Transgenic Production of Cytokinin Suppresses Bacterially Induced Hypersensitive Response Symptoms and Increases Antioxidative Enzyme Levels in *Nicotiana* spp.

B. Barna, A. C. Smigocki, and J. C. Baker

First author: Plant Protection Institute, Hungarian Academy of Sciences, H-1022 Herman O. 15. Budapest, Hungary; and second and third authors: Molecular Plant Pathology Laboratory, USDA-ARS, Beltsville, MD 20705.
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ABSTRACT

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Responses of cytokinin overproducing transgenic *Nicotiana* plants to infections with compatible and incompatible *Pseudomonas syringae* pathovars were compared. Plants used were transformed with the *ipt* (*isopentenyl transferase*) gene that catalyzes the synthesis of cytokinin. In cytokinin overproducing lines that carry the *ipt* gene fused to the CaMV 35S (Nt+*ipt*), the wound-inducible proteinase inhibitor II (Ntx+*ipt*), or the light-inducible Rubisco small subunit protein (Npl+*ipt*) promoter, development of the hypersensitive response (HR) after infection with incompatible bacteria (*P. syringae* pv. *tomato*) was significantly inhibited as compared to the untransformed (Nt) controls. Over a 12 h period following inoculation, *P. syringae* pv. *tomato* populations were slightly reduced in leaves of the cytokinin-overproducing Nt+*ipt* line compared with the Nt

control. When the compatible *P. syringae* pv. *tabaci* was used to infect the *ipt* transformed lines, slight or no significant differences in necrosis development were observed. Following infection, the titer of *P. syringae* pv. *tabaci* increased rapidly in both the transgenic and control lines but was higher in Nt+*ipt* plants. Leaf superoxide dismutase and catalase enzyme activities were about 60% higher in *ipt* leaf extracts than in the controls. This augmented antioxidant capacity likely decreased the amount of H₂O₂ that may be associated with the higher tolerance of plants to pathogen-induced necrosis. In addition, the Nt+*ipt* lines had a significantly lower molar ratio of free sterols to phospholipids. The more stable membrane lipid composition and the higher antioxidant capacity likely contributed to the suppressed HR symptoms in the cytokinin overproducing Nt+*ipt* plants. In conclusion, the overproduction of cytokinins in tobacco appears to suppress the HR symptoms induced by incompatible bacteria.

Additional keywords: antioxidants, membrane lipids, oxidative burst.

One of the earliest reactions of plants to invading pathogens is the rapid accumulation of active oxygen species (AOS). During the hypersensitive response (HR) of plants to challenge by bacteria, two phases of active-oxygen production, an early non-specific and a late specific, have been described (21). AOS are known to be involved not only in signal transduction or in limiting pathogen ingress (14,22,23), but also in the induction of tissue necrosis (4,9,13). In nonstressed plant tissues, antioxidants are able to neutralize the effect of AOS. On the other hand, stress associated with leaf senescence is suggested to be accompanied by a decrease in the antioxidant capacity and a subsequent increase in the production of AOS (17).

Senescing tobacco leaves have been shown to be more susceptible to the necrotizing fungal pathogens *Alternaria alternata* (Fr.) Kreissler (5,31) and *Botrytis cinerea* (Pers.) (5). In addition, older leaves of tobacco are more sensitive to toxins and to cell-wall degrading enzymes produced by necrotrophic pathogens, as well as to autolysis of membrane lipids (5) indicating different strategies of necrotrophs and biotrophs. Namely, while biotrophs induce enhanced cytokinin production because they need the living plant cell, necrotrophs destroy plant tissue by toxins and cell-wall degrading enzymes to get nutrients and to develop (11).

Plant senescence is regulated by a coordinated genetic program in which cytokinins play an important role. In senescing leaves, leaf cytokinin levels are reduced, and external applications of

cytokinins are well-known to delay tissue senescence. Transgenic plants that express the bacterial *ipt* (*isopentenyl transferase*) gene that catalyzes the synthesis of cytokinin exhibit delayed senescence (10,27,28) and increased insect and microbial resistance (29,30,32). In this paper, transgenic tobacco plants with constitutive or inducible promoters that overproduce cytokinins are utilized to analyze their response to infections with compatible and incompatible *Pseudomonas* spp. in order to gain insight into the mechanism of various symptom developments. Changes in activities of antioxidant enzymes and in membrane lipid composition are reported.

