Transcriptional and Posttranscriptional Plant Gene Silencing in Response to a Pathogen

Nadia S. Al-Kaff,* Simon N. Covey, Maria M. Kreike, Anthony M. Page, Rachel Pinder, Philip J. Dale

Plants are able to respond to pathogen attack to restrain development of a systemic infection. The response of *Brassica napus* (oilseed rape) to systemic infection with the DNA virus cauliflower mosaic virus was shown to result in enhancement and subsequent suppression of viral gene expression in parallel with changes in symptom expression. Transgenes with homology to viral sequences were also affected. This phenomenon, which was shown to be mediated by both transcriptional and posttranscriptional mechanisms, might be related to regulation of highly expressed genetic elements.

Plants show a variety of responses to pathogens. Susceptible plants develop symptoms of disease. Resistance is elicited by interaction between incompatible plants and pathogens containing the appropriate genes. Both types of response involve coordinated expression of plant genes, which can include induction of broad-spectrum defense pathways in resistant plants (*1*) and specific activation or suppression of host genes during disease development (*2*).

An additional type of host response to viral infection is characterized by the initial development of systemic symptoms followed by recovery. Recovery is due to inactivation of the virus replication cycle by a host response reminiscent of cosuppression of transgenes and posttranscriptional gene silencing (*3*–*5*). Pathogen-induced posttranscriptional gene silencing does not require the presence of a transgene but the silencing response might affect transgenes. Therefore, we investigated the relationships of disease symptoms, pathogen-induced gene silencing, and transgene expression.

We studied *Brassica napus* (oilseed rape), an amphidiploid (AACC) that originates from one parental species equivalent to *Brassica rapa* (AA), which does not recover from infections by the DNA virus cauliflower mosaic virus (CaMV), and a second species, *Brassica oleracea* (CC), which does recover (*6*). We used *B. napus* transformed with three transgene constructs, T1 to T3 (*7*), to test the effect of virus infection (*8*) on transgene expression (Fig. 1A). Transgenic and wild-type *B. napus* showed the same response to infection with CaMV. Lesions at the site of virus entry were visible 5 to 7 days postinoculation (pi) (Fig. 1B). Symptoms of systemic infection, including chlorotic vein banding interspersed with dark green tissues, were apparent by 10 to 14 days pi. Symptoms were most intense at 30 to 40 days pi and declined thereafter with newly emergent leaves asymptomatic by 50 days pi (Fig. 1C).

When posttranscriptional gene silencing is induced by a transgene, homologous genes or viruses are also subject to silencing through an effect on RNA (*9*). The recovery of virally infected plants mediated by virus-elicited posttranscriptional gene silencing (*3*, *4*) suggests that a transgene with homology to CaMV might also be silenced after CaMV infection. In plants containing the T1 construct, in which the *GUS* transgene is flanked by sequences homologous to CaMV DNA (Fig. 1A), the transgene was indeed silenced in response to CaMV infection. At the site of inoculation, *GUS* silencing was first observed 7 days pi associated with a local lesion. The extent of both silencing and the lesion was increased by 14 days pi (Fig. 1B). In subsequently infected leaves, T1 *GUS* silencing was observed 14 days pi along the veins. Interveinal islands showed an increase in GUS activity 21 and 28 days pi (Fig. 1C), after which silencing of the *GUS* gene expanded from vein borders until its activity in the entire leaf was suppressed by 50 days pi (Fig. 1C). We observed no silencing of the *GUS* gene in uninfected T1 transgenic plants or in infected or uninfected T2 transgenic plants (Fig. 1C), in which the transgene flanking sequences lack CaMV homology.

