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Activation of defense-related gene expression and systemic acquired resistance in cucumber mosaic virus-infected tobacco plants expressing the mammalian 2′5′oligoadenylate system

Brief Report

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Summary. Tobacco plants expressing the mammalian 2′5′oligoadenylate system (2-5A system) exhibit resistance to cucumber mosaic virus (CMV). Here, to characterize the molecular aspect of the resistance to CMV in 2-5A system-expressing tobaccos, the activation of defense-related genes and systemic acquired resistance (SAR) as the markers for the hypersensitive resistance (HR), were elucidated. Northern hybridization analysis indicated that the expression of four pathogenesis-related (PR) protein genes and five HR-related genes were induced in CMV-infected tobaccos expressing 2-5A system. Furthermore, the induction of SAR against *Pseudomonas syringae* pv. *tabaci* as second challenge, was observed on CMV-inoculated tobaccos expressing 2-5A system. These results suggested that the resistance to CMV in tobacco expressing 2-5A system is associated with the establishment of an HR-like response.

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The resistance to plant viruses in tobaccos transformed with mammalian 2′5′oligoadenylate system (2-5A system), has been reported by two independent research groups [18, 21]. At the observational level, this resistance response to virus infection was very similar to the hypersensitive resistance (HR), which is

one of the typical resistance responses of host plants to pathogens, including virus, fungi and bacteria. The resistance response to virus in 2-5A system-expressing tobacco was accompanied with cell death at the primary infection site of virus-inoculated leaves. The 2-5A system consists of two components, 2′5′-oligoadenylate synthetase (2-5Aase) and ribonuclease L (RNase L). Because the replicating intermediates of RNA virus could bind to 2-5Aase thereby activating RNase L in mammalian cells, the same reaction about 2-5Aase and RNase L seems to be also induced by virus dsRNA in 2-5A system-expressing tobacco cells infected with virus. However, the HR-like cell death is not observed in mammalian cells. Furthermore, it has not been identified whether the real HR was induced in virus-infected tobaccos expressing 2-5Aase and RNase L. Therefore, the molecular mechanism of this 2-5A system-mediated virus resistance in the transgenic tobacco still remains to be investigated.

The induction of the HR in virus-infected plants is generally accompanied with several molecular markers [9–11]. The accumulation of pathogenesis-related (PR) proteins seems to be a common marker for the HR, although some abiotic stresses can activate the expression of PR-protein genes in plants [16, 31, 32]. The HR is usually accompanied with programmed cell death [13, 19]. *HIN1* and *HSR203J* are thought to be available markers to monitor the induction of programmed cell death [23, 25].In the HR, the accumulation of phytoalexin is also generally observed [7, 11]. Before increasing the amount of phytoalexin, the expression of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) genes is usually induced [7, 17]. Furthermore, lipid peroxidation increases during the HR in several pathosystems [27]. Because lipid peroxidation can be induced by lipoxygenase (LOX), the activation of *LOX* gene during the HR seems to play a role for causing HR-associated membrane damage [27]. Except for these defense-related gene expressions during the induction of the HR, another typical event concerning the HR is likely to be the induction of systemic acquired resistance (SAR). SAR is the broad resistance response to various pathogens on upper non-inoculated leaves of necrogenic pathogen-infected plants showing necrotic local lesions (NLLs) on their inoculated leaf [9, 11]. Salicylic acid (SA)-mediated signaling pathway is thought to control the activation of many defense-related genes and the induction of SAR [1, 9, 10, 16, 33].

Cucumber mosaic virus (CMV) has the largest host range of any plant viruses and causes severe loss in agricultural production in many countries [22]. *Nicotiana* species are susceptible to CMV. Although the severity of mosaic symptoms in CMV-infected tobacco depends on the strain of CMV, CMV systemically spreads in tobacco plants. Except for two CMV mutants [28, 29], the local infection of CMV with the NLL formation has not been reported in *Nicotiana* species. However, surprisingly, the localization of CMV spread with cell death was observed in 2-5A system-expressing tobaccos [21]. Here, for better understanding the resistance to virus in 2-5A system-expressing tobaccos at the molecular level, the activation of these defense-related genes and the induction of SAR in CMV-infected tobaccos expressing 2-5A system were investigated.

