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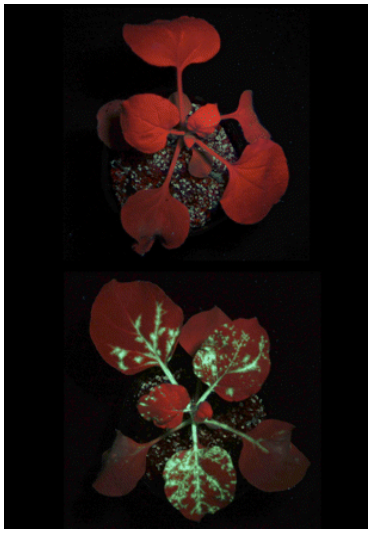

JAX1-MEDIATED RESISTANCE TO PLANT VIRUS

Plants resist pathogen infection with a multilayered defense strategy. Lectins are believed to serve as defensive molecules, but their precise roles have remained unclear. Yamaji et al. (pages 778–793) cloned the novel virus-resistance gene JAX1 from *Arabidopsis thaliana*, which encodes a lectin-like protein with similarity to another virus-resistance protein, RTM1. However, JAX1-mediated resistance differs from other well-characterized virus-resistance machineries such as *R* gene-mediated resistance involving hypersensitive responses or RNA silencing. Whereas RTM1 functions to inhibit viral long-distance movement, JAX1 inhibits viral proliferation at the cellular level. The characterization of JAX1-mediated resistance demonstrates the generality and significance of lectin-mediated resistance to plant viruses. The cover displays the systemic spread of green fluorescent protein fluorescence monitoring virus infection in a wild-type plant (bottom) and strict inhibition of virus infection in a JAX1-expressing transgenic plant (top).

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Lectin-Mediated Resistance Impairs Plant Virus Infection at the Cellular Level [C][W][OA]

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Plants possess a multilayered defense response, known as plant innate immunity, to infection by a wide variety of pathogens. Lectins, sugar binding proteins, play essential roles in the innate immunity of animal cells, but the role of lectins in plant defense is not clear. This study analyzed the resistance of certain *Arabidopsis thaliana* ecotypes to a potexvirus, plantago asiatica mosaic virus (PlAMV). Map-based positional cloning revealed that the lectin gene *JACALIN-TYPE LECTIN REQUIRED FOR POTEXVIRUS RESISTANCE1* (*JAX1*) is responsible for the resistance. JAX1-mediated resistance did not show the properties of conventional resistance (R) protein–mediated resistance and was independent of plant defense hormone signaling. Heterologous expression of JAX1 in *Nicotiana benthamiana* showed that JAX1 interferes with infection by other tested potexviruses but not with plant viruses from different genera, indicating the broad but specific resistance to potexviruses conferred by JAX1. In contrast with the lectin gene *RESTRICTED TEV MOVEMENT1*, which inhibits the systemic movement of potyviruses, which are distantly related to potexviruses, JAX1 impairs the accumulation of PlAMV RNA at the cellular level. The existence of lectin genes that show a variety of levels of virus resistance, their targets, and their properties, which are distinct from those of known *R* genes, suggests the generality of lectin-mediated resistance in plant innate immunity.

INTRODUCTION

Plants have established multilayered defense responses to gain robust, durable resistance to pathogens (Chisholm et al., 2006). The first phase of resistance is induced by the recognition of pathogen-associated molecular patterns (PAMPs) by plant cell surface pattern recognition receptors, which initiates PAMP-triggered immunity that usually halts the infection of pathogens before invasion (Chisholm et al., 2006; Jones and Dangl, 2006). The next phase of plant resistance, resistance (R)-mediated resistance, or effector-triggered immunity, is induced by the direct or indirect recognition of pathogen effector proteins by plant R proteins, which are typically nucleotide binding site–Leu-rich repeat (NB-LRR) proteins (Chisholm et al., 2006; Jones and Dangl, 2006). Effector-triggered immunity usually induces a hypersensitive response (HR) with localized cell death and defense gene expression that suppresses the growth and spread of pathogens postentry (Chisholm et al., 2006; Eitas and Dangl, 2010).

Similar to the plant innate immunity against bacteria, fungi, and oomycetes, the resistance to plant viruses can be divided into multiple stages (Kang et al., 2005). The primary stage of virus resistance is the cellular-level resistance that occurs immediately after entry of the virus into plant cells; this effect, also called extreme resistance, inhibits viral accumulation in the initially invaded cells (Ponz and Bruening, 1986; Kang et al., 2005). A representative example of the cellular-level virus resistance is Rx-mediated resistance against potato virus X (PVX; Bendahmane et al., 1999). Rx, an NB-LRR–type R protein, recognizes the coat protein (CP) of PVX and induces rapid defense signaling reactions, resulting in the inhibition of PVX accumulation at the cellular level (Adams et al., 1986). *Tm-1*, a recently isolated resistance gene from wild tomato (*Solanum habrochaites*), strictly inhibits the replication of tomato mosaic virus, a member of the genus *Tobamovirus*, at the cellular level by inactivating viral RNA-dependent RNA polymerase (Ishibashi et al., 2007). Moreover, the *tm-1* allele of *Tm-1* is responsible for the nonhost resistance to two other tobamoviruses (Ishibashi et al., 2009). Such cellular-level resistance to plant viruses is induced rapidly without HR-like cell death. By contrast, the next phase of resistance to plant viruses is tissue-level resistance, which is usually accompanied by an HR and inhibits virus movement (Kang et al., 2005). R-mediated recognition of viral elicitors from an amplified virus population triggers a variety of defense responses, which usually coincide with HRs (Soosaar et al., 2005; Kachroo et al., 2006). The induced HR usually confines viruses in dead tissues and prevents their spread to surrounding healthy tissues (Lam et al., 2001; Soosaar et al., 2005). R-mediated recognition of a viral elicitor can also trigger systemic-level resistance, such as systemic acquired resistance, which confers virus resistance in tissues distal to the primary infection site (Heil and Ton, 2008).

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A lectin is a protein that reversibly binds carbohydrates (Sharon and Lis, 1989). Lectins exist in most living organisms but were first identified as plant proteins that agglutinate human red blood cells (Van Damme et al., 1998). Since lectins can recognize a specific monosaccharide or oligosaccharide, they have been regarded as self–nonself-discriminating molecules, which suggests that lectins are involved in the recognition of microorganisms, such as pathogens. In fact, some animal lectins, including ficolins and Man binding lectins, recognize pathogens and then activate the complement system, a highly sophisticated innate immunity system of vertebrates and invertebrates (Fujita, 2002). Moreover, c-type lectin receptors (CLRs) form one of the four typical animal pattern recognition receptor families: Toll/interleukin-1 receptors, NOD-like receptors, RIG1-like receptors, and CLRs. CLRs are responsible for the recognition of pathogens, particularly fungi (Pålsson-McDermott and O'Neill, 2007; Willment and Brown, 2008). Although plant lectins possess a diversity of activities, including the ability to recognize cells in a cell surface sugar-specific manner, and serve as antimicrobial and antitumor agents in heterologous animal or in vitro systems, the roles of lectins in plant cells are unclear (Sharon and Lis, 1989; Peumans and Van Damme, 1995; Cowan, 1999; Van Damme et al., 2004; Lam and Ng, 2011). Since most plant lectins appear to be able to bind to exogenous carbohydrate structures but not to plant-originated endogenous ones, they are believed to have roles in defense-related phenomena (Van Damme et al., 2004). Although their biological significance is not clear, a large number of plant lectins are induced by various biotic and abiotic stresses and show antibacterial, antifungal, and anti-insect activities, implying that plant lectins have defensive roles (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995; Van Damme et al., 2004). Plant lectins also may be involved in recognizing pathogenic microorganisms. The soybean (*Glycine max*) lectin β-glucan binding protein shows high-affinity binding activity to β-glucan, a potent PAMP of *Phytophthora sojae* (Mithöfer et al., 2000; Fliegmann et al., 2004). Moreover, the *Arabidopsis thaliana RESTRICTED TEV MOVEMENT1* (*RTM1*) lectin gene inhibits the systemic spread of tobacco etch virus (TEV), a single-stranded RNA plant virus belonging to the genus *Potyvirus*, which is very distantly related to the genus *Potexvirus* (Chisholm et al., 2000). However, very limited evidence exists of the physiological roles of plant lectins in plant cells.

This study reports the identification of *JAX1*, a jacalin-type lectin gene that confers resistance to potexviruses, members of the genus *Potexvirus*. *JAX1* confers resistance in the primary stage of infection by plant viruses, in contrast with another lectin, RTM1, which confers virus resistance in the later stage of virus infection, indicating the important roles of lectin-mediated resistance (LMR) in a variety of plant–virus interactions.

RESULTS

Isolation of *Arabidopsis* Ecotypes Resistant to Plantago Asiatica Mosaic Virus

To identify genes involved in resistance to plant viruses, we screened *Arabidopsis* ecotypes for resistance to the potexvirus plantago asiatica mosaic virus (PlAMV). To discriminate between PlAMV-resistant and -susceptible ecotypes, we constructed a green fluorescent protein (GFP)-tagged PlAMV infectious clone (pPlAMV-GFP; Figure 1A). This infectious vector is derived from a binary vector that enables efficient infection using agroinfiltration (Bendahmane et al., 2000) and produces GFP and coat protein fusion proteins connected with a foot-and-mouth disease virus 2A sequence, resulting in a self-cleavage reaction. For simplicity, we refer to *Agrobacterium tumefaciens* strains containing a binary vector plasmid by the name of the expressed proteins.

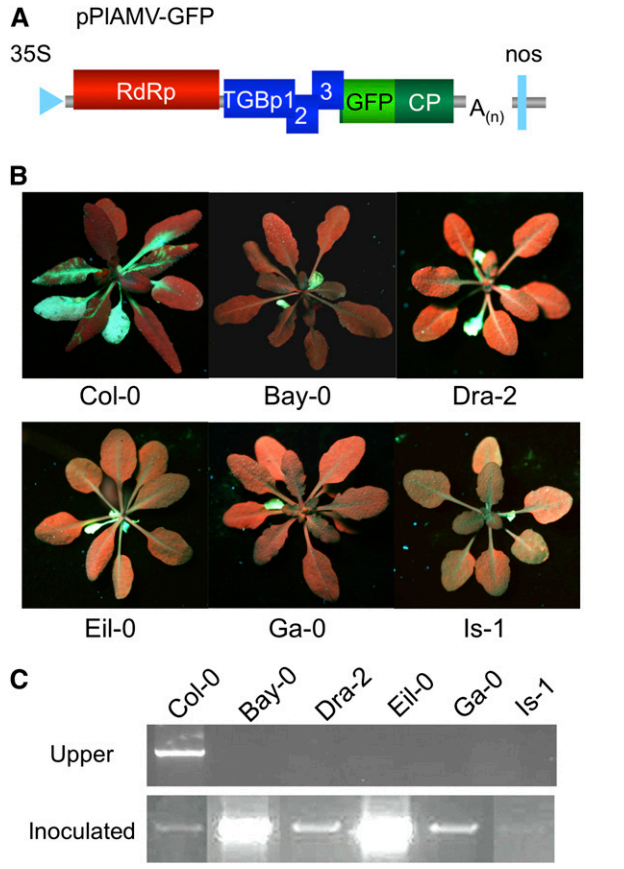

Figure 1. Screening of Resistant *Arabidopsis* Ecotypes.

(A) A schematic of the genomic structure of PlAMV-GFP used for ecotype screening. GFP was expressed as a fusion protein with CP under the control of the CP subgenomic promoter. The PlAMV-GFP infectious cDNA was driven by the 35S promoter and inoculated using agroinfiltration.

(B) The inability of PlAMV-GFP to infect resistant ecotypes systemically. The *Arabidopsis* ecotypes Col-0, Bay-0, Dra-2, Eil-0, Ga-0, and Is-1 were inoculated with PlAMV-GFP by agroinfiltration, and GFP fluorescence was visualized with a UV lamp at 20 DAI. PlAMV-GFP fluorescence spread systemically in Col-0, whereas it localized in the inoculated leaves in Bay-0, Dra-2, Eil-0, Ga-0, and Is-1.

(C) Detection of PlAMV-GFP RNA in inoculated and upper leaves of the ecotypes in **(B)**. PlAMV-GFP RNA was amplified by RT-PCR with a CP-specific primer set.

To screen for virus-resistant *Arabidopsis* ecotypes, we examined 45 distinct ecotypes for PlAMV-GFP susceptibility. Two plants per ecotype were inoculated with PlAMV-GFP in the initial screening. GFP fluorescence was observed under UV light in inoculated and upper leaves at 20 d after inoculation (DAI), and the ecotypes were classified as susceptible or resistant, depending on whether GFP fluorescence was observed systemically. Seven plants per candidate selected in the first screening were inoculated with PlAMV-GFP for the second screening. As a result of the first and second screenings, we selected five ecotypes that did not show GFP fluorescence systemically: Bayreuth-0 (Bay-0), Drahonin-2 (Dra-2), Eilenburg-0 (Eil-0), Gabelstein-0 (Ga-0), and Isenburg-1 (Is-1) (Figure 1B). Most ecotypes, including Columbia-0 (Col-0) and Landsberg *erecta* (L*er*), displayed systemic fluorescence. RT-PCR analysis of PlAMV-GFP RNA showed that PlAMV-GFP accumulated in the inoculated leaves of Bay-0, Dra-2, Eil-0, Ga-0, and Is-1 but could not produce a systemic infection (Figure 1C). Thus, we isolated five resistant ecotypes that PlAMV-GFP cannot infect systemically.

