Plants rendered herbicide-susceptible by cauliflower mosaic virus–elicited suppression of a 35S promoter-regulated transgene

Nadia S. Al-Kaff*, Maria M. Kreike, Simon N. Covey, Robert Pitcher, Anthony M. Page, and Philip J. Dale

*John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK. *Corresponding author (alkaff@bbsrc.ac.uk).*

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Crop plants genetically modified for herbicide tolerance were some of the first to be released into the environment. Frequently, the cauliflower mosaic virus (CaMV) 35S promoter is used to drive expression of the herbicide tolerance transgene. We analyzed the response to CaMV infection of a transgenic oilseed rape line containing the bialaphos tolerance gene (*BAR*) from *Streptomyces hygroscopicus*, regulated by the 35S promoter. Oilseed rape is susceptible to CaMV, but plants recover from infection. CaMV infection altered the expression of the herbicide tolerance gene such that plants became susceptible to the herbicide. The effect on transgene expression differed in infections with viral pathogenic variants typical of those found in natural situations worldwide. Susceptibility to the herbicide was most likely a result of transcriptional gene silencing of the transgene. Our results show that transgene phenotypes can be modified by pathogen invasion.

Keywords: 35S promoter, oilseed rape, transgene expression, CaMV, herbicide tolerance gene

Stability of expression of introduced genes is an important factor in developing transgenic crop plants. A transgene can be subject to inactivation by gene silencing[1,2]. Transcriptional gene silencing (TGS) causes reduced RNA synthesis, whereas posttranscriptional gene silencing (PTGS) accelerates RNA degradation. A silenced gene can influence the expression of related transgenes, endogenous genes, or viral genes by a process of homology-dependent gene silencing[3–5]. In addition, gene silencing can be triggered as a plant defense mechanism by infecting plant viruses[6–8]. In nontransgenic plants, such silencing is directed against the pathogen[6,7], but in transgenic plants where a transgene shares homology with the pathogen, the transgene can be silenced as well[8].

We have previously shown that cauliflower mosaic virus (CaMV) infection of *Brassica oleracea* and *B. napus* (oilseed rape) causes transient symptoms followed by plant recovery due to PTGS[6,7]. During the recovery stage in transgenic oilseed rape plants, reporter transgenes expressing β-glucuronidase (GUS) or antibiotic resistance markers, and which contain transcription elements derived from CaMV, become silenced. Transgenes flanked by the CaMV 35S promoter and RNA terminator were preferentially silenced by PTGS, whereas those with only 35S promoter homology were subject to TGS[8].

The CaMV 35S promoter[9–11] is present in a large proportion of transgenic plants under commercial cultivation in many parts of the world. Cauliflower mosaic virus also occurs worldwide, although its host range is restricted largely to cruciferous plants[12]. The incidence and the importance of virus infections differ from season to season[13]. In the UK in 1991 and 1993, between 14% and 25% of plants sampled from test fields were infected with CaMV[14]. Also, 60% of the naturally occurring wild cabbage (*B. oleracea*) in Dorset (UK), was found to be infected with CaMV[15]. Thus, CaMV has a variable but significant incidence in the United Kingdom and could have a major impact on transgene expression in cruciferous crops around the world[13]. In this paper, we show that CaMV infection can destabilize a commercially important genetic trait in oilseed rape by silencing a *BAR* transgene regulated by the CaMV 35S promoter, altering the plant phenotype from herbicide resistance to susceptibility.

Results

CaMV infection suppresses expression of the *BAR* herbicide tolerance gene in apical leaves of young oilseed rape plants. We used two heterozygous transgenic lines of oilseed rape (*B. napus,* dihaploid genotype: Wester 10) containing a single copy of the *BAR* herbicide tolerance transgene. The *BAR* gene (Fig. 1A) was regulated by the 35S promoter (35Sp-*BAR*-OCSt) in one line and by the *Agrobacterium* nopaline synthase (NOS) promoter (NOSp-*BAR*-OCSt) in the second[16]. *BAR* transgenes in both lines were terminated by the octopine synthase terminator (OCSt). These lines contained the selectable marker gene *NPT II* (Fig. 1A) flanked by the CaMV 35S promoter and OCS terminator (35Sp-*NPT II*-OCSt)[16].