Tobacco (*Nicotiana tabacum* cv. Xanthi nc) was grown in the growth cabinet at 27 °C under 150 μmol m^{-2} s^{-1} for 14 hr light condition. Tobacco plants expressing 2-5Aase and RNase L were obtained from F1 population of a cross between tobaccos transformed with cDNAs to *2-5Aase* (A13) and *RNase L* (HRL27) under the control of CaMV 35S promoter [21]. The presence of cDNAs to *2-5Aase* and *RNase L* in each transgenic plant was confirmed by PCR with two pairs of primers, 5′-ATGGATCTCAGAAATACCCC-3′ and 5′-TGGGGGTTAGGTTTATAGCT-3′ for *2-5Aase* and 5′-CATGGAGAGCAGGGATCATA-3′ and 5′-TTTCCTCAGC CGCTCTTGAT-3′ for *RNase L*, respectively. The genomic DNA for PCR template was isolated from tobacco by the CTAB method [20]. 1 μg of genomic DNA was added to 50 μl of 10 mM Tris-HCl (pH8.3) containing 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 0.2 μM each primers, and 5 units of Taq DNA polymerase (Promega, Madison, WI, U.S.A.) for PCR. The reaction ran with the program: 30 cycles at 94 °C for 1 min, 55 °C for 2 min, and 73 °C for 1 min. PCR product was analyzed by agarose gel electroporesis. A yellow strain of CMV [CMV(Y)] was purified by the method of Lot et al. [15]. Transgenic tobaccos expressing 2-5Aase and RNase L were inoculated with 100 μg/ml of CMV(Y). The development of NLLs on virus-inoculated leaves was observed and the distribution of virus was detected by the tissue printing method [29]. After measuring the average size of fifty NLLs on CMV(Y)-inoculated transgenic leaves, total RNA was extracted from virus-inoculated leaves by the acid guanidium-phenol-chloroform (ASGPC) method [6]. Northern hybridization was performed by the standard procedure [26]. To detect the expression of acidic and basic *PR-1* and *PR-2* genes, respectively, cDNA clones containing ∼200 bp of 3′-untranslated regions of PR-protein genes; 507–737 nucleotide position for acidic *PR-1*, 499–750 nucleotide position for basic *PR-1*, 1,504–1,728 nucleotide position for acidic *PR-2*, and 3,435–3,597 nucleotide position for basic *PR-2*, were used as the probes. The DNA fragments for detecting transcripts from *HIN1*, *HRS203J*, *PAL*, *CHS* and *LOX* by Northern hybridization were amplified by PCR with primers, 5′-GAACGGAGCCTATTATGGCCCTTCC-3′ and 5′-CATGTATATCAATGAACACTAAACGCCGG-3′ for *HIN1*, 5′-CATGAAAA GCAA GTGATAGA-3′ and 5′-GCCACACAGTACAAATAAGG-3′ for *HSR203J*, 5′-CACTGCATCAAGG AATCCAA-3′ and 5′-CATTCCAGCTCTTGAGACAC-3′ for *PAL*, 5′-AATTACTCA TTTGGTCTTTT-3′ and 5′-CTAAGTAGCAACACT GTGGA-3′ for *CHS*, and 5′-ACTGGGTTTTCCCTGAACAA-3′ and 5′-CTATAT TGACACACT GTTAG-3′ for *LOX*. PCR was performed by the procedure described above. The PCR product was purified and cloned into *Eco*RV site of pBluescript SK+ (Stratagene, CA, U.S.A.). To confirm that the expected DNA was cloned, the nucleotide sequence of each insert was determined by the Sanger method using an automated DNA sequencer (ABI model 373A) [26]. To examine the induction of SAR, fully expanded 6th-leaf stage leaf of tobaccos expressing 2-5Aase and RNase L was inoculated with 100 μg/ml of CMV(Y). At 10 days after CMV(Y) inoculation, upper 9th-leaf stage leaf of CMV(Y)-inoculated tobaccos were challenge-inoculated with *Pseudomonas syringae* pv. *tabaci* strain SUPP278, which was grown at 28 °C in Nutrient agar medium

containing 10 g/l beef extract, 10 g/l peptone, 5 g/l NaCl and 20 g/l agar. Cells were resuspended in 10 mM $MgCl_2$, and then used for the inoculum. Upper leaves of CMV(Y)-inoculated tobaccos were inoculated with *P. syringae* pv. *tabaci* strain SUPP278 at a concentration of 2×10^3 cfu/ml using plastic syringe. Quantitative determinations of bacterial growth in leaves were performed by dilution plating of homogenized leaf tissue on Nutrient agar medium containing 12.5 μg/ml rifampicin.

