A Replicase of *Potato virus X* Acts as the Resistance-Breaking Determinant for JAX1-Mediated Resistance

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Lectin-mediated resistance (LMR) has been suggested to comprise an uncharacterized branch of antiviral plant innate immunity. To unveil the feature of resistance conferred by jacalin-type lectin required for potexvirus resistance 1 (JAX1), a recently isolated LMR gene against potexviruses, we analyzed the resistance-breaking variants to find the viral component involved in resistance. We employed grafting-mediated inoculation, a high-pressure virus inoculation method, to obtain *Potato virus X* (PVX) variants that can overcome JAX1-mediated resistance. Whole-genome sequencing of the variants suggested that a single amino acid in the methyl transferase domain of the replicase encoded by PVX is responsible for this resistance-breaking property. Re-introduction of the amino-acid substitution to avirulent wild-type PVX was sufficient to overcome the JAX1-mediated resistance. These results suggest that viral replicase is involved in JAX1-mediated resistance. The residue that determines the resistance-breaking properties was highly conserved among potexviruses, suggesting a general role of the residue in potexvirus–JAX1 interactions.

Plant viral diseases cause serious economic losses in many major crops by reducing their yield and quality (Kang et al. 2005; Reddy et al. 2009). Use of resistant genetic resources can be the most reliable and cost-efficient method to protect crops against viral diseases. To date, over 200 resistance (*R*) gene loci mediating resistance against viruses have been introgressed into commercial crop cultivars and used throughout the world (Kang et al. 2005). However, the use of resistant cultivars with *R* genes often leads to the emergence of resistance-breaking viruses (Harrison 2002). Resistant cultivars usually reduce viral fitness and impose strong selection pressure on viruses, facilitating the survival of resistance-breaking strains from a population of virus variants.

Resistance-breaking viruses have one or more mutations in their genomes and, usually, an encoded protein that enables them to circumvent the host's resistance (Janzac et al. 2010). Such mutations are often found in viral avirulent proteins that

trigger resistance reactions when recognized by *R* proteins. The resistance-breaking determinants for *N*-mediated resistance to *Tobacco mosaic virus* (TMV) are found in the replicase of TMV (Padgett and Beachy 1993), which is an avirulent protein recognized by *N* protein and elicits the resistance responses (Padgett et al. 1997). The resistance-breaking determinants of *Rx*-mediated resistance to *Potato virus X* (PVX) were mapped to the coat protein (CP) (Kavanagh et al. 1992), which has been shown to be the avirulent protein (Kohm et al. 1993). Therefore, determining the mutations responsible for overcoming resistance is beneficial for revealing virus resistance mechanisms, such as how viruses are perceived by the host resistance machinery.

Plant innate immunity against viruses consists of two major defense machineries: RNA silencing and *R*-mediated resistance (Moffett 2009; Soosaar et al. 2005). RNA silencing is triggered when the host's Dicer-like proteins perceive double-stranded RNA of virus origin and digest them into small interfering RNAs (siRNAs) (Ding and Voinnet 2007). RNA-induced silencing complex formed by siRNA loading into a member of Argonaute family proteins targets viral RNA in a sequence-specific manner and degrades it. *R*-mediated resistance is induced when plant nucleotide-binding and leucine-rich repeat (NB-LRR) proteins recognize viral proteins, causing transcriptional activation of defense responsive genes, the production of reactive oxygen species, and hypersensitive responses (HR) (Moffett 2009).