MATERIALS AND METHODS

Plants and pathogens. The following cytokinin overproducing transgenic lines were used in this study: Nt+*ipt*, *Nicotiana tabacum* L. cv. Petit Havana SR1 that carries the *ipt* gene fused to the CaMV 35S promoter (32); Ntx+*ipt*, *N. tabacum* L. cv. Xanthi-nc with the *ipt* gene fused to the wound-inducible proteinase inhibitor II promoter (28,30); and the Npl+*ipt*, *N. plumbaginifolia* Viv. 43A, where the *ipt* is under the control of light-inducible Rubisco small subunit protein promoter (34). T2 generation of homozygous Ntx+*ipt* and Npl+*ipt* plants were grown from seed and used for the experiments. Tobacco seedlings were grown in 10-cm diameter pots for 4 weeks and then transplanted to 25-cm diameter pots. All plants were propagated in the greenhouse at 25 ± 2°C under a 14 h light period during experiments.

Cultures of *P. syringae* pvs. *tomato* 44 and *tabaci* 11528 were obtained from Myron Sasser, Department of Plant Pathology, University of Delaware, Newark, DE. Bacterial cultures were

Corresponding author: B. Barna; E-mail address: bbar@nki.hu

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stored in sterile water or on silica gel (21) at 0°C for long-term storage. Active cultures were maintained on nutrient agar at 30°C. Cultures were transferred to fresh medium 16 to 24 h prior to use. Bacteria were washed by centrifugation and resuspended in deionized water. Bacterial concentration was determined turbidimetrically and/or by dilution plating.

Infiltration of leaves and rating of symptoms. One centimeter diameter areas of fully expanded leaves of tobacco in preflowering stage were infiltrated with bacterial suspensions or water as control using 1-ml needle-less syringe as described previously (21). Necrosis development in the infiltrated area was recorded at 24, 48, or 96 h. Rating of symptoms was made by visual observation and based on a scale of 0 to 5, where 0 = no visible symptoms, 1 = only chlorosis, no necrosis, 2 = 25% necrosis, 3 = 50%, 4 = 75%, and 5 = 100% necrosis (2).

Bacterial populations in leaf tissue. A 0.85-cm diameter disk from leaf areas previously infiltrated with 4.4×10^7 colony forming unit (cfu)/ml bacterial suspension was excised with a cork borer and ground in 1 ml of 0.1 mM NaCl in 25 mM potassium phosphate buffer, pH 7.0 with a mortar and pestle. The bacterial concentration was determined by dilution plating on nutrient agar plates 2, 6, and 12 h after inoculation.

Determination of antioxidative enzyme activities. Enzymes were extracted from fresh tissue as previously described (6). Samples (0.5 g) from fully developed 2nd and 3rd lower leaves (counting up from the bottom) of *N. tabacum* cv. Petit Havana Nt+*ipt* and Nt control plants were ground in 3 ml of ice-cold 50 mM TRIS buffer (pH 7.8) containing 2 mM Na₂EDTA and 5 mM dithiothreitol. Polyvinylpyrrolidone (PVPP, 75 mg) was added to the homogenate which was filtered through one layer of muslin and centrifuged at $15,000 \times g$ for 20 min at 4°C. Glycerol was added to the supernatants to a final concentration of 10% (vol/vol). Polyacrylamide electrophoresis (10% native gel) and specific staining for superoxide dismutase (SOD; EC 1.15.1.1.), peroxidase (POD; EC 1.11.1.7.), and catalase (CAT; EC 1.11.1.6) enzyme activities were carried out as described previously (1,6). For superoxide dismutase (SOD) staining, the polyacrylamide gels were incubated for 20 min in the dark in a solution containing 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 3 mM riboflavin, and 0.25 mM nitroblue tetrazolium chloride, rinsed with distilled water, placed on a glass sheet, and illuminated for about 10 min under a 200 W incandescent lamp. The SOD isoenzymes were visualized as achromatic bands against a blue/purple background. For peroxidase (POD) activity, gels were incubated in 100 mM sodium acetate buffer (pH 5.5) containing 1 mM *o*-dianisidine and 0.03% H₂O₂ for 20 min. POD activity appears as orange bands against a clear background. Catalase (CAT) staining was carried out after electrophoresis by incubating the gels in 30% H₂O₂ for 10 min, rinsing with water, staining for 5 min in 2% FeCl₃ + 2% K₃Fe(CN)₆ (1:1) and then rinsing with water again. Relative enzyme activity was determined by densitometry using a Shimadzu model CS-930, Kyoto, Japan, scanner, and calculated as total integrated intensity in arbitrary units.

