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Characterization of Nonhypersensitive Mutant and Nonpathogenic Mutant of *Xanthomonas campestris* pv. *campestris*

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Xanthomonas campestris pv. *campestris*의 비과민성 돌연변이주와 비병원성 돌연변이주의 특성

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ABSTRACT : We have screened hypersensitive responses of 18 cultivars of *Nicotiana tabacum* to *Xanthomonas campestris* pv. *campestris*. TC500 cultivar produced the most strong hypersensitive response (HR) to *Xanthomonas campestris* pv. *campestris*. By NTG mutagenesis, nonhypersensitive mutants (XHN 514-774, XHN 620-831) were generated, which does not induce hypersensitive response on tobacco leaves (*Nicotiana tabacum* cv. TC500). Also nonpathogenic mutant (XPN 1001), which does not incite any of the black rot symptoms on leaves was generated. We observed that HR⁻ mutants were still pathogenic on cabbage leaves producing black rot symptoms and nonpathogenic mutant induced HR in tobacco leaves. The *in planta* growth of wild type and HR⁻ mutants were examined for up to 120 hrs after inoculation : population of wild type strain increased to 10^8 in 24 hrs, but rapidly declined thereafter; HR⁻ mutants increased to more than 10^6 in 48 hrs after inoculation but subsequently stabilized and slowly decreased. We observed that wild type and these mutants produced similar amounts of degradative enzymes such as protease, pectate lyase, cellulase and amylase.

Key words **: *Xanthomonas campestris* pv. *compestris*, Hypersensitive, Nonhypersensitive mutant, Nonpathogenic mutant.**

Plant pathogenic bacteria have the ability to invade and to multiply within certain plants causing typical disease symptoms. However, disease does not develop on nonhost plant. Plant pathogenic bacteria usually infect only a limited number of plant species. The hypersensitive response (HR) of higher plants is a defense reaction characterized by the rapid and localized necrosis of plant tissues, which is specifically induced by plant pathogenic microorganisms (9, 11). The ability of bacteria to elicit the hypersensitive response (HR) in plants is controlled by *hrp* genes, which are so named because they are required for both the HR (on nonhost plants) and pathogenicity (on host plants) (13). *Hrp* genes have been cloned from phytopathogenic Pseudomonads, Xanthomonads, and Erwiniae (3, 4, 7, 13). In this paper, we report the characterization of HR⁻ mutant and nonpathogenic mutant of *Xanthomonas campestris* pv. *campestris*, the causal agent of the black rot of crucifers.

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MATERIALS AND METHODS

Media and culture condition. *Xanthomonas campestris* pv. *campestris* was grown on Nutrient agar or YDC media (Yeast extract, 10 g; Dextrose, 20 g; $CaCO_3$, 20 g; Bactoagar, 15 g per liter distilled water) at 30°C.

Induction of Hypersensitive response (HR) of tobacco cultivars. The hypersensitivity of tobacco plants (*Nicotiana tabacum*) to *Xanthomonas campestris* pv. *campestris* was tested on 18 different tobacco cultivars. Seeds of tobacco cultivars were germinated in

plates containing gauze moistened with distilled water, transplanted to 18 cm pots and grown in a greenhouse. The cultures of bacteria grown at 30°C were pelleted by centrifugation (7,000 rpm, 15 min, 4°C), suspended in sterile distilled water at a concentration of about 10^8 cfu/m*l* and infiltrated into leaves with a disposable plastic syringe. Inoculated plants were kept in a growth chamber at 28°C with a 12 hr light-12 hr dark cycle. Hypersensitive response on 18 tobacco cultivars was recorded after 48 hr.

Mutagenesis of *Xanthomonas campestris* pv. *campestris*. Portions (1 m*l*) of overnight cultures of *Xanthomonas campestris* pv. *campestris* were centrifuged for 20 min at 20°C (14,000 rpm). The pellet was washed twice with 1 m*l* of 0.1 M sodium citrate buffer (pH 5.5) and mixed with 10 μ*l* of N-methyl-N'-nitro-N-nitrosoguanidine (NTG, 1 mg/m*l* in citrate buffer). Then the tube was left at room temperature for 10~30 min. The mutagen was removed by centrifugation for 1 min and the pellet was washed twice with 0.1 M sodium phosphate buffer (pH 7.0). The mutagenized cells were resuspended in LB broth (5 m*l*) and incubated for 24 hrs at 25°C. Suitable dilutions of the culture were plated on LB media (Bactotryptone, 10 g; Yeast extract, 5 g; Sodium chloride, 10 g; Bactoagar, 15 g per distilled water) (8).

