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RESEARCH ARTICLE

Cauliflower Mosaic Virus Gene VI Controls Translation from Dicistronic Expression Units in Transgenic Arabidopsis Plants

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Transformed Arabidopsis plants were used to study the effect of the cauliflower mosaic virus (CaMV) inclusion body protein on translation of dicistronic RNA. Reporter plants contain a dicistronic transcription unit with CaMV open reading frame VII (ORF VII) as the first and the β-glucuronidase (GUS) reporter ORF as the second cistron. "Transactivator plants" contain CaMV ORF VI under the control of the strong CaMV 35S promoter. The transactivator plants were difficult to regenerate and showed an abnormal phenotype. Expression of GUS activity in the reporter plants was very low, but high GUS activity could be induced by introduction of gene VI, either by crossing with plants containing gene VI as a transgene or by infection with CaMV. Histological GUS assays showed that transactivation occurred in all types of tissue and at all developmental stages. The practical implications of the induction of GUS expression from the dicistronic unit by virus infection are discussed.

INTRODUCTION

The genome of cauliflower mosaic virus (CaMV), a plant pararetrovirus, is shown in Figure 1. It accumulates in the form of ~1000 minichromosomes per nucleus of infected cells during the infection cycle (Olszewski, 1982). These are transcribed by the host RNA polymerase II, resulting in the production of the 19S and the 35S RNAs. The 35S RNA is a particularly complex RNA that is thought to be involved in several basic steps of the virus life cycle: it is packaged into capsids (Fütterer and Hohn, 1987), reverse transcribed to yield circular DNA, and translated into six or seven (poly)proteins. The 35S RNA contains a 600-nucleotide leader sequence with several small open reading frames (sORFs) followed by six tightly arranged larger ORFs that are expressed during infection (reviewed by Mason et al., 1987; Bonneville, 1992).

The translation mechanism from this unusual mRNA has been studied in CaMV and figwort mosaic virus (another member of the caulimovirus group) by isolating and testing polar mutants and their revertants (Sieg and Gronenborn, 1982; Dixon and Hohn, 1984) and by expressing CaMV derivatives in vitro (Gordon et al., 1988) and in plant protoplasts (Baughman and Howell, 1988; Fütterer et al., 1988, 1989, 1990a, 1990b; Bonneville et al., 1989; Gowda et al., 1989; Fütterer and Hohn, 1991; Scholthof et al., 1992). In summary, the results suggest that the translational machinery starts scanning at the cap site in accordance with the central dogma of eukaryotic translation (Kozak, 1989), but then bypasses (shunts) the central region of the leader sequence in a less defined process involving parts of the leader itself and host factor(s) (Fütterer et al., 1989, 1990b). This results in translation of the first coding region (ORF VII). The following ORFs (I, II, III, IV, V, and probably VI) are translated by a reinitiation mechanism which depends on the presence of the CaMV ORF VI protein (Bonneville et al., 1989; Gowda et al., 1989, 1991, 1992; Scholthof et al., 1992). Thus, the gene VI gene product appears to be a transactivator protein, whose initial appearance results from translation of the subgenomic 19S RNA.

The ORF VI protein is an interesting and probably multifunctional protein. It is the major component of the amorphous inclusion bodies accumulating in infected cells (Odell and Howell, 1980; Covey and Hull, 1981). Inclusion bodies have been suggested to be the site of translation of CaMV RNA, accumulation of viral translation products (De Zoeten et al., 1989), reverse transcription (Pfeiffer and Hohn, 1983; Bonneville et al., 1984; Mazzolini et al., 1985; Thomas et al., 1985), and virus assembly. In electron micrographs, polysomes are seen attached to the surface of inclusion bodies, whereas virus particles are inside (Shepherd et al., 1979). The ORF VI protein might also alter the translation machinery by interaction with ribosomes and/or initiation factors.

These properties of ORF VI protein can be expected to affect the host plant, and in fact they determine the severity of

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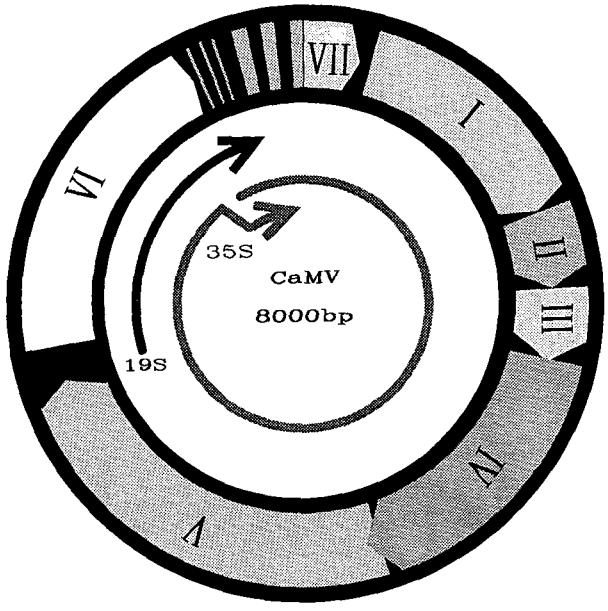

Figure 1. Map of the CaMV Genome.

The black ring symbolizes the 8-kb double-stranded CaMV DNA; the stippled unnumbered blocks within the ring show the set of sORFs; the numbered stippled and white blocks designate the ORFs proper. VII is known to be translated but nonessential (Dixon and Hohn, 1984); I and II code for functions typical of plant viruses, i.e., systemic spreading (Linstead et al., 1988; Citovski et al., 1991) and aphid aquisition factor (Woolston et al., 1983); III codes for a DNA binding protein (Giband et al., 1986). The following ORFs have counterparts in retroviruses: IV (GAG) codes for a set of structural proteins related to retrovirus core proteins; V codes for a polyprotein with protease and polymerase activities (Fütterer and Hohn, 1987); VI encodes a protein that forms large cytoplasmic inclusion bodies (Covey and Hull, 1981) within which the virus particles accumulate; it is also the transactivator protein (Bonneville et al., 1989). The two major viral transcripts are shown as arrows along their respective coding regions. These 19S and 35S RNAs are transcribed clockwise from distinct promoters located just upstream of their respective 5′ ends, but share the same 3′ end (arrowheads).

the cauliflower mosaic disease, the types of symptoms, and, together with other factors, the host range (Daubert et al., 1984; Schoelz et al., 1986; Stratford and Covey, 1989; Daubert and Routh, 1990). Interestingly, expression from ORF VI transgenes also leads to viruslike symptoms (Bálasz, 1988; Baughman et al., 1988; Kiernan et al., 1989; Takahashi et al., 1989; Goldberg et al., 1991; Schoelz et al., 1991). Plants that are not systemic hosts for CaMV are especially affected, whereas certain transformed host plants remain symptomless (Goldberg et al., 1991). It remains to be seen whether these phenotypic effects are caused by modulation of the cell's translation machinery, resulting in overproduction of otherwise poorly translated host proteins, or by other functions of the ORF VI protein.