When double transgenic tobacco plants expressing 2-5Aase and RNase L were inoculated with CMV(Y), NLLs were developed on virus-inoculated leaves (Fig. 1A and 1B). However, individual tobacco plants expressing 2-5Aase or RNase L did not show any symptoms on CMV(Y)-inoculated leaves (Fig. 1A), and yellow mosaic symptoms were developed on their non-inoculated upper leaves, 10 days after inoculation (data not shown). NLLs in the inoculated leaves of double transgenic tobaccos expressing 2-5Aase and RNase L began to appear 24 h after inoculation, and the size of NLLs increased until 36 hr after inoculation (data not shown). The immunological detection of the coat protein within CMV(Y)-inoculated leaves of double transgenic tobacco expressing 2-5Aase and RNase L indicated that virus was just restricted around NLLs on the inoculated leaves (Fig. 1B and 1C).

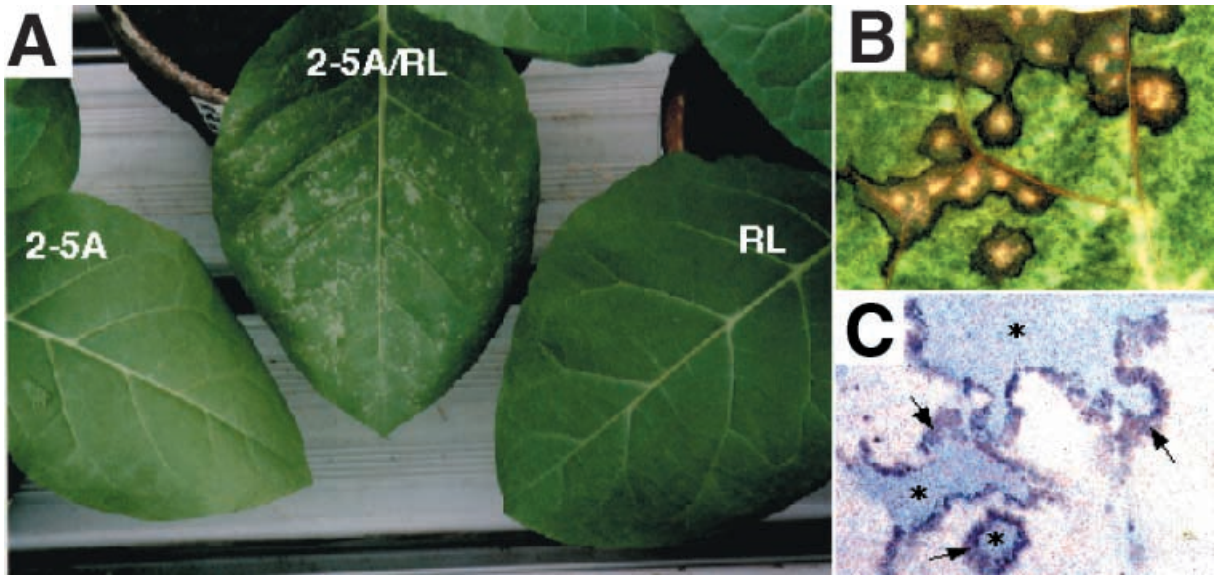

Fig. 1. Response of 2-5A system-transformed tobacco to CMV(Y). **A** Symptoms on transgenic tobacco expressing 2-5Aase (2-5A) or RNase L (RL) and double transgenic tobacco expressing 2-5Aase and RNase L (2-5A/RL) to CMV(Y) infection at 3 days after inoculation. **B, C** At 3 days after CMV(Y) inoculation, necrotic local lesions (NLLs) were developed on the inoculated leaf of double transgenic tobacco expressing 2-5Aase and RNase L (**B**), and the restriction of virus spread around them was detected by the tissue printing method (**C**). The asterisks indicate a central part of NLL from which leaf sap could not be blotted, because the leaf tissue in NLLs was collapsed and dried. The arrows indicate the signal derived from virus coat protein by the tissue printing method

