Supplemental Data

Tomato Brown Rugose Fruit Virus Resistance Generated by Quadruple Knockout of Homologs of *TOBAMOVIRUS MULTIPLICATION1* in Tomato

Masayuki Ishikawa[1], Tetsuya Yoshida[1], Momoko Matsuyama[1], Yusuke Kouzai[2], Akihito Kano[3], Kazuhiro Ishibashi[1]

[1] Crop Disease Research Group and [2]Crop Stress Management Group, Division of Plant Molecular Regulation Research, Institute of Agrobiological Sciences, NARO, 2-1-2, Kannondai, Tsukuba, Ibaraki 305-8602, Japan
[3] Plant Breeding and Experiment Station, Takii and Company, Limited, Konan, Shiga 520-3231, Japan

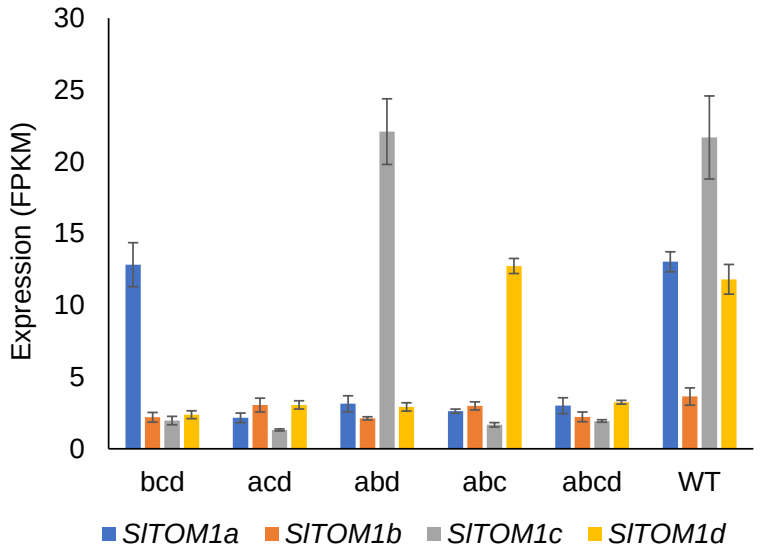
A

```
SlTOM1a   1:-MGRVETAVDPSSTAAVAAYRLHEAISWWDEVNESPIWQDR-IFYVLAILYGVVSAVALV 58
SlTOM1b   1:MVKSISFLLEFAKDYDDGTPRVRPIFDWFNAMMEVSDYEKQAIFYSLSAAYALVSFVALV 60
SlTOM1c   1:-MARLPLGSSPIDIAG-------PVTNWWDHVNESVQWQDG-IFYSLCASYGLVSAVALI 51
SlTOM1d   1:-MGRAEMVVGPSEKVAVVAYHLNDAINWWDDVNRSLDWQNR-IFHVLAVLYGVVAVVALV 58
             . .      .                *.. ....  ..   **. *   *. *. ***.

SlTOM1a  59:QLIRIQMRVPEYGWTTQKVFHFLNFLVNGVRSLVFVFRRDVQKLNPEIIQHILLDMPSLA 118
SlTOM1b  61:QLIRIQLRLSGIGWTTQKVFHLMNFVVCGLRAILFGFYSSVFNLRSKALEMMLLDLPGLL 120
SlTOM1c  52:QLIRIDLRVPEYGWTTQKVFHLMNFVVNGVRAIVFGFHKHVFLLHYKVLTLAILDLPGLL 111