In addition to the above-mentioned resistance mechanisms, plant jacalin-lectin genes have been suggested to compose another class of antiviral resistance genes and have been named lectin-mediated resistance (LMR) (Chisholm et al. 2000; Yamaji et al. 2012). Thus far, two such lectin genes have been isolated. One is restricted *Tobacco etch virus* (TEV) movement 1 (*RTM1*) from *Arabidopsis thaliana* ecotype Col-0, inhibiting the systemic movement of potyviruses including TEV, *Plum pox virus* (PPV), and *Lettuce mosaic virus* (Chisholm et al. 2000; Decroocq et al. 2006). The other is Jacalin-type lectin required for the potexvirus resistance 1 (JAX1) of *A. thaliana* ecotype Bay-0 that impairs cellular accumulation of potexviruses, including *Plantago asiatica mosaic virus* (PIAMV), PVX, *Asparagus virus 3*, and *White clover mosaic virus* (Yamaji et al. 2012). The viral CP has been shown to be responsible for RTM1-mediated resistance, based on a comparative analysis of naturally occurring avirulent and virulent isolates of PPV (Decroocq et al. 2009). However, little is known regarding the molecular mechanisms underlying LMR in either RTM1- or JAX1-mediated resistance.

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In this study, we employed a graft inoculation technique, which was a high-pressure virus inoculation method (Hajimorad and Hill 2001), to obtain variants of PVX that can overcome JAX1-mediated resistance. We found that a single amino acid in the replicase encoded by PVX is responsible for the resistance-breaking property. The reintroduction of the amino-acid substitution enabled avirulent wild-type (WT) PVX to overcome JAX1-mediated resistance. The highly conserved nature of this residue among potexviruses suggests a general role of this residue in potexvirus-JAX1 interactions.

Fig. 1. JAX1-mediated resistance-breaking *Potato virus X* (PVX) strains obtained through a grafting experiment. **A**, Schematic view of the grafting experiment. JAX1-transgenic *Nicotiana benthamiana* was grafted onto wild-type (WT) *N. benthamiana*. PVX was inoculated to the WT rootstock by agroinfiltration. **B**, Inoculated grafts at 4 weeks postinoculation with no visible symptom (NS) or with faint mosaic symptoms (1 to 5) on their scion leaves. **C**, Reverse-transcription polymerase chain reaction analyses detecting transcripts of viral RNA and JAX1. Total RNA extracted from scion leaves of symptomatic grafts (lanes 1 to 5) and of asymptomatic grafts (lane NS) were used as templates.

Fig. 2. Schematic representation of resistance-breaking *Potato virus X* (PVX) (JB1 and JB2) and their parental PVX (wild-type [WT]) sequences. Mutated sites are indicated with arrowheads on a representation of the PVX genome. The position of each mutation is shown on the upper side of each arrowhead, with the amino acid position of the replicase in parentheses. Nucleotide sequences and the amino acid of the resistance-breaking viruses are shown on the lower side of the figure. Mutated nucleotides are indicated in bold.

RESULTS

JAX1-mediated resistance-breaking virus variants obtained by graft inoculation.

We found that PVX could not infect JAX1-transgenic *Nicotiana benthamiana* plants when inoculated mechanically (Yamaji et al. 2012). We also inoculated JAX1-transgenic plants with PVX using agroinfiltration to achieve a higher level of inoculation pressure compared with mechanical inoculation. However, JAX1-mediated resistance toward PVX was so robust and stable that no resistance-breaking variants appeared despite repeated inoculation with the virus using agroinfiltration (data not shown). Therefore, we next tried graft inoculation to achieve higher inoculation pressure. We grafted JAX1-transgenic *N. benthamiana* onto nontransgenic rootstocks and inoculated PVX to the rootstock leaves (Fig. 1A). This allowed the virus to accumulate in the rootstock and JAX1-

Fig. 3. The Q336H mutation in replicase is sufficient to confer JAX1-mediated resistance-breaking ability. **A**, Schematic representation of *Potato virus X* (PVX) mutants. Mutated nucleotides are in bold. **B**, Symptoms of wild-type (WT) PVX and PVX mutants in JAX1-transgenic (JAX1+) or nontransgenic (JAX1-) *Nicotiana benthamiana*. Appropriate virus variants were inoculated to JAX1+ and JAX1- plants and photographs were taken at 14 days postinoculation. **C**, Reverse-transcription polymerase chain reaction analyses detecting viral RNA or JAX1 transcripts.

transgenic scions to be constantly challenged by the virus. As a result, we observed faint mosaic symptoms on the upper scion JAX1-transgenic leaves of five grafts among seven grafts tested 4 weeks postinoculation (Fig. 1B, lanes 1 to 5). The emergence of resistance-breaking PVX variants on the scion leaves of these grafts was confirmed by reverse-transcription-polymerase chain reaction (RT-PCR) of every graft that showed systemic mosaic symptoms (Fig. 1C, lanes 1 to 5).