Before complete *GUS* silencing was apparent, the pattern of transgene expression reflected that of disease symptoms. Suppression and enhancement of *GUS* expression coincided with chlorotic vein borders and dark green islands, respectively (Fig. 2, A and B). Initially, virus accumulated to a greater extent in the dark green islands than in the vein borders (Fig. 2C), which suggests differences in the amount of viral replication. We assessed viral replication by analyzing types of viral DNA (*10*, *11*). Cells containing replicating CaMV accumulate unencapsidated DNA products of reverse

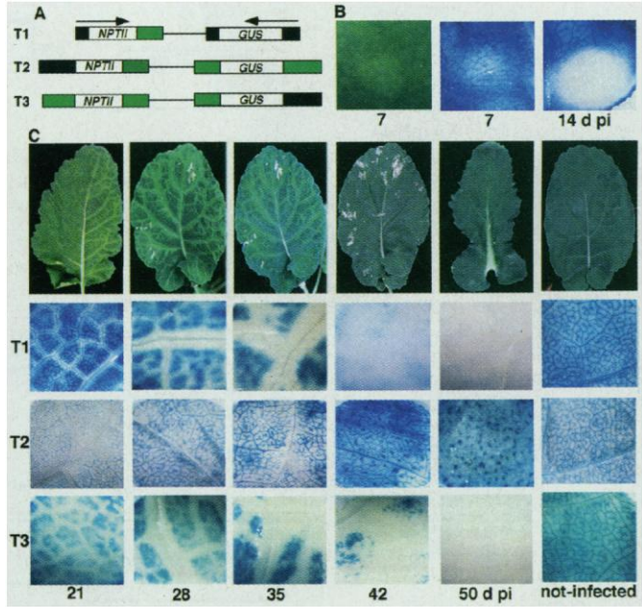

Fig. 1. Response of transgenic *B. napus* plants to virus infection. (**A**) Each of three transgenic lines (T1, T2, and T3) of *B. napus* (cultivar Westar 10) contains the neomycin phosphotransferase transgene (*NPTII*) as a selectable marker and the reporter glucuronidase transgene (*GUS*) expressed in the direction of the arrows. Flanking black blocks represent elements homologous to CaMV (35*S* RNA promoter and RNA terminator) and green blocks represent nonhomologous elements [*Agrobacterium* nopaline synthase gene (*NOS*) promoter and octopine synthase gene (*OCS*) RNA terminator]. Therefore, the transgene structures are as follows: T1 construct, Pr35S-*GUS*-Tr35S::Pr35S-*NPTII*-Tr*OCS*; T2 construct, Pr*NOS*-*GUS*-Tr*OCS*::Pr35S-*NPTII*-Tr*OCS*; and T3 construct, Pr35S-*GUS*-Tr*OCS*::Pr*NOS*-*NPTII*-Tr*OCS* (where Pr and Tr represent promoter and terminator sequences, respectively). (**B**) T1 construct. Left panel, local lesion at the site of CaMV inoculation 7 days (d) pi; middle panel, *GUS* expression (blue product) (*16*) in the same lesion; right panel, *GUS* expression in another lesion 14 days pi. (**C**) Top row, time course of systemic symptoms of CaMV infection in transgenic *B. napus* leaves, including early vein clearing, subsequent vein banding, fading symptoms by 42 days pi, and the appearance of asymptomatic new leaves 50 days pi. The remaining three rows show *GUS* expression in CaMV-infected leaves (area, 1 cm^2) from each of the transgenic plant lines. The final column corresponds to uninfected plants.

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK.

*To whom correspondence should be addressed. E-mail: alkaff@bbsrc.ac.uk

transcription. After replication, viral DNA is largely present in a supercoiled state (*3*, *6*). The dark green tissue containing increased GUS activity also contained viral DNA products indicative of active viral replication. Viral DNA in chlorotic vein borders was predominantly postreplicative (Fig. 2D). Thus, increased *GUS* expression was associated with active CaMV replication, and *GUS* suppression was associated with a postreplicative viral state.