Next, we examined the virus resistance exhibited by the isolated ecotypes. To evaluate the resistance accurately, we inoculated PlAMV-GFP by mechanical inoculation instead of agroinoculation. Since the resistance phenotypes exhibited by the five ecotypes were very similar, we characterized the resistance phenotype of Bay-0 in detail. To compare virus accumulation in the inoculated and upper leaves of Col-0 and Bay-0, we performed RNA gel blot analysis with a virus-specific probe. Consistent with the primary screen (Figure 1), virus accumulation was detected in the upper leaves of Col-0, but not in Bay-0 (Figure 2A). However, the viral RNA accumulation in the inoculated leaves of Bay-0 was restricted to a much lower level than in Col-0. GFP imaging of PlAMV-GFP–inoculated leaves of Col-0 and Bay-0 showed that both the size and number of PlAMV-GFP fluorescent foci were smaller in Bay-0 than in Col-0 (Figure 2B). The number of PlAMV-GFP foci in the inoculated leaves of Bay-0 was significantly lower than that of Col-0 (Figure 2C). The spread of PlAMV-GFP was also impaired in the inoculated leaves of Bay-0 compared with Col-0. PlAMV-GFP foci in the inoculated leaves of Bay-0 included fewer fluorescent cells than those of Col-0 at both 2 and 3 DAI (see Supplemental Table 1 online). After 3 DAI, the spread of PlAMV-GFP was slower in Bay-0 than in Col-0 (see Supplemental Figure 1 online). These results showed that PlAMV-GFP accumulation was inhibited in the inoculated leaves of Bay-0, which resulted in the resistance phenotypes of Bay-0.

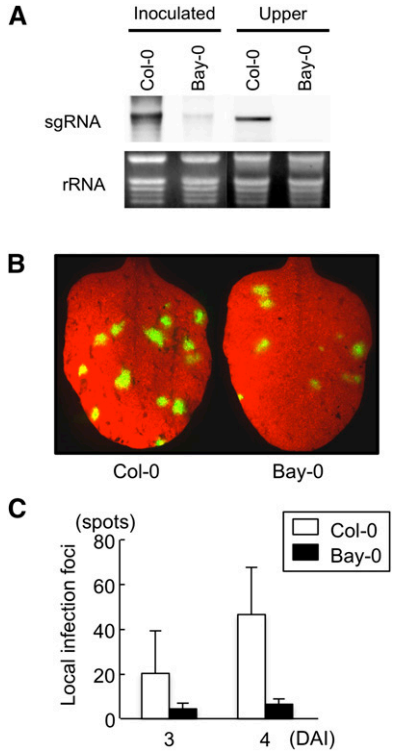

Figure 2. Virus Resistance Exhibited in Bay-0.

(A) Virus accumulation in inoculated and upper leaves of *Arabidopsis* ecotypes Col-0 and Bay-0. To evaluate the virus resistance, extracts from PlAMV-GFP–infected plants were mechanically inoculated into Col-0 and Bay-0. RNA gel blot analysis of PlAMV-GFP was performed on total RNA from inoculated leaves at 4 DAI and upper leaves at 20 DAI using a CP-specific probe to detect the plus-strand viral RNA. The accumulation of viral subgenomic RNA (sgRNA) is indicated. Ethidium bromide–stained rRNA is shown as a loading control.

(B) PlAMV-GFP foci in inoculated leaves of susceptible and resistant ecotypes. PlAMV-GFP–inoculated leaves of Col-0 and Bay-0 in **(A)** were observed under UV irradiation at 3 DAI.

(C) The number of PlAMV-GFP foci in inoculated leaves of susceptible and resistant ecotypes. The numbers of PlAMV-GFP foci in **(B)** were counted from two inoculated leaves of four independent plants at the indicated DAI. The mean number per leaf is indicated with the SD.

[See online article for color version of this figure.]

Mapping and Molecular Cloning of a Gene Required for Resistance

To characterize the genetic basis of virus resistance, Col-0 and Bay-0 were crossed and the progeny were subject to segregation analysis. When the F1 progeny were inoculated with PlAMV-GFP, no F1 progeny were infected systemically, indicating that all of the F1 progeny were resistant to PlAMV-GFP (Table 1). This shows that the resistance phenotype of Bay-0 is dominant. When 100 plants of self-fertilized F2 progeny were inoculated with PlAMV-GFP, PlAMV-GFP infected 29 plants systemically and did not infect 71 plants (Table 1). This ratio (71 resistant to 29 susceptible) was reasonably close to a 3:1 segregation ratio (χ^2 = 0.85; $P > 0.2$), indicating that the resistance phenotype in Bay-0 is caused by a single dominant locus. Segregation analysis using crosses of Col-0 with Dra-2, Eil-0, Ga-0, and Is-1 gave similar results (see Supplemental Table 2 online).

A map-based cloning approach was used to delimit the resistance locus. Since the resistance phenotype is dominant, systemically infected F2 progeny were used for map-based cloning. Initially, we performed linkage analysis using 23 simple sequence length polymorphism (SSLP) markers that distinguish between Col-0 and Bay-0 and are spread throughout the five *Arabidopsis* chromosomes. We found that the resistance locus was most tightly linked to the SSLP marker nga280 on chromosome 1 and that the SSLP markers ciw1 and nF5I14 were cosegregating flanking markers in the centromeric and telomeric

Table 1. Genetic Analysis of the Resistance Phenotype in Bay-0 and the Responses of the Signal Transduction Mutants

Plants	Resistant	Susceptible
Col-0	0	21
Bay-0	21	0
F1 (Col-0 × Bay-0)	10	0
F2	71[a]	29[a]
eds5-1/JAX1[b]	10	0
jar1-1/JAX1[b]	10	0
ein2-1/JAX1[b]	10	0

The indicated plants were inoculated with PlAMV-GFP. Virus infection was evaluated whether the spread of GFP expression from PlAMV-GFP was systemic (Susceptible) or not (Resistant) at 20 DAI.
[a] χ^2 (3:1) = 0.85; $P > 0.2$.
[b] The genetic background of *eds5-1*, *jar1-1*, and *ein2-1* is Col-0.

vicinity of the resistance locus, respectively (Figure 3A). Linkage analysis of F2 populations generated from crosses of Col-0 with Dra-2, Eil-0, Ga-0, and Is-1 using SSLP markers showed that the resistance locus in these ecotypes was also linked most tightly to the SSLP marker nga280 (see Supplemental Table 3 online). Further linkage analysis was performed using single nucleotide polymorphism (SNP) markers to identify the Bay-0 resistance locus. We amplified and sequenced several regions of the Bay-0 genome to develop six new SNP markers that distinguish Col-0 and Bay-0 (see Supplemental Table 4 online). As a result of fine mapping with the SNP markers, we delimited the resistance locus into a 130-kb region between markers SNP21.4 and SNP21.6 (Figure 3A).

We examined annotated genes in the 130-kb region with The Arabidopsis Information Resource (TAIR) database. No NB-LRR–type *R* gene–like gene was observed in the region, but we found a jacalin-type lectin gene locus at At1g58160. This was similar to *RTM1*, a jacalin-type lectin gene involved in the resistance to a potyvirus (Chisholm et al., 2000). Genomic DNA and cDNA fragments of this locus in Bay-0 and Col-0 were sequenced, and nucleotide polymorphisms were identified. The cDNA fragment of Bay-0 included an intact 157–amino acid At1g58160 open reading frame (ORF). By contrast, Col-0 had a stop codon in the first exon, resulting in translational termination that generated an N-terminal 36–amino acid fragment of At1g58160 (Figure 3B; see Supplemental Figures 2A and 2B online). Sequencing At1g58160 cDNAs from other ecotypes also showed that resistant ecotypes (Dra-2, Eil-0, Ga-0, and Is-1) encoded the full-length protein, whereas a susceptible ecotype (L*er*) had the same internal termination codon in At1g58160 ORF as Col-0, resulting in an N-terminal 36–amino acid fragment (see Supplemental Figure 2B online). Since At1g58160 encoded a jacalin-type lectin protein, we named this gene *JAX1* (for *JACALIN-TYPE LECTIN REQUIRED FOR POTEXVIRUS RESISTANCE1*), considering the results of the complementation tests outlined below.

JAX1 contains a single jacalin-type lectin domain similar to RTM1 and jacalin from jackfruit (*Artocarpus heterophyllus*). Jacalin-like proteins form one of the seven plant lectin families (Van Damme et al., 1998). The intact ORF of jacalin from jackfruits encodes a 217–amino acid polypeptide, whereas the mature structure of jacalin consists of four long 133–amino acid α-chains and four short 20–amino acid β-chains that are produced through posttranslational processing (Van Damme et al., 1998). The α-chain of jacalin, which contains the sugar binding region, was conserved in both JAX1 and RTM1, whereas the β-chain was absent (Figure 3C). The *Arabidopsis* genome contains 48 jacalin-type lectin genes, which conserve one to six repeats of the jacalin α-chain domain (Nagano et al., 2008). Of these, only nine jacalin-type lectin proteins have a single jacalin repeat, including JAX1 and RTM1. The protein encoded at At3g16450, known as the myrosinase binding protein (MBP), contains two repeats of the jacalin α-chain domain; it binds specifically to several oligosaccharides (Takeda et al., 2008). JAX1 showed a similarity of 37% with RTM1, 27% with MBP, and 29% with jacalin.

To examine whether *JAX1* confers the resistance phenotype, we performed complementation analysis. The genomic DNA fragment of Bay-0 was transformed into Col-0. An ~3.5-kb fragment including the putative promoter region and the intact ORF of *JAX1* from Bay-0 was cloned to generate a construct, PJAX1-JAX1, and transformed into Col-0 using an *Agrobacterium*-mediated method. When these transgenic plants (*PJAX1-JAX1*) were inoculated with PlAMV-GFP by agroinfiltration, PlAMV could not systemically infect most of the transformants (61 of 73 plants) (Figure 4A). Quantitative real-time RT-PCR analysis using PlAMV-specific primers showed that the accumulation of PlAMV-GFP RNA in inoculated leaves was similar to that in nontransgenic plants (Figure 4B). However, the level of PlAMV RNA in upper leaves of the *PJAX1-JAX1* plants was significantly lower than that in nontransgenic plants. These phenotypes were similar to the resistant phenotype of Bay-0 (Figure 1). Moreover, to overexpress the *JAX1* gene product, the *JAX1* cDNA fragment was fused to the cauliflower mosaic virus 35S promoter to generate a construct, P35S–JAX1, and transformed into Col-0. When PlAMV-GFP was inoculated on these transgenic plants (*P35S-JAX1*), it could not systemically infect any of the transformants (10 of 10 plants) (Figure 4A). Real-time RT-PCR analysis showed that PlAMV RNA was undetectable in both inoculated and upper leaves of *P35S-JAX1* transgenic plants (Figure 4B). These results demonstrate that *JAX1* is the causal gene that confers the resistance to PlAMV-GFP in Bay-0.

Expression Analysis of *JAX1*

To analyze whether *JAX1* shows tissue-specific expression patterns, RNA gel blot analysis was performed on total RNA extracted from the organs of Bay-0 plants using a *JAX1*-specific probe. This detected similar levels of *JAX1* transcripts in rosette leaves and flowers (Figure 5A). By contrast, the *JAX1* mRNA level was elevated in stems, while it was below the detection limit in roots. The spatial expression pattern of *JAX1* was assessed using a histochemical assay of β-glucuronidase (GUS) activity. Binary vectors containing GUS under the control of the *JAX1* promoter or 35S promoter were transformed into Col-0. Plants expressing *P35S-GUS* were stained in most cells of leaves, whereas plants expressing GUS from the *JAX1* regulatory sequence (*PJAX1-GUS*) were stained mainly within vascular

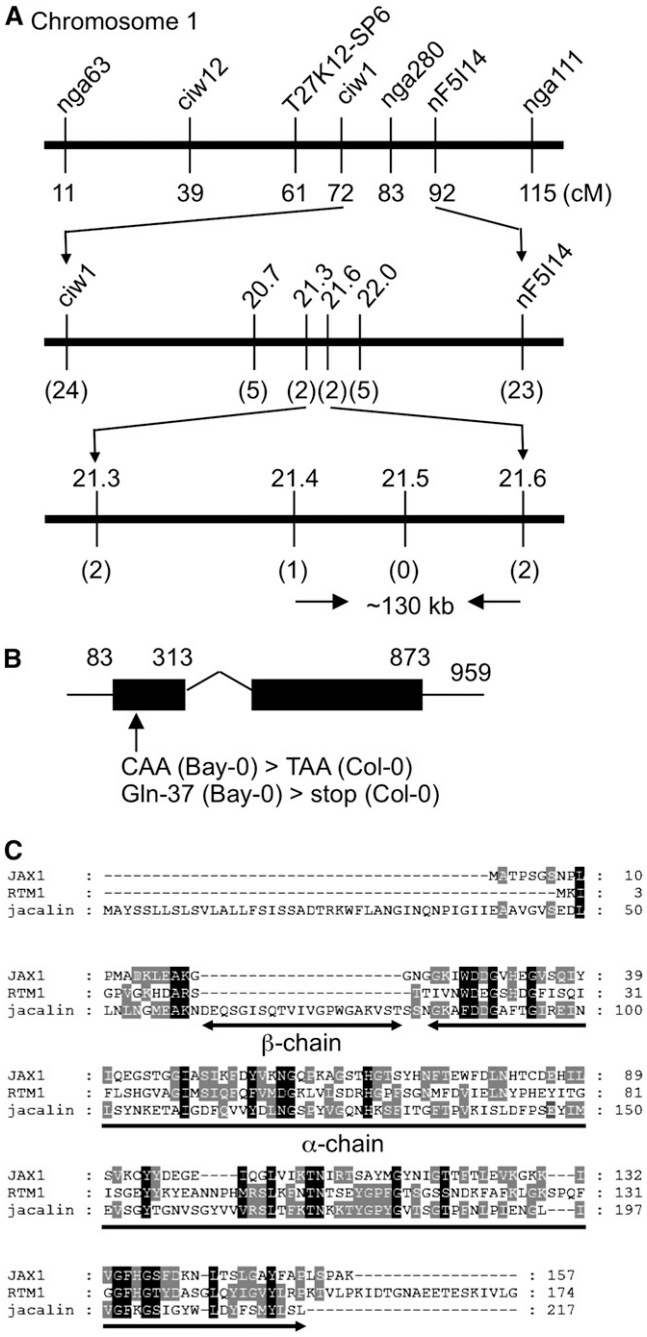

Figure 3. Molecular Cloning of the *JAX1* Gene.

