Changes in Phenol Metabolism Associated with Induced Systemic Resistance to Tobacco Mosaic Virus in Samsun NN Tobacco

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ABSTRACT

Induction of systemic resistance in upper leaves of Samsun NN tobacco following inoculation of the lower leaves with tobacco mosaic virus was not accompanied by changes in activity of phenylalanine ammonium lyase or in concentration of total phenols, orthodihydroxyphenols, or chlorogenic acid. Challenge inoculation of these resistant upper leaves, however, effected an earlier and greater decrease in the concentration of these phenolic compounds during and subsequent to lesion formation than that occurring when nonresistant leaves were similarly inoculated. Also, the abrupt increase in phenylalanine ammonium lyase activity occurring at about the time of initial lesion formation was greater in resistant than in nonresistant leaves. A few hours later, activity of this enzyme decreased abruptly in both types of leaves. These results support the hypothesis that accelerated necrotic and metabolic responses of resistant leaves after challenge inoculation are due to rapid peroxidase-mediated quinone accumulation at points of infection. We suggest that the mechanism responsible for cessation of lesion expansion is the eventual collapse, largely due to quinone accumulation, of a ring of cells just in advance of virus infection or shortly thereafter. Phytopathology 61:1261-1265.

Additional key words: hypersensitivity, local lesions.

Necrotic lesions appear in leaves of tobacco varieties containing the *N* gene for hypersensitivity (3) on the 2nd day after inoculation with tobacco mosaic virus (TMV), expand rapidly for 3-5 days, and enlarge more slowly thereafter; lesion expansion has usually ceased by the 10th day after inoculation. The resistance to TMV multiplication of tissues just beyond the periphery of necrotic lesions is presumably implicated in the limitation of lesion expansion and cell-to-cell spread of the virus.

Formation of TMV lesions in lower leaves of hypersensitive tobacco is followed by development of enhanced hypersensitivity to TMV, referred to as systemic resistance, throughout the plant (9). Development of this condition is accompanied by permanently increased peroxidase and catalase activity (11, 12). After challenge inoculation, the rapid phase of expansion of TMV lesions in the upper leaves of such plants terminates early, and the lesions consequently fail to achieve normal size. When high-titer inocula are used, lesions appear sooner in resistant than in nonresistant leaves (12); also, the metabolic changes concomitant to lesion appearance occur first in resistant leaves (12).

We have hypothesized (11, 12) that necrogenesis in TMV-infected leaves of hypersensitive tobacco is due to peroxidase-mediated accumulation of toxic quinones at infection points, and that the accelerated responses of resistant leaves after TMV-inoculation are a consequence of their high peroxidase activity which, by causing rapid accumulation of quinones to toxic concentration, quickly brings about necrosis and all the events associated with necrosis.

In order to test this hypothesis, estimates were made of the concentration of total reduced phenols, orthodihydroxyphenols, and chlorogenic acid in leaves of hypersensitive tobacco at intervals during the induction of systemic resistance and subsequent to challenge inoculation with high-titer TMV inocula. Because phenylalanine ammonium lyase (PAL-ase) catalyzes the first reaction of the hydroxycinnamic acid pathway, the main avenue of phenol production in plant tissues (6), the activity of this enzyme was also followed during resistance induction and after subsequent inoculation.

MATERIALS AND METHODS.—*Nicotiana tabacum* L. 'Samsun NN', a local lesion host for TMV, was used. Plants were grown in a greenhouse as previously described (12); after inoculation they were held in a controlled environment chamber (12) unless otherwise stated.

Inocula, prepared from frozen clarified juice of Turkish tobacco plants systemically infected with TMV (common strain), were applied with artists' brushes to leaf surfaces previously dusted with Carborundum. Leaves were rinsed immediately after inoculation. In simulated inoculations of leaves of control plants, tap water was substituted for virus inocula.

Plants in the six- to eight-leaf stage were topped and trimmed to provide four adjacent leaves, the two lowest of which were well expanded and the two upper ones still expanding. The two lower leaves were inoculated with TMV inoculum diluted to induce 300-400 lesions/leaf. Challenge inoculations of the two upper leaves were made 6-8 days later with high-titer inocula (juice diluted 10^{-1}).

