Lectin-Mediated Resistance Impairs Plant Virus Infection at the Cellular Level

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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).
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; Wilmont 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 endogenous 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; Fieegmann 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 distant from 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 PIAMV infectious clone (pPIAMV-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 PIAMV-GFP used for ecotype screening. GFP was expressed as a fusion protein with CP under the control of the CP subgenomic promoter. The PIAMV-GFP infectious cDNA was driven by the 35S promoter and inoculated using agroinfiltration.

(B) The inability of PIAMV-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 PIAMV-GFP by agroinfiltration, and GFP fluorescence was visualized with a UV lamp at 20 DAI. PIAMV-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 PIAMV-GFP RNA in inoculated and upper leaves of the ecotypes in (B). PIAMV-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 PIAMV-GFP susceptibility. Two plants per ecotype were inoculated with PIAMV-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 PIAMV-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 (Ler), displayed systemic fluorescence. RT-PCR analysis of PIAMV-GFP RNA showed that PIAMV-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 PIAMV-GFP cannot infect systemically.

Next, we examined the virus resistance exhibited by the isolated ecotypes. To evaluate the resistance accurately, we inoculated PIAMV-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 PIAMV-GFP–inoculated leaves of Col-0 and Bay-0 showed that both the size and number of PIAMV-GFP fluorescent foci were smaller in Bay-0 than in Col-0 (Figure 2B). The number of PIAMV-GFP foci in the inoculated leaves of Bay-0 was significantly lower than that of Col-0 (Figure 2C). The spread of PIAMV-GFP was also impaired in the inoculated leaves of Bay-0 compared with Col-0. PIAMV-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 PIAMV-GFP was slower in Bay-0 than in Col-0 (see Supplemental Figure 1 online). These results showed that PIAMV-GFP accumulation was inhibited in the inoculated leaves of Bay-0, which resulted in the resistance phenotypes of Bay-0.

**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 PIAMV-GFP, no F1 progeny were infected systemically, indicating that all of the F1 progeny were resistant to PIAMV-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 PIAMV-GFP, PIAMV-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 ($\chi^2 = 0.85; P > 0.2$), 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

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**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 PIAMV-GFP–infected plants were mechanically inoculated into Col-0 and Bay-0. RNA gel blot analysis of PIAMV-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) PIAMV-GFP foci in inoculated leaves of susceptible and resistant ecotypes. PIAMV-GFP–inoculated leaves of Col-0 and Bay-0 in (A) were observed under UV irradiation at 3 DAI.

(C) The number of PIAMV-GFP foci in inoculated leaves of susceptible and resistant ecotypes. The numbers of PIAMV-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 s.d. [See online article for color version of this figure.]
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 SNP214.4 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 (Col-0) showed that resistant ecotypes (Dra-2, Eil-0, Ga-0, and Is-1) had the same internal termination codon in At1g58160 ORF (Figure 3C). To analyze 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, P35S–JAX1, and transformed into Col-0 using an Agrobacterium–mediated method. When these transgenic plants (P35S–JAX1) were inoculated with PIAMV-GFP by agroinfiltration, PIAMV could not systemically infect most of the transformants (61 of 73 plants) (Figure 4A). Quantitative real-time RT-PCR analysis using PIAMV-specific primers showed that the accumulation of PIAMV-GFP RNA in inoculated leaves was similar to that in nontransgenic plants (Figure 4B). However, the level of PIAMV RNA in upper leaves of the P35S–JAX1 transgenic 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 PIAMV-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 PIAMV 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 PIAMV-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 structures.
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 PIAMV-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 PIAMV (Figure 5D). These results indicated that JAX1 expression is not induced during the resistance reactions to PIAMV.

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 PIAMV. 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 PIAMV-GFP, bright GFP fluorescence was observed in both the inoculated and upper uninoculated leaves at 20 DAI, indicating systemic infection with PIAMV-GFP (Figure 6A). By contrast, when both lines of P35S-JAX1 transgenic N. benthamiana plants were inoculated with PIAMV-GFP, no GFP fluorescence was observed in either inoculated or upper leaves at 20 DAI, indicating the inhibition of PIAMV-GFP infection by JAX1. Therefore, JAX1 can produce strict resistance to PIAMV in the heterologous plant N. benthamiana.
Next, we investigated whether JAX1 can inactivate PIAMV 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 PIAMV infection in the primary inoculated leaves. In control experiments, when PIAMV-GFP was co-expressed with the vector control by agroinfiltration, GFP fluorescence showing intense accumulation of PIAMV-GFP was observed in infiltrated leaves at 5 DAI (Figure 6B). Strikingly, no GFP fluorescence was detectable in the leaves infiltrated with PIAMV-GFP and JAX1. Consistent with this result, RNA gel blot analysis of viral RNA showed that PIAMV-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 PIAMV-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 PIAMV-GFP infection was inhibited in the inoculated leaves of Bay-0 when it was mechanically inoculated (Figure 2B), these results suggested that JAX1 inhibits PIAMV infection at the early infection step.