We wanted to test whether transactivation by gene VI occurs in all types of plant tissues and at all developmental stages. Because this problem cannot be studied using transient expression in protoplasts, we produced Arabidopsis plants transformed with a dicistronic transcription unit containing CaMV ORF VII as the first and the β-glucuronidase (GUS) reporter gene as the second ORF. GUS expression was low in these plants, but high activity could be induced by crossing with plants containing the ORF VI transgene or by infection with CaMV. Transactivation occurred in all tissues and developmental stages tested and may constitute a useful tool for induced expression and induced resistance in plants.

RESULTS

Production and Testing of Arabidopsis Plants Transformed with Dicistronic Expression Units

Plasmids pMonoGUS and pBiGUS (Bonneville et al., 1989) contain monocistronic and dicistronic GUS expression cassettes, respectively, consisting of the CaMV 35S promoter, the GUS reporter ORF fused to the AUG of CaMV ORF I, and the CaMV polyadenylator. The BiGUS cassette contains an additional ORF, the CaMV ORF VII, between the promoter and the GUS ORF. The expression cassettes from these plasmids, symbolized in Figure 2, were cloned between the T-DNA borders

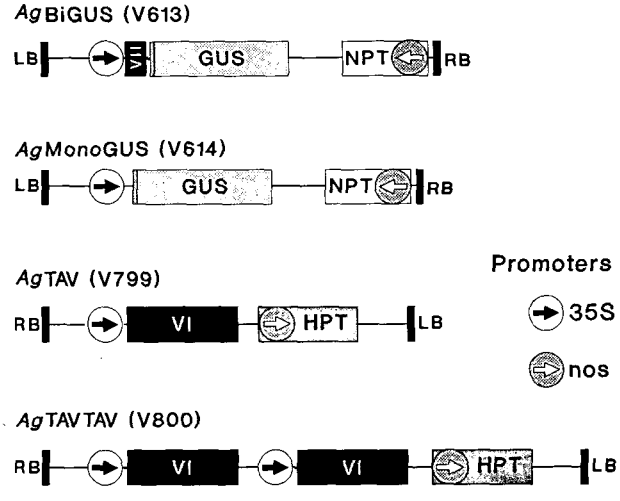

Figure 2. Agrobacterium Lines and Shuttle Plasmids.

Only the T-DNAs are shown. Vectors are pBIN19 for V614 and V613 in *Ag*MonoGUS and *Ag*BiGUS, respectively, and pGSC1704 for V799 and V800 in *Ag*TAV and *Ag*TAVTAV, respectively (see Methods). These binary vectors were transferred from *E. coli* to Agrobacterium. Transgenic plants produced by using these Agrobacterium lines were given an *At* designation with numbers identifying the individual primary transformants. LB and RB, left and right T-DNA borders; NPT, neomycin phosphotransferase II; HPT, hygromycin phosphotransferase; nos, nopaline synthase promoter; 35S, CaMV 35S promoter. V-numbers are collection numbers under which the plasmids are available.

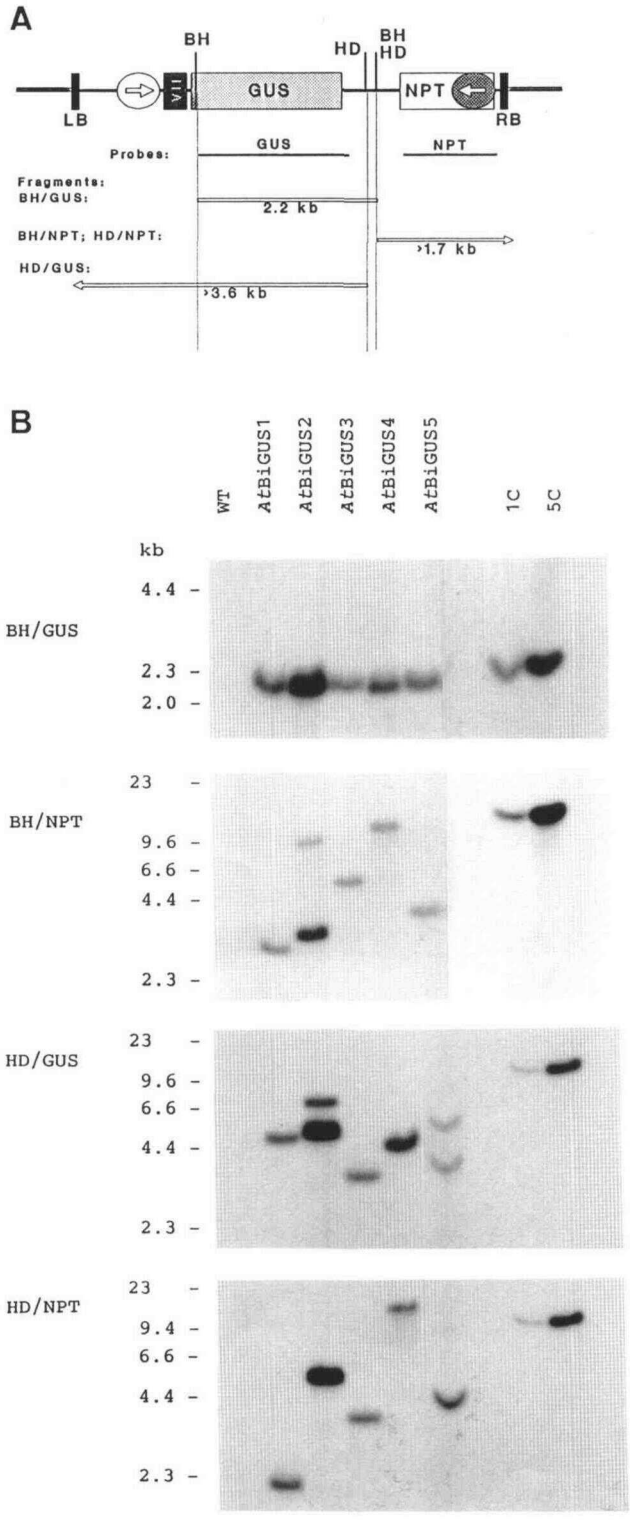

Figure 3. DNA Gel Blot Analysis of Transgenic *At*BiGUS Plants.

(A) T-DNA from *Ag*BiGUS. Arrangement of genes, restriction sites, probes used, and possible restriction fragments produced by digestion with BamHI when probed with GUS (BH/GUS), BamHI when probed with NPT (BH/NPT), HindIII when probed with GUS (HD/GUS), and HindIII when probed with NPT (HD/NPT). LB, left border; RB, right border. The number of T-DNA insertions in an individual transformant were determined by generating border fragments between T-DNA and plant DNA. Border fragments were obtained and characterized by digesting DNA with BamHI or HindIII and probing with the NPT probe or by digesting DNA with HindIII and probing with the GUS probe. In both cases, a single fragment was expected if only one copy of the T-DNA had integrated into the plant DNA.

