JARQ 24, 91-99 (1990)

Inducible Expression of Pathogenesis-Related 1 Protein Gene in Transformed Plants

Yuko OHASHI, Masahiro OHSHIMA and Makoto MATSUOKA

Abstract
Induced expression of tobacco pathogenesis-related (PR) 1 protein genes was analyzed. The base sequence comparison of the active three genes (PR1a, PR1b and PR1c genes) indicated that there was a well conserved region in 5' flanking 180 nucleotides in their promotor sequences. Direct gene transfer into tobacco protoplasts by electroporation with the chimeric gene of PR1a promoter and a reporter gene (B-glucuronidase: GUS), and stable transformation of tobacco with a binary vector mediated Agrobacterium infection with the same chimeric gene suggested that the cis-acting element responding to stress or salicylic acid be present in the 0.3Kb sequence of the 5' flanking region of PR1a gene.

Discipline: Biotechnology
Additional key words: Agrobacterium infection, electroporation, Nicotiana tabacum, stress protein, transformation

Introduction

Pathogenesis-related (PR) proteins are host-coded, novel and soluble stuff induced by hypersensitive responses to a wide range of pathogenes, including viruses, viroids, bacteria and fungi[20]. They are also induced in healthy plants by some chemicals such as salicylic acid[10,21], or plant hormone[1,11]. Some stresses caused by cutting, mechanical injury and high osmotic pressure also induce the accelerated synthesis. This fact indicates that the relevant incidents are associated with the stress-inducible proteins. In a tobacco plant, *Nicotiana tabacum* cv. Samsun NN, more than ten kinds of PR proteins were identified so far, and recently, it has been found that some of them have ß-1, 3-glucanase[7] or chitinase activity[8]. However, the function of tobacco PR1 proteins, consisting of PR1a, PR1b and PR1c as well as of well-defined major PR proteins with low molecular weight, is not clearly identified yet. Based on the finding that they are induced in parallel with acquired resistance against virus infection, a possible role of the PR1 proteins in defence mechanism of tobacco to pathogen is suggested[6,13]. PR1 proteins were found in the intercellular spaces of the tissue[4,11,12,18] and secreted into the medium of tobacco suspension cultures[11].

The authors[9] and others[2,19] have recently isolated cDNA clones of PR1 proteins and characterized their properties. The analysis of cDNA clones and translation experiments *in vitro* reveals that these proteins are synthesized as larger precursors with signal peptides for a secretion signal. PR1 protein genes are encoded by a small multigene family comprising PR1a, 1b and 1c, and that these components are very similar in their amino acid sequences[9].

This paper summarizes experimental results of the analysis of the PR1 gene expression, including: 1) cloning of single PR1 genes in tobacco, characterization and comparison of the regulatory sequences of these genes; 2) direct gene transfer into tobacco protoplasts by electroporation with the chimeric gene of PR1a promoter and a reporter gene; 3) stable transformation of tobacco leaf disc with binary vector system mediated by Agro-infection, and induced expression of the introduced gene by stress or chemical in the transformants; and 4) some possible practical uses of the PR1 gene.

Department of Molecular Biology, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305 Japan)

The multigene family of tobacco PR1 protein genes

1) Genomic cloning of PR1 genes of tobacco

In order to characterize the genes encoding PR1 proteins in Samsun NN tobacco genome, tobacco genomic DNA digested with EcoR1 was hybridized with ^{32}P-labelled PR1a cDNA under the conditions where the probe could also be hybridized with PR1b and PR1c genes, because these genes have a high homology in their base sequences. Several bands were identified on Southern blotting analysis (Plate 1). These fragments were cloned using λgt 11 as a vector, followed by recloning into pSK(+) (Stratagene) for further analysis. From the results of sequencing, it is revealed that the flagment of 2.4 Kb, 3.5 Kb and 6.0 Kb each contains one PR1c gene, one PR1a gene[17] and one PR1b gene, respectively, per haploid tobacco genome. The other positive fragments contain an inactive pseude-gene or another type of PR1-related gene[3,15,16].