Lipid extraction and determination. Phospholipids (PL), galactolipids (GL), and free sterols (St) were extracted from fully developed 2nd and 3rd lower leaves (counting up from the bottom) of *N. tabacum* cv. Petit Havana Nt+*ipt* and Nt control plants as described previously (5). Lipids were extracted by homogenizing the leaves in chloroform/methanol (2:1). Extracts were filtered and then washed with 0.1% NaCl; the organic phase was separated and evaporated to dryness under vacuum. PLs and GLs were separated on precoated thin layer chromatography (TLC) Merck, Germany, Silica Gel 60 plates with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5), were identified by co-chromatography of standards (Sigma) and stained with 3% copper acetate in 5.7% (vol/vol) H₃PO₄. Free sterols were separated from lipid extracts with petroleum ether:ether:acetic

acid (85:15:1) solvent and stained with 50% H₂SO₄. The position of free sterols on the TLC plate was located by using β -sitosterol (Sigma) as standard. The amount of PLs, GLs, and free sterols was estimated by densitometry with a Shimadzu model CS-930 scanner, Kyoto, Japan, and calculated as total integrated intensity as compared to commercial standards.

Statistical analysis. At least three independent experiments were conducted in each case. Measurements were carried out in three or four repetitions. Statistical analysis was performed using Student's *t* test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Effect of the cytokinin biosynthetic gene, *ipt*, on *Pseudomonas*-induced necrosis. Lines *N. tabacum* cv. Petit Havana SR1 either transformed with the *ipt* gene (Nt+*ipt*) or untransformed controls (Nt) were inoculated with varying concentrations of incompatible and compatible pathovars of *P. syringae*. The incompatible pathovar, *P. syringae* pv. *tomato*, produced a hypersensitive response in the untransformed Nt plants with almost complete necrosis of the inoculated site at 24 h at all bacterial concentrations tested (Fig. 1A). However, in the *ipt* transformed line (Nt+*ipt*), the amount of necrosis within the inoculation site after 24 h increased with the concentration of the bacterial inoculum; inoculum concentrations of 10^7 cfu/ml causing only chlorosis while 5×10^7 and 10^8 cfu/ml caused nearly complete necrosis. After 48 h, the amount of necrosis in the Nt+*ipt* line increased, however at the lower concentrations (1 to 2×10^7 cfu/ml) of the inoculum the amount of necrosis was generally less than in the untransformed Nt plants (Fig. 1B).

When the compatible bacterial pathovar *P. syringae* pv. *tabaci* was used to inoculate the tobacco lines no fast HR was found, visible necrotic (wildfire) symptoms, that are mainly due to the production of tabtoxin by the compatible bacteria, developed at a slower rate in both the Nt+*ipt* and Nt control leaves. The incompatible HR is always the consequence of the rapid programmed cell death, while necrosis by *P. syringae* pv. *tabaci* can develop slowly in tobacco leaves when the bacteria produces enough tabtoxin to develop necrosis. At 24 h, necrosis was found only at higher inoculum concentrations in both Nt+*ipt* and Nt controls (Fig. 1C). After 48 h, necrosis occurred at all concentrations with very little difference between the two tobacco lines (Fig. 1D), indicating that the overproduction of the cytokinins had little effect on the compatible bacterial interaction.