(A) Map-based strategy for identifying the resistance locus. SSLP markers on chromosome 1 and the map distances (in centimorgans [cM]) are indicated at the top. SNP markers developed for delimiting the candidate region are shown below. The number of informative recombinants from the mapping population of ~1500 F2 plants is indicated in parentheses. The resistance locus was mapped to an ~130-kb region between SNP21.4 and SNP21.6.

(B) A representation of the *JAX1* cDNA, with the nucleotide positions of the start codon (nucleotide 83), 5′-intron splice site (nucleotide 313), stop codon (nucleotide 873), and 3′-terminal nucleotide (nucleotide 959) indicated. Polymorphisms between Col-0 and Bay-0 identified in the sequence analysis are shown below the intron/exon structure.

(C) Alignment of the deduced Bay-0 *JAX1* sequence, jacalin, and RTM1. Gray and black shading indicate conserved and identical residues, respectively. The positions of the α- and β-chain domains of jacalin are indicated.

tissues (Figure 5B). The GUS staining assay also showed that GUS is highly expressed in the vascular and surrounding tissues in cotyledons (see Supplemental Figure 3 online). GUS was also detected in vascular tissues in roots and extensively in root apical meristems (see Supplemental Figure 3D online).

We also observed the expression pattern of JAX1 in Bay-0 immunocytochemically. To analyze the expression pattern of JAX1 in detail, vertical sections of Col-0 and Bay-0 leaves were immunostained using anti-JAX1 antibody. Although the signals for the expression of JAX1 were below the detection level in Col-0, they were obvious in Bay-0 (Figure 5C). Intense signals indicating JAX1 expression were extensively observed in vascular cells but were also detected in surrounding mesophyll cells of Bay-0. These results indicated that JAX1 expression is specific in Bay-0.

To determine whether *JAX1* is induced by virus inoculation, we prepared total RNA from PlAMV-inoculated leaves collected at several time points and used it to perform RNA gel blot analysis of *JAX1*. Similar to the pattern of mock-inoculated leaves, the level of *JAX1* mRNA transcription was neither upregulated nor downregulated by the inoculation of PlAMV (Figure 5D). These results indicated that *JAX1* expression is not induced during the resistance reactions to PlAMV.

Strict Inhibition of Virus Infection by JAX1 in the Heterologous Plant *Nicotiana benthamiana*

We produced transgenic *N. benthamiana* lines that express *JAX1* under the control of the 35S promoter to investigate whether the *Arabidopsis JAX1* gene can produce virus resistance in a heterologous plant, *N. benthamiana*, which is another host of PlAMV. The 35S promoter–driven *JAX1* fused with a fluorescent amplicon generation (FLAG) peptide tag was introduced into *N. benthamiana* using an *Agrobacterium*-mediated method to generate two lines of transformants (*P35S-JAX1*, lines 3 and 11). Successful transformation was confirmed by PCR analysis of the inserted sequence and immunoblot analysis using anti-FLAG antibody (see Supplemental Figures 4A and 4B online). As a control experiment, when nontransgenic *N. benthamiana* was inoculated with PlAMV-GFP, bright GFP fluorescence was observed in both the inoculated and upper uninoculated leaves at 20 DAI, indicating systemic infection with PlAMV-GFP (Figure 6A). By contrast, when both lines of *P35S-JAX1* transgenic *N. benthamiana* plants were inoculated with PlAMV-GFP, no GFP fluorescence was observed in either inoculated or upper leaves at 20 DAI, indicating the inhibition of PlAMV-GFP infection by JAX1. Therefore, *JAX1* can produce strict resistance to PlAMV in the heterologous plant *N. benthamiana*.

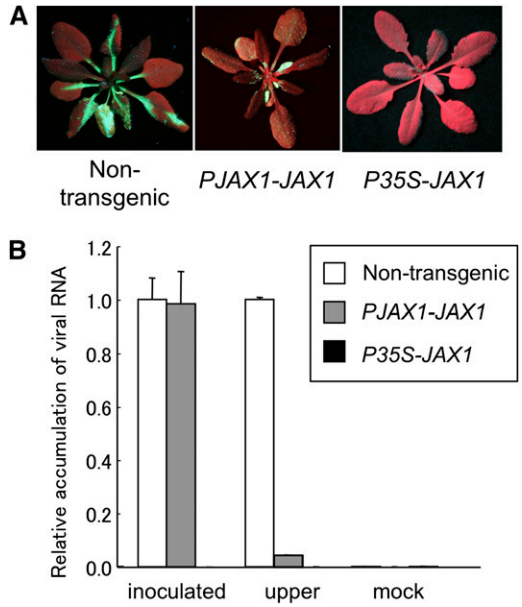

Figure 4. Complementation Analysis of the *JAX1* Gene.

(A) Inhibition of systemic PlAMV-GFP infection in transgenic Col-0 plants expressing JAX1. Nontransgenic Col-0 plants and *PJAX1-JAX1* and *P35S-JAX1* transgenic plants were inoculated with PlAMV-GFP by agroinfiltration. GFP fluorescence indicating virus infection was visualized under UV light at 20 DAI.

(B) Quantitative detection of PlAMV-GFP RNA in *JAX1* transgenic plants. Total RNA was extracted from inoculated leaves at 5 DAI, upper leaves at 20 DAI of six PlAMV-GFP–inoculated plants, and from upper leaves at 20 DAI of three mock-inoculated plants. Real-time RT-PCR analysis was performed on each sample of nontransgenic plants and *PJAX1-JAX1* and *P35S-JAX1* transgenic plants. The accumulation level of endogenous actin mRNA was used as a reference. The mean level of PlAMV-GFP RNA in inoculated leaves of nontransgenic plants was taken as a standard (1.0). The error bars represent the SD.

JAX1 Inhibits Virus Accumulation at the Cellular Level

Next, we investigated whether JAX1 can inactivate PlAMV at an early stage of virus infection because highly expressed JAX1 strictly inhibited virus infection in inoculated leaves in transgenic *Arabidopsis* and *N. benthamiana* (Figures 4A, 4B, and 6A). To this end, we transiently expressed *JAX1* by agroinfiltration in *N. benthamiana* leaves and examined the effect on PlAMV infection in the primary inoculated leaves. In control experiments, when PlAMV-GFP was coexpressed with the vector control by agroinfiltration, GFP fluorescence showing intense accumulation of PlAMV-GFP was observed in infiltrated leaves at 5 DAI (Figure 6B). Strikingly, no GFP fluorescence was detectable in the leaves infiltrated with PlAMV-GFP and JAX1. Consistent with this result, RNA gel blot analysis of viral RNA showed that PlAMV-GFP RNA accumulated in vector-expressing leaves but not at all in *JAX1*-expressing leaves (Figure 6C). These results, along with the immunoblot analysis indicating the expression of JAX1 (Figure 6D), showed that the accumulation of PlAMV-GFP was strictly inhibited by JAX1 in the initially inoculated leaves. Moreover, we showed that when *JAX1* was expressed under its own promoter, virus accumulation was inhibited to a certain extent (see Supplemental Figure 5 online). Together with the result that PlAMV-GFP infection was inhibited in the inoculated leaves of Bay-0 when it was mechanically inoculated (Figure 2B), these results suggested that JAX1 inhibits PlAMV infection at the early infection step.

To investigate the cellular-level effect of JAX1 on virus accumulation, JAX1-mediated resistance to PlAMV was evaluated in *Arabidopsis* protoplasts. Protoplasts extracted from *Arabidopsis* suspension culture (Col-0) were transfected with a plasmid expressing PlAMV-GFP and either an empty vector, a plasmid expressing JAX1 under its own (PJAX1-JAX1), or 35S promoter (P35S-JAX1). At 2 DAI, PlAMV-GFP showed bright fluorescence in a large number of vector-introduced protoplasts, whereas PlAMV-GFP fluorescence showed a certain reduction in PJAX1-JAX1–introduced protoplasts and, furthermore, decreased drastically in P35S-JAX1–introduced protoplasts (Figure 7A). Real-time RT-PCR analysis of PlAMV-GFP RNA was conducted to quantify the influence of JAX1 on virus accumulation. This revealed that the level of virus RNA was ~45 to 65% in PJAX1-JAX1 protoplasts and ~10% in P35S-JAX1 protoplasts compared with vector-introduced protoplasts, indicating a significant decrease in virus accumulation in JAX1-introduced protoplasts (Figure 7B). Moreover, JAX1-mediated inhibition of virus accumulation was compared with the effect of RNA silencing. To induce RNA silencing of PlAMV-GFP, we used a binary vector, pIR-GFP, which includes an inverted-repeat sequence of GFP under the control of the 35S promoter and expresses double-stranded RNA of GFP in plants, resulting in degradation of RNAs, including GFP sequences (Senshu et al., 2009). The inhibitory level of virus accumulation by JAX1 was comparable to the level of virus accumulation inhibition by IR-GFP (Figures 7A and 7B). Note that the virus accumulation level in P35S-JAX1 protoplasts was lower than that in IR-GFP protoplasts. This indicated that JAX1 could inhibit virus accumulation more strictly than double-stranded RNA derived from an inverted-repeat sequence because both P35S-JAX1 and IR-GFP express their downstream sequences under the 35S promoter. Collectively, these data suggest that JAX1 produces a strict resistance to virus accumulation at the cellular level.

JAX1-Mediated Resistance Differs from Other Virus Resistance Machinery

We compared JAX1-mediated resistance with other resistance responses to plant viruses. First, to compare it with the conventional virus resistance mechanism, R-mediated resistance, we analyzed whether the characteristics of R-mediated resistance are observed in JAX1-mediated resistance. As a positive control for R-mediated resistance, we used RCY1-mediated resistance to *Cucumber mosaic virus* (CMV; Takahashi et al., 2004). To detect cell death, Trypan blue staining was performed on CMV-inoculated leaves of *Arabidopsis* ecotype C24 carrying *RCY1*, an NB-LRR–type *R* gene to CMV, at 4 DAI. We detected apparent blue staining in CMV-inoculated leaves of C24. However, no staining was observed with Trypan blue staining of PlAMV-GFP–inoculated leaves of Col-0 and Bay-0 (Figure 8A), showing that JAX1-mediated resistance is not accompanied by cell death

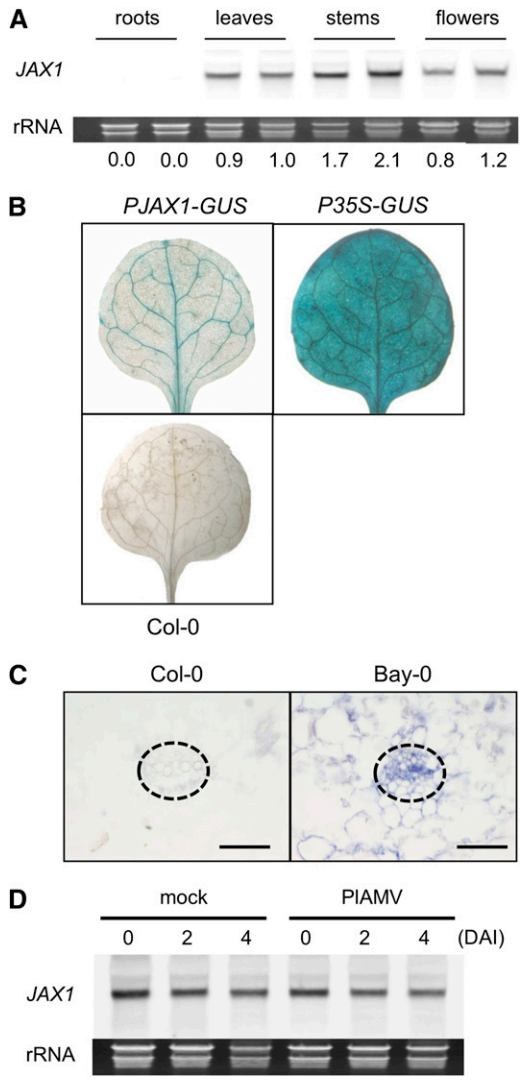

Figure 5. Expression Analysis of *JAX1*.

(A) Tissue-specific expression patterns of *JAX1*. The levels of *JAX1* transcripts were examined by RNA gel blot analysis using total RNA from roots, rosette leaves, stems, and flowers of the Bay-0 ecotype using the *JAX1* cDNA as a probe. Ethidium bromide–stained rRNA is shown as a loading control. Two independent plants were analyzed for each tissue. The relative accumulation of *JAX1* mRNA is indicated at the bottom. The mean value of *JAX1* mRNA in leaves was taken as a standard (1.0).