(B) Autoradiograms of gel blots of DNA extracted from wild-type and *At*BiGUS Arabidopsis plants. DNA was digested with BamHI and HindIII, respectively, and filter hybridized with the GUS and NPT probes, respectively. Lanes 1C and 5C contain DNA from untransformed plants and one and five equivalents, respectively, of the *Ag*BiGUS T-DNA. Blot BH/GUS shows that all transformants had the complete BamHI fragment of ~2.2 kb, whereas no signal was obtained with untransformed tissue. Plants *At*BiGUS1, *At*BiGUS3, and *At*BiGUS4 contain a single copy of *Ag*BiGUS (appearance of single fragments in blots BH/NPT, HD/GUS, HD/NPT). According to the strength of the signals, *At*BiGUS2 contains approximately five copies of the integrated T-DNAs in a head-to-tail arrangement, explaining the appearance of the internal 3.2-kb fragment in blot BH/NPT and the internal 5.3-kb fragment in blots HD/GUS and HD/NPT. The banding pattern of *At*BiGUS5 in blots HD/GUS and HD/NPT cannot be explained by any obvious model. *At*BiGUS5 must, therefore, have rearranged its T-DNA. WT, wild type.

of the Agrobacterium binary shuttle vector pBIN19 (Bevan, 1984) in addition to its resident neomycin phosphotransferase II (NPTII) marker. The resulting plasmids, V614 and V613, were introduced into Agrobacterium by electroporation, and the resulting strains, *Ag*MonoGUS and *Ag*BiGUS, were used to transform Arabidopsis leaf discs.

Kanamycin-resistant calli were regenerated to several normal-appearing "*At*MonoGUS" and "*At*BiGUS" (T_0) Arabidopsis plants over a 4-month period. Seeds from selfed transformed plants were sown on selective medium. Progeny plants giving a 3:1 segregation ratio for kanamycin-resistant seedlings were selected for further analysis, on the assumption that only a single locus had been affected in these transformants. DNA gel blot analysis revealed that most of the *At*MonoGUS plants contained multiple insertions (data not shown), whereas most of the *At*BiGUS plants contained single insertions, as shown in Figure 3. Only plants with single-copy insertions were analyzed further.

GUS activity in the transformed plants was determined either in plant extracts or in extracts from protoplasts. Figure 4 shows that *At*MonoGUS transgenic plants, as expected, contained high levels of GUS activity, whereas no activity was found in untransformed controls. These levels are ~150 times higher than the ones we observed regularly in transfected protoplasts. The GUS activity in *At*BiGUS transgenic plants and in their protoplasts was low but detectable, i.e., ~1% of the activity of *At*MonoGUS transgenic plants. In contrast, the level of GUS activity in wild-type protoplasts transfected with 5 μg of pBiGUS was less than 0.05% of the activity in protoplasts transfected with 5 μg of pMonoGUS (data not shown). The relatively high expression and background levels in transformed

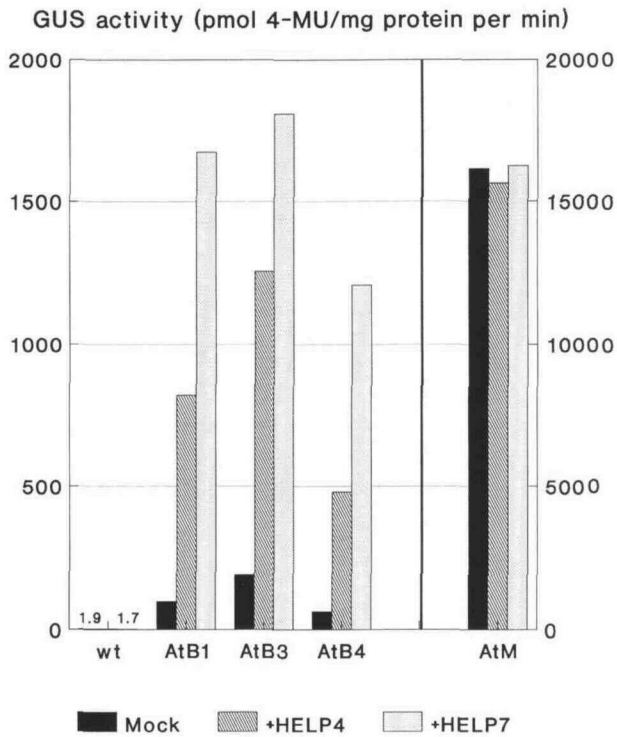

Figure 4. *GUS Expression in Wild-Type and AtBiGUS Protoplasts.*

GUS activities measured in representative Arabidopsis protoplasts after transfection of 50 μg calf thymus DNA (mock), 50 μg calf thymus and 10 μg pHELP4 DNA, and 50 μg calf thymus and 10 μg pHELP7 DNA. GUS activity was measured in samples containing 7 μg of crude protein and is expressed as picomoles of 4-methylumbelliferone (4-MU) per milligram of protein per minute. wt, wild type; AtB1, *At*BiGUS1; AtB3, *At*BiGUS3; AtB4, *At*BiGUS4; AtM, *At*MonoGUS (for which GUS activity is shown on a different scale).

plants compared to transfected protoplasts is probably due to the accumulation of GUS enzyme produced by leaky expression over a long period.

Super-transfection of *At*BiGUS protoplasts with transactivator plasmids pHELP4 or pHELP7 coding for the same ORF VI under 19S and 35S promoter control, respectively, increased GUS activity about eightfold and 10- to 20-fold, respectively (Figure 4), showing that the GUS coding region in the dicistronic expression unit was intact and confirming the results of Bonneville et al. (1989) on the activity of the pHELP plasmids.

Production and Testing of Arabidopsis Plants Transformed with the CaMV Transactivator Gene

The CaMV transactivator (gene VI) cassette from pHELP7 consisting of the 35S promoter, ORF VI, and the polyadenylator was cloned between the T-DNA borders of the binary shuttle vector pGSC1704 (kindly provided by Plant Genetic Systems, Gent, Belgium) in addition to the resident hygromycin phosphotransferase (HPT) marker. Plasmids V799 in *Ag*TAV and V800 in *Ag*TAVTAV contained one and two copies of gene VI, respectively, under the control of the 35S promoter (Figure 2).

After incubation of leaf discs with *Ag*TAV or *Ag*TAVTAV, hygromycin-resistant calli were obtained with the same efficiency as the kanamycin-resistant calli after transformation with *Ag*BiGUS or *Ag*MonoGUS. However, the production of plants expressing ORF VI proved very difficult because only a few of the calli survived and only a small percentage of the survivors regenerated shoots. Many of these shoots died at a later stage and the survivors produced very few or no seeds. Most regenerated hygromycin-resistant plants had the wild-type phenotype, but some transformants produced abnormal rosettes with an unusually large number of leaves. The leaves

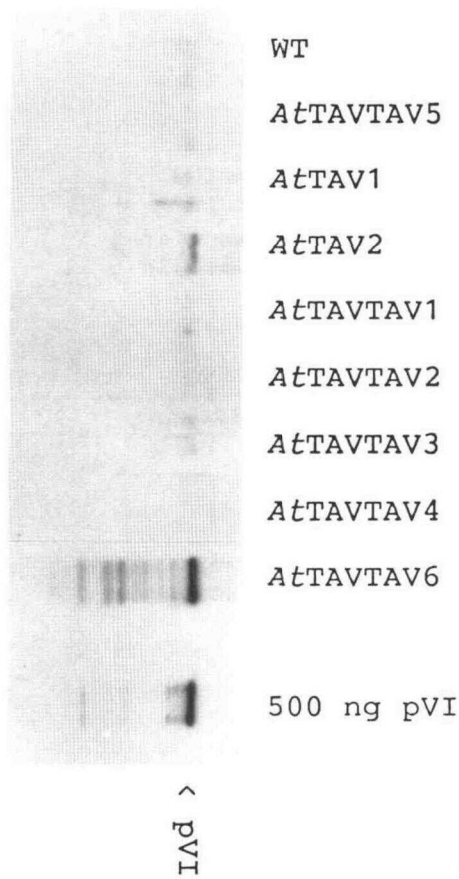

Figure 5. Protein Gel Blotting of Transgenic Arabidopsis.