SlTOM1d  59:QLIRIQMRVPEYGWTTQKVFHFLNFFVNGVRSLVFTFRRDVQKLHPEIVQHIMLDMPSLA 118
            *****. *....*********  ** *.*.*  .* *   *  *          ** * *

SlTOM1a 119:FFTTFALLVLFWAEIYYQARAVSTDALRPSFFTINGVVYAIQIILWLIIWWKPVPVLVIL 178
SlTOM1b 121:FFSTYTLLVLFWAEIFHQARNLPIDKLRPAYYAVNAVVYFIQICIWIFIGVGPASAAVET 180
SlTOM1c 112:FFSTFTLLVLFWAEIYHQARSLPTDKLRISYIAINDAIYFIQACIWVYLWINDNSTVEFI 171
SlTOM1d 119:FFTTYALLVLFWAEIYYQARAVSTDGLRPSFFTINGVVYAIQIILWLIMWWKPIRVLFIL 178
            ** *  *********. ***   .* **..   .* ..* **.  *   .  .

SlTOM1a 179:SKAFFAGVSLFAALGFLLYGGRLFLMLRRFPVESRGRQKKLQEVGYVTTICFSCFLIRCI 238
SlTOM1b 181:AKLFFAVISFTAALGFVMYGGRLFAMLRRFPIESRGRQKKLHEVGFVTGICCICFMIRCV 240
SlTOM1c 172:GKIFMAVVSVIAALGFLLYGGRLFLMLRRFPIESKGRRKKLHEVGSVTAICFTCFLIRCF 231
SlTOM1d 179:SKMFFAGVSLFAALGFLLYGGRLFLMLQRFPVESRGRRKKLQEVGYVTTICFSCFLIRCV 238
            * *.* .*  *****..******.**.*** **.** *** *** ** **. **.***

SlTOM1a 239:MMCFNAFDKAADLDVLYHPMLNFVYYLLVEILPSSLVLFILRKLPPKRGITQYHPIR    295
SlTOM1b 241:MVAVSAFNGNADVDVIDHPVLILFYYVVVEILPSVLVLFILRKLPPKRVSEQYHPIQ    297
SlTOM1c 232:VVVLSAFDSDASLDVLDHPVLNLIYYLLVEILPSALVLYILRKLPPKRVSAQYHPIS    288
SlTOM1d 239:MMCFNAFDKAADLDVLYHPILNLIYYLLVEILPSSLVLFILRKLPPKRGITQYHPIH    295
            .    **.  *..**. ** *.. **..****** ***.*********   *****
```