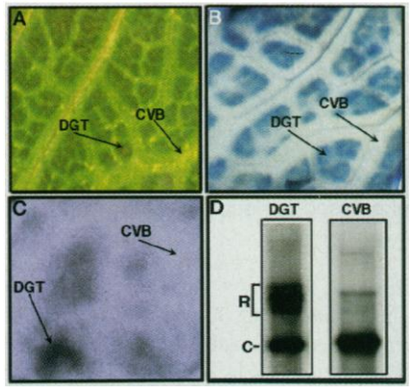

Fig. 2. Symptoms, *GUS* expression, and viral activity in CaMV-infected T1 transgenic plants 21 days pi. (**A**) Systemically infected *B. napus* leaf showing dark green interveinal tissue (DGT) and chlorotic vein border tissue (CVB). (**B**) A similar leaf after removal of chlorophyll to reveal high and low GUS activity in the interveinal and vein border tissue, respectively. (**C**) In situ hybridization (*15, 17*) of an infected transgenic leaf showing greater virus accumulation in the interveinal tissue than in the vein borders. Leaf areas in (A) through (C) are 1 cm^2. (**D**) Southern blot analysis revealed that viral DNA intermediates in dark green tissue contain heterogeneous replication products (R), indicative of active viral replication. Vein borders contain more supercoiled DNA (C) and no heterogeneous replication products, indicative of a postreplicative state.

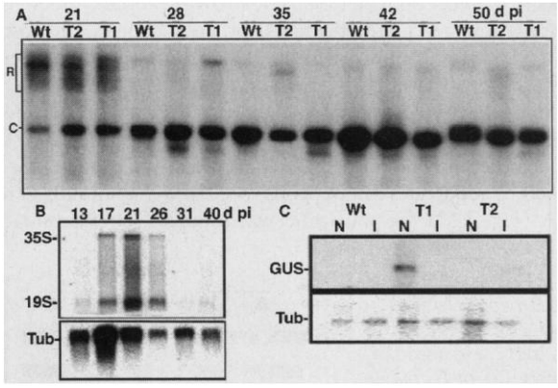

Fig. 3. Analysis of CaMV replication and transcripts in wild-type and transgenic plants. (**A**) Southern blot analysis of unencapsidated CaMV DNA from infected *B. napus* leaves during development of and recovery from disease. Each lane contains 10 μg of total cellular DNA (*10*). Heterogeneous CaMV replication intermediates (R) and supercoiled DNA (C) in leaves of wild-type (Wt) and T1 and T2 transgenic plants are indicated. Viral replication status at any given time was similar in all plants. (**B**) Northern blot analysis of CaMV mRNA (35*S* and 19*S* RNA) and tubulin (Tub) mRNA in T1 transgenic plants. Each lane contains polyadenylated RNA from 25 μg of total cellular RNA. (**C**) Northern blot analysis of *GUS* mRNA in infected (I) and noninfected (N) wild-type and T1 and T2 transgenic plants 50 days pi. Each lane contains 10 μg of total cellular RNA. Different RNAs from the same starting material were analyzed on the same or parallel gels.