Extracts containing 50 μg of total protein from *At*TAV and *At*TAVTAV plants were subjected to gel electrophoresis and electroblotting. A 500-ng sample of purified ORF VI protein (pVI) was loaded as a positive control. An obviously unspecific weak band is observed at about the same position as pVI in all samples including the untransformed control (wild type, wt) and also with other types of antibodies. We consider only the strong bands in *At*TAV2 and *At*TAVTAV6 to be positive signals.

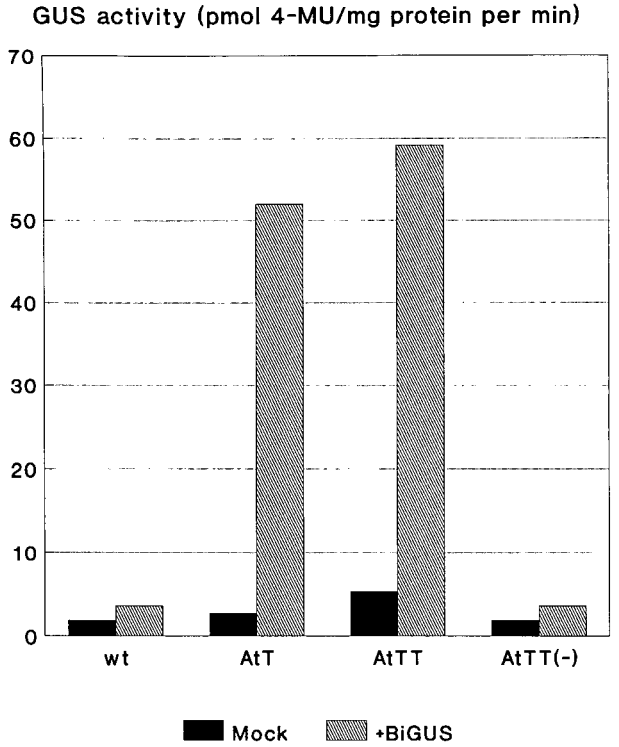

Figure 6. Transactivation of GUS Expression in Transactivator Protoplasts.

GUS activities measured in representative Arabidopsis protoplasts after transfection of 50 μg of calf thymus DNA (mock) and 50 μg of calf thymus and 5 μg of pBiGUS DNA. GUS activity was measured in samples containing 35 μg of crude protein extract and is expressed in picomoles of 4-methylumbelliferone (4-MU) per milligram of protein per minute. wt, wild type; AtT, *At*TAV2; AtTT, *At*TAVTAV6; AtTT(−), *At*TAVTAV3 (a line not expressing ORF VI).

were mottled, chlorotic, and abnormally shaped (C. Zijlstra, N. Hernandez, and T. Hohn, unpublished data). Further characteristics of these plants are stunting, delayed flowering, and reduced fertility. Eight hygromycin-resistant plants were further analyzed, and two of them (*At*TAV2 and *At*TAVTAV6) were found to contain unmodified ORF VI inserts (C. Zijlstra, N. Hernandez, and T. Hohn, unpublished data) and to produce the ORF VI antigen of the expected size, as shown in Figure 5. These two plants had the abnormal phenotype, now called the "ORF-VI-phenotype." The other six plants gave rearranged DNA gel blot patterns of ORF VI or no signals at all, and had no detectable level of ORF VI antigen.

The activity of the ORF VI protein present in the transformed plants was first tested in transient expression experiments. Figure 6 shows a much higher level of GUS activity upon transfection with pBiGUS DNA in protoplasts derived from *At*TAV2 and *At*TAVTAV6 plants than in protoplasts derived from wild-type or the hygromycin-resistant plants with rearranged inserts. The levels were about 50% of those obtained by transfection with pMonoGUS regardless of the presence or absence of the ORF VI transgene (data not shown). Thus, the activation of translation from the dicistronic reporter construct correlates with the presence of an intact gene VI and its protein product. The lower expression levels in this experiment compared to those shown in Figure 4 reflect the lower expression rate of the reporter plasmids in transfected protoplasts compared to transgenic plants already described.

Transactivation in Transformed Plants

To study transactivation in whole plants, double-transformed plants were bred containing both the *At*BiGUS and one of the transactivator constructs. For this purpose, single-copy pure (homozygous) lines of the kanamycin-resistant *At*BiGUS plants were emasculated and pollinated with pollen from the hygromycin-resistant *At*TAV2 or *At*TAVTAV6 plants. The cross was made in this direction because of the low fertility of the transactivator plants. Progeny seeds were germinated on medium containing hygromycin. Further generations were subjected to double selection on medium containing both kanamycin and hygromycin.

The double-transformed seedlings, as well as their monotransformed parents and the *At*MonoGUS plants, were tested for GUS activity by incubation in GUS assay buffer containing 5-bromo-4-chloro-3-indolyl β-D-glucoronide (X-gluc). As shown in Figure 7, the *At*MonoGUS and the double-transformed plants gave the intense blue staining indicative of GUS gene expression, whereas no staining was observed in the transactivator plants. Staining of the *At*BiGUS plants was very weak but still discernible, suggesting that some GUS expression from the dicistronic unit also occurred in the absence of the transactivator.

GUS activity in transformed plants was quantified by assaying extracts from 3-week-old seedlings of monotransformed parents and double-transformed progeny with 4-methylumbelliferyl β-D-glucuronide (MUG) as substrate. The level of GUS activity is shown in Figure 8. It was sevenfold to 43-fold higher in the double-transformed plants than in the *At*BiGUS mother plants. *At*MonoGUS plants showed about 10-fold higher GUS activity than the most active double-transformed plants.

For determination of the cell specificity of GUS expression, the activity was analyzed in more detail by histochemical staining, as shown in Figure 9. Double-transformed plants containing the *At*BiGUS and a transactivator construct showed intensive staining in all types of tissue, indicating that transactivation is not tissue specific. Furthermore, staining was observed in very young (2-day-old) seedlings as well as in mature and senescent plants.