Then, the induction of pathogenesis-related (PR) proteins was examined in CMV(Y)-infected tobaccos expressing 2-5Aase and RNase L or either one by Northern hybridization. Not only the expression of acidic *PR-1* but also of basic *PR-1* was activated in only double transgenic tobacco plants expressing 2-5Aase and RNase L at 72 hr after CMV(Y) inoculation, although these *PR-1* genes were not activated in either transgenic plant expressing 2-5Aase or RNase L (Fig. 2A). Acidic PR proteins are induced via SA-mediated signaling pathway, whereas basic PR proteins are induced via JA and ethylene-mediated signaling pathway [10]. Transcripts from acidic and basic *PR-1* genes were confirmed in tobacco leaves exogenously treated with SA and methyl-JA, respectively (Fig. 2A). To study the timing of the development of NLLs and the expression of PR genes, the activation of acidic and basic *PR-1* and *PR-2* genes in double transgenic tobaccos expressing 2-5Aase and RNase L was analyzed at 24 hr intervals until 5 days after CMV(Y) inoculation. Transcripts from acidic and basic *PR-1* and*PR-2* genes were detected 24 hr after CMV(Y) inoculation, when NLLs began to appear on virus-inoculated leaves (Fig. 2B). The level of these PR gene expressions increased until 5 days after CMV(Y) inoculation (Fig. 2B). Therefore, in double transgenic tobaccos expressing 2-5Aase and RNase L, the expression of both acidic and basic *PR-1* and *PR-2* genes were activated in the progress of NLL development. The accumulation of acidic and basic PR protein transcripts indicated that both SA and JA plus ethylene-mediated signaling pathways were activated by CMV(Y) infection in transgenic tobaccos expressing 2-5Aase and RNase L. The activation of acidic and basic PR genes also occurred in Tobacco mosaic virus-tobacco carrying *N* gene system [33].

HIN1 and *HSR203J* were activated in double transgenic tobaccos expressing 2-5Aase and RNase L, but not in tobacco expressing either one at 48 hr after CMV(Y) inoculation (Fig. 3). Activation of *PAL*, *CHS* and *LOX* also occurred in only double transgenic tobaccos expressing 2-5Aase and RNase L at 48 hr after CMV(Y) inoculation (Fig. 3). These results indicated that many defense-related genes were activated in CMV(Y)-infected tobaccos expressing 2-5A system, as well as in the plant showing HR by the infection of necrogenic pathogens.

The induction of SAR in double transgenic tobaccos expressing 2-5Aase and RNase L to *Pseudomonas syringae* pv. *tabaci* strain SUPP278 was examined. Non-inoculated upper leaves of CMV(Y)-infected double tobaccos expressing 2-5Aase and RNase L and mock-inoculated double transgenic tobaccos as a control, were challenge-inoculated with *P. syringae* pv. *tabaci* strain SUPP278. At 8 days after challenge inoculation, growth of the bacteria in double transgenic plants was inhibited by about 10-fold compared with that in the mock-inoculated control plants (Fig. 4). This observation indicated that SAR was weak but significantly induced in only CMV(Y)-infected tobaccos expressing 2-5A system. As there are many reports that SAR tightly coincides with the accumulation of PR proteins [4, 33], the induction of SAR in CMV(Y)-infected double transgenic tobaccos expressing 2-5Aase and RNase L was to be expected.

The 2-5A system was first studied as a mediator of the antiviral activity of interferon (IFN) in cells of higher vertebrates [5]. In virus-infected cells, viral