PAL-ase extraction and assay were based on described procedures (1). Extracts were prepared from 10 g freshly harvested leaf tissues ground at 4 C for 2 min in a Virtis 45 homogenizer in 20 ml redistilled acetone which had been kept at −20 C until use. The homogenate was filtered through a Büchner funnel; the residue, collected on Whatman No. 5 filter paper, was washed twice with 25 ml of cold acetone and dried first under an air stream for 1 hr, then dried in a bell

jar under reduced pressure for 1 hr at room temperature. The dry acetone powder was stored at −20 C.

Prior to assay, the acetone powder was suspended in 0.1 M borate buffer at pH 8.8 (0.1 g powder/6 ml buffer). The mixture was stirred occasionally in an ice bath for 80 min, then filtered through a double layer of cheesecloth. The filtrate was centrifuged at 3,000 *g* at 4 C for 12 min, then assayed for PAL-ase activity by a method based on the rate of conversion of L-phenylalanine to cinnamic acid. Reaction mixtures, consisting of 1.5 ml of enzyme solution, 2.5 ml of 0.1 M borate buffer (pH 8.8), and 1.0 ml of freshly prepared 0.05 M L-phenylalanine, were incubated for 1 hr in a test tube in a water bath at 40 C. The reaction was stopped by addition of 0.1 ml of 5 M HCl. The acidified reaction mixture was extracted with 7.5 ml of ether, and 5.0 ml of the ether phase was dried under an air stream. The absorbance of the residue dissolved in 4 ml of 0.05 M NaOH was measured at 269 nm in a Beckman DB spectrophotometer, cinnamic acid concentration was estimated by reference to a standard curve, and PAL-ase activities were calculated as milligrams of cinnamic acid formed per gram dry weight of tissue, correction being made for a zero time control.

For total phenol and orthodihydroxyphenol determinations, 9 g freshly harvested interveinal leaf tissue were boiled in 40 ml 95% ethanol for 2 min, then cooled to about 28 C; the leaf material and extract were ground together for 2 min at high speed in a Virtis 45 homogenizer. The resultant slurry was vacuum-filtered through ethanol-washed Whatman No. 5 filter paper, and the residue washed twice with 25 ml 95% ethanol. The filtrate was adjusted to 150 ml 70% ethanol; a 25-ml volume was scrubbed with carbon tetrachloride until free from chlorophyll, then assayed. Extracts for chlorogenic acid estimations were prepared similarly; each was from 5 g leaf tissue in 15 ml 95% ethanol. Filtrate and washings, amounting to about 40 ml, were flash evaporated to about 3 ml and made up to 10 ml with 95% ethanol.

For total phenol estimation, 1 ml 2 N Folin-Ciocalteau phenol reagent (Fisher Scientific Co., Fair Lawn, N.J.) diluted 1:4 with distilled water was added to 4 ml of saturated $NaHCO_3$ and 1 ml ethanol extract in a colorimeter tube. The intensity of the blue-green color that developed was measured 5 min later at 395 nm in a Bausch & Lomb Spectronic 20 colorimeter; a standard curve was used to convert the results to milligram equivalents of chlorogenic acid per gram dry weight. Orthodihydroxyphenol assays were according to a procedure (5) based on measurement at 515 nm in a Bausch & Lomb Spectronic 20 colorimeter of the pink color that appears in a reaction mixture containing 0.5 ml ethanol extract, 0.5 ml 0.5 N HCl, 0.5 ml Arnow's reagent, 5.0 ml distilled water, and 1.0 ml NaOH. Measurement was made 30 sec after addition of the alkali. The extinction was referred to a standard curve, and results were expressed as milligram equivalents of chlorogenic acid per gram dry weight.

Chlorogenic acid in ethanol extracts was separated chromatographically and estimated colorimetrically according to the method of Johnson & Schaal (5). A 50-μliter aliquot of the extract was placed on Whatman No. 1 paper (15 × 15 inches) as a band 15 cm long. Separation was effected by descending chromatography with 1-butanol, acetic acid, and water in a 40:10:20 mixture as solvent. When the chlorogenic acid fraction, visible under ultraviolet light as a strongly fluorescing pale-blue band, neared the end of the chromatogram, the latter was removed from the tank and dried in an air stream at room temperature. The blue-fluorescing band of the chromatogram was eluted with 70% ethanol for 1 hr at room temperature, and extinction of the eluate at 324 nm was determined in a Beckman DB spectrophotometer and referred to a standard curve for estimation of the chlorogenic acid content of the eluate. Results were expressed as milligrams of chlorogenic acid per gram dry weight of leaf tissue.