Effect of the cytokinin biosynthetic gene, *ipt*, on *P. syringae* multiplication. To determine if the reduced symptom development of the Nt+*ipt* line inoculated with *P. syringae* pv. *tomato* was due to reduced multiplication of the bacteria, extracts from infected leaves were titered. Two hours after infiltration of the leaves with 4×10^7 cfu/ml *P. syringae* pv. *tomato* or pv. *tabaci*, no differences were observed in the concentration of bacteria in Nt+*ipt* or control Nt leaves (Fig. 2). By 12 h, the bacterial populations of *P. syringae* pv. *tabaci* increased greatly in both tobacco lines, but was substantially higher in the Nt+*ipt* transformed line. In contrast, populations of the incompatible *P. syringae* pv. *tomato* decreased in the Nt+*ipt* line compared with the Nt control where the populations remained at a similar level over the 12 h period following inoculation (Fig. 2).

Antioxidant enzyme levels in Nt+*ipt* plants overexpressing cytokinin. Because oxidative enzymes have been closely linked to plant stress and pathogenesis, the activity of SOD, CAT, and POD enzymes were compared in the Nt+*ipt* and Nt tobacco plants. Total activity of SOD and CAT isoenzymes in the transformed Nt+*ipt* leaf extracts were 53 and 57%, respectively, higher than that of the Nt leaf extract (Fig. 3). The total POD activity was reduced about 6% in Nt+*ipt* compared with the Nt control, but this difference was not significant.

Phospholipid, galactolipid, and free sterol composition of leaves. Overproduction of the cytokinins influenced the leaf membrane polar lipid and sterol composition. Nt+*ipt* leaves contained more PLs and GLs but less free sterols as compared to Nt control leaves (Table 1). As a consequence, the free sterol/phospholipid ratio in Nt+*ipt* plants was approximately half (0.24) that of the control (0.41), a characteristic generally associated with juvenile plant tissues (Table 1).

Disease symptoms on *ipt* transformed *N. plumbaginifolia* and *N. tabacum* cv. Xanthi plants. Other *Nicotiana* lines transformed with *ipt* gene constructs linked to inducible promoters were tested to determine whether they exhibited a similar delay in HR development. When transgenic *N. plumbaginifolia* (Npl+*ipt*) plants were inoculated with the incompatible *P. syringae* pv. *tomato*, necrosis development was delayed and was not as severe as in the untransformed controls (Fig. 4A and B). Inoculation of Npl+*ipt* plants resulted in no visible HR symptoms after 24 h

(Fig. 4A) and reduced HR symptoms after 48 h (Fig. 4B), compared with inoculations on Npl control plants. No differences in necrosis development were found when transgenic *ipt* *N. plumbaginifolia* and *N. tabacum* plants were inoculated with the compatible *P. syringae* pv. *tabaci* bacteria and compared with their respective controls (Figs. 4C and 5B). When the Ntx-*ipt* cytokinin overproducing Xanthi plants were inoculated with *P. syringae* pv. *tomato*, leaves that exhibited delayed senescence (24,28) also had reduced HR development compared with the Npl plants, but the differences were not significant at $P = 0.05$ level (Fig. 5A).

DISCUSSION

Our earlier results showed that the Nt+*ipt* line that carries the *ipt* gene fused to the CaMV 35S promoter had 130 to 190% more zeatin riboside and isopentenyl adenosyl type cytokinin content than the *N. tabacum* L. cv. Petit Havana SR1 control (32) and

Fig. 1. Symptom development on second and third leaves of *Nicotiana tabacum* cv. Petit Havana SR1 that were either transformed with the *ipt* gene (Nt+*ipt*) or untransformed (Nt). The 55-day-old tobacco were infiltrated with various concentrations of *Pseudomonas syringae* pv. *tomato* or pv. *tabaci*. Necrosis development in the infiltrated area was recorded at 24 and 48 h. Rating of symptoms was made by visual observation and based on a scale of 0 to 5, where 0 = no visible symptoms, 1 = only chlorosis, no necrosis, 2 = 25% necrosis, 3 = 50%, 4 = 75%, and 5 = 100% necrosis (3). Data are average of four replicates of a representative from three independent experiments with similar results. Bars show standard deviations.

