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Cauliflower mosaic virus gene VI causes growth suppression, development of necrotic spots and expression of defence-related genes in transgenic tobacco plants

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Summary. In order to study possible functions of the inclusion body matrix protein (IBMP) encoded by gene VI of cauliflower mosaic virus (CaMV), the *XbaI* fragment containing the gene VI of a Japanese strain of CaMV (CaMV S-Japan) was transferred to tobacco plants by Ti mediated transformation. Eight out of 18 kanamycin resistant plants (40%) expressed detectable levels of IBMP. Those transgenic plants expressing IBMP produced leaves with light green color, and their growth was suppressed as compared with control plants. Symptom-like necrotic spots also appeared on the leaves and stems of the mature transgenic plants. Furthermore, in these transgenic plants, pathogenesis-related proteins 1a, 1b and 1c were highly expressed and the activity of 1,3-β-glucanase was increased up to eightfold. From these results, we concluded that expression of the IBMP is associated with symptom development.

Key words: Cauliflower mosaic virus gene VI – Transgenic tobacco plants – Pathogenesis-related proteins – Stress – Symptom expression

Introduction

Cauliflower mosaic virus (CaMV) belongs to the caulimoviruses having a circular, double stranded DNA genome of approximately 8000 bp in length (for reviews, see Shepherd 1979; Hohn et al. 1982; Howell 1982; Hirth 1986). Three strains of CaMV have been completely sequenced, and in each case six major open reading frames (ORFs; gene I–VI) and two intergenic regions have been identified (Franck et al. 1980; Gardner et al. 1981; Balazs et al. 1982). Two smaller ORFs (VII, VIII) are also present in CaMV, but their significance is unclear. These six ORFs on the CaMV genome are believed to be important for viral multiplication. The polypeptides corresponding to the six ORFs of CaMV have been detected in plant extracts, and their functions have been characterized. Gene IV codes for the coat protein of the virus (Franck et al. 1980; Daubert et al. 1982; Martinez-Izquierdo and Hohn 1987). Insect transmission has been mapped to gene II, which encodes the 18000 daltons (18 kDa) protein component of the inclusion body (Givord et al. 1984; Woolston et al. 1983). Gene V specifies the viral reverse transcriptase (Toh et al. 1983; Pfeiffer et al. 1984; Menissier et al. 1984; Volovitch et al. 1984; Takatsuji et al. 1986; for reviews, see Hohn et al. 1985). Gene III possibly codes for a DNA-binding protein (Giband et al. 1986). Gene I encodes the 41 kDa protein component of the inclusion body. The function of gene I has not yet been identified, but its predicted amino acid has homology with sequences of the TMV 30 kDa protein and with ribonucleotide reductase of *Escherichia coli* (Martinez-Izquierdo et al. 1987). The polypeptides of these five genes are possibly translated from the 35 S RNA transcript, which is slightly greater than the genome length (Dixon and Hohn 1984; Gordon et al. 1988).

The polypeptide encoded by gene VI has been identified as a major protein of the matrix of the inclusion body by cell-free translation studies using mRNA isolated from infected plants (Odell and Howell 1980; Xiong et al. 1982). The inclusion body matrix protein (IBMP) is translated from a 19 S RNA generated by direct transcription of gene VI (Covey and Hull 1981; Xiong et al. 1982). Insertional mutagenesis experiments revealed that the IBMP is essential for virus replication (Daubert et al. 1983; Dixon et al. 1983). Several lines of evidence are accumulating that CaMV replication by reverse transcription occurs in the inclusion body (Modjtahedi et al. 1984; Mazzolini et al. 1985). In addition, the IBMP is implicated in host range control and disease symptoms (Daubert et al. 1984; Schoelz and Shepherd 1988). Although some transgenic plants having gene VI integrated into their chromosomes displayed the mosaic-like symptom (Shewmaker et al. 1985; Young et al. 1987; Baughman et al. 1988), it is not yet established whether the appearance of disease-like symptoms is a general phenomenon in transgenic plants expressing IBMP.

