1 First report of *Pelargonium zonate spot virus* from wild Brassicaceae plants in Japan

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Abstract

Pelargonium zonate spot virus (PZSV) was identified from two wild Brassicaceae plant species, *Arabidopsis halleri* and *Rorippa indica*, in central Japan using RNA-Seq and reverse transcription polymerase chain reaction. The deduced amino acid sequences of RNA-dependent RNA polymerase and coat protein were highly similar to those of previously reported PZSV isolates, with 96.6%–98.2% and 93.7%–98.0% identity, respectively. Mechanical inoculation revealed the pathogenicity of the PZSV isolate to *Nicotiana benthamiana* and *Brassica oleracea*. To the best of our knowledge, this is the first report of PZSV from Japan.

Keywords

Pelargonium zonate spot virus, *Arabidopsis halleri*, *Rorippa indica*, Japan, RNA-Seq, *Brassica oleracea*

Pelargonium zonate spot virus (PZSV) belongs to the family *Bromoviridae* genus *Anulavirus* and consists of three linear, positive-sense single-stranded RNAs (ssRNAs) (Codoner & Elena, 2006). The three RNA segments, RNA-1, RNA-2, and RNA-3, encode protein 1a, protein 2a (RNA-dependent RNA polymerase, RdRP), and two other proteins (movement and coat proteins), respectively (Finetti-Sialer & Gallitelli, 2003). PZSV was isolated from *Pelargonium zonale* (Quacquarelli and Gallitelli 1979 cited in Finetti-Sialer and Gallitelli 2003) and has been reported to be a causal agent of tomato diseases in Italy (Gallitelli, 1982), Spain (Luis-Arteaga & Cambra, 2000), France (Gebre-Selassie *et al.*, 2002), Israel (Lapidot *et al.*, 2010), Australia (Luo *et al.*, 2010), Argentina (Giolitti *et al.*, 2014) and USA (Liu & Sears, 2007). PZSV has also been shown to have a wider host range, including plants from Solanaceae, Actinidiaceae, Brassicaceae, and Asteraceae (reviewed in (Li *et al.*, 2016)). In this study, we report the first cases of PZSV infection in wild Brassicaceae plants in Japan.

We made samplings in two localities where multiple Brassicaceae species co-occur, i.e. Mino-gawa (Mino, Osaka Prefecture, Japan, alt. ca. 200 m, June 18, 2014) and Gongen-dani (Taga, Shiga Prefecture, Japan, alt. ca. 320 m, June 28, 2014). The former and the latter communities contained four [*Arabidopsis halleri* subsp. *gemmifera* (*A. halleri*, hereafter), *Rorippa indica*, *Cardamine scutata* and *C. occulta*] and six (*A. halleri*, *R. indica*, *C. leucantha*, *C. impatiens*, *C. hirsuta* and *Arabis flagellosa*) Brassicaceae species, respectively. At Mino-gawa, *A. halleri* grew by forming patches along the valley, and we collected 17 leaves (Fig. 1a) from 17 plant patches (one sample/ patch) at 15–20-m intervals within a sampling range of approximately 350 m in distance. At Gongen-dani, we collected 9 leaves of *R. indica* (Fig. 1a) along the valley at 5–10-m intervals within a sampling range of approximately 100 m. Immediately after sampling of cauline leaves, each sample was immersed in 1.0 mL RNAlater (Life Technologies, CA, USA) to avoid

RNA degradation. Total RNA was extracted from each sample using 300–600 μL (10 volumes) TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. We conducted RNA-Seq with selective depression of rRNA by thermostable RNaseH (Morlan *et al.*, 2012, Nagano *et al.*, 2015) using a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) at Macrogen Japan. Detailed procedures for the RNA-Seq library preparation, mapping, and virus detection are described in a previous study (Kamitani *et al.* 2016). We obtained 1.8 Gb (giga base) sequence data in total that contained 8,132,772 and 9,820,064 reads from *R. indica* (9 samples) and *A. halleri* (17 samples), respectively. We determined the infecting viruses by considering the coverage of virus genome and the amount of reads which mapped on the virus genome.