RESULTS.—For a preliminary examination of PAL-ase activity during resistance induction in upper leaves of Samsun NN tobacco plants, the lower leaves of 15 plants were inoculated and the procedure simulated with water on 15 comparable control plants. Samples, consisting of five combined 2-g lots, each one of which was from a single plant, were taken from upper leaves of both types of plants at 1, 4, and 7 days subsequent to primary inoculation, and assayed for PAL-ase activity. No differences between samples from inoculated plants and those of the controls were detected at these times.

Upper leaves of resistant (lower leaves inoculated) and nonresistant (lower leaves rubbed) Samsun NN tobacco plants were challenge-inoculated with high-titer TMV inoculum. Sampling was as in the previously described experiment, and was made at a series of intervals after challenge. In a number of experiments, the results of one of which are presented (Fig. 1), the activity of the enzyme remained at the preinoculation level until the approximate time of lesion appearance, when a sharp rise, greater in resistant than in nonresistant leaves, occurred. This rise was brief, and was followed by an equally sharp fall. Shorter intervals between samplings would be necessary to establish whether or not maximum activity in resistant leaves is

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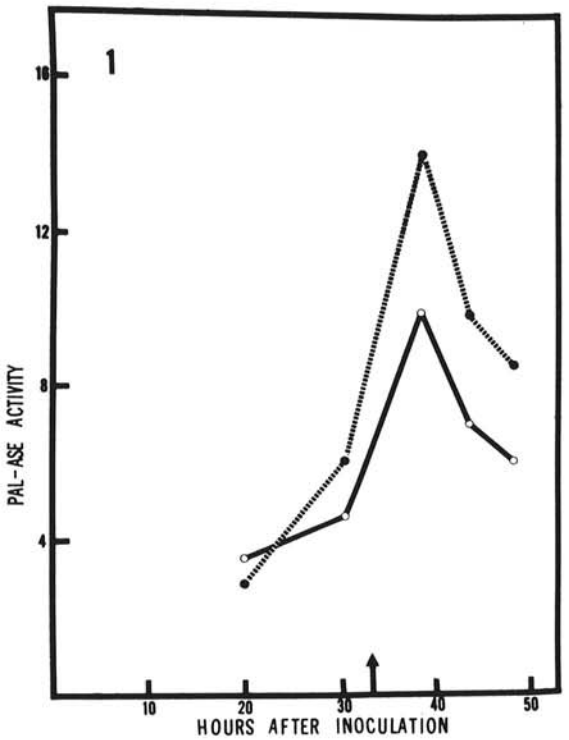
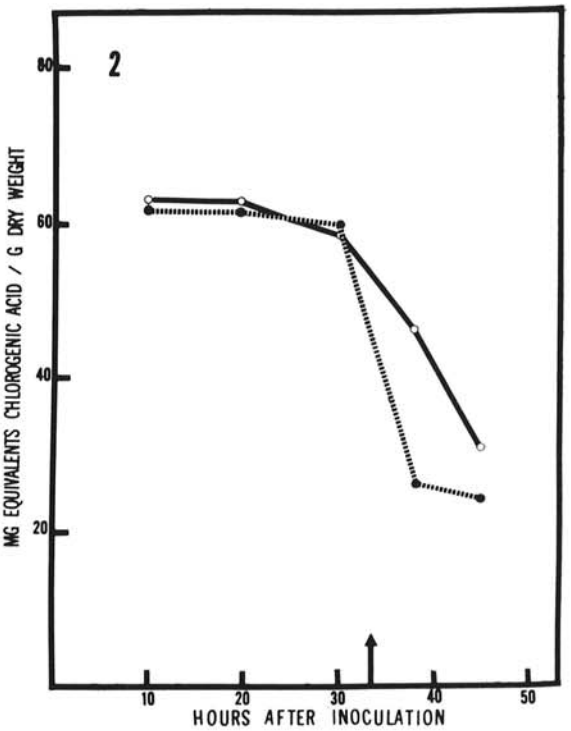
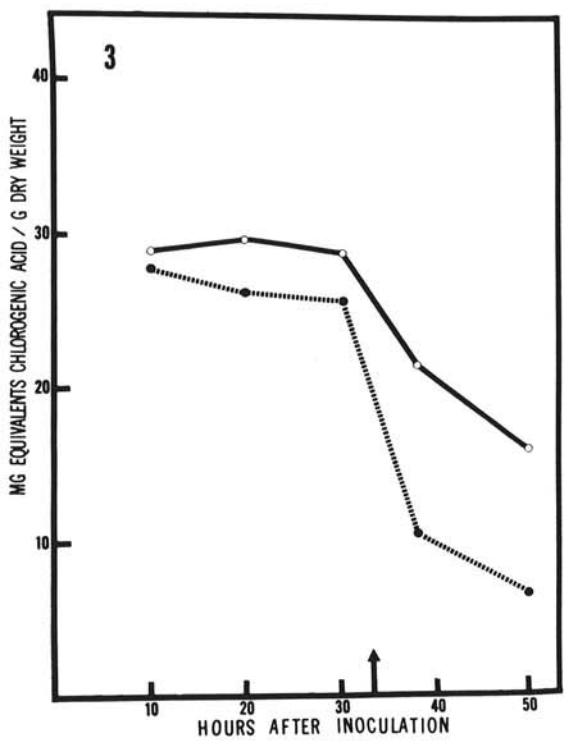