Transactivation by CaMV Infection

Another way to introduce CaMV ORF VI and its product into the *At*BiGUS plants is to inoculate the plants with CaMV. Three- to 4-week-old plants were infected according to the method

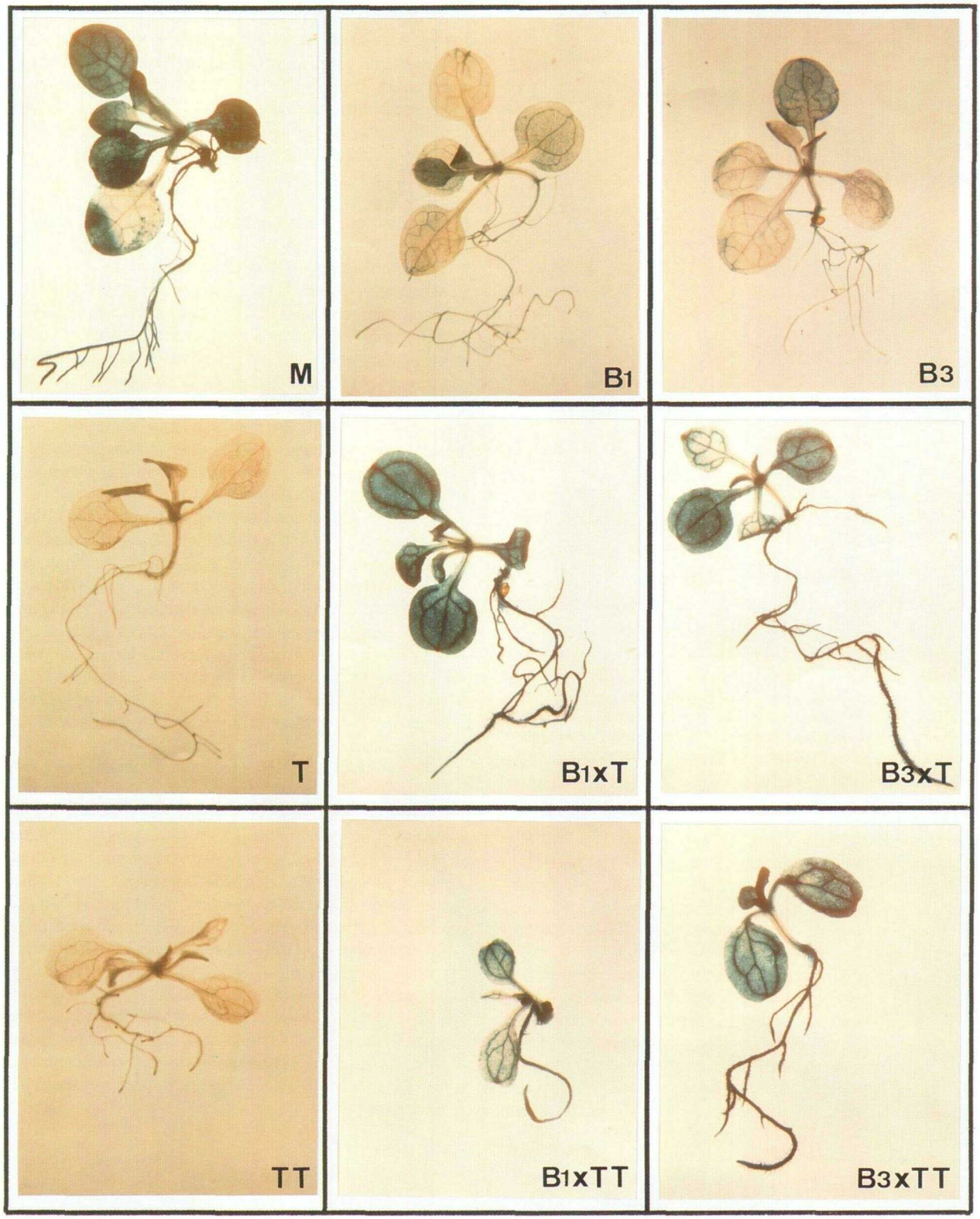

Figure 7. GUS Expression in Transgenic Arabidopsis Plants.

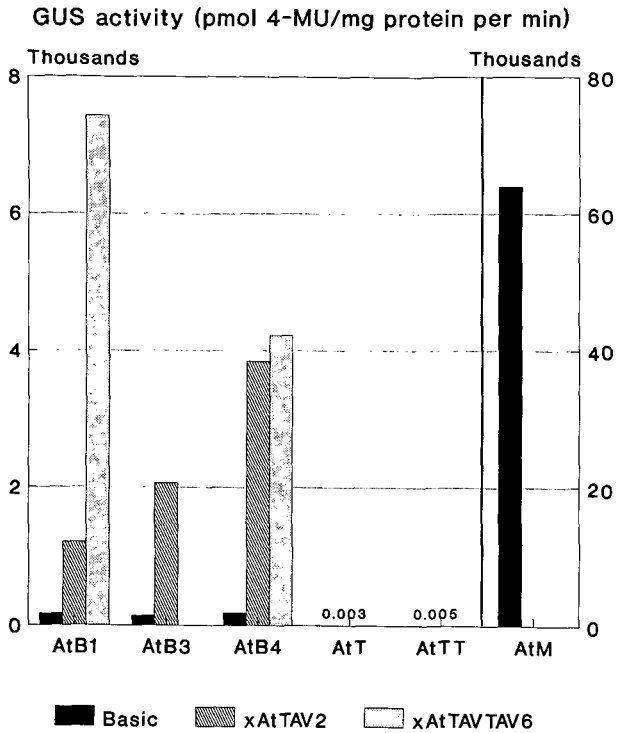

Figure 8. Transactivation of GUS Expression in *At*BiGUS Plants after Crossing with Transactivator Plants.

GUS activities measured in 4-μg protein extract samples from representative 3-week-old transgenic *At*BiGUS seedlings (basic level) and their F_1 progeny after crossing with *At*TAV2 and *At*TAVTAV6. GUS activity is expressed in picomoles of 4-methylumbelliferone (4-MU) per milligram of protein per minute. AtB1, *At*BiGUS1; AtB3, *At*BiGUS3; AtB4, *At*BiGUS4; AtT, *At*TAV2; AtTT, *At*TAVTAV6; AtM, *At*MonoGUS (for which GUS activity is shown on a different scale).

described by Melcher et al. (1989) with CaMV CabbB-JI, which is very virulent in Arabidopsis (A. Bannister and S. N. Covey, unpublished observations). Severe symptoms were observed 2 weeks after inoculation of seedlings of wild-type and *At*BiGUS plants, namely stunting, leaf curling, mosaic formation, and vein clearing. These symptoms differ from those elicited by the gene VI product in transgenic plants. The histochemical stainings of infected *At*BiGUS plants shown in Figure 10 reveal high levels of GUS activity, whereas GUS activity was low once again in the uninfected *At*BiGUS plants. The highest GUS activity was localized in the inner (youngest) leaves of the rosette, whereas lower GUS activity appeared in the outer (older) leaves. The veins were most intensively stained. Leaves that existed before inoculation showed no symptoms, and the inoculated leaf occasionally showed localized staining (Figure 10B). The staining pattern probably reflects the CaMV replication and expression rates in each tissue and the fact that staining of the leaves was restricted to those appearing after inoculation (with the exception of isolated spots on the inoculated leaf) confirms the concept of source-to-sink movement of CaMV (Leisner et al., 1992).