Forty transgenic oilseed rape seedlings were infected in the first true leaf with CaMV isolate Cabb B-JI. Systemic symptoms were observed at 10–12 days post inoculation (d.p.i.), but plants recovered from infection after about 35 d.p.i.; leaves produced in the flowering stage were symptom-free. Cabb B-JI infection caused identical symptoms and recovery in *BAR* transgenic and wild type (wt) plants, as we had found in other transgenic oilseed rape lines[8].

Infected and noninfected transgenic and wt oilseed rape plants were sprayed twice with glufosinate ammonium, the herbicide

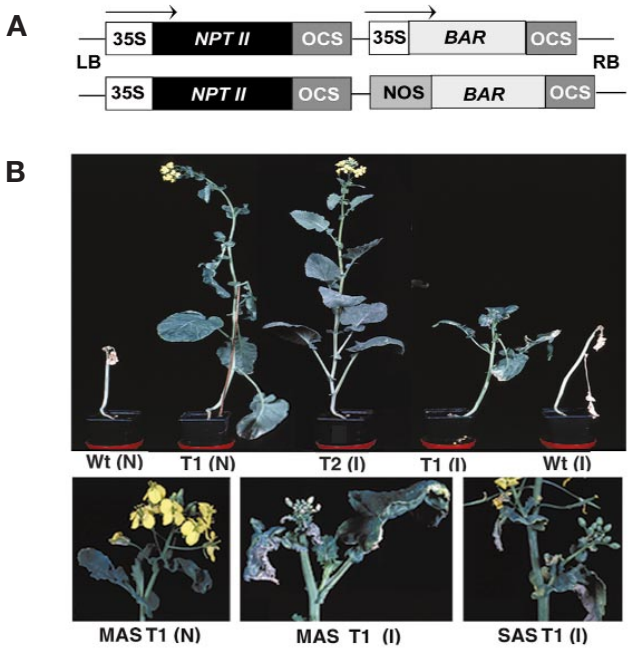

Figure 1. Effect of CaMV infection on herbicide tolerance in transgenic oilseed rape plants. (A) Transgene constructs transformed into oilseed rape. Each contained the selectable marker, neomycin phosphotransferase (*NPT II*), driven by the CaMV 35S promoter, which is similar in size to the 35S promoter driving the *BAR* transgene in T1. The T2 transgene NOSp-*BAR*-OCSt lacked the viral promoter homology. (B) Plants were sprayed with the herbicide Harvest at 35 d.p.i. with virus or following mock inoculation. Nontransgenic (Wt) and transgenic plants containing constructs T1 (35Sp-*BAR*-OCSt) or T2 (NOSp-BAR-OCSt) were infected with CaMV (I) or not infected (N). Effects of herbicide were observed in the main apical shoot (MAS) and the side apical shoot (SAS).