(B) GUS histochemical analysis of the *JAX1* expression patterns. Col-0 plants were transformed with *GUS* genes fused with the *JAX1* promoter region (*PJAX1-GUS*) and the 35S promoter (*P35S-GUS*). The transformants were infiltrated with the histochemical substrate X-gluc and incubated at 37°C for 12 h to visualize the GUS expression patterns. Col-0 plants were used as a negative control.

(C) Immunocytochemical analysis of *JAX1* expression. Transverse sections around vascular tissues were prepared from leaves of Col-0 and Bay-0 and subjected to immunocytochemical analyses using anti-JAX1 antibody. The positions of the vascular tissues are indicated by dotted circles. Bars = 50 μm.

(D) Levels of *JAX1* transcripts in virus-inoculated leaves. RNA gel blot analysis was performed on total RNA from mock- and PlAMV-inoculated leaves at the indicated DAI using the *JAX1* cDNA as a probe. Ethidium bromide–stained rRNA is shown as a loading control.

reactions. Next, we performed 3,3′-diaminobenzidine (DAB) staining to detect hydrogen peroxide (H_2O_2), a typical indicator molecule of R-mediated resistance. In CMV-inoculated leaves of C24 plants, obvious brown staining was detected, whereas no brown staining was observed in Col-0 and Bay-0 leaves inoculated with PlAMV-GFP (Figure 8B), indicating that H_2O_2 is not induced in JAX1-mediated resistance. The expression pattern of the *PR-1* defense gene after virus inoculation was analyzed because it is a representative marker gene of R-mediated defense responses in *Arabidopsis* (Kachroo et al., 2000). RNA gel blot analysis using a *PR-1*-specific probe showed that *PR-1* transcripts were induced in CMV-inoculated leaves of C24 but not in either PlAMV- or mock-inoculated leaves of Col-0 and Bay-0, indicating that *PR-1* is not induced in JAX1-mediated resistance (Figure 8C). These results suggested that plant reactions induced during R-mediated resistance are absent in JAX1-mediated resistance.

Plant hormones are important signaling molecules that regulate developmental processes, but some of them, particularly salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are also essential for plant innate immunity and are regarded as plant defense hormones (Bari and Jones, 2009). To determine whether JAX1-mediated resistance depends on these defense signaling pathways, Bay-0 was crossed with a SA-deficient mutant (*eds5-1*), an ET-insensitive mutant (*ein2-1*), and a JA-insensitive mutant (*jar1-1*). PlAMV-GFP could not systemically infect any of the resulting mutant plants carrying *JAX1* (*eds5-1/JAX1*, *ein2-1/JAX1*, and *jar1-1/JAX1*) (Table 1), indicating that JAX1-mediated resistance was not disrupted in these mutants in plant defense hormone synthesis. These results suggest that JAX1-mediated resistance is independent of SA-, ET-, or JA-dependent defense signaling.

RNA silencing is also an important virus resistance mechanism that recognizes and degrades viral RNA. Since RNA silencing is independent of the HR and can cause strict inhibition of virus accumulation at the cellular level, we examined whether JAX1-mediated resistance is correlated with RNA silencing. If JAX1 is involved in the RNA silencing machinery, JAX1-mediated resistance will be suppressed by a viral RNA silencing suppressor. When we agroinfiltrated PlAMV-GFP with IR-GFP expressing GFP double-stranded RNA and a control vector, GFP fluorescence indicating virus accumulation was not observed (see Supplemental Figure 6 online). When we agroinfiltrated PlAMV-GFP with IR-GFP and tomato bushy stunt virus p19, a strong suppressor of RNA silencing that binds to small RNAs to inactivate them, bright GFP fluorescence was observed in the infiltrated patch, indicating the recovery of virus accumulation by the suppression of RNA silencing. By contrast, when we agroinfiltrated PlAMV-GFP with JAX1 and p19, no GFP fluorescence was observed, which is similar to the patch of PlAMV-GFP where JAX and the vector were agroinfiltrated. These results indicated that JAX1-mediated resistance is independent of the small RNA-triggered cascade of RNA silencing.

JAX1 Confers Broad Resistance to Potexviruses

To determine whether JAX1 confers general resistance to plant viruses, we inoculated several plant viruses belonging to distinct

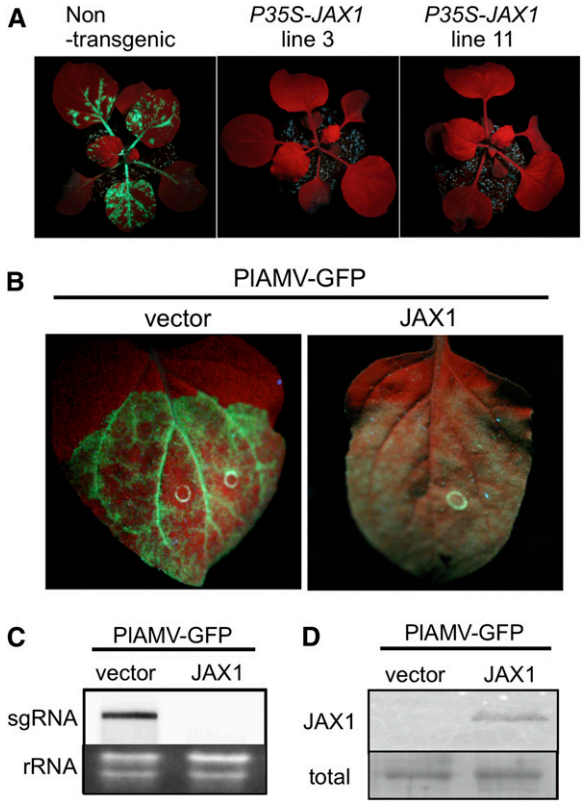

Figure 6. Strict Inhibition of Virus Accumulation in *N. benthamiana* Leaves Expressing JAX1.

(A) Inhibition of PlAMV-GFP infection in transgenic *N. benthamiana* plants expressing JAX1. Nontransgenic plants and two lines of *P35S-JAX1* transgenic plants were inoculated with PlAMV-GFP. GFP fluorescence indicating virus accumulation was visualized under UV light at 20 DAI.

(B) PlAMV-GFP fluorescence in JAX1-agroinfiltrated leaves of *N. benthamiana*. *N. benthamiana* leaves were infiltrated with *Agrobacterium* mixtures containing PlAMV-GFP and a vector expressing either the vector or JAX1. GFP fluorescence indicating virus accumulation was visualized under UV light at 5 DAI.

(C) PlAMV-GFP RNA accumulation in infiltrated leaves. RNA gel blot analysis was performed on total RNA from the infiltrated leaves shown in **(B)** using PlAMV CP cDNA as a probe to detect plus-strand viral RNA. The accumulation of viral sgRNA is indicated. Ethidium bromide–stained rRNA is shown as a loading control.

(D) Accumulation of JAX1 in infiltrated leaves. Immunoblot analysis was performed on total protein from the infiltrated leaves shown in **(B)** using anti-FLAG antibody. Coomassie blue–stained total protein is shown as a loading control.

genera into *P35S-JAX1* transgenic *N. benthamiana* plants, as shown in Figure 6A. In addition to PlAMV, we tested three potexviruses (PVX, white clover mosaic virus [WCIMV], and asparagus virus 3 [AV3]) and plant viruses from other genera, including *Tobacco mosaic virus* (TMV; *Tobamovirus*), CMV (*Cucumovirus*), *Tobacco rattle virus* (TRV; *Tobravirus*), *Turnip mosaic virus* (TuMV; *Potyvirus*), TEV (*Potyvirus*), *Potato virus Y* (PVY; *Potyvirus*), and *Radish mosaic virus* (RaMV; *Comovirus*), all of which can infect *N. benthamiana* systemically and result in obvious symptoms. Infection with these viruses was confirmed by systemic symptoms and RT-PCR with virus-specific primers. When *P35S-JAX1* transformants were inoculated with the potexviruses PVX, WCIMV, and AV3, no symptoms were observed in any of the plants and no virus RNA was detected in either inoculated or upper leaves at 20 DAI (Table 2). Conversely, when plants were inoculated with viruses from genera other than *Potexvirus* (TMV, CMV, TRV, TuMV, TEV, PVY, and RaMV), they showed obvious symptoms characteristic of each inoculated

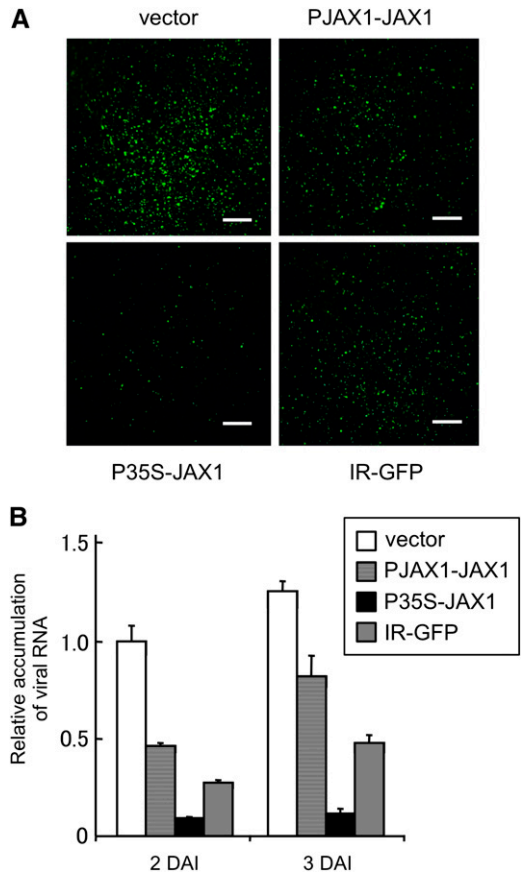

Figure 7. JAX1-Mediated Inhibition of Virus Accumulation in Protoplasts.

(A) Reduction of PlAMV-GFP fluorescence in protoplasts expressing JAX1. DNA mixtures containing PlAMV-GFP and a vector, PJAX1-JAX1, P35S-JAX1, or IR-GFP, were introduced into protoplasts prepared from suspension culture cells of Col-0. PJAX1-JAX1 and P35S-JAX1 express JAX1 under the control of its native and 35S promoters, respectively. IR-GFP expresses the inverted-repeat sequence of GFP to induce RNA silencing of GFP. GFP fluorescence indicating virus accumulation was visualized at 2 DAI under a fluorescence microscope. Bars = 100 μm.

(B) Quantitative real-time RT-PCR analysis of viral RNA. Total RNA was extracted from protoplasts at 2 or 3 DAI and subjected to real-time RT-PCR analysis using CP-specific primers. The PlAMV RNA value was normalized relative to the actin mRNA in each sample. The mean level of PlAMV-GFP RNA in the protoplast expressing vector at 2 DAI was taken as the standard (1.0). The error bars represent the SD.

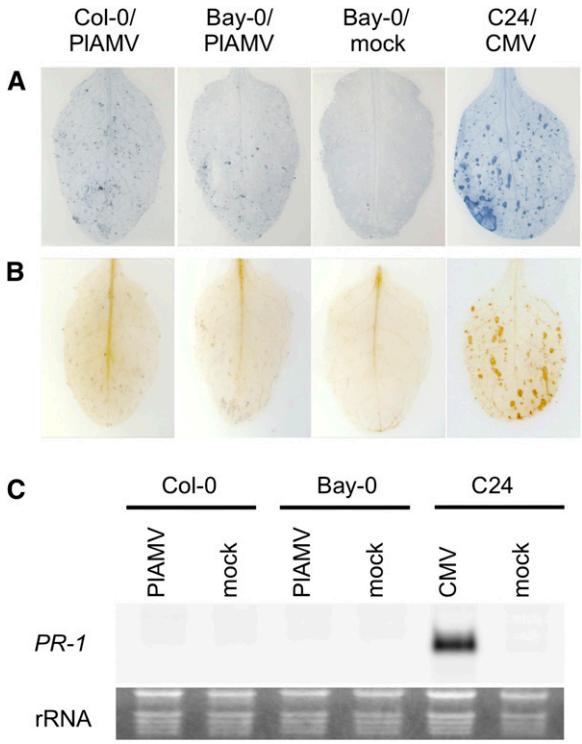

Figure 8. The Characteristics of JAX1-Mediated Resistance Are Distinct from Those of R-Mediated Resistance.

(A) Detection of dead cells with Trypan blue staining. Mock- and PlAMV-inoculated leaves of Col-0 and Bay-0 were stained with Trypan blue to visualize dead cells at 4 DAI. As a positive control, *Arabidopsis* ecotype C24 carrying *RCY1*, an NB-LRR *R* gene acting against CMV, was inoculated with CMV and subjected to Trypan blue staining.

(B) Detection of H_2O_2 by DAB staining. The inoculated leaves of the same plants in **(A)** at 4 DAI were infiltrated with DAB solution. The reaction was stopped when a brown precipitate began to appear in the CMV-inoculated C24 leaves.

(C) RNA gel blot analysis of the defense response gene *PR-1*. Total RNA was extracted from Col-0 and Bay-0 plants that were mock-inoculated or inoculated with PlAMV and C24 plants that were mock-inoculated or inoculated with CMV at 4 DAI. *PR-1* transcripts were detected by RNA gel blot analysis with a *PR-1*-specific cDNA probe. Ethidium bromide–stained rRNA is shown as a loading control.

virus, and viral RNA was detected in both the inoculated and upper leaves by RT-PCR specific to each inoculated virus in all of the plants. These results suggested that *P35S-JAX1* transgenic plants are resistant to all of the potexviruses inoculated but are susceptible to viruses from other genera, indicating that JAX1 confers broad, but specific, resistance to potexviruses.