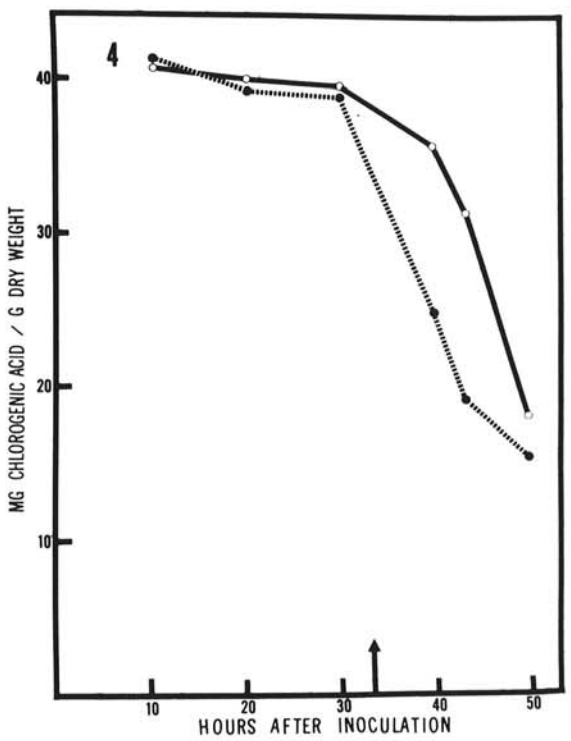
Fig. 1-4. Changes in activity of a phenol-synthesizing enzyme and in concentration of phenolics in resistant (broken line) and nonresistant (solid lines) Samsun NN tobacco leaves at intervals after inoculation with a potent tobacco mosaic virus (TMV) inoculum. Resistance was induced in upper leaves by prior (8 days) inoculation of lower leaves with TMV. Arrow on horizontal axis represents approximate time of lesion appearance following the challenge inoculation. **1)** Phenylalanine ammonium lyase (PAL-ase) activities estimated by rate of conversion of L-phenylalanine *trans*-cinnamic acid by acetone powders. Based on a single experiment, but similar results were obtained in several others. **2)** Changes in concentration of total phenols. Average of three experiments. **3)** Changes in concentration of orthodihydroxyphenols. Average of three experiments. **4)** Changes in concentration of chlorogenic acid. Average of three experiments.


1
PAL-ASE ACTIVITY
16
12
8
4
10
20
30
40
50
HOURS AFTER INOCULATION


2
MG EQUIVALENTS CHLOROGENIC ACID / G DRY WEIGHT
80
60
40
20
10
20
30
40
50
HOURS AFTER INOCULATION


3
MG EQUIVALENTS CHLOROGENIC ACID / G DRY WEIGHT
40
30
20
10
10
20
30
40
50
HOURS AFTER INOCULATION


4
MG CHLOROGENIC ACID / G DRY WEIGHT
40
30
20
10
10
20
30
40
50
HOURS AFTER INOCULATION

coincident with that in nonresistant leaves; it does seem clear, however, that maximum activity in the former is greater than in the latter.