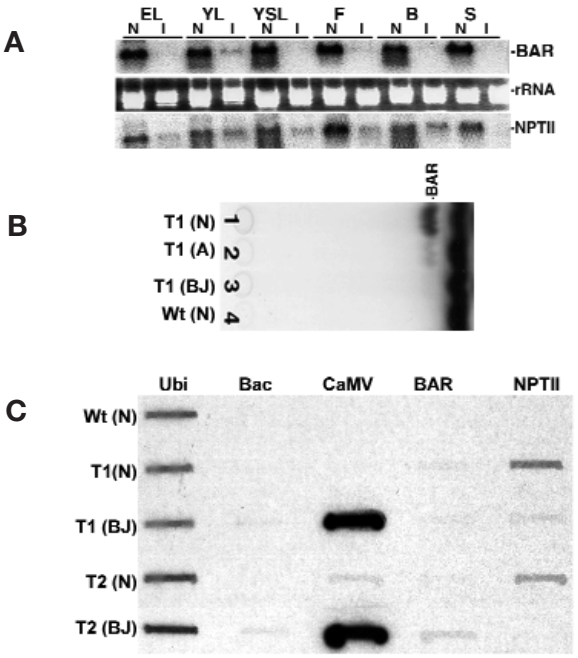

Figure 2. Effect of CaMV infection on *BAR* transgene expression in oilseed rape. (A) Levels of BAR and NPTII transgene mRNAs relative to rRNA standards in a fully expanded leaf (EL), young leaf (YL), leaf from a side branch (YSL), flower (F), buds (B), and stem (S). (B) PAT enzyme activity in nontransgenic (Wt) and in transgenic (T) oilseed rape plants infected with CaMV isolates Cabb B-JI (BJ), and Aust (A), and in noninfected plants. (C) Nuclear run-on transcription assay from infected plants sampled at 50 d.p.i. from Wt, 35Sp-*BAR*-OCSt (T1), and NOSp-*BAR*-OCSt (T2) plants that also contain the 35Sp-*NPT II*-OCSt transgene. Radioactive RNA synthesized in isolated nuclei was used to probe filter slots containing ubiquitin (Ubi) DNA as a positive control, bacterial (Bac) DNA as a negative control, and CaMV DNA.

Harvest (2.5 ml/L), at 35 and 42 d.p.i. Analysis of the sprayed plants was carried out 10 days after the last spraying (52 d.p.i.). The noninfected 35Sp-*BAR*-OCSt plants, as well as the infected and noninfected NOSp-*BAR*-OCSt plants, were all resistant to the herbicide (Fig. 1B). Both infected and noninfected wt plants were herbicide-sensitive and died (Fig. 1B). However, the main apical shoot of infected plants containing the 35Sp-*BAR*-OCSt transgene showed sensitivity to the herbicide, causing its senescence (Fig. 1B). This led to production of more side shoots compared with the noninfected plants.

Virus-induced herbicide sensitivity is caused by suppression of the *BAR* transgene. To determine the effect of CaMV infection on *BAR* transgene expression in the herbicide-sensitive plants, total RNA was extracted from parts of the herbicide-sprayed infected and noninfected transgenic plants. Northern hybridization showed that significantly lower levels of BAR transgene mRNA were detected in the CaMV-infected than in the noninfected plant parts (Fig. 2A). This included parts of the plant that were sensitive to the herbicide such as young leaves, and other primary apical parts such as buds and flowers, as well as parts that were not so sensitive to the herbicide such as fully expanded leaves, young leaves from the side shoots, and stems. Suppression of BAR mRNA resulted in lower enzyme activity of phosphinothricin acetyl transferase (PAT) in infected plants compared with noninfected plants (Fig. 2B). The degree of PAT suppression was greater in plants infected by CaMV isolate Cabb B-JI than by the very severe CaMV isolate Aust (Fig. 2B).

The *BAR* transgene shared homology with CaMV only in the 35S promoter, together with eight bases of RNA homology in the transgene RNA leader (see Fig. 1A). This makes it most likely that suppression of *BAR* expression was as a result of transcriptional downregulation. To check this, we performed nuclear run-on analysis (Fig. 2C). High levels of run-on transcription were observed from the CaMV chromosome in infected plants. However, in several experiments we were unable to detect any *BAR* transgene activity significantly above background, most likely because of the relatively low uridine content of this gene. By contrast, the *NPT II* transgene, again only possessing 35S promoter homology with CaMV, showed significant reduction in transgene expression determined by nuclear run-on analysis at 50 d.p.i. (Fig. 2C). Reduction in NPT II mRNA levels were also observed in different parts of infected as compared with noninfected plants (Fig. 2A). From these experiments, we conclude that suppression of both the *BAR* and *NPT II* transgenes was most likely due to transcriptional downregulation of the 35S promoter. We checked for changes in transgene promoter methylation following CaMV-elicited transgene suppression and found no differences compared with noninfected plants (data not shown). We also observed a stimulatory effect of CaMV on the NOS promoter in the T2 transgene construct (Fig. 2C). This suggests that host responses to pathogen invasion can cause both up- and downregulation of transgenes.