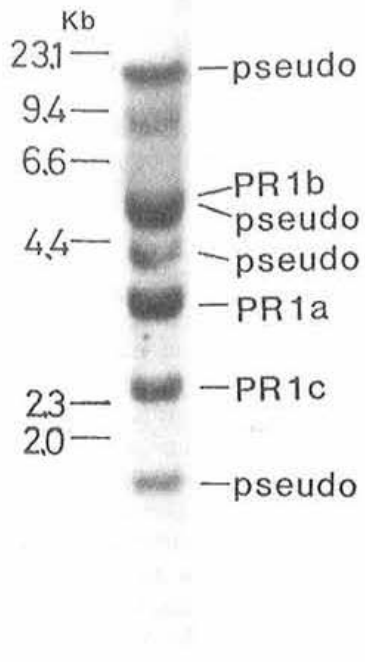


Plate 1. Southern blot analysis of tobacco PR1 protein genes
EcoR1 digested Samsun NN tobacco DNA was probed with PR1a cDNA. Band assignments are described in the text.

Tobacco plant, *Nicotiana tabacum*, is an amphydiploid of *N. sylvestris* and *N. tomentosiformis*. Southern blotting analysis of these two genome donors indicates that PR1a and PR1c are derived from *N. sylvestris* and PR1b from *N. tomentosiformis*[15].

2) Comparison of the regulatory sequences of PR1 genes

Fig. 1 shows the comparison of DNA sequences of PR1 genes which were isolated and sequenced by the authors. The right open box shows coding regions, and its upstream is 5' non-coding region of PR1 gene. All genes have cap site (−29) and TATA box (−63), which are ubiquitous in eucaryote. PR1a and PR1b genes isolated by the authors have an insertion as shown by a solid line between black boxes, that are the target sites of this insertion. PR1c gene also has an insertion shown by a dotted line between shaded boxes. The 5' flanking 180 nucleotides fragment just upstream from coding region are highly conserved in these active three PR1 protein genes. Far upstream of that region, the sequences are not conserved. These results show that a cis-acting element of PR1 gene expression may exist in this conserved region.

Analysis of PR1a promoter in tobacco cells

1) Direct gene transfer of fused PR1a gene

The expression of PR1 gene was analyzed for two different systems. The first system was a direct gene-transfer into tobacco mesophyll protoplasts with electroporation. The second system was a gene-transformation through a binary vector. A chimeric gene consisting of 5' flanking region of PR1a gene and a reporter gene (ß-glucuronidase:GUS) (Fig. 2) were transferred directly into protoplasts. As a positive control, a fusion gene consisted of the promoter sequence of cariflower mosaic virus (CaMV) 35S gene was used. Expression level of the introduced gene was measured by induced GUS activity in the incubated protoplasts.

Fig. 3 shows a GUS activity pattern depending on the time of incubation. Protoplasts were incubated for various periods with or without salicylic acid, which is an inducer of PR1 protein synthesis. The

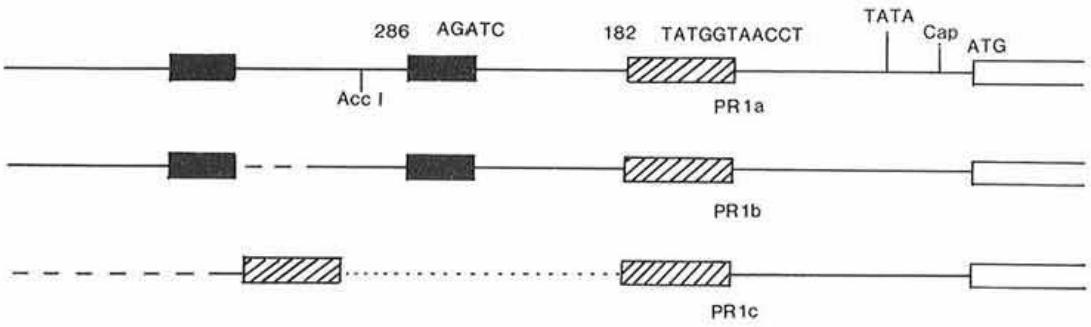


Fig. 1. Base sequence comparison of 5' flanking region of the cloned PR1 protein genes
Direct repeated sequence 'AGATC' is duplicated by insertion observed in PR1a and PR1b, both of which are presented by black boxes. The target sites of insertion in PR1c gene are presented by shaded boxes. 'TATA' and 'Cap' mean TATA-box and capping site, respectively. Open boxes mean protein coding regions.