Resistance-breaking viruses share an amino-acid substitution in their replicase.

Next, to analyze the cause of resistance breaking, we directly sequenced the whole-genomic sequences of these resistance-

breaking viruses. The sequences of viruses obtained from grafts 1, 2, 3, and 4 were completely the same (Fig. 2), having only one base substitution in the replicase gene and no changes in the rest of the sequences compared with their parental strain (WT). This substitution was at the nucleotide position 1,098 (A1098C) and was nonsynonymous, causing a Gln-to-His substitution of viral replicase at the amino-acid position 336 (Q336H). This resistance-breaking virus strain is hereafter referred to as JAX1-mediated resistance breaking 1 (JB1). The virus strain obtained from graft 5 had two base substitutions. One of them was at the position 1,098 (A1098T), which causes the same amino acid substitution (Q336H) as JB1. The other was at position 549 (G549A) and was synonymous. This resistance-breaking virus strain is hereafter referred to as JB2. In summary, all five independent resistance-breaking viruses share the same single amino-acid substitution (Q336H) in replicase, which strongly suggests the importance of this amino-acid residue in JAX1–PVX interactions.

Single amino-acid substitution in replicase conveys the resistance-breaking property.

To investigate the relevance of the Q336H mutation in overcoming JAX1-mediated resistance, we constructed PVX mutants that harbored the Q336H amino acid substitution and tested whether they were able to overcome the resistance using JAX1-transgenic plants (Fig. 3A). Of these mutants, 1092C and 549A1092T were identical to JB1 and JB2, respectively, and 1092T was another mutant harboring Q336H like JB2 but without the 549A substitution. When inoculated into JAX1-transgenic plants by agroinfiltration, 1092C and 549A1092T systemically induced mosaic symptoms on JAX1-transgenic plants at 21 days postinfiltration (Fig. 3B). However, 1092T failed to induce symptoms on JAX1-transgenic plants. To determine whether each mutant virus infected JAX1-transgenic plants, we performed RT-PCR to detect viral RNA in the upper leaves. Consistent with the appearance of mosaic symptoms, RT-PCR analyses successfully detected the accumulation of viral cDNA fragments from 1092C- and 549A1092T- but not

Fig. 4. *Potato virus X* (PVX) with the Q336H mutation accumulated higher than their parental strain in JAX1-transgenic plants. PVX (wild type [WT]) and PVX mutants that harbor the Q336H mutation were inoculated to JAX1-nontransgenic (JAX1–) or JAX1-transgenic (JAX1+) plants. The accumulation of viral RNA was quantified by real-time reverse-transcription polymerase chain reaction at 3 days postinoculation. The amount of viral RNA is presented relative to that of WT PVX on nontransgenic plants. Asterisks indicate statistical significance between each mutant and the WT using a *t* test (* and ** indicate *P* < 0.05 and 0.001, respectively). Data are shown as the mean value of four plants representative of two independent experiments.

Fig. 5. Q336 is broadly conserved in the genus *Potexvirus*. Amino-acid sequences of replicases from the genus *Potexvirus* were aligned. Closed arrowheads and the nucleotide numbers with amino acid numbers in parenthesis were shown on the upper side. The open arrowhead below the alignment indicates the residue corresponding to the Q336 residue of *Potato virus X* that determines resistance-breaking ability.

1092T-challenged JAX1-transgenic plants (Fig. 3C). Moreover, although 1092C and 549A1092C infected and showed systemic symptoms in nontransgenic plants, and viral RNA was detected by RT-PCR, 1092T still showed no symptoms and viral RNA of this mutant could not be detected. These results indicate that the 1092T mutant lacks the ability to infect even susceptible nontransgenic *N. benthamiana* plants independently of JAX1-mediated resistance.