PZSV was detected in five samples of *A. halleri* and four samples of *R. indica* (red bold IDs in Fig. 1a and red circles in Fig. 1b). Some infected leaves showed chlorosis or yellowing, but others did not show visible symptom at a leaf level (red bold IDs in Fig. 1a). Therefore, we could not judge whether symptom-like phenotypes of our samples had been caused by the infection of PZSV. Eight of the nine putative infected samples contained nearly the full length of the PZSV genome sequence, as indicated by the nearly 100% genome coverage by RNA-Seq reads (Fig. 1b). One sample of *A. halleri* showed relatively low coverage (24%) which may be caused by low read number in this sample. In addition to PZSV, *Turnip mosaic virus* (TuMV), *Cucumber mosaic virus* (CMV) and *Brassica yellows virus* (BrYV) were detected from *A. halleri* at Mino-gawa. In 5 PZSV-infected samples, two samples showed coinfections with TuMV (A11 in Fig. 1a) and with TuMV and CMV (A8 in Fig. 1a), respectively. No other virus was detected from *R. indica* at Gongen-dani.

To characterize PZSV sequences from each host species, consensus sequences of RdRP, encoded in RNA-2, and coat protein (CP), encoded in RNA-3, were determined

using the RNA-Seq reads, respectively. The nucleotide and deduced amino acid (AA) sequences from the two hosts were deposited in GenBank (accession numbers were shown in Tables 1 and 2). The two AA sequences of RdRP and CP were similar (> 96% identity and > 93% identity, respectively) to those of three previously reported PZSV isolates (Tables 1 and 2). We found 35 polymorphic AA sites in RdRP across the five sequences (Fig. S1).

To test the pathogenicity of the virus, mechanical inoculation was conducted using two infected leaves of *R. indica* collected at the Gongen-dani site. Each leaf was crushed completely in 500-µL of 0.1M phosphate buffer (pH 7.4, consists of disodium hydrogen phosphate and sodium dihydrogen phosphate) and inoculated onto *Nicotiana benthamiana* using a carborundum (600 mesh). At 28–35 days post-inoculation (dpi), *N. benthamiana* showed a susceptible phenotype with mild chlorosis and stunting relative to the uninfected plant (Fig. 2a, b). The stunting symptom by the infection of PZSV has been reported in *N. benthamiana* (Lapidot *et al.*, 2010). We extracted RNA from inoculated/upper leaves of PZSV-inoculated or buffer (mock)-inoculated plants at 28 dpi. RT-PCR was conducted to confirm the infections by amplifying RNA-3 genome fragment of PZSV (403 bp in length). The RT reaction was conducted using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies) with random primers. PCR was conducted using KOD -plus- (TOYOBO, Japan) with specific primers (forward, 5'-AGATTTTTCCGGGCTCTCTA-3' and reverse, 5'-GTTCAACTGTTTTACCAGGATAG-3'). Based on the RNA 3 sequences from our study sites, the sequence of the primers were modified from a previous study (Choi *et al.*, 2013). The RT-PCR assay detected PZSV from both inoculated leaves and upper leaves (Fig. 2c), suggesting that the virus caused systemic infection in *N. benthamiana*.

Mechanical inoculations were also conducted to test the pathogenicity of the virus in

Solanum lycopersicum (tomato, cultivar 'Momotaro' obtained from TAKII SEED, and another 1 cultivar) and *Brassica oleracea* var. *capitata* (cabbage, cultivar ‘Shoshu’, obtained from TAKII SEED). For all cultivars of *S. lycopersicum* and *B. oleracea*, in total four plant individuals (two for inoculation and two for mock inoculation) were used for the test. From 20 dpi and later, *B. oleracea* showed symptoms of chlorosis and occasional ring patterns along the leaf veins (Fig. 3a) that were distinct from mock-inoculated phenotypes (Fig. 3b). RT-PCR confirmed systemic PZSV infection in inoculated *B. oleracea*, and we continually observed symptoms on newly expanded leaves. Although further quantitative evaluation is required, we judged that PZSV has a potential risk to reduce the cabbage yield. PZSV has been the causal agent of multiple disease outbreaks in commercial tomato crops (Hanssen *et al.*, 2010), but infection was not detected either of the two cultivars of *S. lycopersicum*. We also tested mechanical inoculations on two *A. halleri* plants, but infection was not detected.