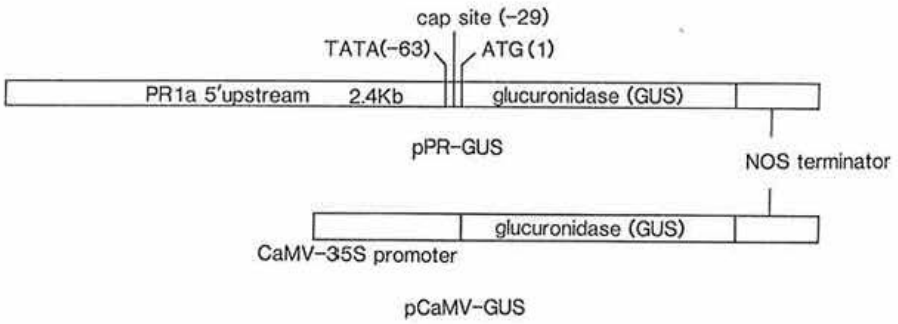
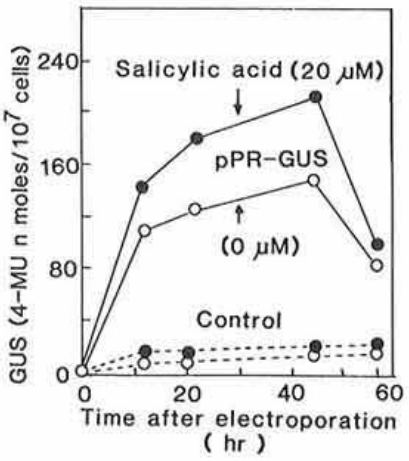


Fig. 2. Structure of chimeric genes; PR-GUS and CaMV-GUS
The left part of PR-GUS is a 2.4 Kb fragment of 5' flanking region of PR1a gene. The promoter sequence of CaMV-35S gene is used as positive control in the experiment of constitutive expression. The right part of these chimeric genes is a coding region of the GUS gene of *E. coli* linked to the fragment of NOS gene including poly-A adding signal.

GUS activity was induced in 12 hr under incubation and increased till 44 hr in the electroporated protoplasts. Salicylic acid activated the GUS induction at all time. Fig. 4 shows salicylic acid-induced GUS activity in pPR-GUS and pCaMV-GUS introduced cells. Protoplasts were electroporated in the solutions containing the fusion gene and various concentration of salicylic acid, followed by incubation in a salicylic acid-free medium. With the increase in the concentration of the salicylic acid, a greater activity of the induced GUS was seen in pPR-GUS-introduced protoplasts in comparison with the case in pCaMV-GUS-introduced cells. These results show that salicylic acid activates expression of the GUS gene under the control of the PR1a gene in the isolated protoplasts.

To define cis-acting element of PR1a gene, 5' deletion series of the fusion gene were transferred into protoplasts by electroporation and the GUS activity was measured after one-day incubation (Fig. 5). The activity induced by a fusion gene with 1.0 Kb fragment in the 5' upstream region of PR1a gene was the same level as that with 2.4 Kb and it decreased to the level of one third with that of 0.3 Kb fragment. This result shows that the 0.3 Kb fragment is enough to salicylic acid-inducible expression of the GUS activity, suggesting that this fragment contain a cis-acting element involved in gene