One of the physiological changes which occurs in pathogen-infected plants is the synthesis of pathogenesis-related (PR) proteins, which have been detected in 16 plant species (van Loon 1985). Of the 10 PR proteins found in tobacco, PR-P and -Q have been recently identified as acidic endochitinases (Legrand et al. 1987) and PR-O, -N and -2 as 1,3-β-glucanases (Kauffmann et al. 1987). However, the functions of PR-1a, -1b and -1c proteins remain unknown. The PR proteins are often detected in plants developing necrotic local lesions (hypersensitive reaction) after infection by viruses, fungi or bacteria. Therefore, the possible correlation of the disease symptom and the induction of PR proteins has been suggested.

Here we report that the synthesis of the IBMP by the Japanese strain of CaMV, CaMV S-Japan, causes the sup-

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pression of plant growth, the induction of the PR proteins and the appearance of necrotic spots in transgenic plants.

Materials and methods

Plasmid construction. CaMV S-Japan DNA cloned in the *Sal*I site of pBR322 was obtained from Dr. H. Hirochika (National Institute of Agrobiological Resources, Japan; Hirochika et al. 1985). Its 3.1 kb *Xba*I fragment containing the terminal portion of ORF V (698 bp), the 19 S promoter, gene VI, the 35 S promoter, a poly(A) addition signal and the proximal portion of ORF VII (54 bp) was inserted into the *Xba*I site of pGA482 (An 1986) and the resulting plasmid, pGA482CaS6, was used for plant transformation. The sequence of the 3.1 kb *Xba*I fragment will be described elsewhere. pGA482CaS6 contains the left and the right borders of T-DNA, and a neomycin phosphotransferase II gene (NPTII) under the control of the nopaline synthetase promoter as a selective marker. It was directly transformed into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) by selecting resistance to rifampicin and tetracycline.

Plant transformation. Leaves of *Nicotiana tabacum* var. Petit Havanna (SR1) grown for 8–10 weeks in a growth chamber were sterilized and infected with *A. tumefaciens* LBA4404 containing pGA482CaS6. The leaf disks were transferred to MS medium (Murashige and Skoog 1962) containing 300 μg/ml kanamycin, 1.0 mg/l naphthylacetic acid (NAA), 0.1 mg/l benzylaminopurine (BAP) and 500 μg/ml carbenicillin. After 1 week, the leaf disks were transferred to the same MS medium but containing 100 μg/ml kanamycin. After 3 weeks, kanamycin resistant callus was transferred to shoot induction medium (0.1 mg/l NAA, 1.0 mg/l BAP) containing 100 μg/ml kanamycin and 500 μg/ml carbenicillin. Kanamycin resistant shoots were rooted on half strength MS medium containing 100 μg/ml kanamycin. After development of the roots, plantlets were transferred to compost for further growth in a growth chamber at 20° C under light (5000 lux, 14 h). When the plants reached the 3–4 leaf stage, analyses of the IBMP and the PR proteins were performed.

Protein analysis of transgenic plants. Samples of leaves (0.2 g) were ground in a chilled mortar with 1 ml of 50 mM TRIS-HCl (pH 9.0) containing 2% SDS, 1% 2-mercaptoethanol, 20% sucrose and bromophenol blue (BPB), incubated at 100° C for 10 min and centrifuged at $10000 \times g$ for 10 min. The supernatants were subjected to electrophoresis in a 10% SDS-polyacrylamide gel (Laemmli 1970) and transferred electrophoretically to nitrocellulose filters (Towbin et al. 1979). The filters were incubated with antiserum raised against the IBMP, and then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Tago, USA). Staining was performed with 0.005% 5-bromo-4-chloro-3-indolyl phosphate, 0.01% nitro blue tetrazolium chloride, 1 mM $MgCl_2$, 0.1 M diethanolamine (pH 9.0). The antiserum against the IBMP was kindly provided by Dr. G. Lebeurier (CNRS, France; Xiong et al. 1982). For analysis of the pathogenesis-related (PR) proteins, samples of leaves (0.2 g) were ground in a chilled mortar with 0.2 ml of 84 mM citric acid, 32 mM Na_2HPO_4 (pH 2.8) containing 14 mM 2-mercaptoethanol and centrifuged at $10000 \times g$ for 30 min. Aliquots of the supernatant were mixed with an equal volume of 100 mM TRIS-HCl (pH 9.0) containing 2% 2-mercaptoethanol, 40% sucrose and BPB and were subjected to electrophoresis in a 15% native polyacrylamide gel. After electrophoresis, the gel was stained with silver according to Morrissey (1981). The aliquots were also mixed with equal volumes of 100 mM TRIS-HCl (pH 9.0) containing 4% SDS, 2% 2-mercaptoethanol, 40% sucrose and BPB, subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel (Laemmli 1970) and transferred electrophoretically to nitrocellulose filters (Schleicher and Schuell). The filters were incubated with antiserum raised against PR-1a protein according to Towbin et al. (1979). The filters were then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Tago) and stained. The antiserum of PR-1a protein was a kind gift from Dr. Y. Ohashi (National Institute of Agrobiological Resources, Japan; Ohashi and Matsuoka 1985). Phosphorylase a (mol. wt. 92.5 kDa), bovine serum albumin (66.0 kDa), aldolase (39.0 kDa), carbonic anhydrase (28.8 kDa), TMV coat protein (17.5 kDa) and cytochrome c (12.3 kDa) were used as the mol. wt. standards.