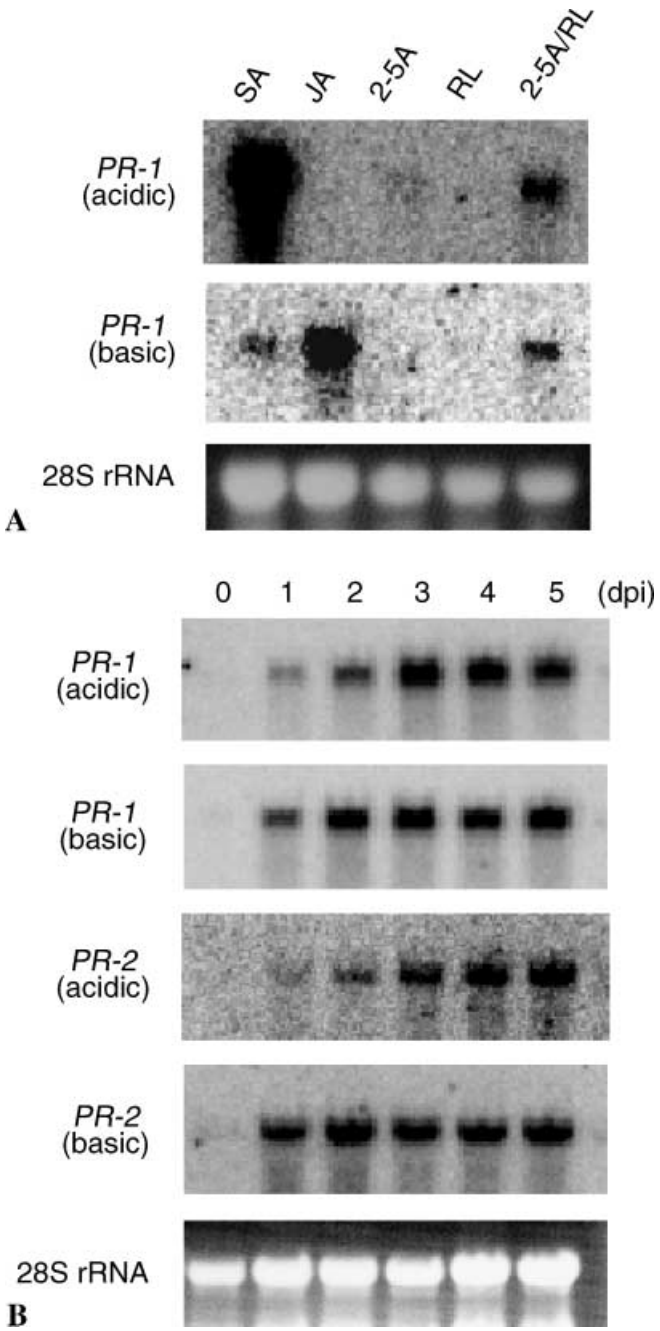

Fig. 2. Expression of acidic and basic *PR-1* genes in CMV(Y)-infected tobacco transformed with 2-5A system. **A** Transcripts from acidic and basic *PR-1* genes at 72 h after inoculation in CMV(Y)-infected transgenic tobacco expressing 2-5Aase (2-5A) or RNase L (RL), and CMV(Y)-infected double transgenic tobacco expressing both (2-5A/RL), were detected by Northern hybridization. As the control, transcripts from SA (SA) and MeJA (JA)-treated tobaccos were also examined. **B** Time course of acidic and basic *PR-1* and *PR-2* gene expression was analyzed during 5days after CMV(Y) inoculation