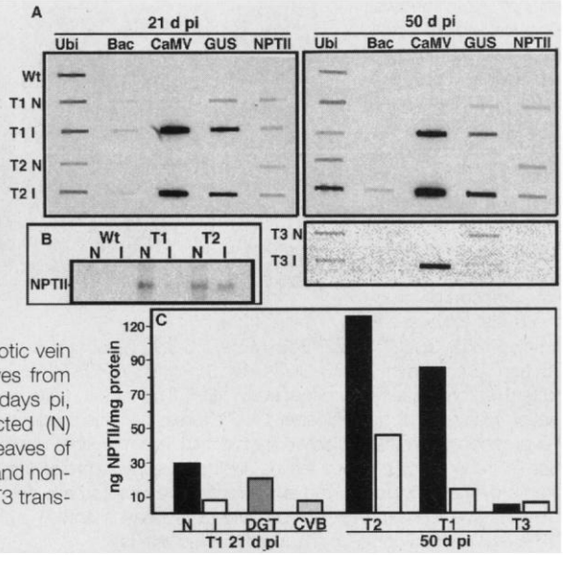

Fig. 4. Analysis of viral and transgene expression. (**A**) Nuclear run-on transcription assays. Radioactive RNA synthesized in isolated nuclei from leaves of wild-type *B. napus* (Wt) or of infected (I) or noninfected (N) T1, T2, or T3 transgenic plants was used to probe filter slots containing ubiquitin (Ubi), bacterial (Bac), CaMV, *GUS*, or *NPTII* DNA. (**B**) Northern blot analysis of *NPTII* transgene mRNA from the same samples as in Fig. 3C 50 days pi. (**C**) Assay of NPTII protein (*14*) in dark green tissue (DGT), chlorotic vein border tissue (CVB), and whole leaves from infected (I) T1 transgenic plants 21 days pi, and in whole leaves from noninfected (N) plants (left). NPTII protein in whole leaves of infected (50 days pi) (open columns) and noninfected (solid columns) T1, T2, and T3 transgenic plants is also shown (right).

To confirm that suppression of viral replication was independent of the transgene, we analyzed CaMV replication in infected leaves of nontransgenic and transgenic plants. The population of viral DNA products 21 days pi was consistent with ongoing viral replication in wild-type plants and in transgenic plants containing the T1 or T2 transgene constructs. However, from 28 to 50 days pi, the preponderance of supercoiled viral DNA indicated cessation of viral replication in all plants (Fig. 3A). Viral 19*S* and 35*S* RNA transcripts were apparent before but not after 26 days pi (Fig. 3B). The amount of T1 *GUS* mRNA, which bears homology to CaMV RNA, was reduced 50 days after CaMV infection in transgenic plants (Fig. 3C). T2 plants, in which the *GUS* transgene is controlled by the *NOS* promoter and lacks CaMV homology, contained only small amounts of *GUS* mRNA (compared with 35*S* RNA promoter constructs), and these amounts were slightly increased by CaMV infection (Fig. 3C). Thus, changes in CaMV replication and gene expression were independent of the presence of transgenes, whereas changes in transgene activity were dependent on the presence of virus. Therefore, the *GUS* transgene that shares sequence homology with CaMV was cosuppressed in the presence of infectious CaMV during plant recovery from disease.

If cosuppression of virus and transgene is mediated by degradation of homologous RNA molecules, then transcriptional activity should be unaffected (*5*, *9*). Nuclear run-on assays (*12*, *13*) revealed that both the CaMV minichromosome and the T1 *GUS* transgene were actively transcribed in infected plants (Fig. 4A). Transcription of the *NOS* promoter-driven T2 *GUS* transgene was less than that of the T1 *GUS* transgene in uninfected plants but was stimulated by CaMV infection. Thus, CaMV infection elicits a response from the plant that silences viral gene expression, as well as that of transgenes with RNA sequence homology to CaMV, by a posttranscriptional mechanism.

We also detected transcriptional suppression of the *NPTII* gene, located adjacent to the posttranscriptionally suppressed *GUS* transgene (Fig. 1), in the T1 construct. The homology of the *NPTII* portion of both the T1 and T2 transgene constructs to CaMV is limited to the CaMV 35*S* RNA promoter. In T1 transgenic plants, the amount of *NPTII* mRNA was reduced by infection at 50 days pi (Fig. 4B). The amount of *NPTII* mRNA was less affected by infection in T2 plants, although the amount of NPTII protein (*14*) was substantially reduced by infection in

both T1 and T2 transgenic plants (Fig. 4C). The amount of NPTII protein was not affected by infection in T3 plants, in which the *NPTII* transgene does not share homology with the CaMV promoter. The distribution of NPTII protein between dark green island and chlorotic vein border tissue of T1 transgenic plants (Fig. 4C) reflected that of GUS activity (Fig. 2B).