Previously, we showed that JAX1 suppressed viral accumulation in inoculated leaves (Yamaji et al. 2012). To quantify the effect of JAX1-mediated resistance to each PVX mutant, we performed real-time quantitative RT-PCR to compare the accumulation level of each mutant viral RNA in the inoculated leaves (Fig. 4). In nontransgenic plants, RNA accumulation levels of all viruses were similar and not statistically significant. In JAX1-transgenic plants, the accumulation of WT PVX RNA drastically decreased and was more than 1,000-fold lower than that in nontransgenic plants. The accumulation of viral RNA from 1092C and 549A1092T, which could infect JAX1-transgenic plants systemically, also decreased in the inoculated leaves of JAX1-transgenic plants (29 and 24%, respectively) compared with those in nontransgenic plants. However, JAX1-transgenic plants had significantly higher accumulation of mutant RNA in the inoculated leaves than of WT PVX RNA. Moreover, 1092T, which could not systemically infect either JAX1-transgenic plants or nontransgenic plants, could accumulate to a certain level in the inoculated leaves of JAX1-transgenic plants and had a significantly higher accumulation of RNA than WT PVX. Note that the detected RNA in JAX1-transgenic plants did not result from the transient expression of viral RNA in agroinfiltrated tissues because the RNA of WT PVX was not detected at a similar level in the agroinfiltrated leaves of JAX1-transgenic plants.

Q336 is widely conserved among potexviruses.

JAX1 is known to confer broad resistance to potexviruses. Therefore, to gain insight into the significance of Q336 from PVX replicase in JAX1-mediated resistance, we investigated the conservation status of the amino acid residue among potexviruses. To this end, we aligned amino acid sequences of potexviral replicases to see whether Q336 is conserved in other potexviruses. Amino acid residue corresponding to Q336 of PVX (Fig. 5, arrowhead) is completely conserved among all the potexviruses tested.

Because the amino acid corresponding to Q336 of PVX is highly conserved in potexviruses, we speculated that this residue would be involved in JAX1–virus interactions in other potexviruses as well. To test this possibility, we introduced a mutation in Q320 of replicase encoded by PIAMV, another potexvirus, which corresponds to Q336 of PVX replicase. We introduced three single-nucleotide mutations into the infectious cDNA of PIAMV. Both 1046T and 1046C substitute Q320 with histidine (Q320H), whereas 1046A did not cause an amino acid change. We inoculated JAX1-transgenic and nontransgenic *N. benthamiana* with these three mutants (1046T, 1046C, and 1046A). As a result, 1046A, which has a synonymous mutation, induced severe mosaic symptoms on nontransgenic plants similar to parental PIAMV (Supplementary Fig. S1A) but did not induce mosaic symptoms on the JAX1-transgenic plants. On the other hand, 1046T and 1046C, which have corresponding substitutions of Q336H, failed to induce symptoms on both nontransgenic and JAX1-transgenic plants, indicating that this substitution disrupted viral infectivity. This result may suggest that Q320 is important for PIAMV infection. However, whether conserved glutamine residue is also a virulence determinant against JAX1-mediated resistance in PIAMV was not determined by this experiment.

DISCUSSION

To date, two plant lectin genes, RTM1 and JAX1, have been reported to function in plant immune systems against viral pathogens. Although both RTM1 and JAX1 encode similar jacalin-type lectin proteins, they have distinct mechanisms of viral inhibition: RTM1 limits systemic viral movement while JAX1 restricts virus accumulation at the cellular level (Chisholm et al. 2000; Mahajan et al. 1998; Yamaji et al. 2012). In RTM1-mediated resistance, viral CP is reported to be a resistance-breaking determinant (Decroocq et al. 2009). Here, we obtained JAX1 resistance-breaking PVX strains and showed that only one amino-acid substitution in replicase is responsible for the resistance-breaking property, suggesting that the replicase of PVX is involved in JAX1-mediated resistance. This study highlights the difference between two antiviral plant lectin genes that have distinct mechanisms of viral inhibition.