The concentration of IBMP and PR-1 proteins in leaf extracts was determined by the indirect enzyme-linked immunsorbent assay (ELISA) method (Koenig 1981). Samples of leaves (20 mg) were ground in a chilled mortar with 50 mM Na_2CO_3 (pH 9.6). The homogenates were placed in wells of polystyrene microtiter plates (Linbro E.I.A. microtitration plates, Flow Laboratories) and incubated at 25° C for 1 h. After incubation, the plate was washed with TTBS (20 mM TRIS-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20, 0.05% NaN_3) 3 times and then incubated with antiserum raised against the IBMP or PR-1a protein. After incubation at 25° C for 1 h, the plate was washed with TTBS 3 times and then incubated with alkaline phosphatase-conjugated goat anti-rabbit antibodies (Tago). After incubation at 25° C for 1 h, the plate was washed with TTBS. Immobilised enzyme was visualized by adding 1 mg/ml p-nitrophenyl phosphate and 10% diethanolamine (pH 9.8). Each sample was assayed in duplicate, and purified IBMP and PR-1a proteins were used as standard controls. The soluble protein concentration was determined by the Bradford dye-binding assay (Bradford 1976).

1,3-β-glucanase assay. 1,3-β-glucanase activity was measured by the procedure of Kauffmann et al. (1987). Total soluble protein was measured according to Bradford (1976) with bovine serum albumin as a standard.

Results

Plant transformation

The 3131 bp *Xba*I fragment from CaMV S-Japan containing the terminal portion of ORF V (698 bp), the 19 S promoter, gene VI, the 35 S promoter, a poly(A) addition signal and the proximal portion of ORF VII (54 bp) was inserted into the *Xba*I site of pGA482 (Fig. 1). The constructed plasmid, pGA482CaS6, was transferred to tobacco by Ti mediated transformation using the leaf disc method. Transformants were selected on kanamycin medium (300 μg/ml). Analysis of the DNA isolated from transformed plants by Southern hybridization revealed the presence of the 3.1 kb *Xba*I fragment containing gene VI in their chromosome (results not shown).

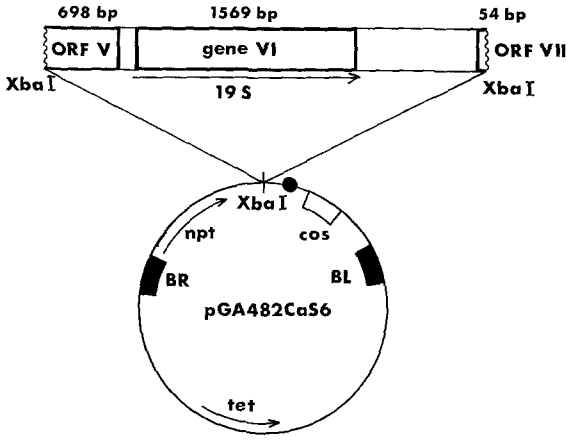

Fig. 1. Structure of pGA482CaS6. BL, T-DNA left border; BR, T-DNA right border; npt, neomycin phosphotransferase gene from Tn*5*: tet, tetracycline resistance gene from RK2; cos, the λ phage cos site; (●), Col-EI origin of replication

