한국식물병리학회지 14(6) : 598-602(1998)
Korean J. Plant Pathol. 14(6) : 598-602(1998)

Screening of Salicylic acid Producing Rhizobacteria Isolated from Plant Roots and Rhizosphere

Min Woong Lee*, P. A. H. M. Bakker[1] and Tae Soo Lee[2]
Department of Applied Biology, Dongguk University, Seoul 100-715, Korea
[1]Department of Plant Ecology and Evolutionary Biology, Utrecht University, Netherlands
[2]Department of Biology, University of Inchon, Inchon 402-749, Korea

식물의 뿌리와 근권으로부터 Salicylic acid를 생성하는 근권세균의 검색

이민웅* · P. A. H. M. Bakker[1] · 이태수[2]
동국대학교 응용생물학과, *[1]Department of Plant Ecology and Evolutionary Biology, Utrecht University, Netherlands,* [2]인천대학교 생물학과

ABSTRACT: Twenty two rhizobacteria were isolated from the roots and rhizosphere of radish, carnation, potato and tomato. These isolates produced a fluorescent pigment in King's B medium and identified as *Pseudomonas* spp. These isolates colonized roots and rhizosphere of the host plants. In the study of cultural characteristics of the bacteria, the pH of the culture broth was changed from neutral (7.0) to alkali (8.8~9.41) and the numbers of cells were increased from 10^6 to 10^8 after 40 hr of incubation in basal standard succinate medium. The salicylic acid production identified by pink color reaction were observed in 7 bacteria. Out of these 7 salicylic acid producing bacteria, only 2 strains of bacteria such as *Pseudomonas fluorescens* RS006, and *Pseudomonas* sp. EN401 were confirmed as salicylic acid producers by optical density measurement. Therefore, for screening of salicylic acid producing bacteria from the roots and rhizosphere, color reaction of the culture medium should be done in the first step, and then optical density measurement of culture extract should be made for the confirmation of salicylic acid production.

***Key words*: *Pseudomonas fluorescens*, rhizobacteria, salicylic acid.**

Some nonpathogenic rhizobacteria can induce physiological changes throughout entire plant life cycles, making them more resistant to pathogens. Some disease resistant plants restrict the spread of fungal, bacterial, or viral pathogens to a small area around the point of initial penetration, where a necrotic lesion appears. This phenomenon, termed induced systemic resistance (ISR), generally has been demonstrated for various rhizobacteria in several plants (12, 14). This is referred to as the hypersensitive resistance (HR) and may lead to systemic acquired resistance (SAR) (8, 22). In some other cases, ISR by rhizobacteria is mentioned by a systemic accumulation of pathogenesis-related proteins that is also associated with an accumulation of pathogenesis-related proteins (10, 14, 20). Since its initial discovery, SAR has been demonstrated in a variety of plant-pathogen interactions.

HR and SAR is commonly associated with the systemic synthesis of several families of serologically distinct, low molecular weight, pathogensis-related (PR) proteins (4, 9). This mechanism is accompanied by induction of number of genes throughout the plant life cycles (24). Evidence is emerging that the products of these genes are causally related with the enhanced resistance of the plant (1). The phenomenon of SAR implies the existence of a signal that spreads systemically from the site of the HR to the rest of the plant. It is well established that resistance to pathogens and the production of PR proteins in plants can be induced by salicylic acid (SA) or acetylsalicylic acid and even in the absence of pathogenic organisms (3, 19, 23, 25), and SA acts as an endogenous signal that triggers local and systemic induction of PR proteins (13, 21, 26). SA affects numerous metabolic processes in plants. In recent years, the importance of this phenolic acid in plant-pathogen interactions has been apparent. When a plant recognizes that it is being infected by a pathogens, a number of defense mechanisms are induced, one of which is in-

*Corresponding author.

creased systemic resistance to subsequent infections.

In this study, we investigated whether the known beneficial rhizobacteria could produce salicylic acid *in vitro* which triggers the induced host resistance against Fusarium wilt disease or not.