In this study, we identified PZSV from two natural Brassicaceae populations in Japan and, to our knowledge, this is the first report of PZSV in Japan. PZSV has been previously reported from Brassicaceae weeds, e.g. *Capsella bursa-pastoris* and *Diplotaxis erucoides* in Europe (Finetti-Sialer & Gallitelli, 2003) and *Cakile maritima* in Australia (Luo *et al.*, 2010), but has not been reported in cabbage. Because we found PZSV pathogenicity to one cultivar of cabbage, further studies are needed to determine whether the Japanese isolates of PZSV in *A. halleri* and *R. indica* can be transmitted to agricultural crops. It has been reported that the PZSV is transmitted from the Brassicaceae weed, *D. erucoides*, to tomato via pollen grains carried by thrips (Vovlas *et al.*, 1989, cited in Finetti-Sialer & Gallitelli, 2003). Our study indicated that surveys of virus infection in wild plants can contribute to improve our knowledge of potential reservoirs of pathogens for crop plants.

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Figure legends

Fig. 1. Detection of PZSV. **a.** Sampled leaves of *A. halleri* (Mino-gawa) and *R. indica* (Gongen-dani). The sample IDs are listed above the photos. The red bold IDs correspond to samples that were found to be infected by PZSV. Asterisks next to the sample IDs indicate the mixed-infection of PZSV with TuMV (*), and with TuMV and CMV (**). **b.** The amount of RNA-Seq reads mapped on the PZSV genome (vertical axis; virus reads per million host reads) and the genome coverage (horizontal axis) plotted for each leaf sample. The red circles indicate samples that were found to be infected by PZSV. The black circles represent samples without any PZSV reads.

Fig. 2. Inoculation experiment and confirmation of PZSV infection in *N. benthamiana*. **a.** Symptoms observed in *N. benthamiana* at 35 dpi in an inoculated plant (*right*) and a mock-inoculated plant (*left*). **b.** Chlorosis observed in a leaf of *N. benthamiana* on 28 and 35 dpi. **c.** Detection of PZSV genome fragments by RT-PCR. The characters “I” and “U” in the panel indicate inoculated leaves and upper leaves of the PZSV-inoculated plant, respectively. “Mock-I” and “Mock-U” indicate the inoculated and upper leaves of the mock (buffer)-inoculated plant, respectively.

Fig. 3. Inoculation of PZSV to *B. oleracea*. **a.** Symptoms observed in an upper leaf of the PZSV-inoculated *B. oleracea*. **b.** An upper leaf of the mock-inoculated *B. oleracea*.