Once Rx-mediated resistance is induced by the recognition of PVX CP, it is also effective against CMV, which is unrelated to PVX (Kohm et al., 1993). Therefore, we hypothesized that JAX1-mediated resistance may be able to cause resistance to viruses unrelated to potexviruses, but this may be activated only when plants are infected by potexviruses. Therefore, we coinoculated Bay-0 with RaMV and PlAMV to investigate whether RaMV infection is influenced by JAX1-mediated resistance that is activated by PlAMV inoculation. At 20 DAI, no PlAMV viral RNA was detected by PlAMV-specific RT-PCR in the upper uninoculated leaves of Bay-0, whereas RaMV RNA was observed using RaMV-specific RT-PCR, indicating systemic infection of Bay-0 with RaMV (see Supplemental Figure 7A online). To further investigate whether JAX1-mediated resistance has some inhibitory effect on RaMV that cannot prevent the systemic spread of RaMV, we quantified the accumulation of RaMV RNA in inoculated leaves of Bay-0. Real-time RT-PCR analysis using RaMV-specific primers showed that similar levels of RaMV RNA accumulated when RaMV was coinoculated with PlAMV compared with when it was inoculated alone (see Supplemental Figure 7B online). These results confirmed that JAX1-mediated resistance is specific to PlAMV and has no effect on RaMV.

Finally, we compared JAX1-mediated resistance with RTM1-mediated resistance. We found that JAX1 inhibits the accumulation of PlAMV at the cellular level, whereas previous studies showed that RTM1 interferes with the long-distance movement of TEV (Chisholm et al., 2001). We performed agroinfiltration analysis to compare JAX1-mediated resistance to PlAMV and RTM1-mediated resistance to TEV. We constructed a binary vector including infectious TEV cDNA under the control of the 35S promoter, which expresses GFP as a fusion protein with HC-Pro. When PlAMV-GFP was coagroinfiltrated with JAX1, no fluorescence was observed at 4 DAI (Figure 9A). By contrast, TEV-GFP fluorescence was obvious when TEV-GFP was coinfiltrated with RTM1. Real-time RT-PCR analysis and immunoblot analysis confirmed this result, indicating that RTM1 cannot produce resistance to TEV in this transient expression system in *N. benthamiana* (Figures 9B and 9C). PlAMV-GFP fluorescence was observed when PlAMV-GFP was coinfiltrated with RTM1. Similarly, bright TEV-GFP fluorescence was observed when TEV-GFP was coinfiltrated with JAX1, indicating that PlAMV and TEV infections were not influenced by RTM1 and JAX, respectively. These results suggested that JAX1 shows a different level of resistance compared with RTM1.

DISCUSSION

In this study, we identified a novel lectin gene that confers resistance to plant viruses. Although more than a dozen dominant genes responsible for resistance to plant viruses have been isolated, most of them are NB-LRR–type *R* genes (Fraile and García-Arenal, 2010). *RTM1* was isolated as the first lectin gene responsible for resistance to a potyvirus (Chisholm et al., 2000), but the importance of lectins in plant immunity to viruses has been debated for more than a decade. Here, we identify the lectin gene *JAX1*, which targets potexviruses, which are distantly related to potyviruses. *JAX1* also exhibits a level of resistance different to that of *RTM1*. Findings of lectin genes showing variety in their targets and levels of resistance strongly suggest the generality of LMR.

Properties of LMR to Plant Viruses

In this study, we showed that JAX1 interferes with virus accumulation in the inoculated leaves of the resistant ecotypes. When

PlAMV-GFP was inoculated into Bay-0 by mechanical inoculation, virus invasion was confined to a small number of local infection foci, and its accumulation was restricted to a much lower level than that in Col-0 (Figure 2). However, when PlAMV-GFP was inoculated into Bay-0 or *PJAX1-JAX1* transgenic plants by agroinfiltration, virus accumulation in the inoculated leaves was comparable to that in Col-0 (Figures 1 and 4). This inconsistency might be caused by the unusually high inoculation pressure of agroinoculation of the virus (Bendahmane et al., 2000), which could partially overcome the resistance by *JAX1* in inoculated leaves of those plants. Since potexviruses are transmitted by mechanical means, the resistance phenotype to the mechanically inoculated virus in Bay-0 should reflect the natural role of *JAX1*.

This study also indicated a significant difference in the levels of virus resistance between JAX1 and RTM1. In Bay-0, the number and size of PlAMV-GFP foci in the inoculated leaves were much lower than in the susceptible ecotype (Figure 2), whereas no difference appears to exist in the number and size of GUS-expressing TEV infection foci in the mechanically inoculated leaves between the *RTM1*-carrying resistant ecotype Col-0 and susceptible ecotype C24 (Mahajan et al., 1998). In addition, while transient expression of JAX1 by agroinfiltration in *N. benthamiana* inhibited the accumulation of PlAMV-GFP in the inoculated leaves, RTM1 expression had little effect on TEV-GFP infection under the same conditions (Figure 9). Considering that JAX1 prevents viral accumulation at the cellular level (Figure 7) and that RTM1 interferes with viral long-distance movement (Chisholm et al., 2001), JAX1- and RTM1-mediated resistance seems to inhibit different phases of viral infection.

We analyzed the expression patterns of JAX1. *PJAX1-GUS* expression was observed extensively in vascular tissues, but a certain level of expression was also observed in mesophyll cells (Figures 5B and 5C). *PJAX1-GUS* expression was also observed

Table 2. JAX1-Mediated Resistance Is Broad and Specific to Potexviruses

		Wild Type		JAX1	
Virus Species[a]	Virus Genus	Inoculated	Upper	Inoculated	Upper
PVX	*Potexvirus*	+	+	–	–
WClMV	*Potexvirus*	+	+	–	–
AV3	*Potexvirus*	+	+	–	–
TMV	*Tobamovirus*	+	+	+	+
CMV	*Cucumovirus*	+	+	+	+
TRV	*Tobravirus*	+	+	+	+
TuMV	*Potyvirus*	+	+	+	+
TEV	*Potyvirus*	+	+	+	+
PVY	*Potyvirus*	+	+	+	+
RaMV	*Comovirus*	+	+	+	+

Plant viruses from several genera were inoculated to wild-type or *JAX1* transgenic *N. benthamiana*. At 20 DAI, virus accumulation was evaluated by RT-PCR using specific primers that amplify fragments of the corresponding viruses. +, Virus-specific band detected; –, nothing detected.

[a]The plant viruses analyzed included PVX, WClMV, AV3, TMV, CMV, TRV, TuMV, TEV, PVY, and RaMV.

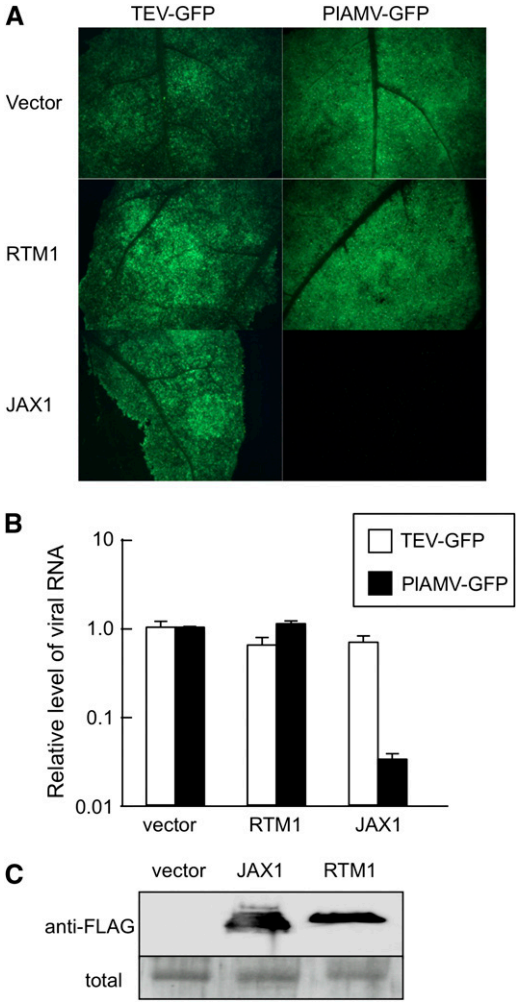

Figure 9. Comparison of JAX1- and RTM1-Mediated Resistance.

(A) Fluorescence images of TEV-GFP or PlAMV-GFP coexpressed with RTM1 or JAX1. *N. benthamiana* leaves were infiltrated with *Agrobacterium* mixtures containing TEV-GFP (left) or PlAMV-GFP (right) along with either the vector (top), RTM1 (middle), or JAX1 (bottom). GFP fluorescence indicating virus accumulation was visualized under a fluorescence microscope at 4 DAI.

(B) Viral RNA accumulation in infiltrated patches. Real-time RT-PCR analysis was performed on total RNA from the infiltrated patches shown in **(A)** using GFP-specific primers. The amount of viral RNA was normalized relative to eEF1A mRNA in each sample. The mean level of TEV-GFP and PlAMV-GFP RNA in patches coinfiltrated with the vector was taken as the standard (1.0). Error bars represent the SD.

(C) The accumulation of JAX1 and RTM1 in infiltrated patches. Immunoblot analysis was performed on total protein from patches infiltrated with TEV-GFP and the vector, RTM1, or JAX1 shown in **(A)** using anti-FLAG antibody. A similar result was obtained with patches infiltrated with PlAMV-GFP and the vector, RTM1, or JAX1. Coomassie blue–stained total protein is shown as a loading control.

in vascular tissues of roots and root apical meristems (see Supplemental Figure 3 online). However, *JAX1* transcription was not detected in roots of Bay-0 (Figure 5A). The most plausible explanation for this discrepancy is that some other root-specific regulation of JAX1 expression in Bay-0 may exist that decreases JAX1 expression in roots.

We revealed that JAX1 confers resistance at the cellular level. Some virus resistance genes responsible for cellular-level resistance, such as *Rx* and *Tm-1*, have been shown to be immune to virus infection (Adams et al., 1986; Ishibashi et al., 2007). When *JAX1* was transgenically expressed from 35S promoter, the resulting plants, including both *Arabidopsis* and *N. benthamiana*, were immune to PlAMV-GFP infection (Figures 4 and 6A). However, when *JAX1* was expressed from its own promoter, the resulting plants were not completely immune (Figure 2). Although JAX1 exhibited certain inhibitory effects on PlAMV-GFP when it was transiently expressed from its own promoter in both *N. benthamiana* leaves and *Arabidopsis* protoplasts, the inhibitory effect was lower than when JAX1 was expressed from the 35S promoter (Figure 7; see Supplemental Figure 5 online). However, this is a similar case to Rsv1, which confers a cellular-level resistance to *Soybean mosaic virus* (SMV) without an HR (Hajimorad and Hill, 2001). Indeed, although the *Rsv1*-carrying cultivar of soybean was immune to SMV when the virus was inoculated mechanically, it induced HR-like lesions when SMV was graft inoculated. A critical reason for this could be the different levels of transcriptional activation between the *JAX1* native promoter and the 35S promoter. Another reason is that since the *JAX1* promoter is extensively activated in vascular tissues, virus infection is less influenced when viruses are in mesophyll tissues and is strictly impaired when they arrive at vascular tissues. This could also possibly be explained by inefficient plant responses to the virus attack because the transcription of JAX1 was not induced by the virus inoculation (Figure 5D).

Generality of LMR to Plant Viruses

Lectins may play important roles in plant innate immunity to viruses. JAX1 and RTM1 produce virus resistance at different phases of plant virus infection, which is the same as with *NB-LRR* genes. Many *NB-LRR* genes are responsible for the tissue-level resistance associated with the HR; however, two potato *Rx* genes (*Rx1* and *Rx2*) and soybean *Rsv1* inhibit virus accumulation at the cellular level without an HR (Bendahmane et al., 1999, 2000; Hajimorad and Hill, 2001). Both Rx- and Rsv1-mediated resistance are dependent on SGT1 and RAR1, which are well-known activators of HR-associated resistance, indicating that they induce virus resistance via a pathway similar to other *NB-LRR* genes responsible for tissue-level resistance (Peart et al., 2002; Liu et al., 2004; Fu et al., 2009). Therefore, some common machinery likely exists underlying both JAX1- and RTM1-mediated resistance. Moreover, we revealed that JAX1 from *Arabidopsis* can also suppress PlAMV infection in a heterologous plant, *N. benthamiana*. This suggests that lectins can confer virus resistance beyond a single plant family, indicating the conserved defensive roles of lectins. Taken together, LMR to plant viruses may occupy an important position in plant innate immunity, just like *NB-LRR* genes.