HR and Pathogenicity test of mutants. The hypersensitivity tests were performed on the leaves of *Nicotiana tabacum* cv. TC500 and the pathogenicity of mutants was tested on cabbage (*Brassica oleracea* cv. 'Hungnong 166'). Bacterial suspensions (10^8 cfu/m*l*) were infiltrated into leaves with a disposable syringe. A total of 4,500 mutants generated by NTG were screened for obtaining HR⁻ mutant and nonpathogenic mutant. Mutants which did not induce a hypersensitive necrotic response on tobacco leaves within 3 days were considered HR⁻ mutants. Mutants which had completely killed the plants within 2 weeks were considered pathogenic and mutants which did not induce any visible symptoms within 3 weeks were considered nonpathogenic.

Measuring bacterial populations in planta. The culture of bacteria was grown to a density of 10^8 cfu/m*l*, represented by an A_{535nm} of 0.06. The cells were pelleted by centrifugation for 2 min and resuspended in 250 m*l* of 10 mM $MgCl_2$. A small amount (20~30 μ*l*) of inoculum was infiltrated into each of sites on tobacco leaves with a 1 m*l* plastic disposable syringe with no needle. Leaf disk samples containing the inoculated area were taken using a 6 mm cork borer on 0 (immediately after inoculation), 9, 12, 24, 48, 72, 96, and 120 hr after inoculation. A series of 10 fold dilutions in sterile distilled water was plated on nutrient agar. The colonies were counted after 3 days at 30°C. Each reported cell count is the average of experiment of three replicates each.

Enzyme assays. The protease, cellulase, amylase and pectate lyase activities of each mutant and of the parent strain were determined. Extracellular protease activity was assayed on skimmed milk agar. Cellulase activity was determined on carboxymethyl cellulose medium. Assay for pectate lyase activity was conducted on sodium polypectate medium. And amylase production was assayed on starch medium.

RESULT

Selection of tobacco cultivar as nonhost plant-hypersensitivity system. Bacterial suspension at 10^8 cfu/m*l* was infiltrated into fully expanded tobacco leaves with disposable syringe. Hypersensitive responses of 18 cultivars of tobacco (*Nicotiana tabacum*) were recorded after 48 hrs. They produced various degrees of hypersensitive response to *Xanthomonas campestris* pv. *campestris* (Table 2). Cultivar TC500 among 18 different *N. tabacum* cultivars produced the most strong hypersensitive response to *Xanthomonas campestris* pv. *campestris* (Fig. 1). Therefore, we used TC500 cultivar of *N. tabacum* as a test plant for HR induction by *Xanthomonas campestris* pv. *campestris* for further studies.

Generation of nonhypersensitive mutants and nonpathogenic mutant. NTG mutagenesis on *Xanthomonas campestris* pv. *campestris* generated HR⁻ mutants (XHN 514-774, XHN 620-831) which did not in-

Table 1. Differential reaction of hypersensitivity of cultivars of *Nicotiana tabacum* to *Xanthomonas campestris* pv. *campestris*

Tobacco cultivar	HR
TC 500	+++
SC 72, CK 319, ZZ-100, CK 254, LAFC 53, NC 82, BY 4, Hicks, NCPY 10, KF 109	+
SOUTH, Va 115, Coker 347, Coker 8, Y.S.A, N.C 2326	±
DG	-

+++ : strongly response, + : moderately response, ± : weakly response, - : nonresponse.