expression of the *ipt* gene was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (24). The T2 generation of homozygous Nt*+**ipt*, *N. tabacum* cv. Xanthi-nc plants with the *ipt* gene fused to the wound-inducible proteinase inhibitor II promoter had characteristic phenotypes associated with cytokinin overproduction and elevated *ipt* transcript levels that were wound-inducible (28). Given these plants were wounded when they were infected with the *Pseudomonas* strain, no further wounding (*ipt* gene induction) was deemed necessary. Similarly, under the relative high light intensity (200 $\mu\text{mol}/\text{m}^2\text{s}$) in the greenhouse, the Np*+**ipt*, *N. plumbaginifolia* 43A plants, where the *ipt* is under the control of light-inducible Rubisco small subunit protein promoter, the zeatin type cytokinins were reported to be two to three times higher than in control plants (34).

The higher cytokinin content of all transgenic lines was also indicated by the slower senescence of the leaves and about a 2-week delay of flowering as well as by the reduced root development.

Inoculation of *P. syringae* pv. *tomato* into the cytokinin-overproducing Nt*+**ipt* transgenic line resulted in suppression or delay of HR necrosis, however, in the compatible interaction with

P. syringae pv. *tabaci*, cytokinin overproduction did not appear to affect symptom development. Bacterial multiplication of *P. syringae* pv. *tomato* was also delayed during the first 12 h in transformed leaves, while multiplication of *P. syringae* pv. *tabaci* was slightly enhanced. Further characterization of these transformed plants demonstrated (i) an increased level of SOD and CAT enzymes, which could enhance the antioxidant capacity, and (ii) a decreased sterol/phospholipid ratio in membrane composition which is associated with juvenile tissue.

The effect of *ipt* incorporation was tested in other *Nicotiana* species as well. In *N. plumbaginifolia*, the *ipt* transformed plants had an even stronger delay in symptom development in response to *P. syringae* pv. *tomato*, while symptom development was somewhat enhanced in response to *P. syringae* pv. *tabaci*. In *N. tabacum* cv. Xanthi, the effect of the *ipt* gene on the symptom development was not as dramatic with either pathogen. There was a slight delay in symptom development with *P. syringae* pv. *tomato* and slight enhancement with *P. syringae* pv. *tabaci*. It is noteworthy, that external applications of cytokinins similarly suppressed the bacterially induced hypersensitive reaction (20).

Fig. 2. Multiplication ($\times 10^3$ colony forming unit ml^{-1}) of *Pseudomonas syringae* pv. *tomato* and *P. syringae* pv. *tabaci* bacteria in leaves of 55-day-old Nt*+**ipt* and Nt tobacco. The bacterial concentration was determined by dilution plating on nutrient agar plates. Data are average of three replicates of a representative from three independent experiments with similar results. Bars show standard deviations.

Fig. 3. Activity (relative units) of superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) enzymes of *Nicotiana tabacum* cv. Petit Havana SR1 tobacco leaves either transformed with the *ipt* gene (Nt*+**ipt*) or untransformed (Nt). Enzyme activities were evaluated by Shimadzu CS-930 scanner. Data are average of three replicates of a representative from three independent experiments with similar results. Bars show standard deviations.

TABLE 1. Comparison of the phospho-(PL) and galactolipid (GL), as well as the free sterol (St) content in transformed (Nt*+**ipt*) or untransformed (Nt) leaves of *Nicotiana tabacum* cv. Petit Havana SR1^a

Lipids ^b	Nt (nmole/g fwt)	Nt <i>+</i> <i>ipt</i> (nmole/g fwt)	% of Nt control
PI	49 ± 22	64 ± 8	130.6
PC	240 ± 20	300 ± 106	125.0
PG	150 ± 30	198 ± 42	132.0
PE	175 ± 31	210 ± 30	120.0
Total PL ^c	614 ± 59	772 ± 57	125.7
MGDG	515 ± 10	658 ± 18	127.8
DGDG	371 ± 77	500 ± 32	134.8
Total GL ^c	886 ± 87	1158 ± 50	130.7
St ^c	251 ± 10	189 ± 22	75.3
St/PL	0.41	0.24	

^a The second and third leaves of 60-day-old plants were used. Data are the average of four replicates of a representative from three independent experiments with similar results.