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Fig. 1

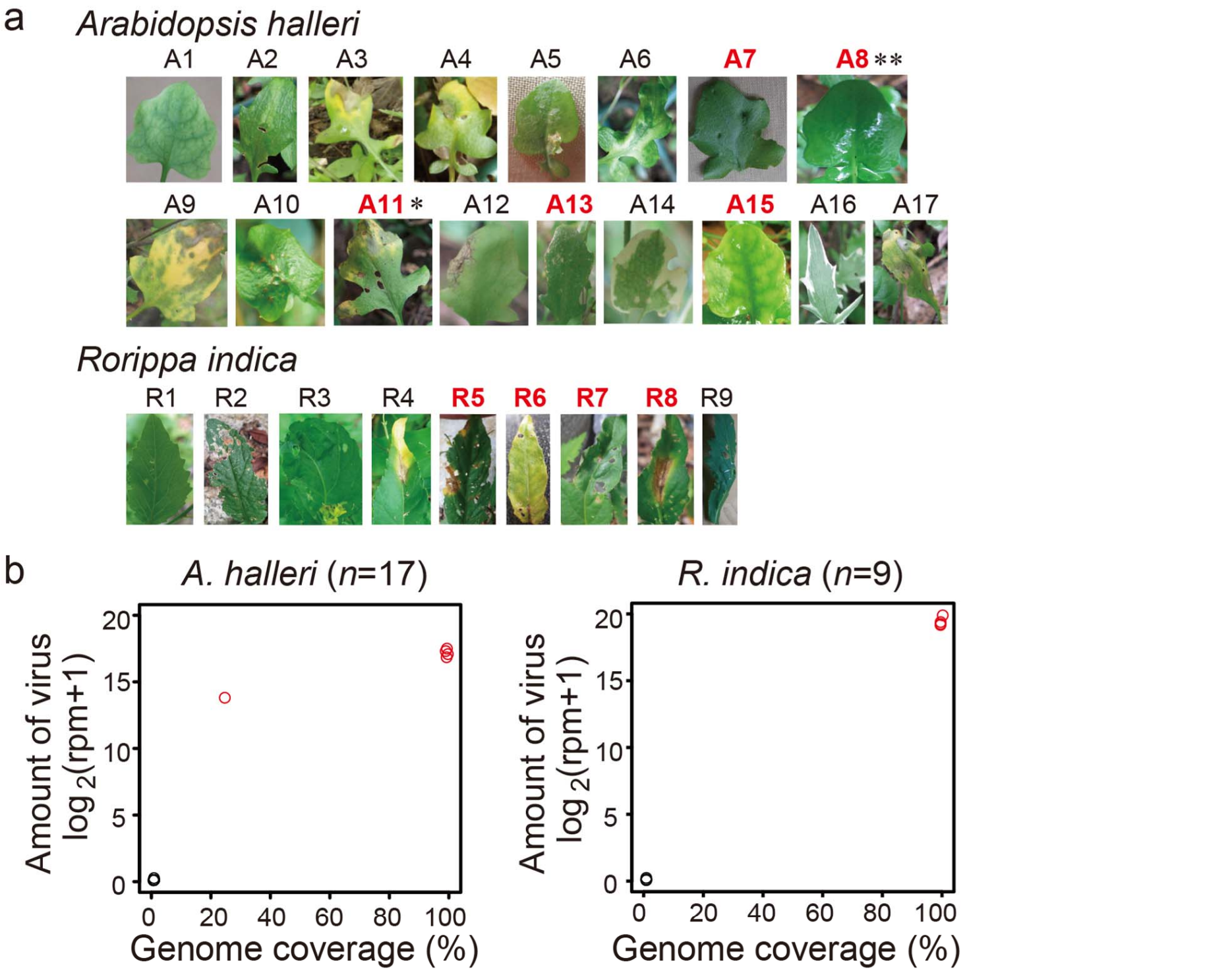

a
Arabidopsis halleri
A1
A2
A3
A4
A5
A6
A7
A8 **
A9
A10
A11 *
A12
A13
A14
A15
A16
A17
Rorippa indica
R1
R2
R3
R4
R5
R6
R7
R8
R9
b
A. halleri (n=17)
R. indica (n=9)
Amount of virus
log 2(rpm+1)
Genome coverage (%)
0
5
10
15
20
0
20
40
60
80
100