DISCUSSION

In the experiments reported here, a dicistronic expression unit in a transformed plant was translated under the control of a plant virus–derived translation activator. This CaMV ORF VI protein could be provided either as a second transgene or as part of an infecting virus. Our results confirm those obtained earlier with transient expression experiments (Bonneville et al., 1989; Gowda et al., 1989; Fütterer et al., 1991). Moreover, they demonstrate that transactivation occurs in the whole plant and is not an isolated phenomenon related to stress or to a particular developmental stage of manipulated plant protoplasts. Furthermore, it was possible using whole plants to ask questions about tissue- and development-stage specificity of transactivation.

The histological assays for GUS showed that transactivation can occur in all types of tissue, i.e., roots, stems, leaves, and flowers, as well as at all developmental stages, i.e., young seedlings and mature and senescent plants. The relatively high intensity of leaf vein and phloem staining is probably related to the higher density of cells in these tissues and to increased availability of the substrate rather than increased transactivation; similarly, the intense staining in the meristematic cells is probably due to their small size, the absence of vacuoles, and higher metabolic activity (Martin et al., 1992). The expression patterns of GUS from *At*BiGUS plants in the presence of ORF VI protein, the transactivator, resemble the expression patterns from *At*MonoGUS plants. Similar patterns have also been observed in tobacco transformed with a comparable monocistronic construct (Benfey and Chua, 1990). It is, therefore, likely that all the variations in staining pattern seen in Figures 7 and 9 reflect variations in promoter activity rather than variations in transactivation. The situation might be different, however, for expression of the original CaMV genes during infection. In that case, transactivator expression is controlled

Figure 7. (continued).

Four-week-old seedlings of transgenic Arabidopsis plants were stained for GUS activity as described in Methods. M, *At*MonoGUS; B1, *At*BiGUS1; B3, *At*BiGUS3; T, *At*TAV2; TT, *At*TAVTAV6; B1 × T, B3 × T, B1 × TT, and B3 × TT are the F_1 progenies of crosses between female reporter and male transactivator plants.

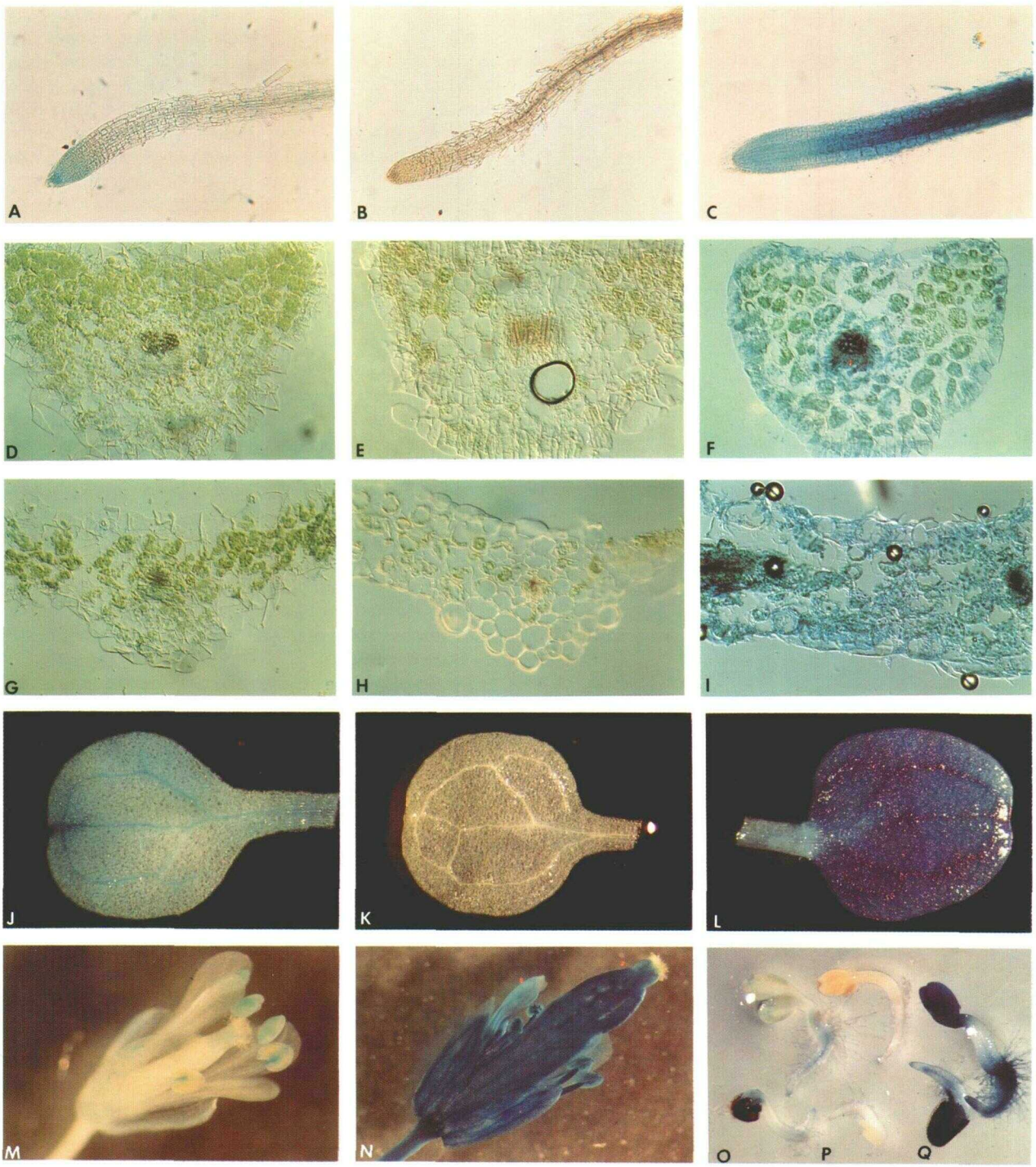

Figure 9. Histochemical GUS Analysis.

Representative histochemical staining shows cell specificity of GUS expression.
(A) to **(C)** Roots.
(D) to **(F)** Cross-sections of petioles.
(G) to **(I)** Cross-sections of leaves.

by the 19S rather than the 35S promoter, by CaMV DNA-copy number, possibly by positive feedback control (M. Driesen, T. Hohn, and J. Fütterer, manuscript in preparation), and by partitioning of CaMV RNA into pools for packaging, reverse transcription, and translation.

Arabidopsis plants containing the CaMV (strain CM4-184) ORF VI as a transgene were difficult to obtain, and those that survived and expressed ORF VI had an abnormal phenotype, indicating that the ORF VI protein interferes with certain metabolic or developmental plant processes. Such difficulties were also encountered by Schoelz et al., (1991) with ORF VI–transformed *Nicotiana bigelovii*, and abnormal phenotypes were observed in several types of nonhost plants by Bálasz (1988), Baughman et al. (1988), Kiernan et al. (1989), Takahashi et al. (1989), and Goldberg et al. (1991). The basis of such phenotypic alterations in plants transformed with gene VI will be discussed in detail elsewhere (C. Zijlstra, N. Hernandez, and T. Hohn, manuscript in preparation).