^b PI = phosphatidylinositol, PC = phosphatidylcholine, PG = phosphatidylglycerol, PE = phosphatidylethanolamine, MGDG = monogalactosyldiglyceride, DGDG = digalactosyl-diglyceride.

^c Least significant differences at *P* = 0.05 for total PL, GL, and St are 113, 134, and 31 nmol g fwt⁻¹, respectively.

In regards to the effect of the inhibition of plant senescence by cytokinins on multiplication of bacteria in leaf tissue, again, differences were found in compatible and incompatible interactions. Although bacterial multiplication does appear to be reduced in the incompatible reaction, suppression of necrotization (i.e., a suppressed HR response) may actually be associated with de-

creased resistance. This suggests that cytokinin overproduction suppresses both necrotization (Figs. 1A and 4A) and bacterial multiplication in incompatible, but not in compatible interactions, when the bacterial multiplication is rather increased (Fig. 2).

One of the reasons for the elevated tolerance of cytokinin overproducing tissues to bacteria-induced necroses is probably the reduced accumulation of H_2O_2 , which is likely a consequence of the augmented antioxidant capacity (Fig. 3). In addition, it has to be emphasized that less H_2O_2 was detected by 3,3'-diaminobenzidine (DAB) staining in Nt+*ipt* than in Nt leaves and more H_2O_2 accumulated in *Tobacco necrosis virus* infected Nt than in Nt+*ipt* tobacco lines, which was probably due to the spectrophotometrically measured higher antioxidant capacity in cytokinin overproducing tobaccos (24). Senescence of plant tissues was accompanied by elevated H_2O_2 production and reduced antioxidant activity (12,15,16,33) and higher antioxidant activity was found in cytokinin overproducing tobacco line (P_{SAG12}-IPT) with a senescence-specific transgene promoter (8,10).

The other important factor of the higher resistance of tobacco lines with delayed senescence to the bacterially induced hypersensitive reaction is the more stable membrane lipid composition.

Fig. 4. Symptom development on second and third leaves of *Nicotiana plumbaginifolia* that were either transformed with the *ipt* gene (Npl+*ipt*) or untransformed (Npl). The 55-day-old tobacco were infiltrated with various concentrations of *Pseudomonas syringae* pv. *tomato* or pv. *tabaci*. Necrosis development in the infiltrated area was recorded at **A and C**, 24 and **B**, 48 h. Rating of symptoms was made by visual observation and based on a scale of 0 to 5, where 0 = no visible symptoms, 1 = only chlorosis, no necrosis, 2 = 25% necrosis, 3 = 50%, 4 = 75%, and 5 = 100% necrosis (3). Data are the average of four replicates of a representative from three independent experiments with similar results. Bars show standard deviations.

Fig. 5. Symptom development on second and third leaves of *Nicotiana tabacum* cv. Xanthi that were either transformed with the *ipt* gene (Ntx+*ipt*) or untransformed (Ntx). The 55-day-old tobacco were infiltrated with various concentrations of *Pseudomonas syringae* **A**, pv. *tomato* or **B**, pv. *tabaci*. Necrosis development in the infiltrated area was recorded at 24 h. Rating of symptoms was made by visual observation and based on a scale of 0 to 5, where 0 = no visible symptoms, 1 = only chlorosis, no necrosis, 2 = 25% necrosis, 3 = 50%, 4 = 75%, and 5 = 100% necrosis (3). Data are the average of four replicates of a representative from three independent experiments with similar results. Bars show standard deviations.

Significant decrease in molar ratio of free sterols to phospholipids was measured in Nt+*ipt* than in Nt tobacco leaves (Table 1). It has been reported earlier that senescence is associated with the breakdown of membrane phospholipids, which is mainly attributed to phospholipases (5,7,19). A lipid-derived senescence retardant inhibits phospholipase D, the key enzyme involved in degradation of membrane phospholipids during the early stages of plant senescence (25). The significant and complex role of lipids and lipid-modifying enzymes in plant disease resistance was emphasized very recently (26).