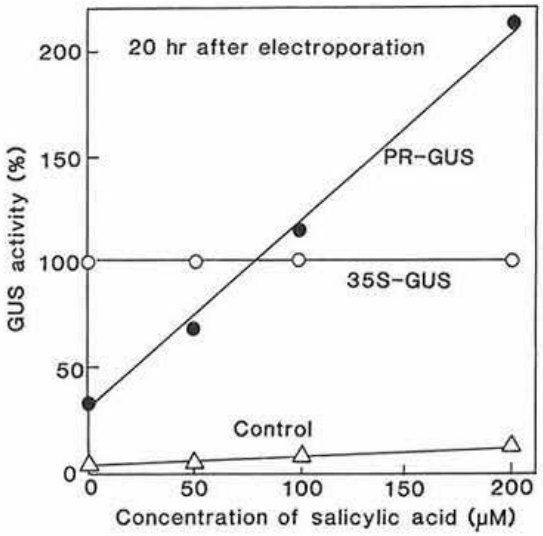


Fig. 3. (left) GUS activity in tobacco protoplasts after electroporation with pPR-GUS fusion gene
Tobacco mesophyll protoplasts (2×10^5 cells) were electroporated with 100 μg of cDNA and 10 μg plasmid DNA in 0.5 ml of 0.5 M mannitol. The washed cells were then incubated in culture medium with (●) or without (○) 20 μM salicylic acid at 25°C. After an appropriate incubation period, protoplasts were harvested, and homogenized with lysis buffer. GUS activity was measured by the fluorometric method described by Jefferson et al.[5]

Fig. 4. (right) Effect of salicylic acid on the transient expression of GUS-fusion gene
Tobacco protoplasts were electroporated in the presence of salicylic acid with 10 μg of plasmid DNA containing GUS fusion gene.

regulation. The 5' flanking sequence upstream rather than 0.3 Kb fragment may be necessary for the more effective expression of the gene[14,17].

2) Transformation of tobacco by pPR-GUS using a binary vector system

The chimeric gene, pPR-GUS, or 5' deletion series of the fusion gene, was ligated to a binary vector plasmid, pTRA 415, which had a kanamycin resistance marker gene (Fig. 6). Tobacco plants were transformed by the standard leaf co-cultivation method, and then transformants were selected by their kanamycin resistance. Southern blot analysis probed with the GUS coding region demonstrated the presence of the introduced chimeric gene in the kanamycin resistant plants. A positive signal was identified at 4.5 (Plate 2), 3.1 or 2.8 Kb fragments (data not shown), which was the expected size of each insert in the plants transformed by the chimeric gene with 5' flanking region of 2.4, 1.0 or 0.3 Kb, respectively. No signal was identified in the non-transformed control plants under the same condition as above. The comparative analysis with the band intensity of standard markers, suggested that the copy number of the introduced chimeric gene of transformants E-2, E-4, E-6, E-10 and E-14 be one each per haploid genome. And the kanamycin resistant and sensitive phenotypes of the self-pollinated progeny of these transgenic plants segregated 3 to 1, suggesting that one chimeric gene be introduced. Other transformants with more strong signals have two or more copies per haploid genome.

Further analysis related to the expression level of the introduced chimeric gene in the transgenic plants, which had one introduced gene per haploid genome (Fig. 6). The GUS activities in the leaf discs were measured immediately as well as in two days after incubation with water or salicylic acid (Fig. 7). In the control leaf discs of non-transformant, no GUS activity was identified in both dates irrespective of

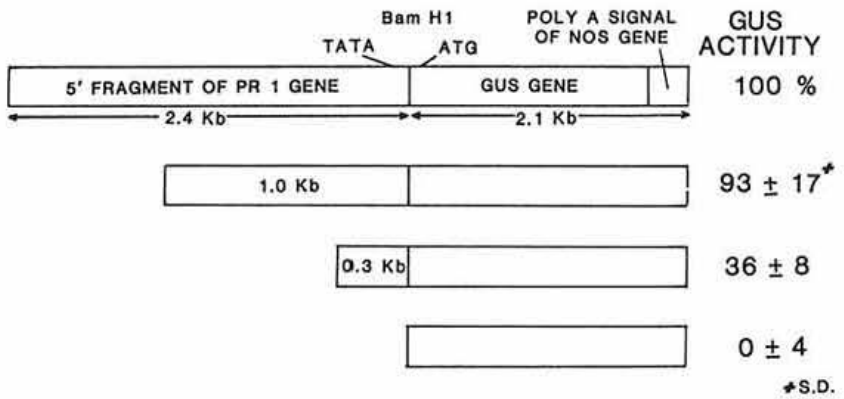


Fig. 5. Expression of GUS-fusion genes with 5' deletion series of 5' flanking region of PR1a gene in electroporated tobacco protoplasts