Table 1. Amount of the CaMV inclusion body matrix protein (IBMP) in the transgenic plants

Plant	Amount of the IBMP (ng/mg soluble protein)[a]
Transformant 21-3	280
Transformant 24-7	320
Transformant 13-3	350
Transformant 32-1	160
Transformant 45-1	130
CaMV-infected[b]	7500
control[c]	0

[a] The concentration of the IBMP on leaf extract was determined by ELISA and soluble protein concentration was determined by the Bradford dye-binding assay
[b] CaMV-infected; turnip leaf infected with CaMV S-Japan
[c] Control; tobacco leaf transformed with pGA482

Expression of the IBMP in transgenic plants

Expression of the IBMP was examined by an immunoblot analysis of proteins extracted from young leaves (Fig. 2A). The expression of IBMP was detected in 8 out of 18 kanamycin resistant plants. The IBMP in the leaves of transformants was identical in size to the IBMP found in CaMV S-Japan infected turnip plants. The amount of the IBMP expressed in several transformants was determined by ELISA (Table 1). The amount of the protein varied from 130 ng/mg total soluble protein to 350 ng/mg total soluble protein among transformed plants. In addition to the leaf extract, the IBMP was also detected in the root and stem of the transgenic plants (Fig. 2B).

Effect of the IBMP expression on plant growth

The transgenic tobacco plants expressing the IBMP displayed a unique phenotype. The leaves of most of the plants showed light green color and their growth was strongly suppressed as compared to the control plants transformed with the pGA482 (Fig. 3A). Furthermore, there was a correlation between the degree of the suppression of plant growth and the levels of the IBMP expression (data not shown). Although the growth of the transgenic plants was generally suppressed, six out of eight transformed plants expressing IBMP developed to mature plants. Of those six mature plants, five produced symptom-like necrotic spots in their leaves (Fig. 3B) and stems (Fig. 3C). Development of the necrotic spots appeared to be influenced by the growth conditions. When the transgenic plants expressing high levels of IBMP were grown at 10–15° C, the necrotic spots developed slowly. However, the plants growing at 30° C developed very severe necrosis at the young leaf and bud stage and finally died. Since the symptom-like necrotic spots were only observed in those plants expressing the IBMP, the expression of the IBMP was likely to be associated with the development of the symptom.