In conclusion, the overproduction of cytokinins in plant tissues induced by the expression of the cytokinin biosynthesis gene *ipt* appears to suppress the HR symptoms induced by incompatible bacteria. The reduced HR is likely due, at least in part, to the higher antioxidant capacity and more stable membrane lipid composition of the *ipt* transformed tissues. On the other hand, we do not know the role of this type of symptom resistance in the interaction between disease resistance proteins and effectors of bacteria, which is still an open question (3,18).

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LITERATURE CITED

1. Ádám, A. L., Bestwick, C. S., Barna, B., and Mansfield, J. W. 1995. Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction to *Pseudomonas syringae phaseolicola*. *Planta* 196:240-249.
2. Baker, C. J., Atkinson, M. M., Roy, M. A., and Collmer, A. 1986. Inhibition of the hypersensitive response in tobacco by pectate lyase. *Physiol. Mol. Plant Pathol.* 29:217-225.
3. Baker, C. J., Mock, N. M., Whitaker, B. D., Roberts, D. P., Rice, C. P., Deahl, K. L., Aver'yanov, A. A. 2005. Involvement of acetosyringone in plant-pathogen recognition. *Biochem. Biophys. Res. Commun.* 328:130-136.
4. Baker, C. J., and Orlandi, E. W. 1995. Active oxygen in plant pathogenesis. *Annu. Rev. Phytopathol.* 33:299-321.
5. Barna, B., and Györgyi, B. 1992. Resistance of young versus old tobacco leaves to necrotrophs, fusaric acid, cell-wall degrading enzymes and autolysis of membrane lipids. *Physiol. Mol. Plant Pathol.* 40:247-257.
6. Barna, B., and Pogány, M. 2001. Antioxidant enzymes and membrane lipid composition of disease resistant tomato plants regenerated from crown galls. *Acta Physiol. Plant.* 23:273-277.
7. Borochoy, A., Halevy, A. H., and Shinitzky, M. 1982. Senescence and the fluidity of rose petal membranes. *Plant Physiol.* 69:296-299.
8. Dertinger, U., Schaz, U., and Schulze, E. D. 2003. Age-dependence of the antioxidative system in tobacco with enhanced glutathione reductase activity or senescence-induced production of cytokinins. *Physiol. Plant* 119:19-29.
9. Elstner, E. F., and Osswald, W. 1994. Mechanism of oxygen activation during plant stress. Pages 131-154 in: *Oxygen and Environmental Stress in Plants*, 102B. R. M. M. Crawford, G. A. F. Hendry, and B. A. Goodman, eds. The Royal Society of Edinburgh, Edinburgh, UK.
10. Gan, S., and Amasino, R. M. 1997. Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. *Plant Physiol.* 113:313-319.
11. Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43:205-227.
12. Jiménez, A., Hernández, J. A., Pastori, G., del Río, L. A., and Sevilla, F. 1998. Role of the ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. *Plant Physiol.* 118:1327-1335.
13. Király, L., and Király, Z. 2006. To die or not to die – Is cell death dispensable for resistance during the plant hypersensitive response? *Acta Phytopathol. Hun.* 41:11-21.
14. Király, Z., Barna, B., Kecskés, A., and Fodor, J. 2002. Down-regulation of antioxidative capacity in a transgenic tobacco which fails to develop acquired resistance to necrotization caused by TMV. *Free Radical Res.* 36:981-991.
15. Király, Z., El-Zahaby, H., Galal, A., Abdou, S., Ádám, A., Barna, B., and Klement, Z. 1993. Effect of free radicals on plant pathogenic bacteria and fungi and on some plant diseases. Pages 9-19 in: *Oxygen Free Radicals and Scavengers in the Natural Sciences*. Gy. Mózsik, I. Emerit, J. Fehér, B. Matkovics, and Á. Vincze, eds. Akadémia Kiadó, Budapest, Hungary.