B

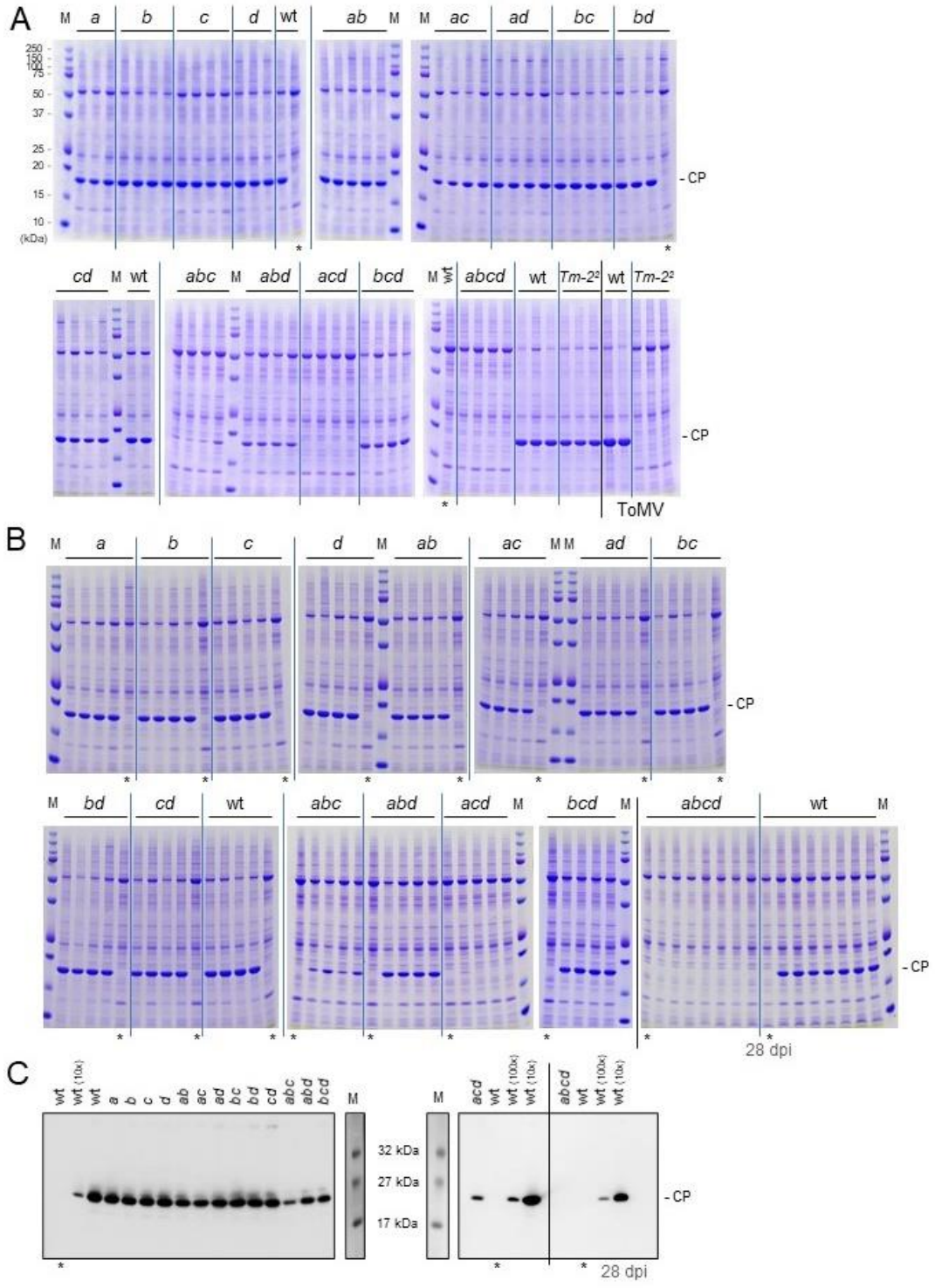
	SlTOM1b	SlTOM1c	SlTOM1d
SlTOM1a	55% (153/277)	63% (171/269)	86% (255/295)
SlTOM1b		64% (175/273)	53% (148/277)
SlTOM1c			62% (169/269)

Supplemental Figure S1. Comparison of amino acid sequences of SlTOM1 proteins.
A. Alignment of amino acid sequences of SlTOM1 proteins. Sequences were aligned using the Multiple Sequence Comparison by Log-Expectation algorithm provided in the GENETYX ver. 12 software. Amino acid residues identical among the four and three proteins are marked by asterisks and dots, respectively.
B. Identity of amino acid residues between SlTOM1 proteins. Identity of amino acid residues (%) was calculated using the fastp program provided in the GENETYX ver. 12 software. The number of identical amino acid residues per the length of aligned regions are shown in parentheses.



Supplemental Figure S2. Expression of *TOM1* homologs in wt, *Sltom1* quadruple-, and triple-mutant tomato plants.