The fact that GUS expression from the dicistronic unit can be induced by virus infection has practical implications. For example, one could trace the route of systemic virus spread by analyzing transactivated expression of GUS following an initial infection event. One could also use transgenes to induce virus resistance: translation of a ribosome-inactivating endotoxin gene (Stirpe et al., 1992) or a special RNase gene, such as barnase (Hartley, 1991; Natsoulis and Boeke, 1991), located on a dicistronic expression unit and, therefore, dependent for its expression on the transactivator, would be induced upon CaMV infection. Production of the toxin would specifically eliminate the infected cells, thereby inhibiting virus spread. This process would thus resemble the hypersensitive reaction, a natural plant defense mechanism.

We are aware that the relatively high background of GUS expression in our plants containing the dicistronic transgenic unit might be a problem for this type of application. This background was higher in transformed plants than in transfected protoplasts, probably due to accumulation of the GUS enzyme during the life span of the transformed plants. The problem might be overcome by the use of dicistronic units with first cistrons other than the sORF VII used. In preliminary experiments in our laboratory (J. Vlasák, unpublished data), dicistronic transgenes containing the NPTII gene as the first cistron did not express detectable levels of the second cistron until transactivated.

METHODS

Plasmids Used for Transient Expression in Plant Protoplasts and for Further Constructions

The cauliflower mosaic virus (CaMV) sequences used were derived from strain CM4-184. Plasmid pMono-β-glucuronidase (GUS) consists of a CaMV open reading frame (ORF) I–GUS translational fusion, pBiGUS of ORF VII and the ORF I–GUS translational fusion, and pHELP7 of ORF VI, all provided with the CaMV 35S promoter and polyadenylator. pHELP4 consists of the CaMV 19S promoter, ORF VI, and polyadenylator (Bonneville et al., 1989).

Agrobacterium Constructs for Plant Transformation

***Ag*BiGUS V613.** This plasmid was used in *Ag*BiGUS (Figure 2) and was obtained by cloning the expression unit from pBiGUS as a PvuII fragment into the SmaI cloning site within the T-DNA of the binary shuttle vector pBIN19; pBIN19 confers kanamycin resistance to transformed plants (Bevan, 1984).

***Ag*MonoGUS V614.** This plasmid was used in *Ag*MonoGUS and is an ORF VII-less derivative of *Ag*BiGUS. It was obtained by ligating the 2227-bp ScaI-HindIII fragment from pBiGUS containing the ORF I–GUS fusion with the 3180-bp SmaI-HindIII fragment of the plant expression plasmid pDH51 (Pietrzak et al., 1986), resulting in intermediate plasmid V594. From this plasmid, the monocistronic GUS expression cassette was isolated as a PvuII fragment and cloned into the T-region of pBIN19, as given above.

***Ag*TAV.** To construct V799, the plasmid present in *Ag*TAV, first a transactivator expression cassette suitable for integration into the binary *Agrobacterium tumefaciens* vector pGSC1704 was constructed by combining two existing ORF VI expression plasmids. The 35S promoter and the first part of ORF VI were isolated as an XhoI-AvaI fragment from V709 (J. M. Bonneville, personal communication) containing ORF VI as an XhoI cassette in the multiple site cloning vector pMTL23 (Chambers et al., 1988). The second part of ORF VI and the polyadenylator were isolated as an AvaI-XhoI fragment from pHELP7. Both fragments were ligated into the T-region of pGSC1704 opened at SalI and HindIII. pGSC1704 was kindly provided by Plant Genetic Systems and contains the HPT cassette, described by Van den Elzen et al. (1985),

Figure 9. (continued).

(J) to **(L)** Cotyledons, all of 4-week-old plantlets.
(M) and **(N)** Flowers of 12-week-old plantlets.
(O) to **(Q)** Whole 2-day-old seedlings.
Representative *At*BiGUS **(A, D, G, J, M,** and **O)** and *At*TAV **(B, E, H, K,** and **P)** plants and their F_1 progeny **(C, F, I, L, N,** and **Q)** are shown.

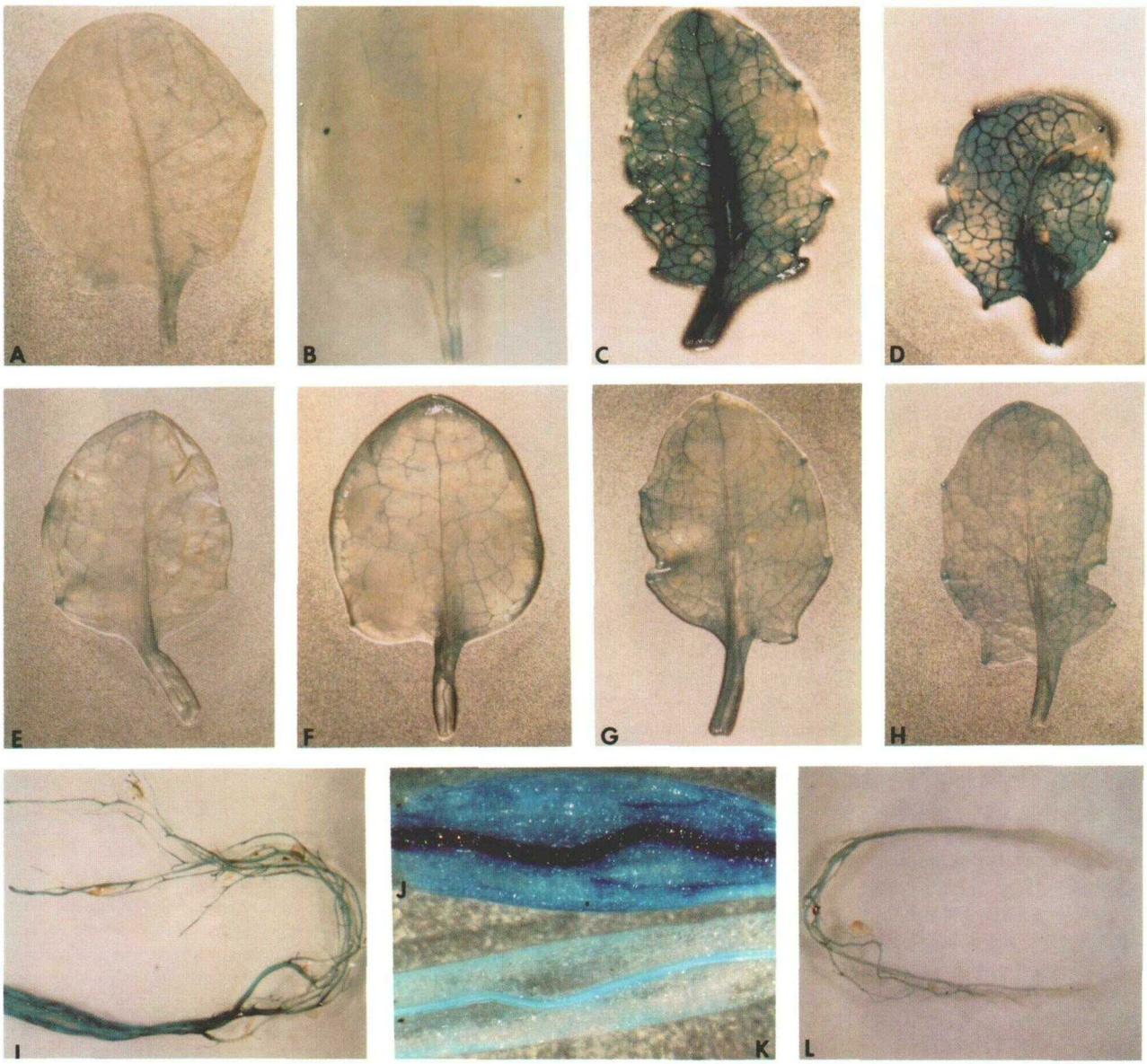

Figure 10. GUS Expression in CaMV-Infected *At*BiGUS Plants.

