPs were detected by the immunoblotting method (upper panels). Control samples derived from mock-inoculated wt protoplasts were run in the left-most lanes (M). Bands of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RuBisCOL) detected by Coomassie brilliant blue staining are shown as loading controls (lower panels). (b) Accumulation of virus-related RNAs. Protoplasts were harvested at 2 and 20 h.p.i. and the accumulation of viral RNAs was examined by Northern blotting and hybridization (upper panels). ToMV RNAs were detected as described by Kubota et al. (2003) except that the *NcoI–MluI* fragment of ToMV cDNA was used as a probe. TMV-Cg and CMV RNAs were detected as described by Yoshii et al. (1998). Positions of tobamovirus genomic RNA (G) and CP subgenomic (sg) RNA are indicated on the left and those of CMV genomic RNAs 1, 2 and 3, and sg RNA4 are indicated on the right. rRNA bands detected by ethidium bromide staining are shown as loading controls (lower panels).
For ToMV multiplication, 2a/h1/h3, 2a/h2/h3 and h1/h2/h3 triple mutations did not show any drastic effect. However, because the expression of TOM2A in the toms2a toms2b double mutant increases ToMV multiplication (Tsujimoto et al., 2003), TOM2A has some function to support ToMV multiplication at least in the toms2b genetic background. It is possible that TOM2A and its homologues (at least TOM2AH1 and TOM2AH2) play a parallel role in ToMV multiplication and the presence of one of these genes is enough for ToMV to accumulate to wild-type levels.

It is also possible that, in the wild-type TOM2B background, none of the TOM2A family members participates in ToMV multiplication. Even if this is the case, TOM2A should be positioned in close proximity to ToMV replication proteins, because TOM1 (and its homologues), which is absolutely required for the multiplication of several tobamovirus species including ToMV (Yamanaka et al., 2002; Asano et al., 2005; Fujisaki et al., 2006), interacts with both TOM2A and the replication proteins of tobamoviruses (Yamanaka et al., 2002; Tsujimoto et al., 2003). If crucifer-infesting tobamoviruses have evolved from a ToMV-like prototype virus, it seems plausible that the toboamovirus prototype gained the ability to specifically utilize TOM2A during this adaptation to cruciferous plants and achieved highly efficient multiplication in them.

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