MATERIALS AND METHODS

Microbial culture and inocula. Rhizobacteria spp. used in this study were obtained from Department of Plant Ecology and Evolutionary Biology, Utrecht University, Netherlands and their relevant characteristics listed in Table 1. The bacteria were maintained in King' B medium (KB) and transferred to basal standard succinate medium (SSM) adjusted to pH 7.0 (0.1 N NaOH) at 25~28°C for 15 hr for their exponential growth for inoculum source (15). Fifteen ml of the bacterial inoculum growing in SSM were inoculated to 35 ml of basal SSM in triangle flask for their growth. These inocula were cultured for 60 hr in shaking incubator at room temperature. Growth of the each bacterium in the culture was measured by optical density (OD) value using spectrophotometer (Beckman DU-64) and pH change of the the culture was measured by pH meter with 1 ml of the broth.

Table 1. Bacterial isolates used in this study

Bacterial strains	Sources of bacterial spp.*
Pseudomonas putida RE008	Radish, endophyte
P. putida RE010	Radish, endophyte
P. florescens RS006	Radish, rhizosphere
P. putida RS009	Radish, rhizosphere
P. florescens RS015	Radish, rhizosphere
P. putida RS011	Radish, rhizosphere
P. putida RE015	Radish, endophyte
P. putida RS002	Radish, rhizosphere
P. fluorescens RS154	Radish, rhizosphere
P. fluorescens CE005	Radish, endophyte
P. putida RE 014	Carnation, endophyte
P. putida RS 153	Radish, rhizosphere
P. fluorescens CE 006	Carnation, endophyte
P. fluorescens CS 101	Carnation, rhizosphere
P. fluorescens CS 108	Carnation, rhizosphere
P. fluorescens WCS 358	Potato
P. fluorescens WCS 374	Potato
Pseudomonas sp. EN401	Tomato
Pseudomonas sp. RS112	Radish, rhizosphere
Pseudomonas sp. EN415	Tomato
Pseudomonas sp. EN7	Tomato
Pseudomonas sp. RS111	Radish, rhizosphere

Regends: CE; isolated from endophyte of carnatioin, CS; isolated from rhizosphere of carnation, RS; isolated from endophyte of radish, EN; isolated from tomato and RS; isolated from rhizosphere of radish.

* : Bacterial spp. were obtained from Dept. of Plant Ecology and Evolutionary Biology, Utrecht Univ. Netherlands.

Screening of salicylic acid production. For both qualitative and qantitative analyses of SA production, the strains of *Pseudomonas* spp. were grown in SSM broth for 48 hr at 200 rpm shaker. Salicylic acid production was identified by purple-colored iron complex in the medium (12). For qualitative detection of SA, a sample of stationary phase of culture supernatants (4 ml) was acidified with 1 M HCl to pH 2 and the SA was extracted into $CHCl_3$ by vigorous shaking modified by Meyer *et al.* (16) and Leeman (12). Culture supernatants were centrifuged in refrigerated centrifuge (Beckman) in 8000 rpm for 10 min. Supernatant was harvested after centrifugation in a screw cap tubes. To make the supernatant acidify, 1.6 ml of 1 M HCl was added to the supernatant, and 4 ml of these mixtures was mixed again with the same volume of chloroform in screw cap tubes. These screw cap tubes were vigorously shaked by hand for 30 second. After that, the tubes was centrifuged for 5 min at 3500 rpm. Upper part of water layer of aliquot was removed by pipetting, and added again the 4 ml of chloroform to the supernant and shaked again as mentioned above. Leaving the tubes for 5 min, and then 4 ml of organic phase in lower part of the tube was transferred carefully to new cap tube using pasteur pipette. One ml of this sample was used for scanning for the wave length interval of 200~400 nm if need. Three ml of the sample was mixed with the same volume of 1 M of $FeCl_3$ solution. After that, 3 ml of distilled water was added for the observation of color change. The tubes were centrifuged again for 5 min at 3500 rpm. One ml of this solution was pipetted to cuvett and optical density (OD) was measured in spectrophotometer at 527 nm (12, 16).