Leaves shown in **(A)** to **(D)**, the root shown in **(I)**, and the petiole in **(J)** are from 10-week-old CaMV-infected *At*BiGUS transgenic plants grown under short day conditions. Leaves shown in **(E)** to **(H)**, the root shown **(L)**, and petiole in **(K)** are from 10-week-old noninfected *At*BiGUS transgenic plants grown under short day conditions.

(A) and **(E)** Leaf number 3.

(B) and **(F)** Leaf number 5.

(C) and **(G)** Leaf number 11.

(D) and **(H)** Leaf number 14.

(I) and **(L)** Roots.

(J) and **(K)** Petioles.

At 4 weeks, number 5 leaves were inoculated with CaMV strain CabbB-JI or were mock inoculated with water. The leaf in **(B)** is enlarged twice as much as the other leaves to show the localized histochemical staining more clearly.

between its T-DNA borders, which confers hygromycin resistance to transformed plant tissue.

***AgTAVTAV* V800.** This plasmid is present in *AgTAVTAV* and was obtained by serendipity in the procedure used to obtain *AgTAV*. It contains two copies of the XhoI ORF VI expression cassette. The binary vectors originally grown in *Escherichia coli* JM101 (Yanisch-Perron et al., 1983) were introduced into Agrobacterium C58C1, containing helper plasmid pGV2260 (Deblaere et al., 1985), by electroporation as described by Mattanovich et al. (1989). Integrity of the vectors was verified by analyzing total DNA from Agrobacterium by the method of Dhaese et al. (1979).

Stable Transformation of Arabidopsis

Arabidopsis thaliana ecotype C24 was transformed with Agrobacterium containing the shuttle and helper plasmids using the method described by Van Lijsebettens et al. (1991).

DNA Gel Blot Analysis

Genomic DNA was isolated from lyophilized plants or callus using a CTAB extraction procedure (modified after Murray and Thompson, 1980, as described by Mittelsten-Scheid et al., 1991). Samples were cleaved with restriction enzymes (10 to 20 units per μg of DNA) and separated on a 0.8 to 1.2% agarose gel. DNA was transferred to a Hybond N membrane (Amersham International) according to Southern (1975). After fixation of the transferred DNA by UV cross-linking, the membrane was prehybridized for 6 hr in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, 0.025% BSA), 0.5% SSC, and 0.05% salmon sperm DNA at 65°C and subsequently hybridized at 65°C overnight with ^{32}P-labeled probes in prehybridization solution containing 10 mM EDTA. Probes were labeled using the random prime labeling kit from Boehringer Mannheim, based on the method of Feinberg and Vogelstein (1983). After hybridization, the membrane was rinsed in 2 × SSC and then washed successively for 15 min in 2 × SSC, 0.1% SDS at room temperature, in 0.5 × SSC, 0.1% SDS at 65°C, and finally in 0.1 × SSC, 0.1% SDS at 65°C. To reuse blots, old probes were removed by repeatedly washing the membrane with a boiling solution of 0.05 × SSC, 0.1% SDS, and 0.01 M EDTA, pH 8, which was allowed to cool down to room temperature after being poured onto the membrane.

Electrophoresis and Immunological Detection of Proteins

Total plant proteins were isolated using the method described by Mayer et al. (1987). Protein concentrations were determined as described by Ramagli and Rodriguez (1985). SDS-PAGE was performed as described by Laemmli (1970). The amount of protein loaded was 50 μg per lane. The proteins were transferred from the electrophoresis gel to an Immun-Lite membrane (Bio-Rad) via semidry electroblotting with an apparatus from JKA Biotech (Vaerløse, Denmark) for 1 hr at 1 mA/cm^2 of gel. Reaction with antisera and anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Immunoblot kit) was performed as described by the manufacturer.

Genetic Crosses

Genetic crossings were carried out as described by Mittelsten-Scheid et al. (1991). Harvested seeds were stored at 28°C for 3 weeks and kept at 4°C for another week.

Preparation of Protein Extracts from Plant Tissue for GUS Assays

Plant tissue samples (5 to 50 mg fresh weight) were homogenized with 200 μL of extraction buffer (50 mM NaH_2PO_4, pH 7, 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl, 10 mM β-mercaptoethanol). Soluble proteins were obtained by centrifugation of the homogenates for 5 min in a table centrifuge.

Transformation of Protoplasts and Preparation of Extracts for GUS Assays

Mesophyll protoplasts were isolated from in vitro-grown Arabidopsis plants as described by Damm and Willmitzer (1988). The protoplasts were used for PEG transformation as described by Damm et al. (1989). Protoplasts were harvested by centrifugation and soluble extracts were prepared as described by Fütterer et al. (1989).

Fluorometric assays were performed with samples of equivalent protein content. Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard. GUS activities were assayed in 96-well microtiter plates in 300-μL reaction samples containing 1 mM 4-methylumbelliferyl β-D-glucuronide (4-MUG, Sigma) (Jefferson et al., 1987); 50-μL aliquots were taken at four successive time points and the appearance of fluorescence was followed using a Titertek Fluoroscan II apparatus (Jefferson et al., 1990).

Histochemical Staining

Whole plants or detached plant organs were placed into a 24-well culturing plate containing 100 mM phosphate buffer, pH 7, and 1 mg of β-D-glucuronide (X-gluc) per mL. After initial vacuum infiltration for about 15 min, the plant material was incubated in the dark at 37°C for 3 to 12 hr. Plant material was fixed for 10 min in a solution containing 5% formaldehyde, 5% acetic acid, and 20% ethanol. When necessary the samples were bleached to remove chlorophyll by overnight treatment with 70% ethanol.

Tissue Sectioning

Small tissue blocks stained for GUS activity were prepared for cryosectioning according to a modified procedure described by Sohn and Sauter (1991). After incubation of the plant tissue in the X-gluc solution, it was fixed overnight at 8°C with 4% (w/v) formaldehyde and 0.1% glutaraldehyde in a buffer containing 100 mM potassium phosphate buffer, pH 7, supplemented with 0.8% NaCl and 0.2% NaN_3 (PBS). The samples were immersed consecutively for 2 hr at room temperature in 0.25, 0.5, 1.0, and 1.5 M sucrose in PBS, followed by a 15-min vacuum infiltration. The samples were then kept overnight in 1.5 M sucrose-PBS, which was renewed before cryosectioning was performed. Cryosections of 30-μm thickness were produced with a Cryostat

microtome (Model 830/C; American Optical Company, New York) at −25°C.

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