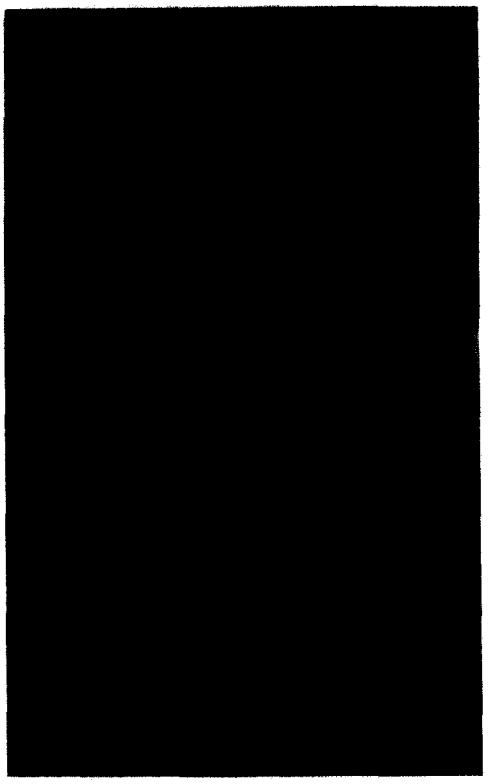

Fig. 1. HR⁻ mutants (XHN 514-774, XHN 620-831) of *Xanthomonas campestris* pv. *campestris* on tobacco (*Nicotiana tabacum* cv. TC 500) leaf. Leaf panels were infiltrated with bacteria at a concentration of 10^8 cfu/m*l* in sterile distilled water. Leaf was photographed 3 days after inoculation.

duce hypersensitive response on nonhost plant (Fig. 2). Also nonpathogenic mutant (XPN 1001) was generated by NTG mutagenesis and did not incite any of the black rot symptoms on cabbage (*Brassica oleracea* cv. 166) (Fig. 3).

Interaction of mutants with tobacco and cabbage plants. The reaction of nonpathogenic mutant to nonhost plant and the reaction of HR⁻ mutants to host plant were observed. When XPN1001 was infiltrated into tobacco leaves, XPN 1001 induced a brown necrosis of an HR similar to the wild type after 2 days. Also XHN 514-774 and XHN 620-831 were able to produce a black rot symptoms similar to the wild type when infiltrated in cabbage leaves. These results suggest that these mutants are independent of each other (Table 2).

Enzyme assays. Nonpathogenic mutant (XPN 1001)

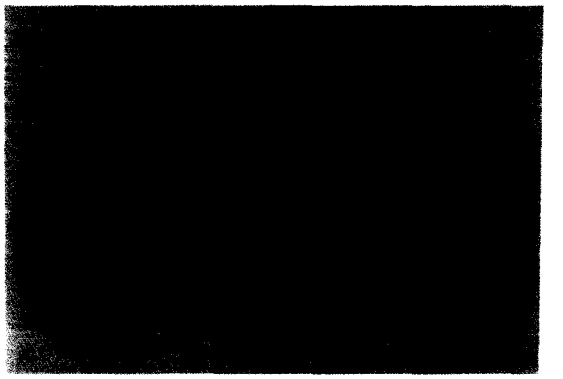

Fig. 2. Nonpathogenic mutant (XPN 1001) of *Xanthomonas campestris* pv. *campestris* on cabbage (*Brassica oleracea* cv. 166) leaf. Leaves were injected with bacterial suspensions of 10^8 cfu/m*l* in sterile distilled water. The photographs were taken 3 weeks after injection. A: Wild type B: Nonpathogenic mutant C: Sterile distilled water.

and HR⁻ mutants (XHN 514-774, XHN 620-831) were similar to the wild type strain in exopolysaccharide and degradative enzyme production (Table 3).

In planta growth of nonhypersensitive mutants vs. wild type. Bacterial suspensions were used to infiltrate into tobacco leaves and leaf disks were periodically excised. The growth of wild type and HR⁻ mutants (XHN 514-774, XHN 620-831) was examined for up to 120 hrs after inoculation. Population of wild type increased to 10^8 in the 24 hr following inoculation but rapidly declined after 24 hr in the leaf tissue. The mutants (XHN 620-831, XHN 514-774) increased to more than 10^6 above the initial population by 48 hr aft-

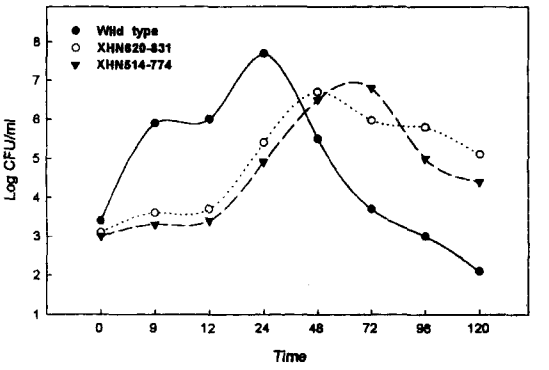

Fig. 3. Multiplication of wild type and nonhypersensitive mutants (XHN 514-774, XHN 620-831) of *Xanthomonas campestris* pv. *campestris* in tobacco leaves.