RESULTS

After 48 hr of incubation in a shaking culture, the yields of bacterial cells and pH of the different strains of rhizobacteria were fluctuated between 0.0011 in *Pseudomonas putida* and 0.978 in *Pseudomonas* sp. EN415 by OD measurement. This result seems to indicate that the number of all strains of rhizobacteria tested were ranged from 10^8 to 10^9. The acidity of the culture after 48 hr of incubation were changed from pH 7.0 to pH 8.8~9.4 (Table 1).

The SA production by tested strains such as *P. putida* RE011, RS015, *P. fluorescens* WCS 374 and *P. fluo-*

Table 2. Salicylic acid production assay by bacterial isolates after 3 day of shaking culture in standard succinate medium

Tested bacteria	pH	OD (527 nm)		Color reaction
		Growth	SA	
1. *Pseudomonas putida* RE008	9.23	0.708	0.0040	−
2. *P. putida* RE010	8.80	0.784	0.0005	−
3. *P. fluorescens* RS006	9.06	0.605	0.0205	−
4. *P. putida* RS009	9.36	0.712	0.0035	−
5. *P. fluorescens* RS015	9.27	0.890	0.0010	+
6. *P. putida* RE011	9.23	0.765	0.0040	+
7. *P. putida* RE015	9.25	0.838	0.0010	−
8. *P. putida* RS002	9.24	0.761	0.0020	−
9. *P. fluorescens* RS154	9.27	0.784	0.0020	−
10. *P. fluorescens* CE005	9.28	0.045	0.0070	−
11. *P. putida* RE014	9.35	0.011	0.0045	−
12. *P. putida* RS153	9.36	0.006	0.0013	−
13. *P. fluorescens* CE006	9.39	0.013	0.0010	−
14. *P. fluorescens* CS101	9.37	0.014	0.0013	−
15. *P. fluorescens* CS108	9.41	0.469	0.0014	−
16. *P. putida* WCS358	9.21	0.560	0.0025	−
17. *P. fluorescens* WCS374	9.26	0.431	0.0905	++
18. EN401	9.06	0.813	0.0185	+
19. RS112	9.31	0.889	0.0013	+
20. EN415	9.28	0.978	0.0012	+
21. EN7	9.38	0.631	0.0034	+
22. RS111	9.39	0.746	0.0015	−

rescens 417, *P. fluorescens* RS015, *P. putida* RE011, RS112 and EN415 and EN7 was qualitatively demonastrated by purple color reaction in their culture media (Table 2). Even though bacterial strains such as *P. fluorescens* RS015, *P. putida* RE011, RS112, EN415 and EN7 showed positive color reaction of SA in the culture medium, SA production from these bacteria was not confirmed through OD measurement in extracted culture supernatant (Table 2). On the contrary, color reaction in the medium and spectrophotometric measurement of extracted culture supernatant of 4 bacterial strains such as *P. fluorescens* RS006 (OD; 0.0205), *P. fluorescens* WCS 417 (OD; 0.0115) and EN401 (OD; 0.0185) showed positive reaction for SA production in both tests. This result seems to indicate that only 4 bacterial strains produced the SA in the SSM. Especially, *P. fluorescens* WCS 374 which is known to as good SA producer (12).

The SA production by *Pseudomonas putida* RS006 and *Pseudomonas* sp. EN 401 strains was qualitatively demonstrated by spectrophotometric detection when compared with *P. fluorescens* WCS374 (Fig. 1).