In the JAX1–PVX interaction, the resistance-breaking determinant, Q336H of replicase, is located in the methyltransferase (MET) domain (1 to 421 amino acids [aa]) but not in the seven conserved motifs (I, It1, II, IIt1, IIt2, IIt3, and IV) encompassing 60 to 224 aa (Rozanov et al. 1992). Although it was located outside the core region of the MET domain, the residue was highly conserved among various potexvirus species, implying its functional importance. Consistent with this observation, the mutation of the conserved residue impaired PIAMV infectivity. It is often the case that acquisition of the resistance-breaking property results in the diminishing of general fitness in susceptible host plants. For example, in the case of PVX mutants that overcome Rx-mediated resistance, the accumulation of viral RNA in susceptible protoplasts decreased compared with that of WT PVX (Goulden et al. 1993). In the case of *Potato virus Y* isolates that overcome Pvr4-mediated resistance, such variants could not compete with avirulent strains when co-inoculated to susceptible host plants (Janzac et al. 2010). In our study, introduction of the resistance-breaking mutation into PIAMV invariably disrupted its infectivity but was not critical for PVX infection. Further studies are required to evaluate such fitness costs in PVX infection.

Among the three PVX mutants examined in this study, 1092C and 549A1092T, corresponding to JB1 and JB2, respectively, could infect *N. benthamiana* systemically but 1092T could not. Unexpectedly, the accumulation of 1092T in the inoculated leaves was similar to those of WT PVX and the other two mutants. These results indicate that 1092T has a defect in terms of systemic movement. Because all three mutants had the same amino acid substitution (Q336H), this amino acid change in replicase may not be the cause of 1092T's loss of systemic movement. Moreover, 549A1092T restored infectivity with an additional mutation at the position 549 nucleotides (nt), which is a mutation synonymous with 1092T. These results suggest that the RNA level defect of 1092T causes the inability of systemic movement and that RNA sequences at approximately 1,092 and 549 nt may have some unknown interaction that facilitates viral systemic movement. To our knowledge, this is the first indication that the PVX genomic region encoding replicase is involved in viral systemic movement and that the RNA sequence or structure is involved.

In this study, we employed a graft inoculation method to enhance inoculation pressure. Differences in inoculation procedures can critically affect the viral infectivity (Zhang et al. 2011). TMV and *Soybean mosaic virus* cannot usually infect resistant plants with the *N* and *RsvI* gene, respectively; however, they can overcome this resistance when they are inoculated by grafting (Hajimorad and Hill 2001; Zaitlin 1962). We took advantage of graft inoculation and successfully obtained resistance-breaking virus variants from five of seven inoculated

plants. A similar method was previously used to examine whether PVX strains could overcome both *Nx* and *Nb* resistance of potato (Jones 1985). Our result confirmed the efficiency of grafting experiments in selecting resistance-breaking strains. This frequent resistance breakdown in grafting experiments can be explained as follows. In infected plants, RNA viruses are supposed to consist of genetically diverse populations termed “quasispecies” resulting from their small genomes, short replication cycle, and replicases that lack proofreading activity (Elena and Sanjuan 2005). Genetic diversity within a virus population is believed to contribute to the viral survival in a rapidly changing environment by providing variants with potentially favorable phenotypes (Lauring and Andino 2010; Quer et al. 1996). When PVX was inoculated mechanically or by agroinfiltration, the accumulation of PVX would be rapidly inhibited in the primary infected cell and, therefore, only a low level of genetic diversity could be achieved within virus population. In contrast, by using graft inoculation, PVX accumulated in susceptible rootstocks enough to achieve genetic diversity capable of producing virus variants with JAX1 resistance-breaking ability.