LMR may affect resistance to a broad spectrum of plant viruses. JAX1 produced resistance to all of the potexviruses we studied. However, JAX1 did not produce resistance to plant viruses from genera other than genus *Potexvirus*. JAX1 also had no effect on infection by RaMV, a plant virus distantly related to potexviruses, even in the same tissues in which JAX1 strongly inhibited PlAMV infection (see Supplemental Figure 7 online). These results indicated that JAX1-mediated resistance was broad, but specific, to potexviruses. By contrast, RTM1 conferred resistance to several potyviruses and not to other genera of viruses, indicating that RTM1 was specific to potyviruses (Decroocq et al., 2006). Such universal and specific resistance to a limited group of plant viruses suggests that LMR targets and inhibits some common pattern that is shared within the group of viruses. Similarly, *NB-LRR* genes show resistance to multiple viruses in the same genus. The *N* and *Rx* genes induce resistance to multiple members of the genus *Tobamovirus* and genus *Potexvirus*, respectively, whereas they have no effect on unrelated viruses (Tobias et al., 1982; Baurès et al., 2008). This suggests that each gene responsible for virus resistance acts on a specific group of viruses, which enables plants to cover all of the innate immune responses to a vast diversity of viruses. Although RTM1 and JAX1 are the only known examples of lectins involved in virus resistance, other lectin-type genes may confer unidentified resistance responses to plant viruses because many resistance loci show resistance to a wide variety of plant viruses independent of HRs (Solomon-Blackburn and Barker, 2001; Kang et al., 2005).

Mechanism of LMR to Plant Viruses

As lectins are regarded as self–nonself-recognizing molecules, they may recognize plant viruses, just like NB-LRR–type R proteins, via a currently unknown mechanism. *Arabidopsis* encodes 48 jacalin-lectin genes, and one of them, MBP (encoded by At3g16450), can specifically interact with several sugars (Nagano et al., 2008; Takeda et al., 2008). JAX1 and RTM1 share substantial similarity with MBP and thus can probably bind sugars. The most attractive hypothesis is that JAX1 and RTM1 can recognize a glycosylated viral protein because lectins recognize glycosylated proteins in animal innate immune systems (Fujita, 2002). Indeed, the N-terminal region of CP encoded by PVX, a potexvirus whose infection is inhibited by JAX1, is glycosylated (Baratova et al., 2004). Moreover, the CP N-terminal region of *Plum pox virus*, which is a *Potyvirus* affected by RTM1-mediated resistance (Decroocq et al., 2006), is also glycosylated in virus-infected cells (Fernández-Fernández et al., 2002). Since the glycosylated N-terminal region of the PPV CP overlaps the viral avirulent region required for RTM1-mediated resistance (Decroocq et al., 2009), RTM1-mediated resistance may be induced by the recognition of glycosylated CP by RTM1. JAX1 may also recognize a glycosylated region of potexvirus CPs, although the possibility that JAX1 recognizes other viral or host proteins cannot be excluded.

In the animal complement system, lectin-mediated recognition of PAMPs activates a sequence of proteolytic reactions by Ser proteases, which makes the pathogen susceptible to phagocytosis, or lectins more directly impair the pathogen by causing it to aggregate (Fujita, 2002). In addition, CLR-mediated recognition

of PAMPs reportedly activates innate immune signaling, including the generation of inflammatory cytokines and chemokines (Willment and Brown, 2008). Therefore, one possible explanation for the mechanism of JAX1-mediated resistance to potexviruses is that the recognition of viruses activates resistance responses, resulting in the inhibition of viral infection. Since JAX1-mediated resistance is not associated with the properties of conventional resistance responses, including HR and defense gene expression and defensive plant hormone signaling, it may trigger currently unknown resistance pathways. In fact, RTM1-mediated resistance requires a small heat shock–like protein (RTM2) and a MATH domain–containing protein (RTM3), which are proteins of unknown functions (Whitham et al., 2000; Cosson et al., 2010). Alternatively, because JAX1-mediated resistance impairs viral accumulation at the cellular level, JAX1 may cause aggregation of the replicase or replicase-associated bodies of potexviruses, resulting in their inactivation.

It is also noteworthy that some studies have reported that plant lectins show inhibitory effects on the infection of animal viruses to their host animal cells (Balzarini et al., 1992; Cowan, 1999; Lam and Ng, 2011). In these reports, because glycoproteins are usually displayed on the surface of viral envelope structures, plant lectins have been postulated to recognize and bind to viral glycoproteins, resulting in the inhibition of animal viral infection. Plant lectin inhibition of animal virus infection might originate from the inhibitory effect of plant lectins on plant viruses. Evidence for the generality of LMR to plant viruses proposed in this study strongly supports this idea. Therefore, future studies analyzing the mechanism of LMR might uncover not only conserved defense mechanisms against plant viruses but also common strategies for inactivating invasive agents shared by animal and plant innate immunity.

METHODS

Plant Materials

Seeds of *Arabidopsis thaliana* ecotypes and the signal transduction mutants *eds5-1* (Glazebrook et al., 1996), *ein2-1* (Alonso et al., 1999), and *jar1-1* (Staswick et al., 1992) were provided by the ABRC (Ohio State University, Columbus, OH). *Arabidopsis* and *Nicotiana benthamiana* plants were grown in growth chambers with 16-h-light/8-h-dark conditions at 23 and 25°C, respectively.

Plasmid Constructions

A binary vector that expresses PlAMV fused with GFP, pPlAMV-GFP, was derived from pPlAMV-GFPΔCP, a movement-deficient PlAMV infectious cDNA that expresses GFP but lacks CP (Ozeki et al., 2009). CP cDNA fused with the foot-and-mouth-disease virus (FMDV) 2A peptide sequence (Santa Cruz et al., 1996) at its 5′ terminus was inserted between GFP and the 3′-untranslated region at the *Spe*I site of pPlAMV-GFPΔCP using primers containing *Spe*I restriction sites, resulting in the expression of a GFP-FMDV 2A–CP fusion protein under the control of the CP subgenomic promoter. GFP-FMDV 2A–CP is partially processed to generate CP in planta (Santa Cruz et al., 1996), which enables the systemic infection of PlAMV-GFP in plants.

To construct a binary vector that expresses TEV-GFP, a full-length of TEV strain HAT obtained from the American Type Culture Collection (PV-633) was cloned into pCAMBIA1301 by replacing the *GUS* gene to generate pCAMBIA-TEV. A 12-nucleotide sequence (5′-CCCGGGA-GATCT-3′) was inserted between the cleavage site of P1 and HC-Pro in pCAMBIA-TEV by PCR to introduce a multicloning site that included *Sma*I and *Bgl*II sites. GFP cDNA was cloned into the *Sma*I site of the modified pCAMBIA-TEV vector to generate pTEV-GFP.

To construct some binary vectors, we used LR Clonase reaction-mediated recombination into the pEarleyGate system (Earley et al., 2006). *JAX1* cDNA and *RTM1* cDNA were amplified from total RNA of Bay-0 and Col-0 by RT-PCR using the primer sets JAX1-F with JAX1-R and RTM1-F with RTM1-R (see Supplemental Table 4 online) and cloned into pENTR1A to generate pENTR-JAX1 and pENTR-RTM1, respectively. The resultant plasmids, pENTR-JAX1 and pENTR-RTM1, were recombined using the LR Clonase reaction (Invitrogen) into pEarleyGate301 to generate the binary vectors pEarley-JAX1 and pEarley-RTM1, respectively. A 3.5-kb genomic fragment including *JAX1* and the putative promoter region of *JAX1* was amplified by PCR with primers JAX1UP-F and JAX1-R (see Supplemental Table 4 online) from total DNA of Bay-0 and cloned into pCAMBIA1301 by replacing the 35S promoter region and GUS sequence to generate pJAX1-JAX1. The 2-kb putative promoter region of *JAX1* was PCR amplified with primers JAX1UP-F and JAX1UP-R (see Supplemental Table 4 online) and cloned into pCAMBIA1301 by replacing the 35S promoter region to generate pJAX1-GUS. Construction of pIR-GFP, a binary vector containing the inverted repeat sequence of GFP, was described previously (Senshu et al., 2009). pBin-P19, a binary vector containing the sequence of tomato bushy stunt virus p19, was kindly provided by D.C. Baulcombe (University of Cambridge, Cambridge, UK).

Virus Inoculation and Agroinfiltration

Plants were inoculated with PlAMV-GFP and TEV-GFP using agroinfiltration as described previously (Takahashi et al., 2006). Rosette leaves of 2-week-old *Arabidopsis* seedlings or young leaves of 4-week-old *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* culture carrying pPlAMV-GFP and pTEV-GFP. *Arabidopsis* plants were also inoculated mechanically with an extract of PlAMV-GFP–infected *N. benthamiana* plants, which was prepared by grinding infected leaf tissues in 0.1 M phosphate buffer, pH 7.0, as described previously (Senshu et al., 2009). *N. benthamiana* plants were also inoculated with AV3 (Hashimoto et al., 2008), CMV (Suzuki et al., 1991), PVX (Komatsu et al., 2010), PVY (Hidaka et al., 1992), RaMV (Komatsu et al., 2007), TMV (Yamaji et al., 2006), TRV (Ratcliff et al., 2001), TuMV (Nomura et al., 2004), or WClMV (Nakabayashi et al., 2002) mechanically. These viruses were detected by RT-PCR with total RNA isolated from the upper leaves of virus-inoculated plants at 20 DAI using the primers indicated in Supplemental Table 4 online.

Genetic Analysis

Col-0 plants were crossed with Bay-0 plants, and the resulting F1 plants were allowed to self-fertilize to generate F2 mapping populations. Genomic DNA was isolated using the DNeasy plant mini kit (Qiagen) from ~1500 F2 plants infected systemically with PlAMV-GFP. Then, 23 SSLP genetic markers anchored throughout the five *Arabidopsis* chromosomes were used for rough mapping of the resistance locus. For fine mapping, we generated six novel SNP markers during the course of mapping, which were identified by partial sequencing of the Bay-0 genome and a comparison with the Col-0 genomic sequence. The SNP markers SNP20.7 and SNP22.0, which flank the SSLP markers ciw1 and nF5I14, respectively, were primarily used to analyze the F2 plants. The F2 plants that proved to be recombinants of the primary SNP markers were analyzed using the secondary SNP markers SNP21.3 and SNP21.6, which flank SNP20.7 and SNP22.0, respectively. This process was repeated once more using additional SNP markers SNP21.4 and

SNP21.5, resulting in the mapping of the resistance locus to a 130-kb region. SNP analysis was performed as described previously (Kawachi et al., 2006). Primer information for the SSLP markers was obtained from the TAIR database (http://www.Arabidopsis.org/). The primer sequences for the SNP markers are given in Supplemental Table 4 online. The genomic sequence and cDNA of *JAX1* were amplified by PCR with total DNA and by RT-PCR with total RNA from Bay-0 using primers JAX1-F and JAX1-R (see Supplemental Table 4 online) and sequenced in at least three replicates to identify the base differences between Bay-0 and Col-0.

RNA Isolation and Detection

RNA was isolated from *Arabidopsis* plants and protoplasts using the RNeasy plant mini kit (Qiagen). RNA isolation from *N. benthamiana* and RNA gel blot analysis were performed as described previously (Komatsu et al., 2010). The probe for detecting PlAMV RNA was described previously (Komatsu et al., 2010). Probes for detecting *JAX1* and *PR-1* were prepared by amplifying *JAX1* cDNA using JAX1-F and JAX1-R and *PR-1* cDNA using PR-1F and PR-1R (see Supplemental Table 4 online), respectively. The quantitative real-time RT-PCR analysis was performed using SYBR Premix Ex Taq II (Takara) after cDNA synthesis using AMV reverse transcriptase (Life Technologies) and detected by the Thermal Cycler Dice real-time system (Takara) as described previously (Komatsu et al., 2010). At least three replicates of RNA samples from plant leaves or protoplasts were subjected to the analysis. Primers used to detect PlAMV RNA and *N. benthamiana* eEF1A were as described previously (Komatsu et al., 2010). Primers used to detect *Arabidopsis* actin and RaMV RNA are listed in Supplemental Table 4 online.

Immunodetection

Protein extraction and immunoblotting were performed as described previously (Kagiwada et al., 2005). Mouse monoclonal antibody to the FLAG peptide tag was obtained from Cell Signaling Technology. To prepare antibody against JAX1, hexahistidine-tagged JAX1 was expressed in *Escherichia coli* and purified as described previously (Yamaji et al., 2006). Polyclonal antibody against JAX1 was raised in a rabbit using the purified protein as antigen.

Plant Transformation

Arabidopsis Col-0 plants were transformed with *Agrobacterium* strain EHA105 carrying pEarley-JAX1, pJAX1-JAX1, pJAX1-GUS, and pCAMBIA1301 to generate the transformants *P35S-JAX1*, *PJAX1-JAX1*, *PJAX1-GUS*, and *P35S-GUS*, respectively. *Arabidopsis* was transformed using the floral dip method, as described previously (Hoshi et al., 2009). T1 plants transformed with pEarley-JAX1 were selected by spraying BASTA herbicide (Earley et al., 2006). T1 plants transformed with pJAX1-JAX1, pJAX1-GUS, and pCAMBIA1301 were isolated by kanamycin selection. Transformation of *N. benthamiana* to generate the transformant *P35S-JAX1* was performed using the leaf disk method, as described previously with *Agrobacterium* carrying pEarley-JAX1 (Yoshii et al., 2008). T1 plants transformed with pEarley-JAX1 were selected by applying BASTA (Earley et al., 2006) and PCR using primers JAX1-F and JAX1-R from the total DNA extracted.