In absorbance spectrum of the *P. putida* RS006 and *Pseudomonas* sp. EN401, the characteristic peaks were found at 245 and 313 nm respectively. The spectrum

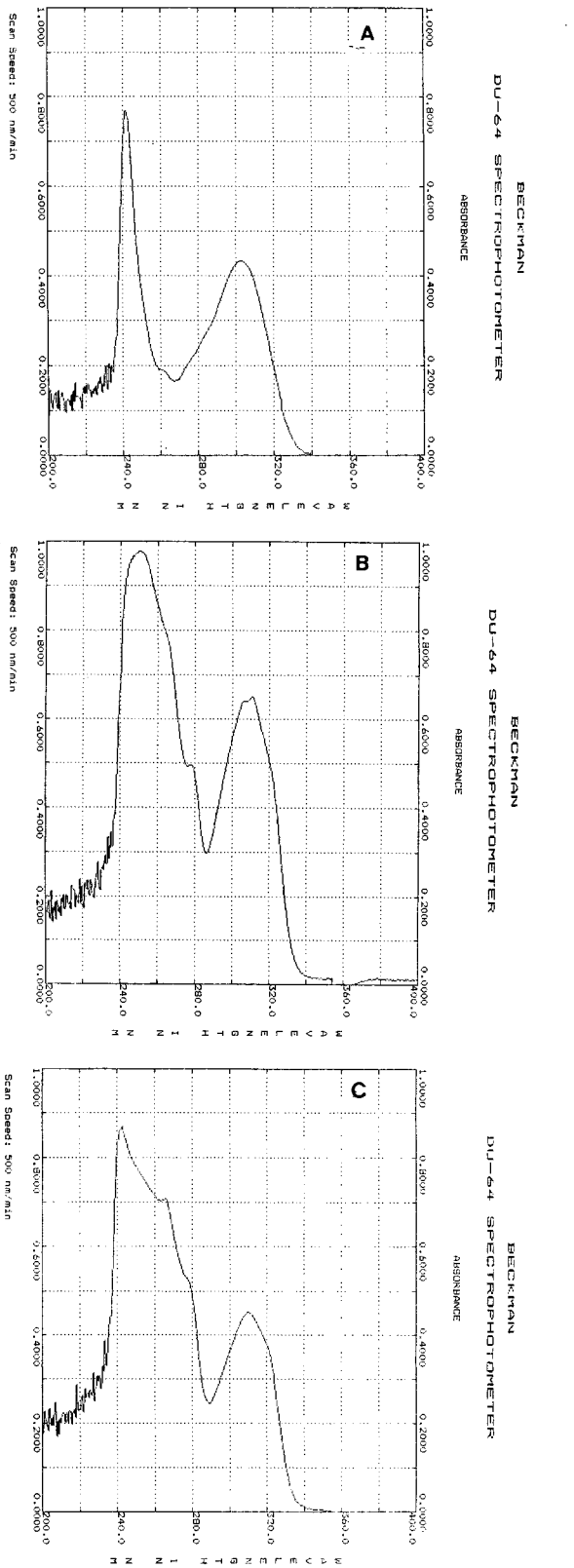

Fig. 1. Absorbance spectrum of culture supernatant of *P. fluorescens* WCS374 (A), *P. fluorescens* RS006 (B) and EN401 for salicylic acid in SSM using spectrophotometry.

of extracted culture supernatant of these two bacterial strains were identical to *P. fluorescens* WCS374 which was used for reference.

DISCUSSION

Fluorescent *Pseudomonas* species are characterized by the production of a fluorescent, yellowish green, water soluble pigment and pyoverdine (18). This compound is chemically defined as a hydroxyquinoline chromophore attached to a peptide chain containing hydroxymate groups (6). This structure exhibits a high affinity for iron (III) (15). This iron is related to properties of pyoverdine which is recognized as a siderophore for some bacteria (17). Under iron starvation, some fluorescent *Pseudomonas* sp. as well as non-fluorescent *Pseudomonas* spp elaborate another siderophore, pyochelin, which is chemically unrelated to pyoverdine since it is composed of a salicylic-substituted cysteinyl peptide (5). Salicylic acid is considered to be an intermediate in pyochelin synthesis which included SA as the first intermidiate in the pathway (2, 5). In the SSM shaking culture, the cell yield of the different strains after 40 hr ranged between 10^8 and 10^9 CFU per ml of SSM, which resulted in absorbance reading of approximately 1 at 660 nm. The production of SA was qualitatively demonstrated by direct observation of color reaction. *P. fluorescens* RS015, *P. putida* RE011, *P. fluorescens* WCS374, EN401, RS112, EN 415 and EN 7 appeared to produce SA.