Resistance-breaking viruses have contributed to the elucidation of mechanisms underlying host resistance. In this study, we found that resistance-breaking determinants of JAX1-mediated resistance were found in the replicase of PVX. Previously reported was that the resistance-breaking determinants of *RTM1*, another gene responsible for LMR, were found in the CP of PPV (Decroocq et al. 2009). Moreover, the CP region where *RTM1* resistance-breaking determinants were mapped was predicted to be glycosylated. Therefore, it is attractive to speculate that JAX1 and *RTM1* recognize the sugar chain bound to the replicase of potexviruses and the CP of potyviruses, respectively. However, the resistance-breaking determinant of JAX1-mediated resistance was glutamine, which has not been shown to be glycosylated, indicating that this residue might not be directly involved in sugar chain recognition. Substitution of Q336H may inhibit the sugar chain recognition of JAX1 by disrupting the proper folding of the replicase protein. JAX1 may recognize viral replicase indirectly via another host protein because viral avirulent proteins have been indirectly recognized by NB-LRR proteins. For example, the recognition of p50 of TMV by N and the CP of PVX by Rx is mediated by a chloroplastic protein NRIP1 and a Ran GTPase-activating protein RanGAP2, respectively (Caplan et al. 2008; Sacco et al. 2007; Tameling and Baulcombe 2007).

Alternatively, viral replicase may be a target of JAX1-mediated resistance. In the case of *Tm-1*, an atypical *R* gene that inhibits the replication of *Tomato mosaic virus* (ToMV), the resistance-breaking determinant is replicase of ToMV (Yamafuji et al. 1991), and *Tm-1* directly interacts with ToMV replicase to inactivate its activity (Ishibashi et al. 2007). A resistance-breaking virus, *Lta1*, can multiply in *Tm-1*-expressing protoplasts even when it is co-inoculated with a virulent virus (Yamafuji et al. 1991), suggesting that the replicase of ToMV is not an elicitor and, instead, is a target of *Tm-1*-mediated resistance. Therefore, JAX1 may target replicase to disturb viral replication. Moreover, the N-terminal portion of viral CP, the resistance-breaking determinant for *RTM1*, is involved in systemic movement of potyviruses (Dolja et al. 1994; Lopez-Moya and Pirone 1998), which is specifically inhibited in *RTM1*-mediated resistance. Taken together, these resistance-breaking determinants may be targets of LMR.

MATERIALS AND METHODS

Plant material.

P35S-JAX1, a transformant of the *N. benthamiana* line that highly expresses *JAX1* under the *Cauliflower mosaic virus* 35S

promoter, has been described previously (Yamaji et al. 2012). *N. benthamiana* plants were grown in growth chambers under conditions of 16 h of light and 8 h of darkness at 25°C throughout the assays.

Construction of vectors.

A binary PVX vector, pCAMV, was previously described by Hoshi and associates (2009). This vector expresses infectious cDNA of PVX derived from pP2C2S kindly provided by D. Baulcombe (Sainsbury Laboratory, Norwich, U.K.). All PVX mutants (pCAM-1092C, pCAM-1092T, and pCAM549A-1092T) were generated from pCAMV by site-directed mutagenesis PCR with the following primers: CXVF (5'-CGC GGA TCC GAG CTA CAT AGT GGA GAC A-3') and CXVRt (5'-CCC TTA ATT AAA TGT CTG ACT TTG GCA AAA-3'), CXVF and CXVRc (5'-CCC TTA ATT AAG TGT CTG ACT TTG GCA AAA-3'), and CXVmutF (5'-CGC GGA TCC AAG CTA CAT AGT GGA GAC A-3') and CXVRt, respectively.

An infectious cDNA of PIAMV Li1 strain (pLi1) has been previously described (Ozeki et al. 2006). The PIAMV mutants (pLi1046A, pLi1046C, and pLi1046T) were generated from pLi1 by introducing a single-nucleotide substitution at 1,046 nt of the Li1 genome by site-directed mutagenesis PCR based on a recombinant PCR technique (Komatsu et al. 2011). The following primers were used for mutagenesis with each complementary reverse primer: Li1046AF (5'-CA AAA TTC AGA CAA CTC ATC-3'), Li1046C (5'-CA AAA TTC AGA CAC CTC ATC-3'), and Li1046TF (5'-CA AAA TTC AGA CAT CTC ATC-3').