Effect of CaMV on herbicide sensitivity of flowering oilseed rape. We tested the herbicide-sensitivity of CaMV-infected *BAR* transgenic oilseed rape plants at the flowering stage (70 d.p.i.). Large plants are more difficult to spray uniformly with herbicide. Therefore, leaves were treated locally by applying herbicide to filter paper disks. After 10 days of herbicide treatment, damage was observed only in herbicide-treated areas of the infected plants (Fig. 3A). No damage was found in the noninfected herbicide-

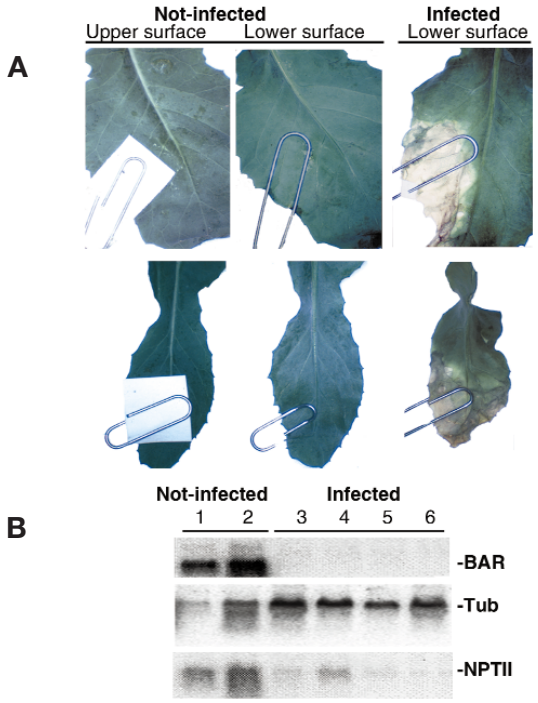

Figure 3. Effect of CaMV infection on herbicide tolerance and transgene expression in leaves of flowering transgenic oilseed rape plants. (A) Fully expanded leaves (upper row) and young cauline leaves (lower row) treated locally with herbicide. (B) Levels of BAR and NPT II transgene mRNAs relative to tubulin mRNA standards in leaves from mature plants showing samples from two noninfected plants (1 and 2), and four samples from infected plants (3–6).