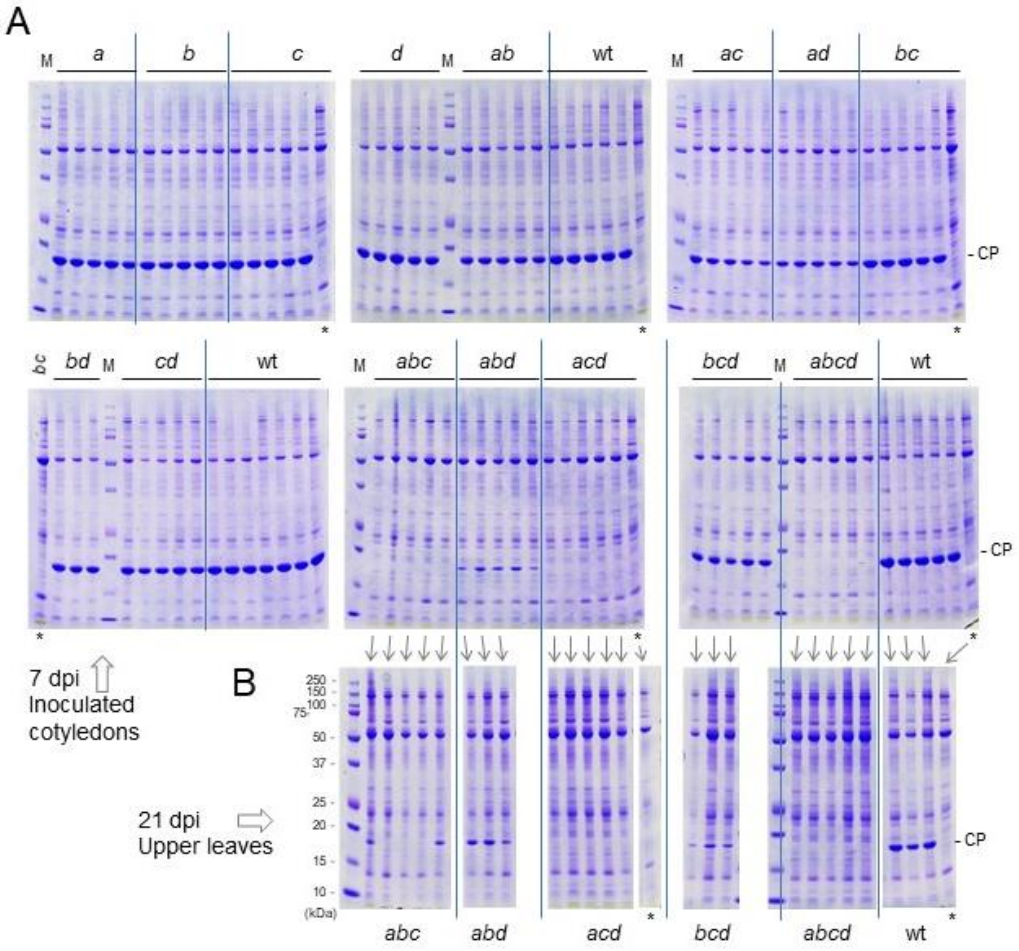
RNAs extracted from leaves from each genotype were analyzed by RNA sequencing. Total RNA was extracted from young true leaves of tomato (19 days after imbibition) with RNAiso Plus (TaKaRa). Library preparation and RNA-seq analysis were carried out by BGI GENOMICS. Briefly, poly(A)$^+$ RNA was purified by oligo dT selection. The poly(A)$^+$ RNA was fragmented and first-strand cDNA was synthesized using random N6 oligo DNA as primers, followed by a second-strand cDNA synthesis with dUTP. The synthesized cDNA was subjected to end-repair and then was 3' adenylated. Adaptors were ligated to the ends of these 3' adenylated cDNA fragments, and the dUTP-marked second-strand cDNA was selectively degraded by Uracil-DNA-Glycosylase. Resultant single-stranded cDNA was used to prepare DNA nanoball (Drmanac et al. 2009. Science. 327: 78–81.) and sequenced using the DNBSEQ platform (paired end, sequence length: 150). Clean reads were obtained using the filtering software SOAPnuke (Chen et al. 2018. Gigascience 7: 1–6.), mapped to the reference genome (Species: Solanum_lycopersicum_4081; Reference Genome Version: GCF_000188115.4_SL3.0) using the Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) software (Kim et al. 2015. Nature Methods 12: 357–360.), and aligned to the reference genes using the Bowtie2 software (Langmead et al. 2012. Nature Methods 9: 357–359). Means ± SEM of the numbers of fragments per kilobase of exon per million reads mapped (FPKM) from three independent plants are indicated. Small letters indicate the genes disrupted. WT: wild-type.



Supplemental Figure S3. ToBRFV CP accumulation in wt, *Sltom1* single-, double-, triple-, and quadruple-mutant and *Tm-2²* plants.