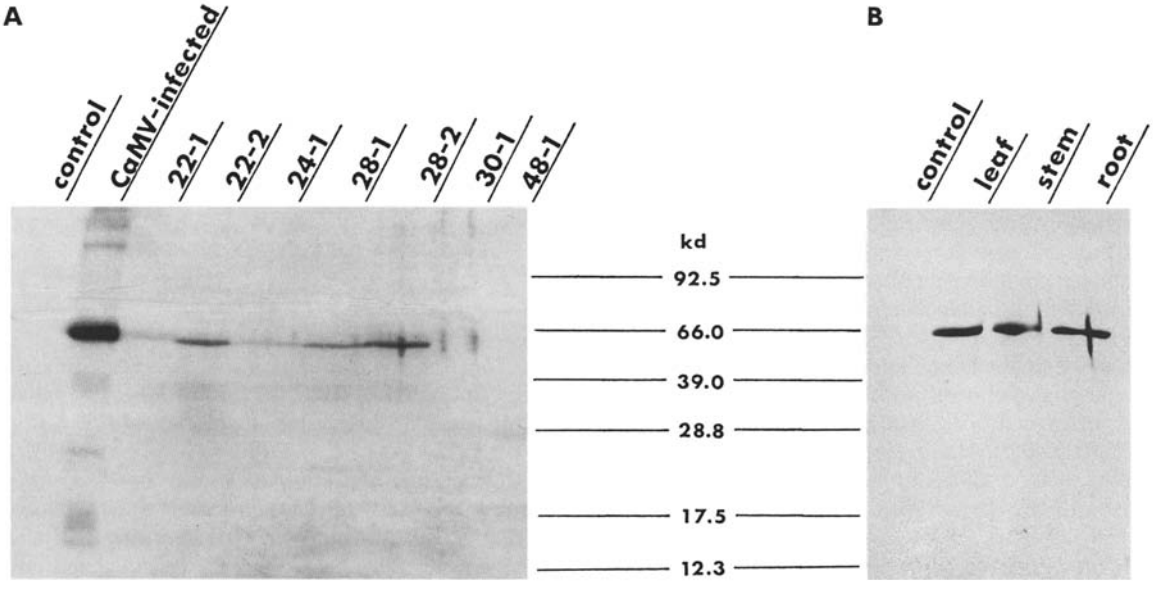

Fig. 2A and B. Expression of CaMV S-Japan (cauliflower mosaic virus) gene VI in transgenic plants. **A** Detection of the inclusion body matrix protein (IBMP) in the transgenic plants by immunoblotting. Young leaves of plants transformed with pGA482CaS6 (22-1; 22-2; 24-1; 28-1; 28-2; 30-1; 48-1), of plants transformed with pGA482 (control) or of CaMV-infected turnip (CaMV-infected) were used for the analysis. **B** Detection of the IBMP in tissues of a transgenic plant 22-1 by immunoblotting