Suppression of the *NPTII* gene might have occurred through interference from the adjacent *GUS* gene. Alternatively, CaMV infection might result in host regulation of the 35S RNA promoter. Therefore, we tested the effects of CaMV infection on expression of the *GUS* transgene of the T3 construct (Fig. 1A), for which viral homology is limited to the CaMV 35S RNA promoter sequence. CaMV infection suppressed *GUS* expression in T3 transgenic plants with the same symptomatic pattern as that in T1 transgenic plants (Fig. 1C). However, silencing in T3 transgenic plants was not likely mediated by posttranscriptional mechanisms because the construct lacked viral RNA homology. Nuclear run-on experiments revealed that transcription of the T3 *GUS* transgene was inhibited in infected plants, despite concurrent transcription of the CaMV minichromosome (Fig. 4A). We suggest that transcriptional silencing of the 35S RNA promoter in the CaMV minichromosome does not occur in the presence of posttranscriptional silencing. However, transcriptional suppression of the CaMV 35S RNA promoter in the T3 construct suggests that viral transcription could potentially be down-regulated in those infections that do not result in recovery from symptoms as in *B. rapa*. Such regulation could explain the differential accumulation of CaMV in chlorotic and dark green tissue observed in the absence of posttranscriptional silencing (*15*).

Thus, plants respond to pathogen invasion by regulating pathogen gene expression, apparently at both transcriptional and posttranscriptional levels. Posttranscriptional suppression of viral genes results in posttranscriptional cosuppression of transgenes that share sequence homology with the virus. Sequences homologous to the viral promoter can be silenced at the transcriptional level. Posttranscriptional suppression of gene expression appears to take precedence over transcriptional regulation, possibly by preventing transcriptional suppression of the same gene, thereby linking cytoplasmic and nuclear gene regulatory mechanisms.

Most gene silencing phenomena that have been described in plants occur as a result of transformation with transgenes (*5*, *9*). Gene silencing can also be elicited by viruses in the absence of transgenes (*3*, *4*). It is not clear whether this response is antipathogenic or whether it is more broadly related to regulation of highly expressed genetic elements.

REFERENCES AND NOTES

1. L. Godiard, M. Grant, R. Dietrich, S. Kiedrowski, J. Dangl, *Curr. Opin. Genet. Dev.* **4**, 662 (1994).
2. D. W. Wang and A. J. Maule, *Science* **267**, 229 (1995); M. A. Aranda, M. Escaler, D. W. Wang, A. J. Maule, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15289 (1996).
3. S. N. Covey, N. S. Al-Kaff, A. Lángara, D. S. Turner, *Nature* **835**, 781 (1997).
4. F. Ratcliff, B. D. Harrison, D. C. Baulcombe, *Science* **276**, 1558 (1997).
5. R. B. Flavell, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3490 (1994). M. A. Matzke and A. J. Matzke, *Plant Physiol.* **107**, 679 (1995); P. Meyer and H. Sadler, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47**, 23 (1996); C. B. Taylor, *Plant Cell* **9**, 1245 (1997).
6. S. N. Covey, D. S. Turner, A. P. Lucy, K. Saunders, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 1633 (1990); K. Saunders, A. P. Lucy, S. N. Covey, *J. Gen. Virol.* **71**, 2603 (1991).
7. J. D. G. Jones *et al.*, *Transgenic Res.* **1**, 285 (1992).
8. Infection of *B. napus* was established by mechanical inoculation of seedlings (second true leaf) with 1 μg of CaMV virions (isolate Cabb B-JI) in 10 μl of 10 mM sodium phosphate buffer (pH 7.0) containing Celite (Celite Corp.) abrasive. Plants were propagated in a containment greenhouse supplemented with illumination to 16 hours per day at 18° to 22°C.
9. D. C. Baulcombe and J. E. English, *Curr. Opin. Biotechnol.* **7**, 173 (1996); D. C. Baulcombe, *Plant Cell* **8**, 1833 (1996).
10. Total nucleic acid was extracted as described (*11*). CaMV DNA and RNA were analyzed by Southern and Northern blotting, respectively, with the appropriate probes.
11. R. Hull and S. N. Covey, *Nucleic Acids Res.* **11**, 1881 (1983); D. S. Turner, D. G. McCallum, S. N. Covey, *J. Virol.* **70**, 5414 (1996).
12. K. H. Cox and R. B. Goldberg, in *Plant Molecular Biology: A Practical Approach*, C. H. Shaw, Ed. (IRL, Oxford, 1988), pp. 1–35.
13. Nuclei were isolated as described (*3*). Incorporation of uridine 5′-[^{33}P]triphosphate (DuPont Biotechnology Systems) was determined by probing 1 μg of the appropriate DNA samples immobilized as slots on Hybond-N$^+$ membranes (Amersham).
14. Plant protein was extracted and NPTII was measured with an enzyme-linked immunosorbent assay (CP Laboratories).
15. N. S. Al-Kaff and S. N. Covey, *Mol. Plant-Microbe Inter.* **9**, 357 (1996).
16. Leaf disks (12 mm in diameter) were collected and treated as described [R. A. Jefferson, T. A. Kavanagh, M. W. Bevan, *EMBO J.* **6**, 3901 (1987)] for histochemical detection of GUS activity.
17. In situ hybridization to detect CaMV was performed as described (*15*) with the same leaf disks as those used for histochemical detection of GUS activity.