To investigate the cellular-level effect of JAX1 on virus accumulation, JAX1-mediated resistance to PIAMV was evaluated in Arabidopsis protoplasts. Protoplasts extracted from Arabidopsis suspension culture (Col-0) were transfected with a plasmid expressing PIAMV-GFP and either an empty vector, a plasmid expressing JAX1 under its own (PJAX1-JAX1), or 3SS promoter (P3SS-JAX1). At 2 DAI, PIAMV-GFP showed bright fluorescence in a large number of vector-introduced protoplasts, whereas PIAMV-GFP fluorescence showed a certain reduction in P3SS-JAX1-introduced protoplasts and, furthermore, decreased drastically in P3SS-JAX1-introduced protoplasts (Figure 7A). Real-time RT-PCR analysis of PIAMV-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 P3SS-JAX1 protoplasts and ~10% in P3SS-JAX1 protoplasts compared with vector-introduced protoplasts, indicating a significant decrease in virus accumulation in P3SS-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 PIAMV-GFP, we used a binary vector, pIR-GFP, which includes an inverted-repeat sequence of GFP under the control of the 3SS 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 P3SS-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 P3SS-JAX1 and IR-GFP express their downstream sequences under the 3SS 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 PIAMV-GFP–inoculated leaves of Col-0 and Bay-0 (Figure 8A), showing that JAX1-mediated resistance is not accompanied by cell death.

![Figure 4. Complementation Analysis of the JAX1 Gene.](image)

(A) Inhibition of systemic PIAMV-GFP infection in transgenic Col-0 plants expressing JAX1. Nontransgenic Col-0 plants and P3SS-JAX1 and P3SS-JAX1 transgenic plants were inoculated with PIAMV-GFP by agroinfiltration. GFP fluorescence indicating virus infection was visualized under UV light at 20 DAI.

(B) Quantitative detection of PIAMV-GFP RNA in JAX1 transgenic plants. Total RNA was extracted from inoculated leaves at 5 DAI, upper leaves at 20 DAI of six PIAMV-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 P3SS-JAX1 and P3SS-JAX1 transgenic plants. The accumulation level of endogenous actin mRNA was used as a reference. The mean level of PIAMV-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
Plants were transformed with the plant hormone 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). PIAMV-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.

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). PIAMV-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 PIAMV-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 PIAMV-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 PIAMV-GFP with JAX1 and p19, no GFP fluorescence was observed, which is similar to the patch of PIAMV-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 families and tested their infectivity to JAX1-expressing plants. We selected potexviruses (Potyviridae) because they are small RNA viruses with a linear single-stranded RNA genome and often cause systemic infections. To test the broad resistance of JAX1, we inoculated several potexviruses, including cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), potato leafroll virus (PLRV), potato virus Y (PVY), potato virus X (PVX), potato plant virus (PPV), and potato virus S (PVS). The results showed that JAX1-mediated resistance was not disrupted in any of these potexviruses (Figure 8), indicating that JAX1 confers broad resistance to potexviruses.

**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 PIAMV-inoculated leaves at the indicated DAI using the JAX1 cDNA as a probe. Ethidium bromide–stained rRNA is shown as a loading control.
genera into P35S-JAX1 transgenic N. benthamiana plants, as shown in Figure 6A. In addition to PIAMV, 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 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 6. Strict Inhibition of Virus Accumulation in N. benthamiana Leaves Expressing JAX1.

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

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

(C) PIAMV-GFP RNA accumulation in infiltrated leaves. RNA gel blot analysis was performed on total RNA from the infiltrated leaves shown in (B) using PIAMV 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.