Fig. 2

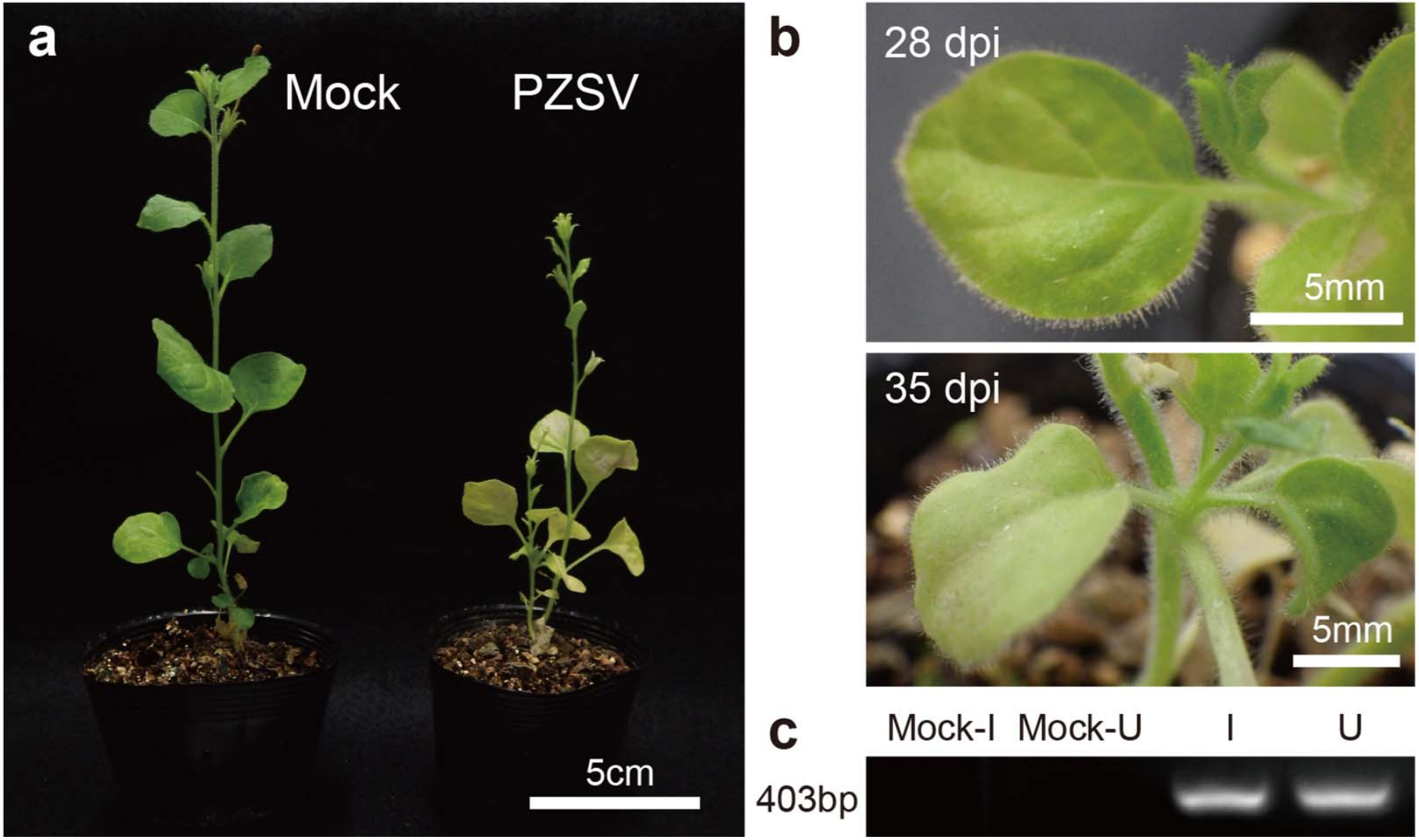
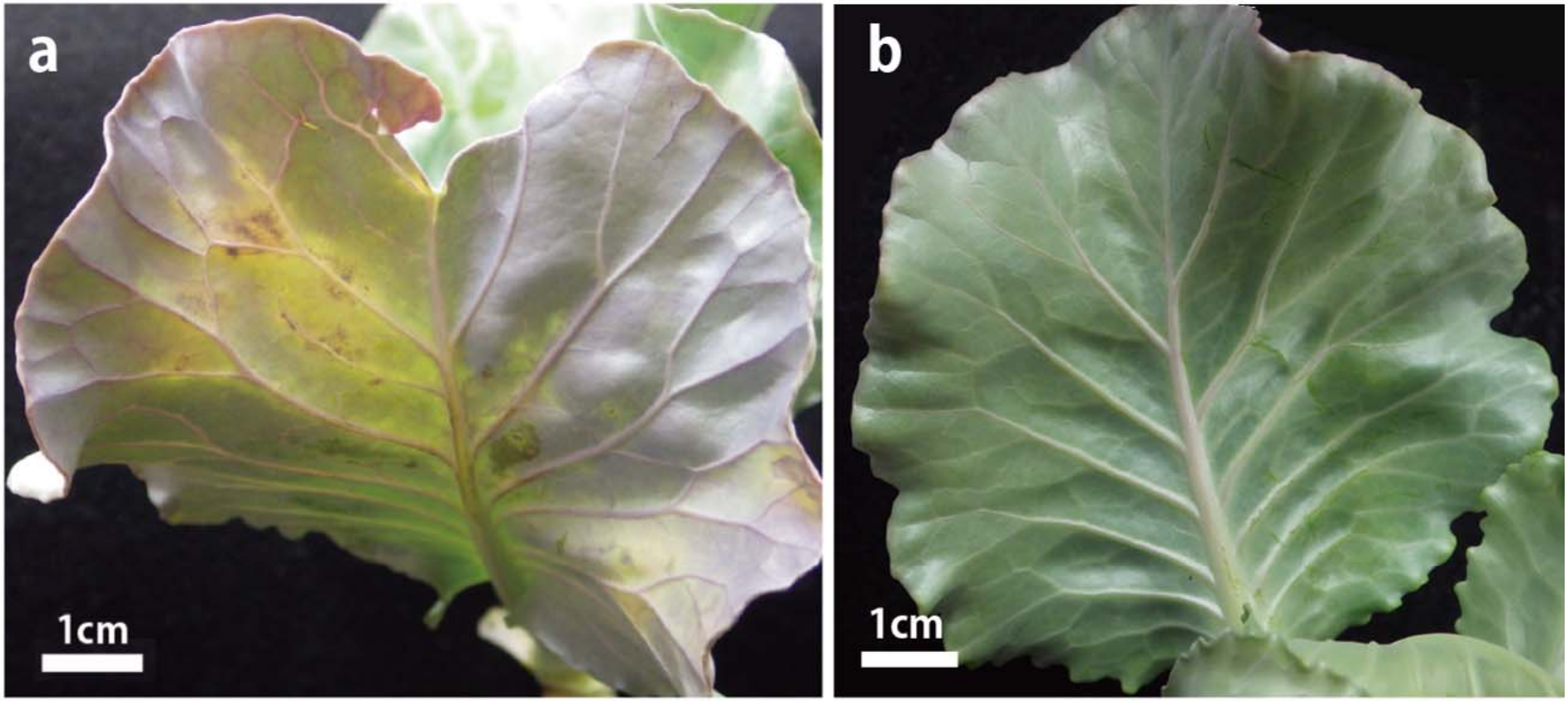