Protoplast Analysis

Arabidopsis suspension culture cells (Mathur and Koncz, 1998) were kindly provided by S. Hasezawa (University of Tokyo, Kashiwa, Chiba, Japan). The detailed conditions for cell culture were as described previously (Oda et al., 2005). Protoplast isolation from *Arabidopsis* suspension cells and transfection were performed as described with some modifications (Abel and Theologis, 1994). First, 20 mL of suspension cells was collected by centrifugation and washed with 0.4 M mannitol. Cells were collected again and incubated with 10 mL enzyme solution (1% cellulase Onuzuka R-10 [Yakult], 0.2% Macerozyme R-10 [Yakult], 0.4 M mannitol, 10 mM $CaCl_2$, and 20 mM MES-KOH, pH 5.7) for ~90 min at 25°C. The cells were washed twice with W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM Glc, and 1.5 mM Mes-KOH, pH 5.6) and filtered through a 100-μm nylon mesh to separate the protoplasts, which were stored on ice for 30 min before transfection. The protoplasts were counted in a hemocytometer and prepared at a density of $5 \times l0^6$ protoplasts per mL. The protoplasts were collected and resuspended in the same volume of MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 5 mM Mes-KOH, pH 5.6). Then, 300 μL protoplast solution was mixed with 100 μg salmon sperm carrier DNA, 10 μg pPlAMV-GFP, and 10 μg pEarley-JAX1, pJAX1-JAX1, pIR-GFP, or pEarleyGate301. Next, 300 μL polyethylene glycol-CHS solution [0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, and 40% polyethylene glycol 4000] was added to the protoplast-plasmid mixture and incubated for 30 min at room temperature. Then, 10 mL W5 buffer was added slowly to the mixture and washed with W5 buffer twice. The transfected protoplasts were resuspended in 2 mL W5 buffer and incubated in the dark at 23°C.

Cell Death Analysis

Cell death assays, including Trypan blue and DAB staining, were performed as described previously (Komatsu et al., 2010).

Immunohistochemical Analysis and Microscopy

Immunohistochemical analysis was performed as described previously (Hoshi et al., 2009). Leaf tissues, including the vascular system, were excised from Col-0 and Bay-0 plants. The tissues were fixed, sectioned, and reacted with anti-JAX1 antibody. The localization was detected using the alkaline phosphatase–mediated reporter system. Tissues were observed with AxioImager microscopy (Carl Zeiss).

Fluorescence microscopy to detect GFP fluorescence of PlAMV-GFP and TEV-GFP was performed using an MZ16F fluorescence stereomicroscope (Leica).

Sequence Analysis

Multiple sequence alignment was performed using ClustalW multiple alignments (gap open penalty, 10.0; gap extension penalty, 0.20; selected weight matrix, BLOSUM) available from the DNA Data Bank of Japan.

Accession Numbers

Sequence data from this article can be found in GenBank/EMBL data libraries or the Arabidopsis Genome Initiative under the following accession numbers: *Artocarpus heterophyllus* jacalin, AAA32680; *Arabidopsis* ecotype Col-0 *RTM1*, At1g05760; MBP, At3g16450; cDNA of At1g58160 in Col-0, AB638773; L*er*, AB638774; Bay-0, AB638775 (*JAX1*); Dra-2, AB638776; Eil-0, AB638777; Ga-0, AB638778; and Is-1, AB638779.

Supplemental Data

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

Supplemental Figure 1. Comparison of the Spread of PlAMV-GFP in the Inoculated Leaves between Col-0 and Bay-0.

Supplemental Figure 2. Sequence Analysis of At1g58160 cDNA.

Supplemental Figure 3. Detailed Observation of GUS Expression in *PJAX1-GUS* Transgenic Plants.

Supplemental Figure 4. Confirmation of Transformation with Transgenic *N. benthamiana* Plants Expressing *JAX1* under the Control of the 35S Promoter.

Supplemental Figure 5. A Certain Level of Inhibition of Virus Accumulation in *N. benthamiana* Leaves by JAX1 Expressed from Its Own Promoter.

Supplemental Figure 6. JAX1-Mediated Resistance Is Unaffected by an RNA Silencing Suppressor.

Supplemental Figure 7. Coinfection Assay of PlAMV and RaMV in JAX1-Expressing Plants.

Supplemental Table 1. Comparison of the Size of PlAMV-GFP Foci in the Inoculated Leaves between Col-0 and Bay-0.

Supplemental Table 2. Genetic Analysis of the Resistant Phenotype in Dra-2, Eil-0, Ga-0, and Is-1.

Supplemental Table 3. Linkage Analysis Using SSLP Markers on Chromosome 1 of Dra-2, Eil-0, Ga-0, and Is-1.

Supplemental Table 4. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

Y.Y., K.M., K.K., and S.N. designed the research. Y.Y., K.M., K.K., T.S., Y.O., M.H., K. S., Y.N., N.M., C.M., and M.H. performed the research. Y.Y., K.M., K.K., and S.N. analyzed the data. Y.Y. and S.N. wrote the article.

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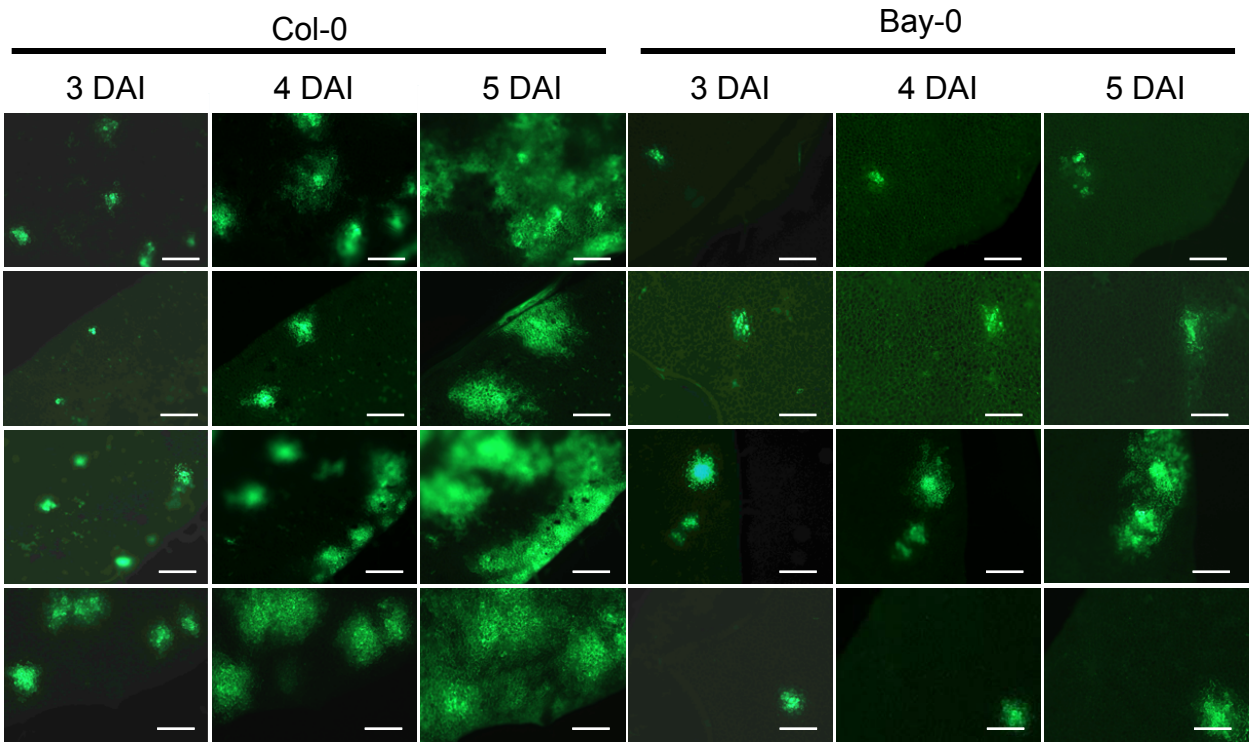
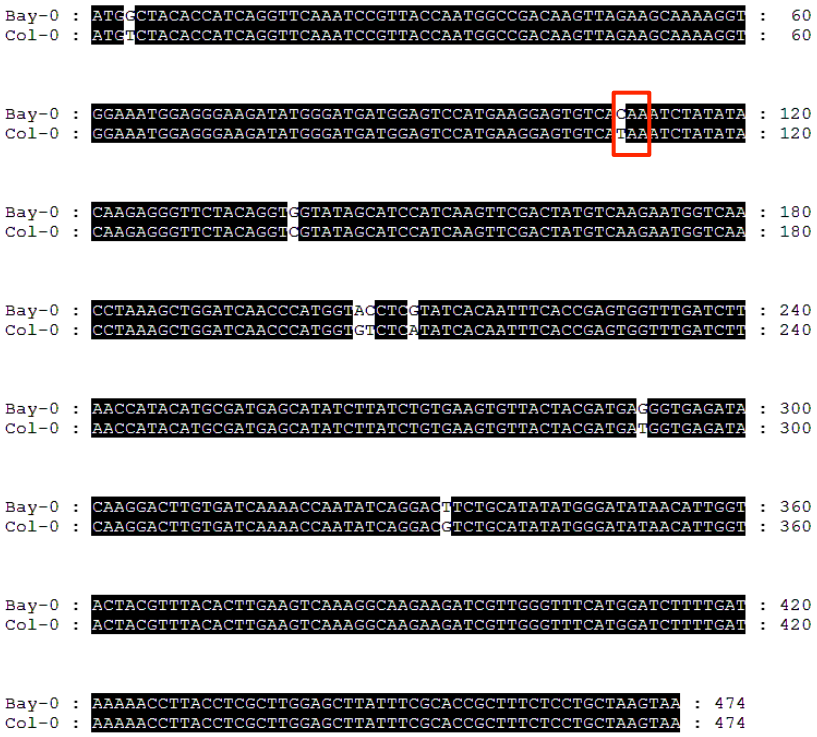


Supplemental Figure 1. Comparison of the Spread of PlAMV-GFP in the Inoculated Leaves between Col-0 and Bay-0.

Extracts from PlAMV–GFP-infected plants were mechanically inoculated to Col-0 and Bay-0. Inoculated leaves were excised from plants and incubated in humid airtight box. The same tissues were observed under the fluorescent microscopy at the indicated consecutive days. Four independent experiments were performed. The bar represents 0.5 mm.

A

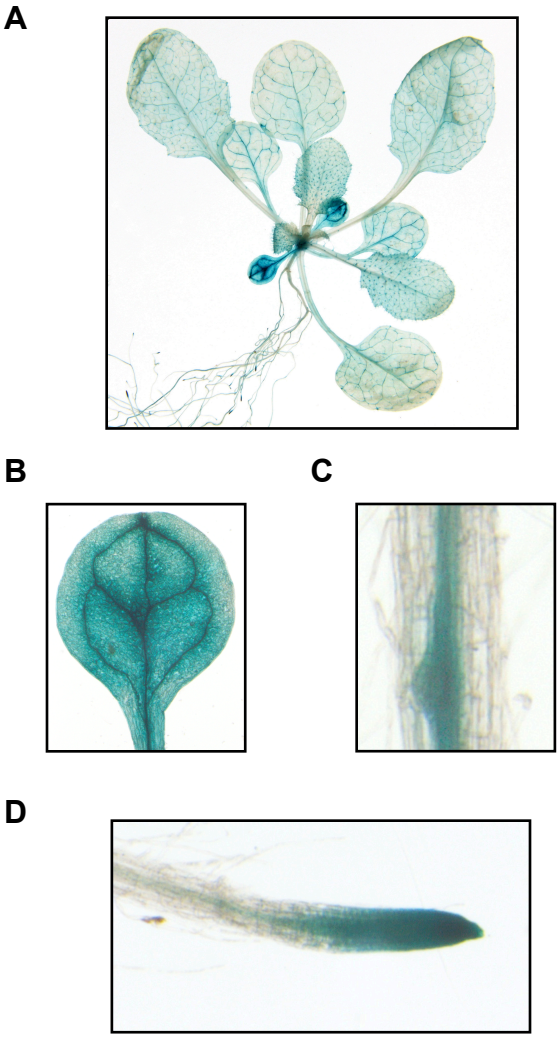


B

Supplemental Figure 2. Sequence Analysis of At1g58160 cDNA.

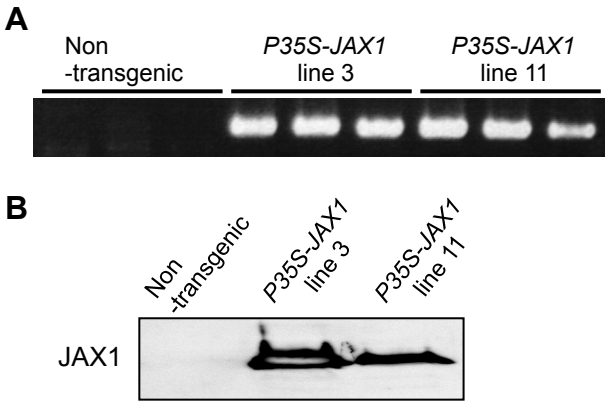
(**A**) The nucleotide sequence alignment of At1g58160 cDNA from Col-0 and Bay-0. At1g58160 cDNAs amplified from total RNA of Col-0 and Bay-0 were sequenced and aligned. The red box indicates the position of the internal translational termination codon found in the At1g58160 cDNA of Col-0.

(**B**) Amino acid sequence alignment of At1g58160-encoded proteins. The putative amino acid sequences encoded by the At1g58160 cDNAs of Bay-0, Ga-0, Dra-2, Eil-0, Is-1, Col-0, and Ler were aligned. The PlAMV-resistant ecotypes (Bay-0, Ga-0, Dra-2, Eil-0 and Is-1) encoded 157-amino-acid proteins, whereas the susceptible ecotypes (Col-0 and Ler) encoded N-terminal 36-amino-acid fragments.



Supplemental Figure 3. Detailed Observation of GUS Expression in *PJAX1–GUS* Transgenic Plants.