Table 2. Reactions of mutants on tobacco (*Nicotiana tabacum* cv. TC500) and cabbage (*Brassica oleracea* cv. 166) leaves

Bacterial strain	HR	Pathogenicity
Wild type	+	+
XPN 1001	+	−
XHN 514-774	−	+
XHN 620-831	−	+

XPN 1001: Nonpathogenic mutant, XHN 514-774 and XHN 620-831 : HR⁻ mutant.

er inoculation but subsequently stabilized and slowly decreased (Fig. 3).

DISCUSSION

Most of the works so far on hypersensitive response of plant pathogen bacteria to nonhost plants, exclusively, if not all, are focused on *Pseudomonas*, presumably due to the distinct reaction in 24 hr. All phytopathogenic bacteria (except those pathogenic to bean) induced the formation of light or dark brown necroses at the inoculation site. The pods treated with the saprophytes remained symptomless. The bean pathogens caused water-soaked spots which appeared considerably later. The necroses produced by *Pseudomonas* appeared rapidly and were severe, whereas those induced by *Xanthomonas* species appeared more slowly, i. e., in one to four days (Klement and Goodman, 1967). Therefore, we have developed a nonhost *Nicotiana tabacum-Xanthomonas campestris* pv. *campestris* system firstly by selecting Tobacco cultivar TC 500 most suitable to screen massively for hypersensitive reactions. Secondly, by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis, nonhypersensitive mutants and nonpathogenic mutant were selected and reconfirmed by subjecting to TC500 for HR and susceptible cabbage, *Brassica oleracea* cv., 'Hungnong 166', for nonpathogenicity. We also attempted transposon mutagenesis as suggested by Shaw *et al.* (14), but no relevant mutants was obtained.

From the study of population dynamics of wild type and mutant in plant, it was found that threshold levels of bacterial cells *in planta* were essential threshold population for HR to occur. Our result is agreeable to the result of Klement and Goodman (12). They observed that, in incompatible combination, the initial rate of multiplication of *Ps. syringae* in tobacco was practically same as that of *Ps. tobaci*, but multiplication stopped in 8 to 24 hrs. Klement (11) suggested that antimicrobial compounds (phytoalexins) might accumulate and diffuse into the intercellular spaces and, in turn, inhibit bacterial multiplication or cause bacteriostasis during the necrotization process. Turner and Novacky (15) detected dead plant cells by HR response in symptomless tobacco leaves by staining with Evans blue. Kim (1996) detected the plant cell death at 6 hrs after inoculation by light microscopy during of hypersensitive response of this *Xanthamonas campestris* pv. *campestris*, and apparent macroscopic leaf tissue collapse and desiccation in 48 hrs, which could be determined or the latent period according to Klement (11).

Our preliminary result indicated that both HR⁻ mutant and nonpathogenic mutant produced wild type levels of extracellular enzymes by plate assay. Arlat *et al.* (1, 2) also found that the loss of pathogenicity of *Xanthomonas campestris* pv *campestris* Hrp⁻ mutants is not due to a failure to produce extracellular enzyme known to be required for pathogenicity. Felt *et al.* (5) compared the cell surface charge and hydrophobicity of *Ps. syringae* pv. *phaseolicola* wild type and *hrp* mutants and concluded that such an altered phenotype was not due to the changes in the ability to produce exopolysaccharides or to an altered composition of cell surface polysaccharide (LPS and EPS). Both types of mutants in this study were characterized to see if both characters are related or independent. We found that HR⁻ mutants were all pathogenic on susceptible host cultivars and a nonpathogenic mutant was HR positive. This observation was similar to the result of Boucher *et al.* (4) who worked on *Ps. solanacearum*. Therefore, this could be referred to as *dsp*, which is the second group of genes (4), that both characters are independent of each other. However, it still requires further verification since most of the *hrp* genes are known that they are involved in pathogenesis and eliciting HR (1-3, 5, 6, 9).