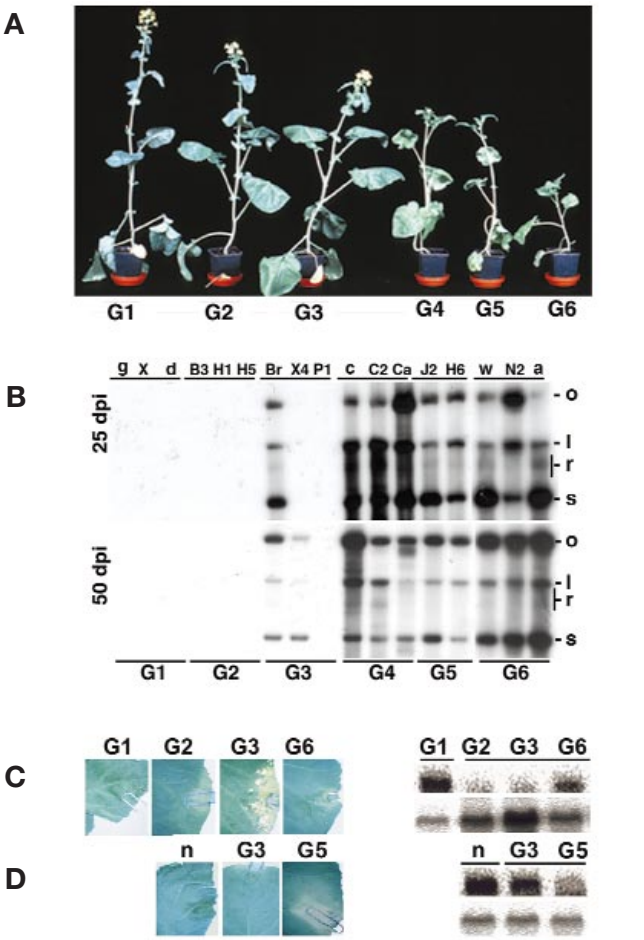

Figure 4. Effects of CaMV pathogenic variants on herbicide suppression. (A) Symptoms caused by representative members of six groups (G1–G6) of CaMV pathogenic variants in oilseed rape. (B) Southern analysis of CaMV DNA replication intermediates produced in infections by viral variants. Two to three variants of CaMV were randomly selected to represent each group. Group 1: variants Greece-2 (g), XJ (X), Datura (d); group 2: Bari-3 (B3), H-1 (H1), H-5 (H5); group 3: Brass-6 (Br), pXJ-4 (X4), pCa24nt-1 (P1); group 4: Camb (c), Cabb-B2 (C2), Calif (Ca); group 5: Jap-2 (J2), H-6 (H6); group 6: w, NZ-2 (N2), Aust (a). DNA was extracted at 25 and 50 d.p.i. Different forms of CaMV unencapsidated DNAs include: open circular (o), linear (l), reverse transcription intermediates (r), supercoiled DNA (s) forms, respectively. (C) Effects of herbicide on leaves of plants infected at first true leaf with CaMV variants (groups G1, 2, 3, and 6) and on levels of BAR transgene (right, top row) and tubulin mRNAs (right, bottom row). (D) Effects of herbicide on leaves of plants infected at the four-leaf stage with CaMV variants (groups G3 and G5) and in noninfected (n) plants and on levels of BAR transgene (right, top row) and tubulin mRNA (right, bottom row).

treated plants. Northern analysis showed lower levels of BAR and NPT II mRNAs in leaves from infected plants than the noninfected plants (Fig. 3B).

Oilseed rape shows a range of responses to CaMV pathogenic variants. There are many CaMV isolates distributed worldwide[17,18]. We assessed the effects on oilseed rape of a wide range of CaMV isolates likely to be encountered in natural situations. Nontransgenic oilseed rape W10 was infected with 67 CaMV variants, and these produced a range of responses that we divided into six groups according to their pathogenic characteristics (Table 1). CaMV variants in group 1 that were asymptomatic in oilseed rape caused symptomatic infections in a highly susceptible indicator host, *B. rapa*. All plants infected with groups 2–4 CaMV variants induced barely discernible symptoms through to moderate symptoms. Group 5 and 6 CaMV variants caused severe and very severe symptoms, respectively (Table 1). Stunting of plant growth was observed in plants infected with all CaMV variants except group 1 (Fig. 4A and Table 1). Plants infected with all of the symptomatic CaMV variants (groups 2–6) showed signs of recovery after 35 d.p.i. Some plants made a complete recovery from infection whereas others showed only a partial recovery with persistence of mild symptoms (Table 1).

Recovery from CaMV infection is associated with changes in the composition of subcellular viral DNA forms[6,19,20]. In particular, an increase in supercoiled DNA occurs when CaMV becomes silenced[6]. We compared viral DNAs at 25 d.p.i. (from leaf 7) and at 50 d.p.i. (from leaf 12) from plants infected with representative viral variants from each group. No viral DNA was detected in plants infected with groups 1 and 2 variants. Little viral DNA was found in group 3 infections, but there was more DNA in leaves infected with group 4–6 variants (Fig. 4B). Most variants showed abundant supercoiled DNA at both sampling times, suggesting that viral replication had been arrested relatively early in all cases. These results show that essentially all cases of recovery were associated with viral arrest, regardless of the severity shown during the symptomatic phase.

Suppression of the herbicide tolerance transgene depends on the viral pathogenic variant and time of infection. In the field, we expect that transgenic oilseed rape will be challenged with CaMV variants at different stages of plant development. To test these variables on expression and effectiveness of the *BAR* transgene, five plants were infected with representative CaMV variants Bari-1 (group 1), Bari-4 (group 2), Camb-M (group 3), Calif (group 4) (data for group 4 not shown), and Aust (group 6). No symptoms or transgene silencing was observed in transgenic plants infected with Bari-1 (Fig. 4C). However, significantly reduced levels of BAR mRNA were found in plants infected with Bari-4, Camb-M,

Table 1. Responses of oilseed rape to CaMV variants[a]