Virus inoculation.

Agroinfiltration was performed as previously described (Senshu et al. 2009). Briefly, bacterial cells with an appropriate plasmid were harvested and resuspended in an infiltration buffer to a final optical density at 600 nm of 1.0.

Cleft grafting.

Rootstocks were prepared from 3-week-old *N. benthamiana* plants by cutting off the stems above the fourth leaves. An approximately 1-cm vertical cut was then made in the center of the stem. For scions, 5- to 7-cm apical shoots of the same old P35S-JAX1 plants were excised and the leaves removed, except for the apical one. The bases of scions were trimmed to wedges and placed onto the stem of rootstocks. The grafted plants were covered with a plastic bag for approximately 1 week to avoid dehydration.

Sequence analyses.

To determine the complete genome sequence of PVX, the cDNA fragments that correspond to 1 to 2,046, 981 to 3,070, 1,936 to 4,340, and 4,320 to 6,524 nt were amplified by one-step RT-PCR by using the Superscript One-Step RT-PCR System with Platinum Taq (Life Technologies, Gaithersburg, MD, U.S.A.) following the manufacturer's instructions. Amplified cDNA were then sequenced directly with 13 primer sets that recognized the following regions of PVX: 1 to 21, 981 to 1,000, 1,788 to 1,807, 1,936 to 1,952, 2,493 to 2,512, 2,026 to 2,046, 3,037 to 3,070, 3,100 to 3,123, 4,320 to 4,340, 4,433 to 4,452, 5,148 to 5,166, 5,540 to 5,560, and 6,500 to 6,524. Sequence analyses were performed using an ABI 3130xl Genetic Analyzer (Life Technologies), and the obtained sequences were assembled by using ATGC software (ver. 4.3.3; Genetyx, Tokyo).

RNA isolation and virus detection.

For virus detection, inoculated or noninoculated upper leaves were harvested at the indicated time points and total RNA

was isolated from them with Isogen (Nippon Gene, Tokyo). The cDNAs of PVX or PIAMV were then amplified by one-step RT-PCR as described above. Primers that recognize the 5,540 to 5,560 and 6,500 to 6,524 regions of the PVX genome were used for the detection of PVX, and primers PIAMV1F (5'-GAA AAC AAA CCT ACA CAA ACC A-3') and PIAMV-1006R (5'-TTG ACG GAC TTA ATG TAG AAG A-3') were used for the detection of PIAMV. The transcript of the JAX1 transgene was also detected by RT-PCR, as described elsewhere (Yamaji et al. 2012). For quantitative real-time PCR, total RNA was extracted from the infiltrated leaves at 3 dpi with Sepasol-I (Nakalai Tesque, Kyoto, Japan) following the manufacturer's instructions. Real-time PCR analyses were performed on total RNA from infiltrated patches using CP-specific primers, as previously described (Senshu et al. 2011). The amount of viral RNA was normalized relative to ubiquitin mRNA in each sample.

Multiple sequence alignment.

Amino acid sequences of the replicase of various potexvirus species were obtained from the National Center for Biotechnology Information database (corresponding reference numbers): PIAMV (AB360765), *Tulip virus X* (NP_702988), *Cassava common mosaic virus* (NP_148778), *Clover yellow mosaic virus* (NC_001753), *Papaya mosaic virus* (NP_044330), *Cactus virus X* (NP148778), *Bamboo mosaic virus* (NP_042582), *Fox-tail mosaic virus* (NP_040988), *Cymbidium mosaic virus* (NP_054025), *Potato aucuba virus* (NP_619745), *Narcissus mosaic virus* (NP_001441), *Scallion virus X* (SVX; NP_570726), *Pepino mosaic virus* (CAD27508), *White clover mosaic virus* (NP_620715), and *Strawberry mild yellow edge virus* (NP620642). All were aligned with ClustalW multiple alignments at the DNA Data Bank of Japan (Thompson et al. 1997).

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