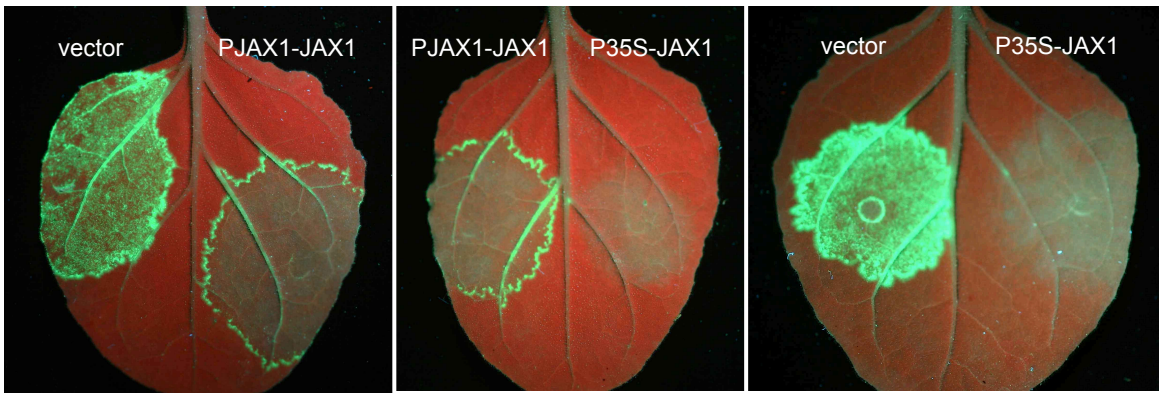
Images of the entire plant (**A**), cotyledon (**B**), stem (**C**) and root apical meristem (**D**) of *PJAX1-GUS* transgenic Col-0 plants indicated in Figure 5B are shown.



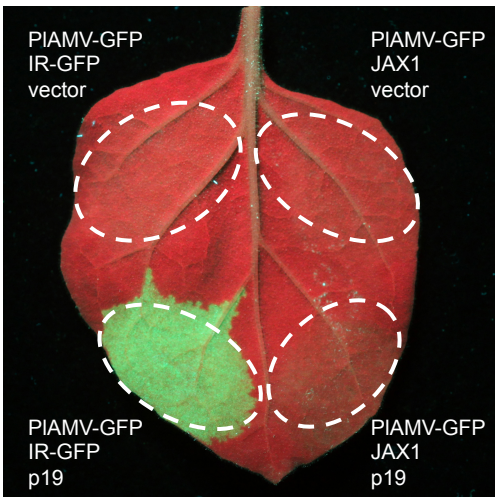
Supplemental Figure 4. Confirmation of Transformation with Transgenic *N. benthamiana* Plants Expressing *JAX1* under the Control of the 35S Promoter.

(**A**) *JAX1* was amplified by PCR from total DNA of non-transgenic plants and two lines of *P35S-JAX1* transgenic plants (lines 3 and 11) using *JAX1*-specific primers.

(**B**) Expression of JAX1 in transgenic plants. Immunoblot analysis was performed on total protein from non-transgenic plants and two lines of *P35S-JAX1* transgenic plants using anti-FLAG antibody.

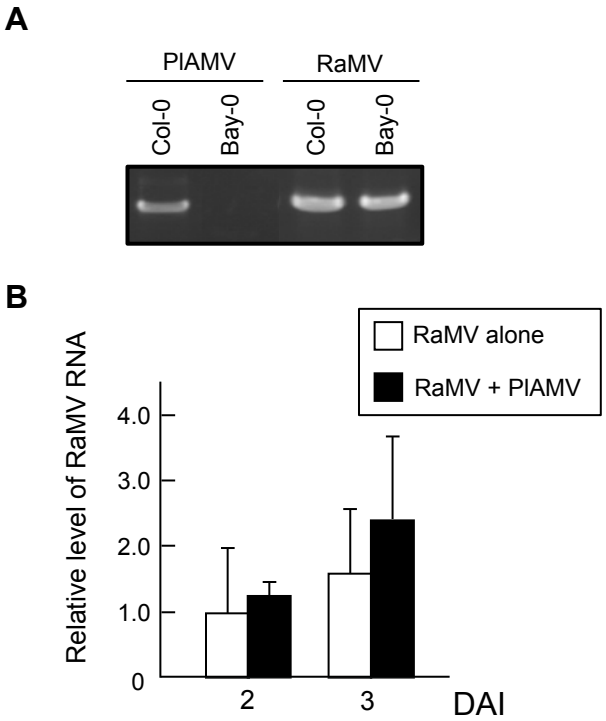


Supplemental Figure 5. A Certain Level of Inhibition of Virus Accumulation in *N. benthamiana* Leaves by JAX1 Expressed from Its Own Promoter.
N. benthamiana leaves were infiltrated with *Agrobacterium* mixtures containing PlAMV–GFP and indicated vectors. GFP fluorescence indicating virus accumulation was visualized under UV light at 5 DAI.



Supplemental Figure 6. JAX1-mediated Resistance Is Unaffected by an RNA Silencing Suppressor.

N. benthamiana leaves were infiltrated with *Agrobacterium* mixtures carrying PlAMV–GFP together with the indicated vectors. p19 is a strong RNA silencing suppressor encoded by TBSV. GFP fluorescence indicating virus accumulation was visualized under UV light at 4 DAI. Infiltrated patches are indicated by white dotted circles.



Supplemental Figure 7. Coinfection Assay of PlAMV and RaMV in JAX1-expressing Plants.
(**A**) Systemic infection of RaMV in Bay-0 plants co-inoculated with PlAMV. Col-0 and Bay-0 were co-inoculated with PlAMV and RaMV by agroinfiltration. Total RNA from upper uninoculated leaves was analyzed by RT–PCR using PlAMV- and RaMV-specific primers at 20 DAI.
(**B**) RaMV accumulation in Bay-0 plants co-inoculated with PlAMV. Bay-0 was inoculated with RaMV alone or with RaMV and PlAMV. RaMV RNA accumulation in the inoculated leaves at 2 or 3 DAI was analyzed using quantitative real-time RT–PCR with RaMV-specific primers. The accumulation of actin mRNA was used as a reference. The mean level of RaMV RNA in leaves inoculated with RaMV alone at 2 DAI was taken as the standard (1.0). The error bars represent the SD. The levels of RaMV RNA in leaves inoculated with RaMV alone and in leaves co-inoculated with RaMV and PlAMV were not significantly different ($P > 0.05$) at both 2 and 3 DAI.

Supplemental Table 1. Comparison of the Size of PlAMV-GFP Foci in the Inoculated Leaves between Col-0 and Bay-0.

Time (DAI)	Ecotype	Total [a] (n)	1 cell [b] [n (%)]	2 cells [n (%)]	3 cells [n (%)]	≥ 4 cells [n (%)]
2	Col-0	55	12 (22)	5 (9)	6 (11)	32 (58)
	Bay-0	6	2 (33)	1 (17)	1 (17)	2 (33)
3	Col-0	33	3 (9)	1 (3)	0 (0)	29 (88)
	Bay-0	11	4 (36)	1 (9)	0 (0)	6 (55)

Extracts from PlAMV–GFP-infected plants were mechanically inoculated to Col-0 and Bay-0, and viral fluorescent foci in the inoculated leaves were observed under the fluorescent microscopy at the indicated DAI.

[a] Total number of PlAMV–GFP fluorescent foci observed.

[b] Number and percentage of fluorescent foci including indicated number of fluorescent cells.

Supplemental Table 2. Genetic Analysis of the Resistant Phenotype in Dra-2, Eil-0, Ga-0 and Is-1.

Plants	Resistant	Susceptible
Dra-2	7	0
Eil-0	7	0
Ga-0	7	0
Is-1	7	0
F_1 (Col-0 X Dra-2)	4	0
F_2 (Col-0 X Dra-2)	87 [a]	28 [a]
F_1 (Col-0 X Eil-0)	NT [b]	NT
F_2 (Col-0 X Eil-0)	90 [c]	19 [c]
F_1 (Col-0 X Ga-0)	5	0
F_2 (Col-0 X Ga-0)	89 [d]	22 [d]
F_1 (Col-0 X Is-1)	5	0
F_2 (Col-0 X Is-1)	103 [e]	21 [e]

Indicated plants were inoculated with PlAMV–GFP. Virus infection was evaluated whether the spread of GFP expression from PlAMV–GFP systemically (susceptible) or not (resistant) at 20 DAI.

[a] c^2 (3:1) = 0.026; $P > 3$

[b] NT ; not tested

[c] c^2 (3:1) = 3.33; $0.02 > P > 0.01$

[d] c^2 (3:1) = 1.59; $P > 0.05$

[e] c^2 (3:1) = 4.30; $0.01 > P > 0.005$

Supplemental Table 3. Linkage Analysis Using SSLP Markers on Chromosome 1 of Dra-2, Eil-0, Ga-0 and Is-1.

Resistant ecotypes	nga63	ciw1	nga280	nga111
Dra-2	17/40	4/40	0/42	NT [a]
Eil-0	15/38	NT	0/36	8/36
Ga-0	NT	NT	0/38	NT
Is-1	NT	2/28	0/36	NT

Resistant ecotypes were crossed with susceptible Col-0 plants and the resulted F_1 plants were self-fertilized to generate F_2 populations. PlAMV–GFP -susceptible F_2 plants were analyzed by SSLP markers anchored on chromosome 1. Number of recombinants per total plants is indicated.

[a] NT ; not tested

Supplemental Table 4. Primers Used in This Study.

Primer	Sequence (5' -> 3')
JAX1-F	TTTGGATCCCTGGAAGATCCATCGGGTAC
JAX1-R	TTTCTCGAGGAATTCTTACTTAGCAGGAGA AAG CGG
JAX1UP-F	AAAGTCGACGGATCCGATTGATGGGTTCCATGGAAA
JAX1UP-R	CCTGATGGTGTAGCCATAGAATTGG
RTM1-F	CCCGTCGACATGAAGATAGGACCTGTAGGGAAGCATG A
RTM1-R	CCACTCGAGTCAGCCCAGTACAATTTTTGACTCTGTTTC C
AV3-F [a]	CCATGGAAATTAGTTATATAGTAGAT
AV3-R [a]	CTAAGAGCCCCGATGGCG
CMV-F [b]	ATGGACAAATCTGAATCAACCAG
CMV-R [b]	TCAGACTGGGAGCACTCCAG
PVX-F [c]	ATTTCAATTGCATCAGCACCAGCTAGCACAACACAG
PVX-R [c]	TAAAAACTAGTTGGTGGTGGTAGAGTGACAAC
PVY-F [d]	GTGCCAAAGCTTGGAACCTGG
PVY-R [d]	TCCTCCTTCTCTGAAAGGTGAT
RaMV-F [e]	ATGAATTCCATATGTGCTACAACAGTGGAGTACG
RaMV-R [e]	AATTCTCGAGAGGCGAAGTGGCATCAACATC
TMVW-F [f]	ATGTCATACAACATCACGAACTCG
TMVW-R [f]	CTATTTAGCCGGCGCAGTAG
TRV1F [g]	GTTGATCAACTCGTTGTTCGGTCC
TRV1R [g]	CAGCTCTCTGTGCCTTCTTCC
TuMV-F [h]	TGGAATTCCCGATCAAACCG
TuMV-R [h]	CTCACCACATGCGCTAACAC
WClMV-F [i]	AAACATATGGCAACCACCACAGCAAC
WClMV-R [i]	AAACTCGAGCTGGGGATAGGTAATAAGGG
RaMV-F [j]	TCTGCCAATGAAACGGAGG
RaMV-R [j]	CCCCTGCCATTACCTTTGTG
AtActin-F [k]	TGGCATCACACTTTCTACAA
AtActin-R [k]	CCACTGAGCACAATGTT
AtPR1-F [l]	CAACTTAGAAAAATGAATTTTACT
AtPR1-R [l]	GAAAGACATTAACTATAATAATTAATTAT
AT1G20726301F [m]	TAGTATCTTCTTTCCTTCTCCA
AT1G20727800R [m]	CTGAATCTCTACAATTGCTCG
AT1G21387101F [n]	TTTGTCTGCAGCTCCACTTTA
AT1G21388400R [n]	CTCAGTTCTCACTTGGTTCTT
AT1G21493416F [o]	CCCATCAATAGTGACTGATCCACTCTG
AT1G21495195R [o]	GGGTGAAGATACTGCAAGAGGTATGTG
AT1G21520196F [p]	GAGCTTACGGACCCGTGATCTTTGT
AT1G21521340R [p]	CCTGTTCTGGTCTGGTTGTCTGAAT
AT1G21609001F [q]	GTTCCAATATTATTCTTTTCTTC
AT1G21610400R [q]	ATTTTGTGTGTAACAATTCTATG
AT1G22051301F [r]	TCGATCATCGTAGTTTCTATTTC
AT1G22053100R [r]	CTGAATGTATCTTTGCTGATTG

[a-i] Primers for RT-PCR of AV3 [a], CMV [b] , PVX [c], PVY [d], RaMV [e], TMV [f], TRV [g], TuMV [h] and WClMV [i].

[j] Primers for real-time RT–PCR of RaMV.

[k] Primers for real-time RT–PCR of *A. thaliana* actin.

[l] Primers for RT–PCR of PR-1.

[m-r] Primers for SNP20.7 [m], SNP21.3 [n], SNP21.4 [o], SNP21.5 [p], SNP21.6 [q] and SNP22.0 [r].