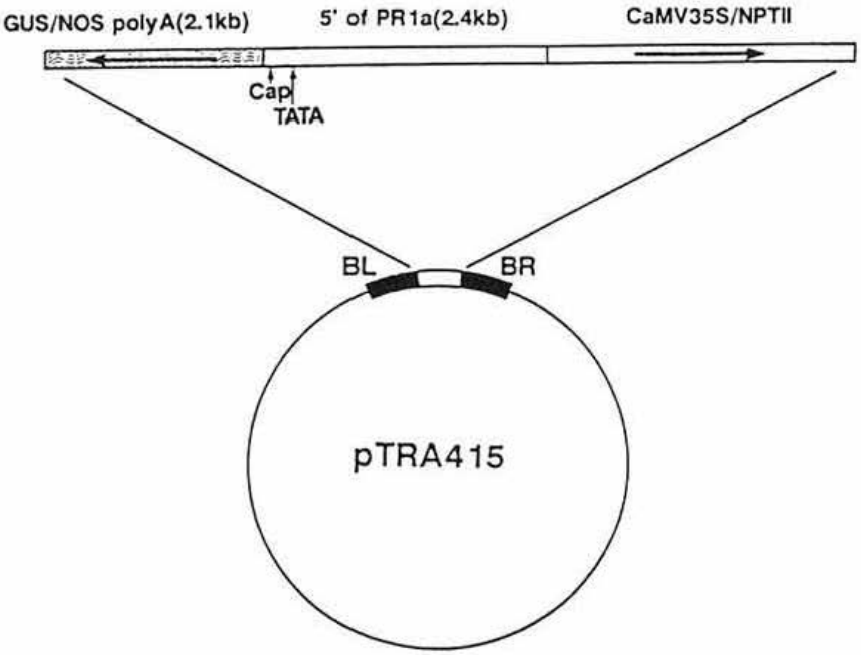


Fig. 6. Binary vector plasmid used for stable transformation mediated by Agro-infection PR-GUS is the same as described in Fig. 3. The right part of the schematic plate is a selectable marker gene which consists of a promoter sequence of CaMV 35S gene, and Neomycin phosphotransferase of *E. coli* Tn 5 (NPTII) and the fragment containing poly-A adding signal of Tml-gene of octopin type Ti plasmid, pTi 15955. Arrows indicate the direction of transcription. BL and BR mean the border sequence of T-DNA region of Ti plasmid.

with and without salicylic acid. The GUS activity was induced by the stress of cutting; its high level was induced by the treatment with salicylic acid in all PR (2.4 Kb)/GUS transformed plants. The induction of GUS activity by salicylic acid was correlated with the increase in the amount of PR1 protein in the same tobacco leaf. These results clearly show that the GUS activity is controlled by the promoter of PR1a gene[16,17].

The hypersensitive reaction-induced expression of the introduced chimeric gene with each of the 2.4, 1.0 or 0.3 Kb fragments of PR1a promoter was analysed cytochemically in the transformed Samsun NN tobacco plants. In three days after TMV-inoculation, local lesions developed well in the leaf discs with TMV-infection. The discs were subjected to GUS-active staining, using X-glucuronide as a substrate. Blue narrow rings developed clearly just around the local lesions on the leaf discs transformed by the chimeric gene with 2.4 Kb fragment as a consequence of product-accumulation of GUS (Plate 3A), while broad blue rings developed on the discs transformed by 0.3 Kb fragment (Plate 3B). No blue rings appeared in the control leaf disc of the non-transformant (Plate 3C). The cytochemical evidence shows that the 0.3 Kb 5' flanking region of PR1a gene regulates the gene expression in the formation of local lesions caused by TMV infection.