Groups	1	2		3		4		5		6	
Symptoms	-	VVM	R	VM	R	M	R	S	R	VS	R
Variants	Bari-1	Bari-2	C	Brauns	C	Belt	C	BSE	P	Aust	P
	Belt-7*	Bari-3	C	Brass-6	P	Cabb-BD	C	Cabb B-JI	P	Hung-1	P
	Cabb-B	Bari-4	C	CM4-184	P	Cabb-B2	P	GCRI	P	NCH-1	P
	Datura	Baj-1	P	Greece-1	P	Calif	P	H6	P	NY8153	P
	Greece-2*	Baj-13	C	PXJ-4	C	Camb	P	Jap-2	P	NZ-1	P
	H-4	Baj-30	C	BJINT	C	H2	P	UK-23	P	NZ-2	P
	KEN-1	Baj-31	C	p11/3-3	C	IG	C	Phatak	P	UK-30	P
	PXJ-2*	Baj-32	C	p11/3-4	P	Nome	P	PRTI	P	Yug-3	C
	PXJ-5*	H-1	C	Camb-M	C	Stras	C	p11/3-5	C	W	P
	UK-29	H-5	C			AustNT	C	P11/3-6	P		
	XJ	Pud-1	C			PXJ-1	P	P11/3-8	P		
						PXJ-3	P	P11/3-9	P		
						P11/3-2	P				
						P11/3-10	P				
						UK-2	C				
						11/3	C				

[a]An asterisk denotes no symptoms in the control plants *B. rapa* cv. Just Right. – No symptoms; VVM, very very mild; VM, very mild; M, mild; S, severe; VS, very severe; R, recovery; C, complete recovery; P, partial recovery.

and Calif (data for Calif not shown). Such plants became susceptible to the herbicide after 35 d.p.i. (Fig. 4C). Surprisingly, plants infected with the severe CaMV isolate Aust were more tolerant to the herbicide than plants infected with less virulent viruses (Fig. 4C) and showed higher levels of BAR mRNA (Fig. 4C) and enzyme activity (Fig. 2B).

To study the consequences of late CaMV infection on transgene expression, plants were infected in the fourth true leaf with CaMV variants Camb-M (group 3) and Cabb B-JI (group 5). Mild vein clearing was observed in leaves 9 and 11, respectively, and all plants recovered. Leaves 12 and 13 of recovered plants were treated locally with the herbicide as before (Fig. 4D). Less herbicide damage and more BAR mRNA, respectively, was observed in plants infected with Camb-M compared with more severe damage and lower BAR mRNA levels found Cabb B-JI infected plants (Fig. 4D). These experiments show that suppression of transgene expression and herbicide-tolerance can be triggered by different CaMV pathogenic variants infecting plants at different stages of development.

Discussion

Stability of transgene expression is an important character in genetically modified crops. Transgene instability leading to silencing of the transgene and loss of the expected phenotype can be due to a high transgene copy number or transgene rearrangement[1–3]. Our experiments have shown that virus infection can lead to loss of herbicide tolerance in transgenic plants expressing the *BAR* gene. This effect is a consequence of a host defense response to CaMV infection causing inactivation of virus replication by gene silencing leading to silencing of a transgene homologous to the invading virus[6–8]. From analysis of different transgenic lines, we have found no evidence that transgene position effects or copy number play a role in the virus-elicited suppression of *BAR*, *NPT II*, or *GUS* transgenes reported here or previously[8]. Gene silencing in cruciferous hosts of CaMV can affect viral functions at the transcriptional and posttranscriptional levels, with a concomitant effect on transgenes sharing promoter or RNA homology with the virus[8]. Since our constructs contained only significant promoter homology, we conclude that suppression was at the promoter level rather than on RNA turnover. Thus, the *BAR* transgene controlled by the 35S promoter in oilseed rape plants becomes downregulated following CaMV infection. We show that gene silencing elicited by a common worldwide pathogen can compromise the phenotype of economically useful transgenic plants through an effect on the 35S promoter present in many transgenic crops. Since CaMV appears to initiate a host defense response in crucifers that operates optionally at transcriptional or posttranscriptional levels, transgenes bearing either promoter and/or RNA terminator sequences derived from CaMV are potentially at risk from CaMV infection.