요 약

십자화과 식물에 검은썩음병을 일으키는 *Xanthomonas campestris* pv. *campestris*에 대하여 담배 18품종 중 가장 과민반응을 강하게 나타내는 품종(*Nicotiana tabacum* cv. TC500)을 과민반응 비기주식물로 선발하였다. NTG 돌연변이를 실시하여 얻은 약 4,500개의 mutant들을 10^8 cells/ml의 농도로 주사 접종하여 비기

주식물인 담배 잎에서 과민반응을 유도하지 않는 HR⁻ mutants(XHN 514-774, XHN 620-831)를 선발하였고, 기주식물인 양배추 잎에서 병원성을 나타내지 않는 비병원성변이주(XPN 1001)를 선발하였다. HR⁻ mutant를 기주식물인 양배추에 접종한 결과 병원성을 나타내었고, nonpathogenic mutant를 비기주식물인 담배 잎에 접종한 결과 과민반응을 유도하였다. 담배 잎에서 HR⁻ mutant와 wild type의 population을 조사한 결과 wild type은 24시간 이후 급격히 감소하는 반면, HR⁻ mutant는 일정수준을 유지하였으나 72시간 이후에 감소하였다. 그리고 mutant들의 protease, cellulase, amylase, polygalacturonate lyase 분비여부를 조사한 결과 wild type과 비슷하게 분비하였다.

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REFERENCES

1. Arlat, M., Gough, C. L., Barber, C. E., Boucher, C. and Daniels M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. *Mol. Plant-Microbe Interact.* 4 : 593-601.
2. Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A. and Boucher, C. A. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. *Mol. Plant-Microbe Interact.* 5 : 187-193.
3. Bauer, D. W., Bogdanove, A. J., Beer, S. V. and Collmer, A. 1994. *Erwinia chrysanthemi hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. *Mol. Plant-Microbe Interact.* 7 : 573-581.
4. Boucher, C. A., Gijsegem, F. V., Barberis, P. A., Arlat, M. and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *J. Bacteriol.* 169 : 5626-5632.
5. Fett, W. F., Osman, S. F., Dunm, M. F. and Panopoulos, N. J. 1982. Cell surface properties of *Pseudomonas syringae* pv. *phaseolicola* wild-type and *hrp* mutants. *Phytopathology* 135 : 135-152.
6. Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W. and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants. *J. Bacteriol.* 170 : 4748-4756.
7. Huang, H. C., Xiao, Y., Lin, R. H., Lu, Y., Hutcheson, S. W. and Collmer, A. 1993. Characterization of the *Pseudomonas syringae* pv. *syringae* 61 *hrpJ* and *hrpI* genes : Homology of *hrpI* to a superfamily of proteins associated with protein translocation. *Mol. Plant-Microbe Interact.* 6 : 515-520.
8. Hwang, I. G., Lim, S. M. and Shaw, P. D. 1992. Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glycines*. *J. Bacteriol.* 174 : 1923-1931.
9. Kamoun, S. and Kado, C. I. 1990. Plant-inducible gene of *Xanthomonas campestris* pv. *campestris* encodes an exocellular component required for growth in the host and hypersensitivity on nonhosts. *J. Bacteriol.* 172 : 5165-5172.
10. Kim, M. H. 1996. Characterization of non-hypersensitive mutant and non-pathogenic mutant of *Xanthamonas campestris* pv. *campestris*. M. S. Thesis, Gyeongsang National University.
11. Klement, Z. 1982. *Hypersensitivity in Chapter 8 of Phytopathogenic Prokaryotes*. 2 : 149-177. Academic Press, Inc., New York.
12. Klement, Z. and Goodman, R. N. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. *Ann. Rev. Phytopathol.* 5 : 17-44.
13. Lindgren, P. B., Peet, R. C. and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168 : 512-522.
14. Shaw, J. J., Settles, L. G. and Kado, C. L. 1988. Transposon Tn4431 mutagenesis of *Xanthomonas campestris* pv. *campestris* : Characterization of a nonpathogenic mutant and cloning of a locus for pathogenicity. *Mol. Plant-Microbe Interact.* 1 : 39-45.
15. Turner, J. G. and Novacky, A. 1974. The quantitative relation between plant and bacterial cells involved in the hypersensitive reaction. *Phytopathology* 64 : 885-890.

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