Utilization of the inducible promoter of PR1 gene

In normal tobacco plants, PR1 proteins are induced in the course of formation of local lesions caused by TMV infection, cutting stress, or treatment with salicylic acid, and accumulated only around the local lesions or in the treated area. In pPR-GUS transformants, the GUS activity is also

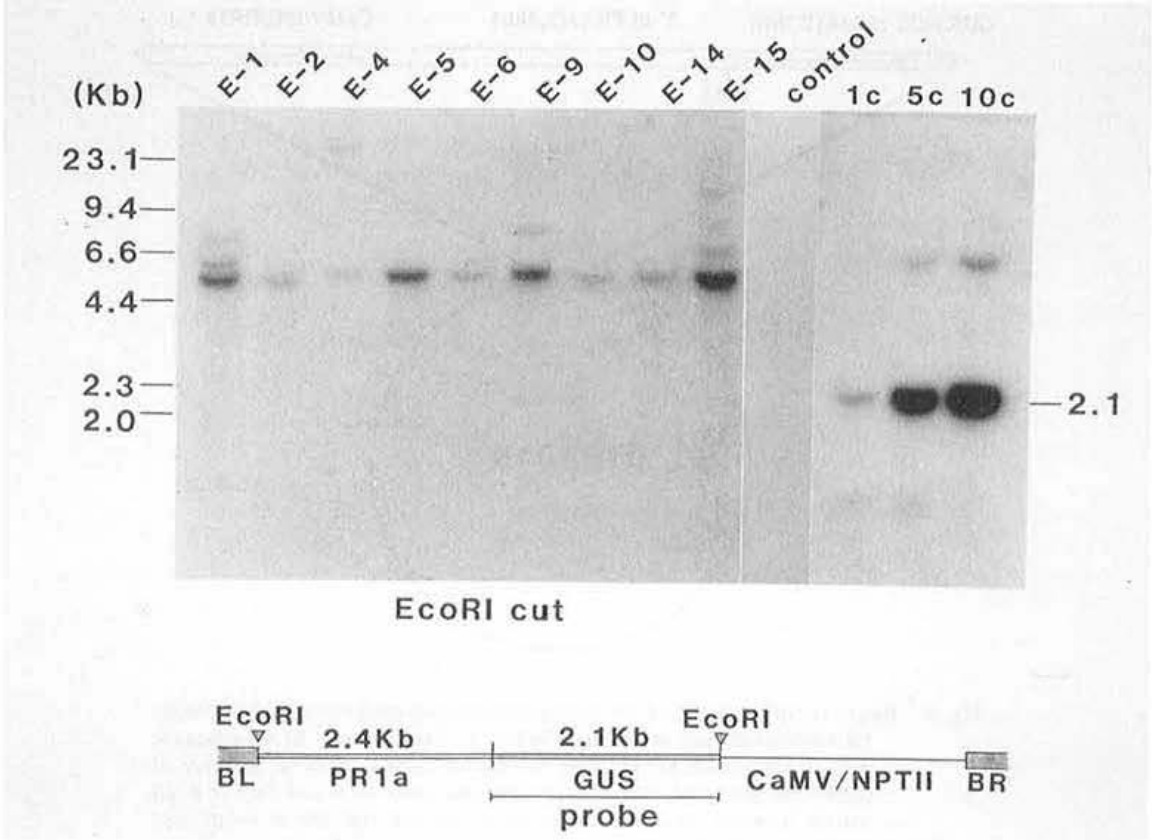


Plate 2. Southern blot analysis of EcoR1 digested transformed tobacco DNA probed with GUS gene
Band assignments are described in the text.

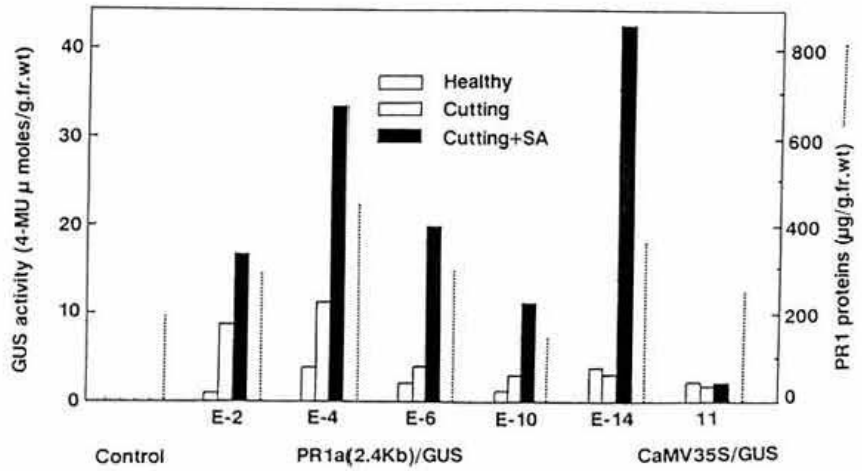


Fig. 7. Expression of the introduced chimeric gene in the transgenic plant by cutting and salicylic acid treatment

The GUS activity was measured by fluorometric assay[5].