A. Whole Coomassie blue-stained gel images used to make Figure 2A. For details, refer to the legend to Figure 2A.

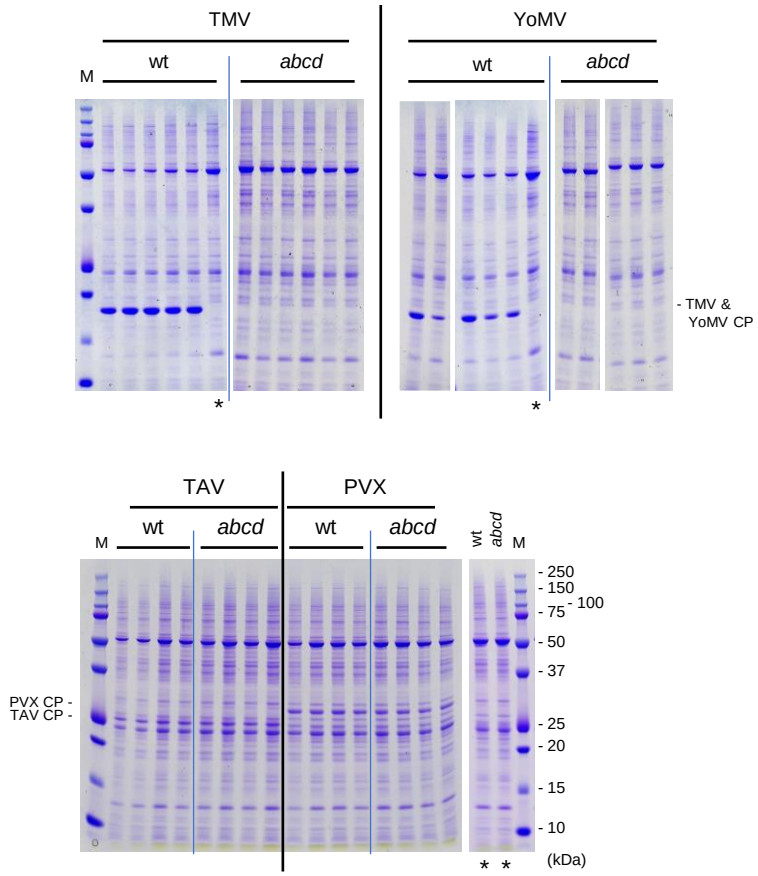
B. ToBRFV CP accumulation in another set of experiment. In *Sltom1* single-, double-, and triple mutants, CP accumulation in inoculated cotyledons at 7 dpi was analyzed. In *Sltom1* quadruple mutant, CP accumulation in uninoculated first true leaves of cotyledon-inoculated *Sltom1* quadruple-mutant plants at 28 dpi was analyzed (the gel marked with '28 dpi'. CP accumulation was analyzed and presented as in panel A.
C. Detection of ToBRFV CP by immunoblotting. For each *Sltom1* genotype (indicated above the blots; also see the legend to Figure 2A), equal volumes of the 4 protein samples appeared in panel B of this figure were mixed and CP accumulation was examined by SDS-PAGE (NuPAGE; Invitrogen, 12%) and immunoblotting using anti-ToBRFV antibody (AS1236, DSMZ). Samples prepared in parallel from non-inoculated plants were analyzed for comparison and are indicated by asterisks. Samples from ToBRFV-infected plants that were diluted 10-fold or 100-fold were simultaneously analyzed (marked with '10x' and '100x', respectively). From comparison of CP signal intensity, CP accumulation in *Sltom1abc* and *Sltom1acd* triple mutant plants was roughly estimated to be 10% and 1% of that in wild-type plants, respectively at 7 dpi. In *Sltom1abcd* quadruple mutant plants, CP was not detected also by immunoblotting (< 1% of CP accumulation in wild-type plants) at 28 dpi. M: DynaMarker Protein MultiColor (BioDynamics Laboratory Inc.). Positions of ToBRFV CP bands are indicated by 'CP'. Numbers represent approximate molecular masses of the marker proteins in kDa.