16. Lacan, D., and Baccou, J. C. 1998. High levels of antioxidant enzymes correlate with delayed senescence in nonnetted muskmelon fruits. *Planta* 204:377-382.
17. Leshem, Y. 1988. Plant senescence processes and free radicals. *Free Radical Biol. Med.* 5:39-49.
18. Martin, G. B., Bogdanove, A. J., and Sessa, G. 2003. Understanding the functions of plant disease resistance proteins. *Annu. Rev. Plant Biol.* 54:23-61.
19. McKersie, B. D., Lepock, J. R., Kruuv, J., and Thompson, J. E. 1978. The effects of cotyledon senescence on the composition and physical properties of membrane lipid. *Biochim. Biophys. Acta* 508:197-212.
20. Novacky, A. 1972. Suppression of the bacterially induced hypersensitive reaction by cytokinins. *Physiol. Plant Pathol.* 2:101-104.
21. Orlandi, E. W., Hutcheson, S. W., and Baker, C. J. 1992. Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspensions treated with *Pseudomonas syringae* pv. *glycinea*. *Physiol. Mol. Plant P.* 40:173-180.
22. Ouf, M. F., Gazar, A. A., Shehata, Z. A., Abdou, E. S., Király, Z., and Barna, B. 1993. The effect of superoxide anion on germination and infectivity of wheat stem rust (*Puccinia graminis* Pers. f. sp. *tritici* Eriks. and Henn.) uredospores. *Cereal Res. Commun.* 21:31-37.
23. Peng, M., and Kuc, J. 1992. Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disks. *Phytopathology* 82:696-699.
24. Pogány, M., Koehl, J., Heiser, I., Elstner, E. F., and Barna, B. 2004. Juvenility of tobacco induced by cytokinin gene introduction decreases susceptibility to *Tobacco necrosis virus* and confers tolerance to oxidative stress. *Physiol. Mol. Plant Pathol.* 65:39-47.
25. Ryu, S. B., Karlsson, B. H., Özgen, M., and Palta, J. P. 1997. Inhibition of phospholipase D by lysophosphatidylethanolamine, a lipid-derived senescence retardant. *Proc. Natl. Acad. Sci. USA* 94:12717-12721.
26. Shah, J. 2005. Lipids, lipases, and lipid-modifying enzymes in plant disease resistance. *Annu. Rev. Phytopathol.* 43:229-260.
27. Smigocki, A. C. 1991. Cytokinin content and tissue distribution in plants transformed by a reconstructed isopentenyl transferase gene. *Plant Mol. Biol.* 16:105-115.
28. Smigocki, A. C. 1995. Expression of a wound-inducible cytokinin biosynthesis gene in transgenic tobacco: Correlation of root expression with induction of cytokinin effects. *Plant Sci.* 109:153-163.
29. Smigocki, A. C., Neal, J. W., Jr., McCanna, I., and Douglass, L. 1993. Cytokinin-mediated insect resistance in Nicotiana plants transformed with the *ipt* gene. *Plant Mol. Biol.* 23:325-335.
30. Smigocki, A., and Wilson, D. 2004. Pest and pathogen resistance enhanced by heterologous suppression of a cytochrome P450 gene *CYP72A2*. *Biotechnol. Lett.* 26:1809-1814.
31. Staveland, J. R., and Slana, L. J. 1971. Relation of leaf age to the reaction of tobacco to *Alternaria alternata*. *Phytopathology* 61:73-78.
32. Székács, A., Hegedus, G., Tóbiás, I., Pogány, M., and Barna, B. 2000. Immunoassays for plant cytokinins as tools for the assessment of environmental stress and disease resistance. *Anal. Chim. Acta* 421:135-146.
33. Takahama, U., Hirotsu, M., and Takayuki, O. 1999. Age-dependent changes in levels of ascorbic acid and chlorogenic acid, and activities of peroxidase and superoxide dismutase in the apoplast of tobacco leaves: Mechanism of the oxidation of chlorogenic acid in the apoplast. *Plant Cell Physiol.* 40:716-724.
34. Thomas, J. C., Smigocki, A. C., and Bohnert, H. J. 1995. Light-induced expression of IPT from *Agrobacterium tumefaciens* results in cytokinin accumulation and somatic stress symptoms in transgenic tobacco. *Plant Mol. Biol.* 27:225-235.