The amount of PR1 proteins was determined by rocket-immuno-electrophoresis using anti-PR1a antibody[10].

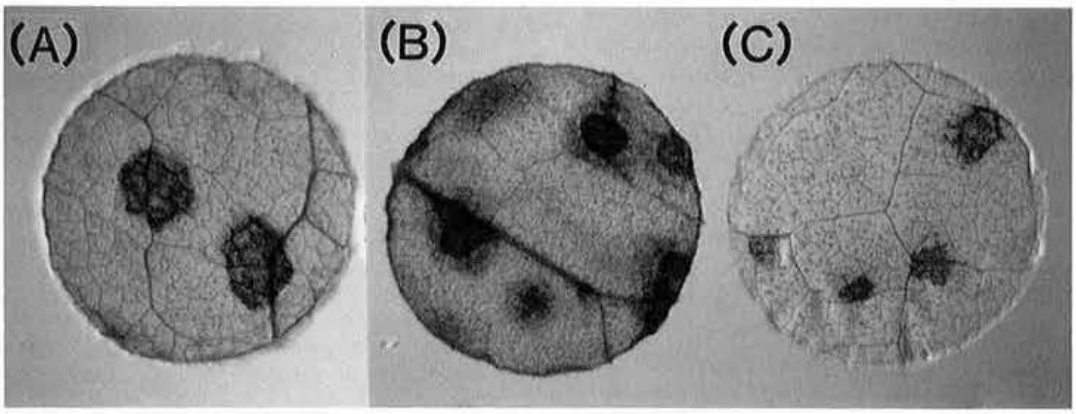


Plate 3. GUS-active staining on the transformed tobacco leaves

TMV was inoculated on the transformed Samsun NN tobacco leaves and incubated at 20°C for 3 days. Local lesions developed on the leaves with a TMV infection.

Discs were prepared from the leaves and vacuum-infiltrated in a 1 mM X-glucuronide solution. In 12 hr after incubation at 37°C, the discs were treated with ethanol to remove chrolophyll, and photographed. Reaction product GUS was represented by blue color. (A): transformant introduced by chimeric gene constructed with 2.4 Kb fragment of 5' flanking region of PR1a gene and GUS gene, (B): transformant introduced by that with 0.3 Kb, and (C): control non-transformant.

induced by these treatments predominantly around the local lesions or in the treated area as shown in Plate 3 or Fig. 7. In case where an anti-pathogenic gene, such as an antisence strand of plant virus, a coat protein gene of plant virus or a gene of an anti-bacterial peptide is linked with a PR1 promoter, instead of GUS gene, and introduced into plants, the transgenic plants may produce plenty of anti-pathogenic gene-products predominantly on such regions of the plants as shown in the GUS activities in Plate 3 or Fig. 7. These transgenic plants may effectively acquire the resistance against pathogen attacks.

Conclusion

All the PR1 genes in Samsun NN tobacco were cloned and their characteristics were identified. The comparison of the 5' flanking region of PR1 genes suggests that the common 180 bp upstream region from the first ATG of coding region possibly play an important role in regulating expression of PR1 genes. By two gene-transfer methods, i.e. a direct gene transfer into protoplasts and a stable transformation by a binary vector, it has been confirmed that the cis-acting element of PR1a gene is contained in the 0.3 Kb fragment of the 5' upstream of the gene.

The use of such an inducible promoter provided a bright prospect in the field of plant breeding. In the putative transformants introduced by the chimeric gene comprising a PR1 promoter and a useful gene, the product of the useful gene could be synthesized in special regions by the induction treatment like a stress. Such an inducible promoter may have a greater advantage than the use of a promoter with constitutive expression, i.e. CaMV 35S promoter. Because the inducible promoter may function more effectively in the limited area, though the latter may function at any time, even when the expression is not necessary.