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

(A) Reduction of PIAMV-GFP fluorescence in protoplasts expressing JAX1. DNA mixtures containing PIAMV-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 PIAMV RNA value was normalized relative to the actin mRNA in each sample. The mean level of PIAMV-GFP RNA in the protoplast expressing vector at 2 DAI was taken as the standard (1.0). The error bars represent the SD.
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 PIAMV to investigate whether RaMV infection is influenced by JAX1-mediated resistance that is activated by PIAMV inoculation. At 20 DAI, no PIAMV viral RNA was detected by PIAMV-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 PIAMV compared with when it was inoculated alone (see Supplemental Figure 7B online). These results confirmed that JAX1-mediated resistance is specific to PIAMV and has no effect on RaMV.

Finally, we compared JAX1-mediated resistance with RTM1-mediated resistance. We found that JAX1 inhibits the accumulation of PIAMV 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 PIAMV 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 PIAMV-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 confirmed this result, indicating that RTM1 cannot produce resistance to TEV in this transient expression system in N. benthamiana (Figures 9B and 9C). PIAMV-GFP fluorescence was observed when PIAMV-GFP was coinfiltrated with RTM1. Similarly, bright TEV-GFP fluorescence was observed when TEV-GFP was coinfiltrated with JAX1, indicating that PIAMV 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 NBS-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
PIAMV-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 PIAMV-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 PIAMV-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 PIAMV-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 |
|---|---|---|---|---|
| Virus Species | Virus Genus | Wild Type | JAX1 |
| PVX | Potexvirus | ++ | + | ++ |
| WCIMV | Potexvirus | ++ | + | + |
| AV3 | Potexvirus | + | + | + |
| TMV | Tobamovirus | + | + | + |
| CMV | Cucumovirus | ++ | + | + |
| TRV | Tobaviruses | ++ | + | + |
| TuMV | Potexvirus | + | + | + |
| TEV | Potexvirus | ++ | + | + |
| PVY | Potexvirus | ++ | + | + |
| RaMV | Coronaviridae | ++ | + | + |

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.

*The plant viruses analyzed included PVX, WCIMV, AV3, TMV, CMV, TRV, TuMV, TEV, PVY, and RaMV.
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 transiently expressed from 35S promoter, the resulting plants, including both Arabidopsis and N. benthamiana, were immune to PIAMV-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 PIAMV-GFP when it was transiently expressed from its own promoter in both N. benthamiana leaves and Arabidopsis protoplasts, the inhibitory effect was lower 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 impared 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 PIAMV 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 PIAMV 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 PAMV fused with GFP, pPAMV-GFP, was derived from pPAMV-GFPΔCP, a movement-deficient PAMV 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 of the SpeI site of pPAMV-GFPΔCP using primers containing SpeI 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 PAMV-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-833) was cloned into pCAMBIA1301 by replacing the GUS gene with a 12-nucleotide sequence (5′-CCCGGGGA-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 SmaI and BglII sites. GFP cDNA was cloned into the SmaI 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 pRl-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 PAMV-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 pPAMV-GFP and pTEV-GFP. Arabidopsis plants were also inoculated mechanically with an extract of PAMV-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), PYY (Hidaka et al., 1992), RaMV (Komatsu et al., 2007), TMV (Yamaji et al., 2006), TRV (Ratcliffe et al., 2001), TuMV (Nomura et al., 2004), or WCIMV (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 PAMV-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 nF51l4, 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) 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 P3SS-JAX1, PJAX1-JAX1, PJAX1-GUS, and P3SS-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 P3SS-JAX1 was performed using the leaf disk method, as described previously with Agrobacterium carrying pEarley-JAX1 (Yoshi 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 Onozuka R-10 [Yakult], 0.2% Macerozyme R-10 [Yakult], 0.4 M mannitol, 10 mM CaCl2, 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 CaCl2, 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 × 105 protoplasts per mL. The protoplasts were collected and resuspended in the same volume of MaMg solution (0.4 M mannitol, 15 mM MgCl2, 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 pEarleyGata301. Next, 300 μL polyethylene glycol-CHS solution [0.4 M mannitol, 0.1 M Ca(NO3)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).

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; Ler, 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 3SS 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 PIAMV and RaMV in JAX1-Expressing Plants.

Supplemental Table 1. Comparison of the Size of PIAMV-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


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Correction


Johji Ozeki has been added to the list of authors.

The Author Contributions section has been amended accordingly.

AUTHOR CONTRIBUTIONS