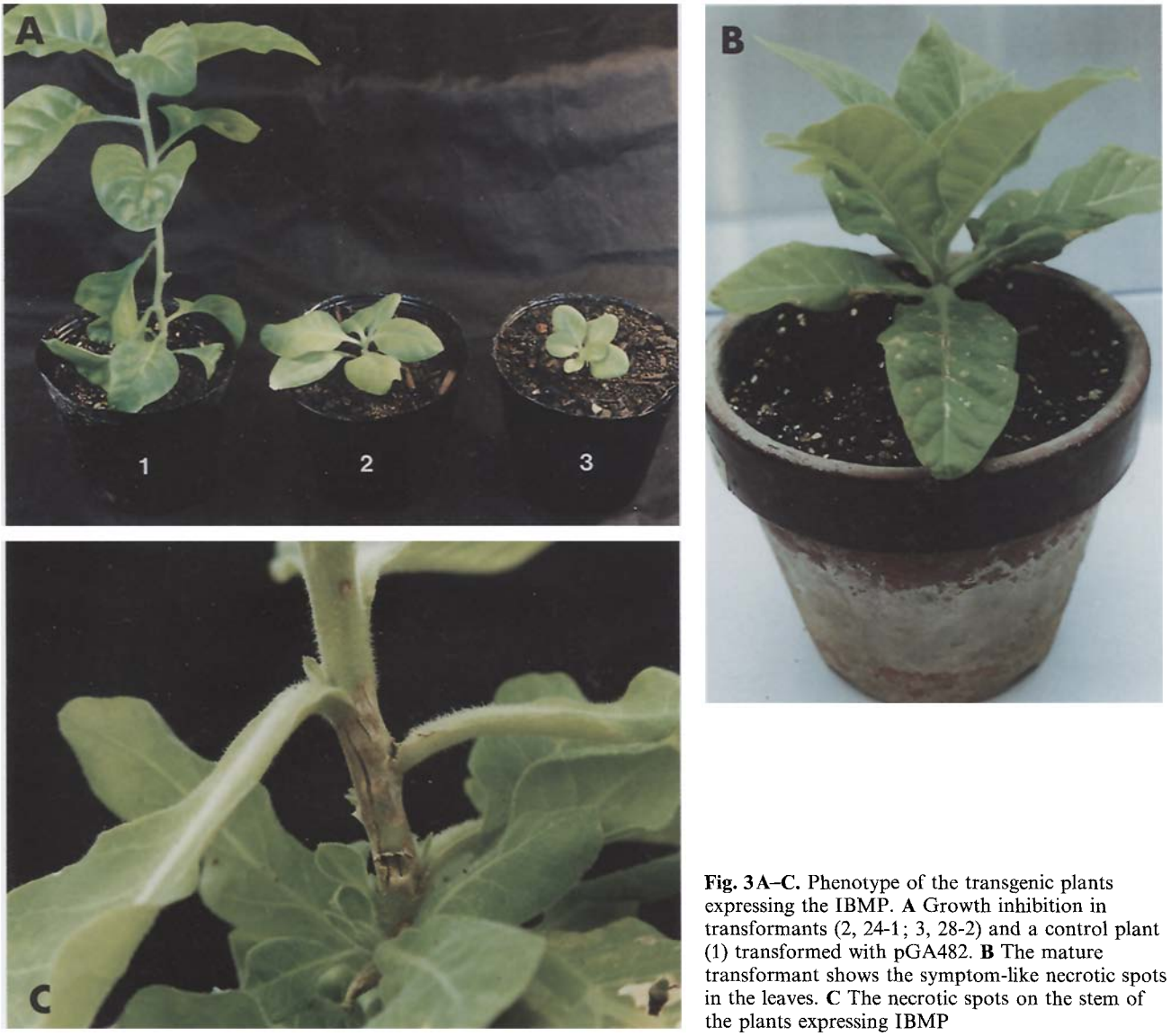

Fig. 3A–C. Phenotype of the transgenic plants expressing the IBMP. **A** Growth inhibition in transformants (2, 24-1; 3, 28-2) and a control plant (1) transformed with pGA482. **B** The mature transformant shows the symptom-like necrotic spots in the leaves. **C** The necrotic spots on the stem of the plants expressing IBMP

Induction of the pathogenesis-related proteins and 1,3-β-glucanase in transgenic plants

The induction of the PR proteins is considered to be a general plant response to stress and disease. To examine possible induction of PR proteins in the transformed tobacco plants, acid soluble proteins were extracted from the leaves of the transgenic plants expressing the IBMP. The resulting protein fractions were electrophoresed in non-denaturing polyacrylamide gels and silver stained, or in SDS-polyacrylamide gels and blotted onto nitrocellulose paper. New, fast migrating proteins were detected in the non-denaturing gel and their size corresponded to the size of the PR-1 proteins induced in TMV-infected tobacco (Fig. 4A). Immunoblot analysis of these proteins further confirmed that they are the PR-1 proteins (Fig. 4B). Although the relative amounts of PR-1a, -1b and -1c proteins were different between transgenic plants (Fig. 4A), the degree of induction of PR-1 proteins was correlated with the amount of IBMP in the transgenic plants (Tables 1, 2).

Among the PR proteins, PR-O, -N and -2 have been identified as 1,3-β-glucanases (Kauffmann et al. 1987). Therefore, we examined the activity of 1,3-β-glucanase in the leaf extracts of five transformed plants (Table 2). Of the three transformants expressing relatively large amounts of IBMP, plants 21-3 and 13-3 exhibited eight- and fivefold increases in activity, respectively. Plant 24-7 contained twice the activity found in the control. In the other two transformants that expressed lower amounts of IBMP, only slight increases of activity were found. From these results, we concluded that the introduced IBMP caused not only the suppression of plant growth but also the induction of the PR proteins and 1,3-β-glucanase.

Discussion

We have shown that gene VI from the CaMV S-Japan genome causes growth suppression and induces the synthesis of the PR proteins in transgenic plants. The suppression