References

1) Antoniw, J. F. et al. (1981): The presence of pathogenesis-related proteins in callus of Xanthinc tobacco. *Phytopath. Z.*, **101**, 179–184.
2) Cornelissen, B. J. C. et al. (1986): Molecular characterization of messenger RNAs of 'pathogenesis-related proteins 1a, 1b and 1c' induced by TMV infection of tobacco, *EMBO J.*, **5**, 37–40.
3) Cornelissen, B. J. C. et al. (1987): Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. *Nucl. Acids Res.*, **15**, 6799–6811.
4) Fortin, M. G., Parent, J. G. & Asselin, A (1985): Comparative study of two groups of b proteins (pathogenesis-related) from the intercellular fluid of *Nicotiana* leaf tissue infected by tobacco mosaic virus. *Can. J. Bot.*, **63**, 932–937 (1985).
5) Jefferson, R. A., Burgess, S. M. & Hirsh, D. (1985): ß-Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Nat. Acad. Sci. USA*, **83**, 8447–8451.
6) Kassanis, B., Gianinazzi, S. & White, R. F. (1974): A possible explanation of the resistance of virus-infected tobacco plants to second infection. *J. Gen. Virol.*, **23**, 11–16.
7) Kauffmann, S. et al. (1987): Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have ß-1, 3-glucanase activity.: *EMBO J.*, **6**, 3209–3212.
8) Legland, M. et al. (1987): Biological function of pathogenesis-related proteins: Four tobacco pathogenesis-related proteins are chitinase. *Proc. Nat. Acad. Sci. USA*, **84**, 6750–6754.
9) Matsuoka, M. et al. (1987): Classification and structural comparison of full-length cDNAs for pathogenesis-related proteins. *Plant Physiol.*, **85**, 942–946.
10) Ohashi, Y. & Matsuoka, M. (1985): Synthesis of stress proteins in tobacco leaves. *Plant Cell Physiol.*, **26**, 473–480.
11) Ohashi, Y. & Matsuoka, M. (1987): Induction and secretion of pathogenesis-related proteins by salicylate or plant hormones in tobacco suspension cultures. *Plant Cell Physiol.*, **28**, 573–580.
12) Ohashi, Y. & Matsuoka, M. (1987): Localization of pathogenesis-related proteins in the epidermis and intercellular spaces of tobacco leaves after their induction by potassium salicylate or tobacco mosaic virus infection. *Plant Cell Physiol.*, **28**, 1227–1235.
13) Ohashi, Y., Shimomura, T. & Matsuoka, M. (1986): Acquisition of resistance to TMV coincident with induction of pathogenesis-related proteins by TMV infection and chemical treatment in tobacco leaves. *Ann. Phytopath. Soc. Jpn.*, **52**, 626–635.
14) Ohashi, Y. et al. (1988): Analysis of the promoter activity of tobacco pathogenesis-related (PR) protein gene by transient expression system. *In* 5th Int. Con Plant Pathol., 273 (Abstract).
15) Ohshima, M. et al. (1987): Nucleotide sequence of the PR-1a gene of *Nicotiana tabacum*. *FEBS Letters*, **225**, 243–246.
16) Ohshima, M. et al. (1990): The nucleotide sequence of pathogenesis-related (PR) 1b protein gene of tobacco. *Nucl. Acids Res.* **18**, 181.
17) Ohshima, M. et al. (1990): Analysis of stress-induced

or salicylic acid-induced expression of the pathogenesis-related la protein gene in transgenic tobacco. *Plant Cell*, **2**, 95–106.

18) Parent, J. G. & Asselin, A. (1984): Detection of pathogenesis-related proteins (PR or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. *Can. J. Bot.*, **62**, 564–569.

19) Pfitzner, U. M. & Goodman, H. M. (1987): Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. *Nucl. Acids Res.*, **15**, 4449–4465.

20) Van Loon, L. C. (1985): Pathogenesis-related proteins. *Plant Mol. Biol.*, **4**, 111–116.

21) White, R. F. (1979): Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, **99**, 410–412.

(Received for publication, Jan. 25, 1990)