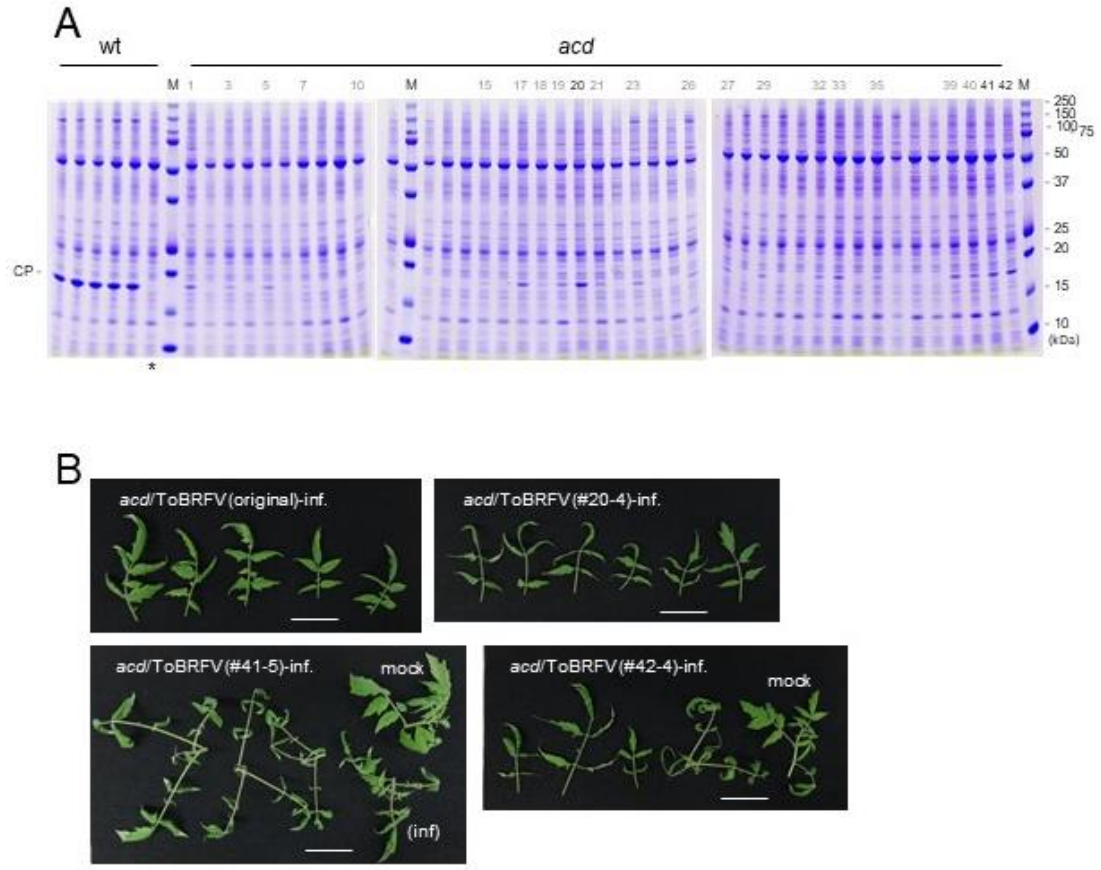
Supplemental Figure S4. ToMV CP accumulation in wt, *Sltom1* single-, double-, triple-, and quadruple-mutant plants.

A. ToMV CP accumulation in inoculated cotyledons at 7 dpi. A part of the rightmost 12 lanes of the second row was used to make Figure 3A. CP accumulation was examined and presented as described in Figure 2A legend. Each lane corresponds to an individual plant. Samples prepared in parallel from non-inoculated plants were analyzed for comparison and are indicated by asterisks. M: Protein Marker Precision Plus Protein Dual Color Standards (Bio-Rad).