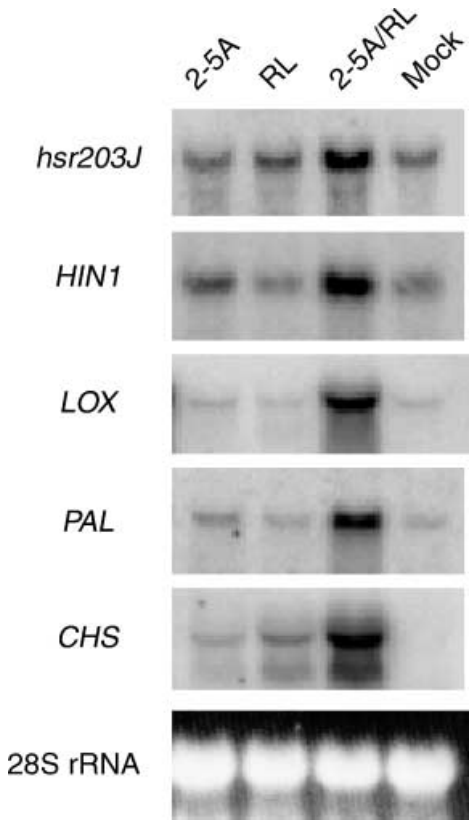

Fig. 3. Expression of defense-related genes in CMV(Y)-infected double transgenic tobacco expressing 2-5Aase and RNase L. Transcripts of other defense-related genes; *hsr203J*, *HIN1*, lipoxygenase (*LOX*), phenylalanine ammonia lyase (*PAL*) and chalcone synthase (*CHS*), in CMV(Y)-infected transgenic tobaccos expressing 2-5Aase (*2-5A*) or RNase L (*RL*), CMV(Y)-infected double transgenic tobacco expressing both (*2-5A/RL*), and mock-inoculated tobacco as the control (*Mock*), were detected by northern hybridization

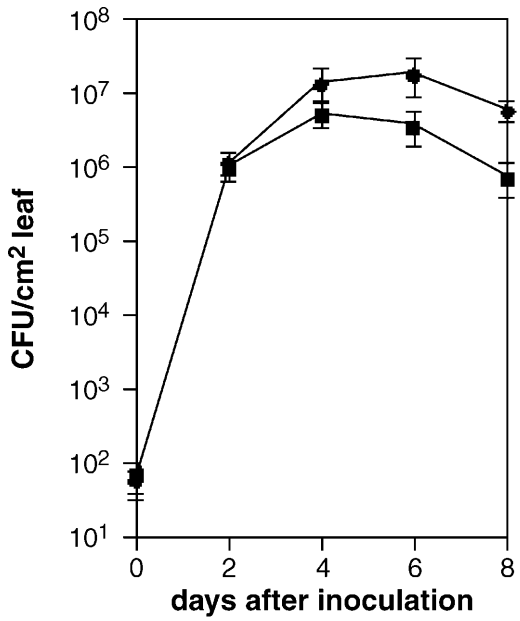

Fig. 4. Systemic acquired resistance (SAR) in CMV(Y)-infected transgenic tobacco transformed with 2-5A system. The double transgenic tobaccos expressing 2-5Aase and RNase L were inoculated with CMV(Y) (■) and mock-inoculated with phosphate buffer as the control (●). At 10 days after inoculation, non-inoculated upper leaves of them were challenge-inoculated with *P. syringae* pv *tabaci* strain SUPP278. At 0, 2, 4, 6 and 8 days after challenge-inoculation, growth of bacteria was measured

RNA appears to be targeted for degradation by RNase L, possibly through a localized activation of the 2-5A system by viral dsRNA [2, 14]. However, the cell death similar to HR in plants has not been observed in the mammalian cells. Although, apparently, plants lack an equivalent of the mammalian 2-5A system, 2-5A system-transformed tobacco exhibited complete resistance to CMV with the NLL formation and activation of genes encoding PR proteins, HIN1 and HSR203J. Currently, PR-proteins are classified in 14 groups, and antimicrobial activity of many PR-proteins has been identified [32]. *HIN1* is inducible by harpins and bacteria that elicit HR in tobacco [12]. *HIN1* belongs to *HIN1/NDR1* family that potentially functions in plant response to pathogen infection at the downstream of signal recognition [8]. *HSR203J* whose activation is rapid, highly localized and specific, has been shown to be strongly correlated with programmed cell death occurring in response to diverse pathogens [24]. Sequence analysis and functional characterization of *HSR203J* gene product demonstrated that HSR203J is a serine hydrolase that displays an esterase activity [3]. *HSR203J* antisense suppression in tobacco accelerates development of the HR cell death, indicating that *HSR203J* may play a functional role in the control and/or execution of the HR cell death [30]. Therefore, the induction of these defense-related genes suggested that, at the molecular level, the HR was induced in 2-5A system-expressing tobacco. Furthermore, the activation of SAR and other genes which were associated with phytoalexin synthesis and lipid peroxidation, gave further evidence that the HR was induced in 2-5A system-transformed tobacco infected with CMV. Therefore, in addition to degradation of CMV RNA by RNase L in 2-5A system transformed tobaccos infected with CMV(Y), 2-5A system may activate the signaling pathway for the HR thereby restricting CMV at the primary infection site with the NLL formation. The 2-5A system is not only useful to produce CMV-resistant plants but is also valuable for studying the molecular mechanism of the HR induction in CMV-infected plants.

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