Fig. 3



Tabel 1 Identity of RdRP amino acid sequences between PZSV isolates

	Accession in Genbank	*A. halleri*, Mino-gawa	*R. inidica*, Gongen-dani	Isolate tomato	Isolate SW13
A. halleri, Mino-gawa[a]	LC178561	-	-	-	-
R. inidica, Gongen-dani[a]	LC178560	0.983	-	-	-
Isolate tomato, Italy[b]	NC_003650.1	0.966	0.966	-	-
Isolate SW13, Australia[c]	KF790761.3	0.979	0.978	0.969	-
Isolate parana, Argentina[d]	JQ350739.1	0.978	0.982	0.970	0.979

(a) this study, (b) Gallitelli (1982), (c) Luo *et al.* (2010), (d) Giolitti *et al.* (2014)

Tabel 2 Identity of CP amino acid sequences between PZSV isolates

	Accession in Genbank	*A. halleri*, Mino-gawa	*R. inidica*, Gongen-dani	Isolate tomato	Isolate SW13
A. halleri, Mino-gawa[a]	LC269013	-	-	-	-
R. inidica, Gongen-dani[a]	LC269014	0.966	-	-	-
Isolate tomato, Italy[b]	NC_003651.1	0.937	0.961	-	-
Isolate SW13, Australia[c]	KF790762.4	0.975	0.980	0.956	-
Isolate parana, Argentina[d]	JQ350737.1	0.956	0.971	0.951	0.980

(a) this study, (b) Gallitelli (1982), (c) Luo *et al.* (2010), (d) Giolitti *et al.* (2014)