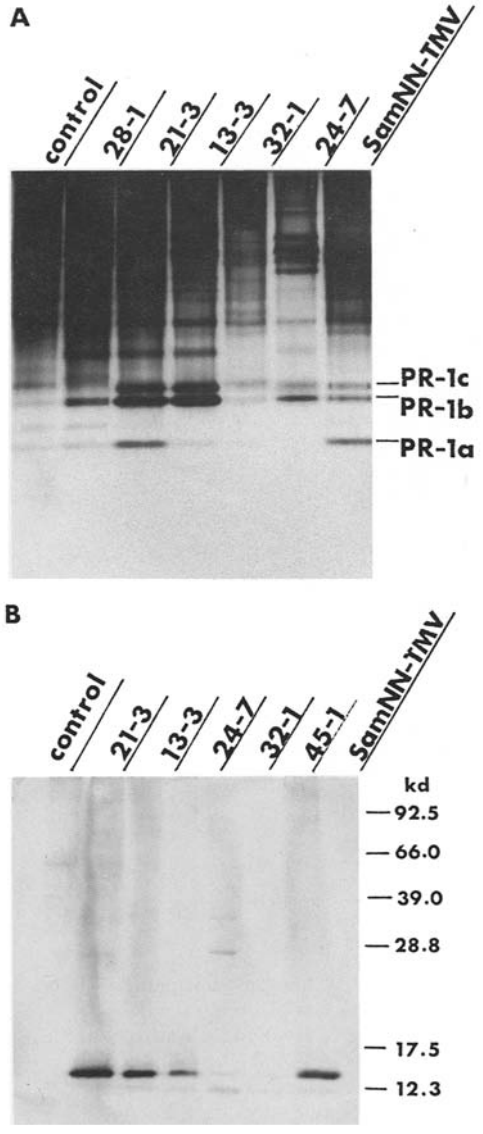

Fig. 4A and B. Electrophoretic analysis of PR-1 (pathogenesis-related) proteins extracted from transformants. Young leaves of the transformants (28-1; 21-3; 13-3; 32-1; 24-7; 45-1), of plants transformed with pGA482 (control) or of TMV-infected tobacco (*Nicotiana tabacum* Samsun NN; SamNN-TMV) were used. **A** The supernatant was subjected to electrophoresis in a 15% native polyacrylamide gel and the gel was stained with silver. The positions of PR-1 proteins are indicated (PR-1a, -1b and -1c). **B** The supernatant was electrophoresed in a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose filters and immunodetected

of growth in transgenic plants indicated that the expression of the IBMP caused stress on the growth of plant cells. Although transgenic plants expressing large amounts of IBMP were lethal, those expressing smaller amounts of the protein could grow to maturity, and most of them showed symptom-like necrotic spots in both leaves and stems. It is highly likely that the necrosis in stems was caused by expression of the transferred gene VI, because IBMP was also observed at the stems in the transgenic plants. These results suggest that virus-like symptom expression in transgenic plants is a general response to the IBMP. Although Baughman et al. (1988) and Goldberg et al. (1988) did not

Table 2. Amount of the PR-1 proteins and activity of 1,3-β-glucanase in transgenic plants

Plant	Amount of PR-1 proteins (ng/mg soluble protein)[a]	Activity of 1,3-β-glucanase (μKat/mg soluble protein)[b]
Transformant 21-3	510	2.4
Transformant 24-7	610	0.6
Transformant 13-3	530	1.5
Transformant 32-1	120	0.5
Transformant 45-1	60	0.4
SamNN-TMV[c]	180	1.8
control[d]	20	0.3

[a] PR-1 protein concentration on leaf sap was determined by ELISA and soluble protein concentration was determined by the Bradford dye-binding assay
[b] A katal (Kat) was defined as the enzyme activity catalysing the formation of 1 mol glucose equivalents/s
[c] SamNN-TMV; tobacco leaf infected with TMV
[d] Control; tobacco leaf transformed with pGA482

mention the influence of IBMP expression on the growth of the transformed plants, different gene VIs from different CaMV isolates might cause different types of symptoms in tobacco plants. CaMV normally has a host range limited to the Cruciferae, however, some strains can infect solanaceous plants (Hills and Campbell 1968; Schoelz et al. 1986). The CaMV S-Japan also induces necrotic spots on *Nicotiana clevelandii* and chlorotic spots on *N. benthamiana* (H. Takahashi, unpublished results). These differences might be due to the difference in amino acid sequence between the gene VIs of different CaMV strains, because approximately 10% of amino acids of gene VI are different between CaMV S-Japan and CaMV CM1841 (Gardner et al. 1981; H. Takahashi, unpublished results). Alternatively different cultivars of tobacco may react to endogenously synthesized IBMP in a different manner.

Expression of specific plant genes in response to the synthesis of IBMP is another interesting problem. The pathogenesis-related (PR) proteins have been described in plants infected with a variety of potential pathogens (van Loon 1985). We have shown the synthesis of PR-1 proteins and the increased activity of 1,3-β-glucanase in transgenic plants. The relative amounts of PR-1a, -1b and -1c proteins synthesized were, however, different in each transgenic plant. Moreover, not all transgenic plants expressing the IBMP and PR-1 proteins contained elevated levels of 1,3-β-glucanase activity. Although the induction of the PR proteins is thought to be a general response to stress, these results suggest that the mechanism, of the induction of PR proteins by IBMP might be different from that induced by stress caused, for example, by chemical treatments. Thus, these transgenic plants should provide useful material for studies on the induction of the PR proteins. Furthermore, it will be interesting to study the reaction of these transgenic plants to infection with various pathogens. Such studies are in progress.

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