Experiments to examine total phenol content of leaf tissues during the induction of systemic resistance were conducted in the greenhouse. In each of four experiments, the lower leaves of 24 plants were inoculated and extracts of upper leaves (each 9-g sample consisting of 3 g from each of three plants) were prepared at daily intervals. Their total phenol contents compared with those of comparable controls. Although great variation, both among and within individual experiments, was encountered, differences between contrasted treatments were not consistent and showed no evident trend; we concluded, therefore, that resistance in leaf tissues is not associated with changes in total phenol content.

Total phenol, orthodihydroxyphenol, and chlorogenic acid concentrations in ethanol extracts (of resistant and nonresistant leaves) made at intervals after challenge inoculation were determined. Similar trends were shown by the three categories of compounds (Fig. 2-4). With each, concentrations remained uniform and at pre-inoculation levels until the approximate time of lesion appearance, when a sharp decrease occurred. In all cases (Fig. 2-4), the rate of decrease was greater initially in resistant than in nonresistant leaves; the rate later leveled off in resistant leaves, but no leveling off, except possibly in a single case (Fig. 3), was detected in nonresistant leaves within the sampling period.

DISCUSSION.—Induction of PAL-ase in plants seems to be a nonspecific response to injury (15), and the greater enhancement of this enzyme in resistant leaves than in nonresistant ones at lesion formation (Fig. 1) suggests that the extent of PAL-ase induction may be directly correlated with the rate and extent of necrogenesis in the early stages of infection (12). In the later stages of infection, however, a reversal occurs in the relative amounts of necrosis in the two kinds of leaves (12); after 40 hr, necrosis becomes progressively more extensive in nonresistant than in resistant leaves. The absence of corresponding reversal in the relative PAL-ase activities of the two types of leaves suggests that the observed changes are not restricted to lesion cells, actual or potential, but also occur in living cells beyond the lesion edge. That localized infections can induce various kinds of changes in advance of infection has been amply established (4, 8, 10, 13, 14). Thus, we infer that capacity for biosynthesis of phenols is increased in living cells bordering lesions at a time presumed critical with respect to induction in such cells of mechanisms that eventually operate to limit lesion size. The sharp decreases in PAL-ase, which cannot be attributed entirely to loss of functional cells, are suggestive of endproduct repression (15) or protein inhibition of PAL-ase synthesis (16); also, the decreases could be due to a rapid rate of protein turnover, with the PAL-ase activation (presumably stimulated enzyme synthesis) being only temporary.

As neither phenol concentration nor PAL-ase activity changes during or subsequent to resistance induction, when peroxidase activity is enhanced (11), spatial or functional separation of peroxidase and its phenolic substrates prior to challenge inoculation is indicated. This suggests that increased capacity for oxidation of phenols in resistant tissues constitutes a potential for a rapid hypersensitive response that is not expressed until virus-induced cellular disruption brings the enzyme into contact with its substrates. After infection, the capacity of cells to oxidize phenols is still further enhanced by an infection-induced stimulation of phenol-oxidizing enzymes (11, 12), and this additional stimulation is greater in resistant than in nonresistant tissues. If, as is postulated, infection brings enzymes and substrates together, it is not surprising that phenols were found to disappear more rapidly from resistant leaves than from nonresistant ones (Fig. 2-4). Moreover, the capacity of resistant tissues for phenol synthesis becomes greater during this period than does that of nonresistant tissues. Consequently, the difference between the rates of phenol oxidation in the two types of leaves may be greater than indicated by the actual data (Fig. 2-4).

We believe that our data support the conclusion of others (2, 7) that phenol oxidation is involved causally in necrotic lesion development. Also the data indicate that quinones may be among the principal agents bringing about, in cells just beyond the edge, the changes that ultimately block the advance of infection. Quinones certainly could be involved if the barrier eventually formed at the periphery of a lesion is a ring of collapsed cells (4, 10, 12), and they conceivably could be involved if the barrier is severance of intercellular cytoplasmic connections as a result of formation of paramural bodies between the plasmalemma and cell wall (14). Furthermore, our data strongly support the hypothesis (11) that the increased ability of tobacco leaves with virus-induced resistance to localize virus is due to their greatly enhanced ability to oxidize phenols after challenge inoculation.

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