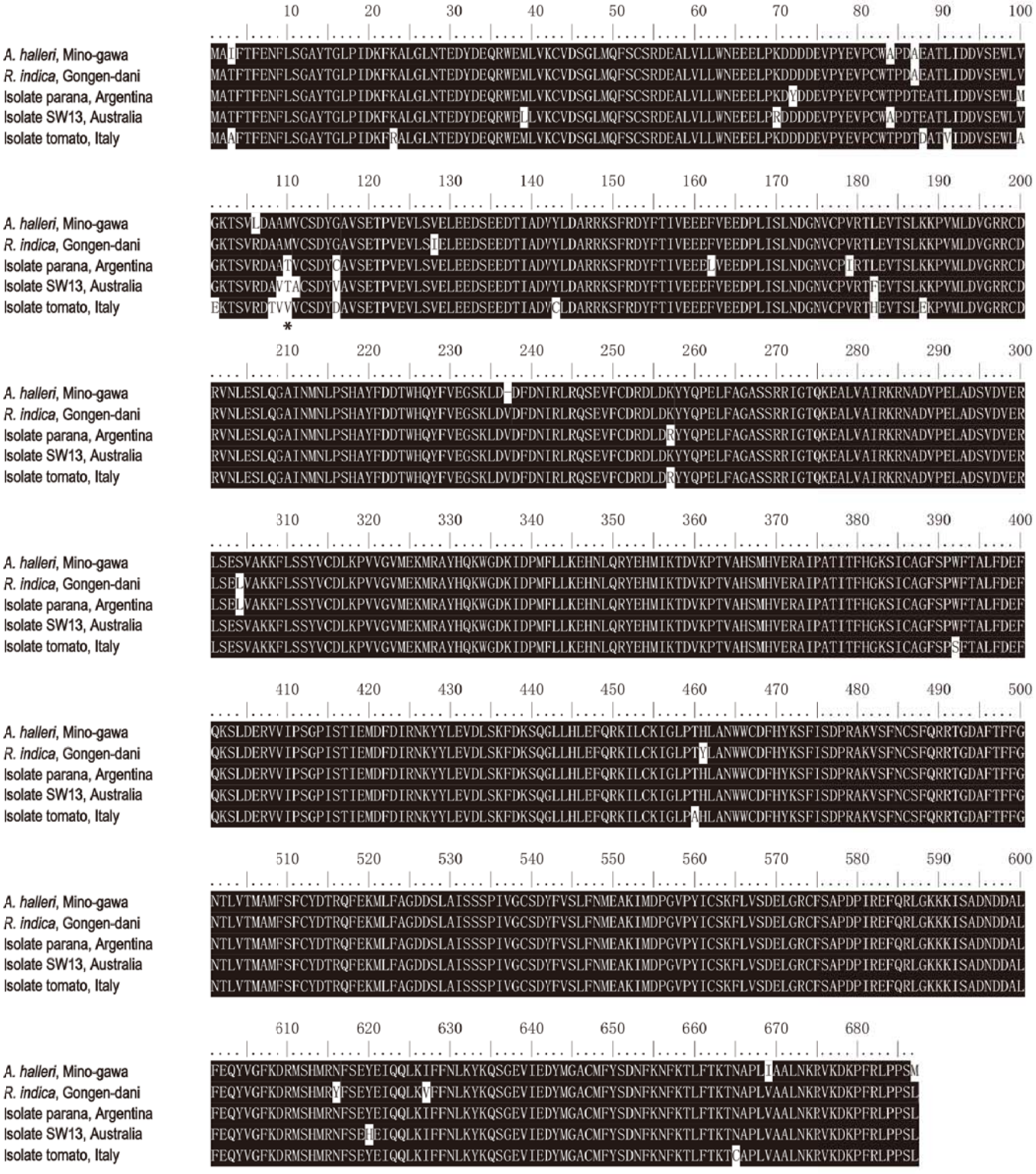


Fig. S1 Comparison of deduced amino acid (AA) sequences of RdRP obtained in this study and those reported in NCBI/Genbank (accession numbers in Table 1). Black shade indicates the major AA residule (n≥2) at the position. An astarisk under the sequence (AA position 110) indicates the position with two major AA (M and T).