B. ToMV CP accumulation in uninoculated first true leaves at 21 dpi. Arrows indicate that the gel samples were prepared from the same plants. CP accumulation was analyzed and presented as in panel A. Numbers represent approximate molecular masses of the marker proteins in kDa.



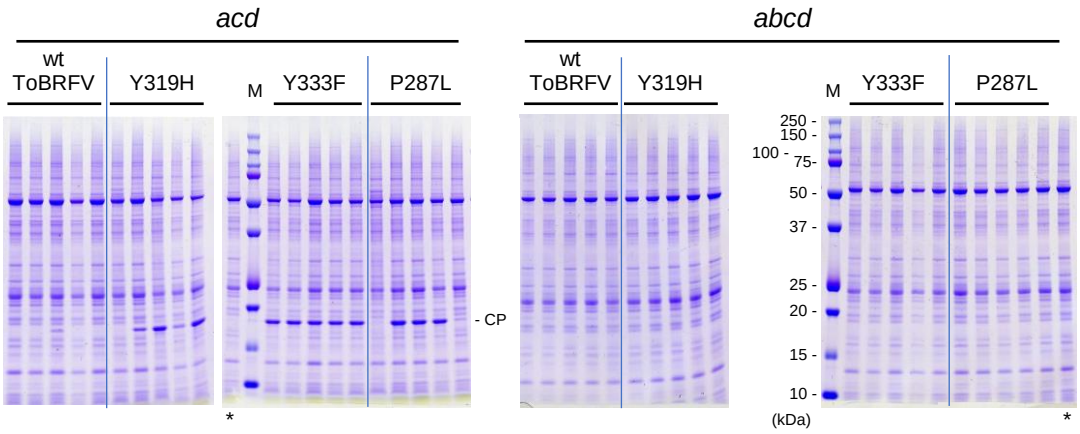
Supplemental Figure S5. TMV, YoMV, TAV, and PVX CP accumulation in wt and *Sltom1* quadruple-mutant plants. Whole Coomassie blue-stained gel images used to make Figure 3 are shown. For details, refer to the legend to Figure 3.



Supplemental Figure S6. Emergence of ToBRFV mutants in tomato *Sltom1acd* triple-mutant plants.

A. ToBRFV coat protein (CP) accumulation in wild-type (wt) and *Sltom1acd* triple-mutant (*acd*) plants. Upper uninoculated leaves of cotyledon-inoculated plants were harvested at 18 dpi, and CP accumulation was examined and presented as described in Figure 2A legend. Each lane represents an individual plant. A sample from a non-inoculated plant grown in parallel is shown for comparison (asterisk). *Sltom1acd* plants corresponding to the numbered lanes accumulated the CP. M: Protein Marker Precision Plus Protein Dual Color Standards (Bio-Rad).

B. Upper uninoculated leaves of *Sltom1acd* triple-mutant plants to which ToBRFV virions propagated in wild-type (original) or *Sltom1acd* triple-mutant plants (#20-4, #41-5 and #42-4; see text) were inoculated. Photographs were taken at 22 dpi. Scale bars represent 5 cm.



Supplemental Figure S7. CP accumulation of ToBRFV mutants in *Sltom1acd* triple-mutant and *Sltom1* quadruple-mutant plants. Whole Coomassie blue-stained gel images used to make Figure 4B are shown. For details, refer to the legend to Figure 4B.

Supplemental Table 1. DNA oligonucleotides used to construct the sgRNA sequence-containing entry clones.

target gene	orientation	sequence
SlTOM1a & *d*	forward	ATTGTTGTGAAGAATGCAAGACT
SlTOM1a & *d*	reverse	AAACAGTCTTGCATTCTTCACAA
SlTOM1b	forward	ATTGTCAAAGATGGGGCGAACTCG
SlTOM1b	reverse	AAACCGAGTTCGCCCCATCTTTGA
SlTOM1c	forward	ATTGCCGATTGACATCGCCGGTC
SlTOM1c	reverse	AAACGACCGGCGATGTCAATCGG