Leeman (12) demonstrated that production of SA by strains of *P. fluorescens* WCS and WCS417 *in vitro* under low iron availability decreased with addition of iron to the medium. SA may serve as a siderophore for the tested strains. Meyer *et al.* (16) reported that pyoverdine deficient mutant of strain CHA0 produced more SA than that of wild type, which was explained by the assumption that the lack of pyoverdine is compensated by increased production of SA, which serve as a siderophore.

In this experiment, we observed that levels of SA production and color reaction of the tested strains were not matched with culture condition. Thus, we concluded that for the effective screening of SA producing rhizobacteria from natural habitats, screening procedures could be modified by two steps such as the measurement of color reaction of the culture medium in the first step and spectrophotometric measurement of SA from supernatant of culture extracted in the second step.

요 약

22종의 근권세균이 무, 카네이션, 감자, 토마토의 뿌리와 근권에서 분리되었다. 이들 세균은 King's B 배지에서 형광성 색소를 생산하였으며, *Pseudomonas*속의 세균으로 동정되었다. 이들 세균은 기주식물의 뿌리 내부와 근권에 정착하고 있었다. 이들 세균의 배양적인 특성을 알아 보기 위해 중성으로 조절된 standard succinate medium에서 40시간 배양한 후 배지의 pH와 증식된 균의 수를 조사해본 결과 pH는 중성(7.0)에서 알카리성(8.8~9.41)으로 높아졌으며 균의 수는 10^6에서 10^8로 증가하였다. 이들 22종 세균의 salicylic acid 생산 여부를 배지의 색갈 변화를 이용해서 조사한 결과 7 종류 세균의 배지 색갈이 분홍색으로 변해 salicylic acid를 생산하는 것으로 나타났다. 그러나 이들 7 종류 세균의 배양액에서 추출한 물질을 분광광도계를 이용해 optical density를 조사한 결과 *Pseudomonas fluorescens* RS006과 *Pseudomonas* sp. EN 401 등 2개의 균주만이 salicylic acid를 생산하는 것으로 확인되었다. 따라서 salicylic acid를 생산하는 균으로부터 salicylic acid의 생산 여부를 알기위해서는 일차적으로는 대상 근권세균의 배지 색갈변화를 조사하고 이차적으로는 이들 균의 배지추출액의 optical density를 분광광도계를 이용하여 조사하면 salicylic acid를 생산하는 균근세균을 선발할 수 있을 것으로 생각된다.

REFERENCES

1. Alexander, D., Goodman, R.M., Gut-Rella, M., Glascook, C., Weymann, K. Friederich, L., Maddox, D., Ahl G.P., Lutenz, T., Ward, E. and Ryals, J. 1993. Increased tolerance to two oomycetes pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. USA* 90 : 7327-7331.
2. Ankenbauer, R.G. and Cox, R. 1988. Isolation and characterization of *Pseudomonas aeruginosa* mutants requiring salicylic acid for pyochelin biosynthesis. *J. Bact.* 170 : 5364-5367.
3. Antoniw, J.F. and White, R.F. 1980. The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. *Phytopath. Z.* 98 : 331-341.
4. Carr, J.P. and Klessig, D.F. 1989. The pathogenesis-related proteins of plants. In genetic engineering, Vol. 11. J. K. Setlow.ed.(New York, Plenum Press), pp.65-109.
5. Cox, C.D., Rinehart, K.L., Moore, M.L. and Cook, J.C. 1981. Pyochelin: novel structure of an iron-chelating growth promoter for Pseudomonas aeruginosa. *Natl. Acad. Sci. USA* 78 : 4256-4260.
6. Demange, P., Wendenbaum, S., Bateman, A., Dell, A., Meyer, J.M. and Abdallah, M.A. 1986. Bacterial siderophore: structure of pyverdines and related compounds, pp.

131-141. In T. R. Swinburne (ed.), Iron, siderophores and plant diseases. Plenum Publishing Co. New York.