We tested a large number of pathogenic variants of CaMV that are representative of those found in different parts of the world and found that the recovery phenotype that is associated with the transgene silencing is a general characteristic of the interaction between virus and host plant. However, the degree of *BAR* transgene suppression depended upon the virulence of the virus variants. Very mild or very severe viral isolates had little observed effect on transgene expression. However, CaMV variants in the broad range of intermediate severity efficiently triggered transgene downregulation.

As yet, we do not know the consequences of these effects for the transgene phenotype at the field level. CaMV is widespread in the United Kingdom oilseed rape crop[14] and in wild *Brassica* species[15]. In addition, CaMV is prevalent in essentially all countries where cruciferous crop plants are grown[13]. Moreover, CaMV infection often occurs with other viral infections, complicating the potential interaction with 35S promoter-driven transgenes. The 35S promoter is often considered to be constitutively expressed, and this, together with its high level of activity, are characteristics favored for applications in plant biotechnology. However, the 35S promoter is clearly regulated by pathogen-elicited plant pathways, a situation that can result in both upregulation[21] and downregulation[8]. The degree to which the 35S promoter is directly or indirectly modulated more generally by pathogen or pest invasion at the field level is not yet known. Gene expression can be subject to a range of responses to pest and pathogen attack, so that use of promoters or transgene regulators other than those derived from CaMV or other pathogens might still be subject to instability of expression. A clearer understanding of these interactions will be obtained when the effects of pathogen and pest invasion on transgenic plants grown under field conditions are investigated.

Experimental protocol

Transgenic plants. Two transgenic lines of *B. napus* Westar 10 were used: 35Sp-*BAR*-OCSt and NOSp-*BAR*-OCSt. Both contained a single copy of the herbicide bialaphos tolerance *BAR* gene regulated by the CaMV 35S or NOS promoter and terminated by the OCSt. Both lines contained the selectable

marker gene *NPT II* driven by the 35S promoter and terminated by the OCSt. T-DNAs are originated from SLJ constructs[16].

Plant propagation and infection. Plants were propagated in a containment glasshouse supplemented with illumination to 16 h/day at 18–22°C. Mechanical inoculation of CaMV variants (described previously[8]) was carried out when the seedlings had one true leaf of about 1 cm in length. The inoculum used was virion (1 µg virion in 10 µl buffer) or with sap (1 mg infected tissues in 2 ml buffer). The buffer used was 10 mM phosphate buffer, pH 7.0, and celite was used as an abrasive.

Nucleic acid extraction. Samples were collected at different days post inoculation (d.p.i.). Leaf samples were ground in liquid nitrogen and divided into two. The first sample was for DNA extraction, and it was carried out by the Kirby methods[8]. The second part of the sample was used to extract total RNA by the RNeasy Plant Mini Kit from Qiagen (Valencia, CA) with some modifications. An extra spin for 2 min after the last column wash and 5 min incubation at room temperature with the elution buffer before centrifugation. DNA and RNA were analyzed by Southern and northern blotting using the appropriate probes.

Herbicide treatments. The herbicide Harvest (glufosinate ammonium; Hoechst & Schering AgrEvo, King's Lynn, UK) was used at 2.5 ml/L (by spraying the whole plants or by treating a local area of the leaf by clipping 1 cm^2 of 3 mm filter paper for chromatography (Whatman, Maidstone, UK) with paper clip. The filter paper then was treated with 100 µl of the herbicide.

PAT assay. Two disks of plant tissue (1 cm in diameter) were used to extract the protein. Protein was labeled with ^{14}C and run on a silica gel TLC (thin-layer chromatography) Plate (Sigma, Dorset, UK)[22].

Nuclear run-on transcription assay. Nuclei were isolated as described by Shepherd, with some changes[12]. Incorporation of uridine 5′-[^{33}P]triphosphate (NEN Life Science Products, Hounslow, UK) was determined by probing 2 µg of the appropriate DNA immobilized as slots on Hybond N+ membranes (Amersham, Buckinghamshire, UK).

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