18. We thank N. Owen and C. Jones for producing transgenic lines, J. Jones for transformation constructs, and A. Lángara for advice on nuclear run-on assays. This work was supported by the U.K. Biotechnology and Biological Sciences Research Council and covered by license PHF 1491/982/34 of the Ministry of Agriculture, Fisheries and Food.

17 October 1997; accepted 4 February 1998

Contingency and Determinism in Replicated Adaptive Radiations of Island Lizards

Jonathan B. Losos,* Todd R. Jackman, Allan Larson, Kevin de Queiroz, Lourdes Rodríguez-Schettino

The vagaries of history lead to the prediction that repeated instances of evolutionary diversification will lead to disparate outcomes even if starting conditions are similar. We tested this proposition by examining the evolutionary radiation of *Anolis* lizards on the four islands of the Greater Antilles. Morphometric analyses indicate that the same set of habitat specialists, termed ecomorphs, occurs on all four islands. Although these similar assemblages could result from a single evolutionary origin of each ecomorph, followed by dispersal or vicariance, phylogenetic analysis indicates that the ecomorphs originated independently on each island. Thus, adaptive radiation in similar environments can overcome historical contingencies to produce strikingly similar evolutionary outcomes.

The theory of historical contingency proposes that unique past events have a large influence on subsequent evolution (*1–3*). A corollary is that repeated occurrences of an evolutionary event would result in radically different outcomes (*4*). Indeed, faunas and floras that have evolved in similar environments often exhibit more differences than similarities (*5–7*). These differences in evolutionary outcome probably result from clade-specific factors that cause taxa to respond to similar selective factors in different ways, as well as from unique historical events and subtle environmental differences in the different areas (*2*, *8*). Here we show that such factors will not always lead to disparate outcomes.

Anolis lizards are a dominant element of the Caribbean fauna. On each of the islands of the Greater Antilles (Cuba, Hispaniola, Jamaica, and Puerto Rico), lizard assemblages are composed of species that differ in

J. B. Losos, T. R. Jackman, A. Larson, Department of Biology, Campus Box 1137, Washington University, St. Louis, MO 63130–4899, USA.
K. de Queiroz, Division of Amphibians and Reptiles, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA.
L. Rodríguez-Schettino, Instituto de Ecología y Sistemática, CITMA, Carretera de Varona km 3.5, Boyeros, La Habana 10800, Apartado Postal 8029, Cuba.

*To whom correspondence should be addressed. E-mail: losos@biodec.wustl.edu