7. Gianinazzi, S. 1984. Genetic and molecular aspects of resistance induced by infections or chemicals. In Plant and Microbe Interactions-Molecular and Genetic Perspectives, Vol. 1. E. W. Nester and T. Kosuge., eds. (Macmillan). pp. 321-342.
8. Gilpatrick, J.D. and Weitraub, M. 1952. The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. *Science* 115 : 701-702.
9. Hoffland, E., Hakulinen, J. and van Pelt, J.A. 1996. Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas species. Phytopathology* 86 : 757-762.
10. Hoffland, F., Pieterse, C. M. J., Bik, L. and van Pelt, J. A. 1995. Induced systemic resistance in radish is not associated with accumulation of pathogenisis-related proteins. *Physiological and molecular plant pathology* 46 : 309-320. Hooft van Huijsduijnen, Albas, R.A.M., De Rijk, R.H. and Bol, J.F. 1986. Induction by SA of pathogensis-related proteins and resistance to alfalfa mosaic virus infection in various plant species. *J. gen. Virol.* 67 : 2143-2153.
11. King, E.O., Ward, M.K. and Raney, D.E. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* 44 : 301-307.
12. Leeman, M., den Ouden, F.M., van Pelt, J.A., Dirks, F.P. M., Steiji, H. Bakker, P.A.H.M. and Schippers, B. 1996. Iron availability affects induction of systemic resistance to Fusarium wilt of radish by *Pseudomonas fluorescens. Phytopathology* 86 : 149-155.
13. Malamy, J., Carr, J.P. and Klessig, D.F. 1990. Salicylic acid-a likely endogenous signal in the resistance response to tobacco to viral infection. *Sciene* 250 : 1001-1004.
14. Maurhofer, M., Hase, C., Meuwly, P., Metraux, J.P. and Defago, G. 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by root-colonizing *Pseudomonas fluorescens* strain CHAO: Influence of the gacA gene and pyoverdine production. *Phytopathology* 84 : 139-146.
15. Meryer, J.M. and Abdallah, M.A. 1978. The fluorescent pigment of *Pseudomonas fluorescens*; biosynthesis, purification and physiological properties. *J. gen. Microbiol.* 107 : 319-328.
16. Meyer, J.M., Azelvandre, P. and George, C. 1992. Iron metabolism in *Pseudomonas* : salicylic acid, a siderophore of *Pseudomonas fluorescens* CHAO. *BioFactors* 4 : 3-27.
17. Meyer, J.M. and Hornsperger, J.M. 1978. *J. Gen. Microbiol.* 107 : 329-331.
18. Palleroni, N.J. 1984. In Krig, N.R.(ed.), Bergey's manual of systemic bacteriology. Williams and Wilkins, Baltimore, Vol. 1, pp.141-199.
19. Pennazio, S., Colariccio, D., Roggero, P. and Lenzi. R. 1987. Effect of salicylate stress on the hypersensitive reaction of asparagus bean to tobacco necrosis virus. *Physiol. Mol. Plant Pathol.* 30 : 347-357.
20. Pieterse, C.M., Van Wess, S.C.M., Hoffland, E., van Pelt, J.A. and van Loon, L.C. 1996. Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8 : 1225-1237.
21. Ponstein, A.S., Bres-Vloermans, S.A., Sela-Buurlage, M. B., Van den Elzen, P.J.M., Melchers, L.S. and Cornelissen, B.J.C. 1994. A novel pathogen and wound inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. *Plant Physiology* 104 : 109-118.
22. Ross, A.F. 1961. Systemic aquired resistance induced by localized virus infections in plants. *Viorology* 14 : 329-339.
23. van Loon, L.C. and Antoniw, J.F. 1982. Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco. *Neth. J. Plant Pathol.* 88 : 237-256.
24. Ward, E. R., Ukness, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3 : 1085-1094.
25. White, R.F. 1979. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. *Virology* 99 : 410-412.
26. Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. and Raskin, I. 1991. Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. *Plant Cell* 3 : 809-818.

(Received October 25, 1998)