Enhanced Peroxidase Activity Associated With Induction of Resistance to Tobacco Mosaic Virus in Hypersensitive Tobacco

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In hypersensitive tobacco inoculated with tobacco mosaic virus (TMV), lesion development is accompanied by induction of localized resistance just beyond the lesion edge (10); presumably this resistance plays a part in virus localization in the hypersensitive reaction. Lesion formation in lower leaves is followed by the development of resistance in upper leaves, although the virus remains confined to the necrotic area of such lesions (11). This systemic resistance, or increased hypersensitivity, is manifested by failure of lesions, induced by subsequent inoculation, to develop to normal size. Resistance to TMV has been reported to result from infection of tobacco leaves with different pathogenic agents (3, 4, 8), and from injection of tobacco leaves with heat-killed bacteria (6). Since increased levels of peroxidase activity (PA) were associated with TMV-induced resistance of tobacco to infection by *Pseudomonas tabaci* in tobacco leaves (7), we investigated the possibility that TMV-induced local and systemic resistance to subsequent inoculation with TMV in hypersensitive tobacco might be associated with increased activity of this enzyme. We believe that such an association is indicated by the data here reported.

Plants of *Nicotiana tabacum* L. 'Samsun NN' grown in 4-inch pots in a greenhouse were used in all experiments. After inoculation they were placed in a growth chamber maintained at 21 C and illuminated 16-hr/day with 1,400 ft-c of cool-white light, mostly from Sylvania VHO fluorescent tubes. Plants, chosen for vigor and uniformity, were decapitated and trimmed to four well-expanded leaves 1 day before inoculation. Crude juice of systemically infected tobacco diluted with distilled water was the source of TMV. Inoculation of leaves with a soft-haired artist's brush was preceded by dusting of the leaves with 400-mesh Carborundum, followed by immediate rinsing with tap water. Leaf samples for PA assay, consisting of 75 discs (diameter 2.1 cm) cut from interveinal tissues with a sharp cork borer, were ground immediately in 15 ml of cold 0.01 M phosphate buffer (pH 6.0) with a Virtis "45" homogenizer at high speed for 90 sec. The homogenate was filtered rapidly through glass wool and centrifuged at 3,300 g for 30 min. The supernatant was decanted into test tubes and kept in an icebath until assayed. Extracts stored at 4 C for 2 weeks lost only 7-9% of their peroxidase activity.

PA was estimated by the method of Cohen as described by Loebenstein & Linsey (5), in which the rate of purpurogallin formation from pyrogallol in the presence of leaf homogenate and H_2O_2 is followed colorimetrically. Pyrogallol reagent, made fresh before use, consisted of 10 ml of 0.5 M pyrogallol added to 12.5 ml of phosphate buffer (pH 6.0, 0.066 M) and diluted in 100 ml with distilled water. Diluted leaf homogenate (1:3 with 0.01 M phosphate buffer pH 6.0) was added in 0.2-ml portions to 5 ml of reagent and 0.5 ml 1% H_2O_2 in a cuvette which was inserted in a Bausch & Lomb "Spectronic 20" colorimeter, and the change in optical density at 420 mμ timed between 0.1 and 0.3.

Absolute PA values were calculated as (sec/cm² leaf area)$^{-1}$; results were sometimes expressed as the ratio of calculated absolute activities of extracts to those of comparable leaf extracts of noninoculated plants.

To study changes in PA in inoculated lower leaves and in upper leaves of the same plant during the period of resistance induction, each of two lower leaves of trimmed Samsun NN tobacco plants was inoculated with TMV (juice diluted 10^{-4}), and corresponding leaves of comparable control plants were rubbed with water. At intervals, homogenates for peroxidase assay were prepared from pooled samples of discs cut randomly from five leaves (15 discs/leaf).

Prior to each sampling, two upper leaves on each of a group of six inoculated plants subjected to the same conditions and treatment as those sampled for PA assay, and 6 comparable noninoculated control plants, were inoculated with TMV inoculum diluted to give 40-60 lesions/inoculated leaf. The diam of 200 lesions chosen randomly from each inoculated leaf was measured 7 days later with a microscope fitted with an ocular micrometer. The ratio of average diam of lesions in challenged leaves of previously inoculated plants to that of lesions in those of the control plants was used as a measure of the level of systemic resistance induced; the smaller the ratio, the greater the level of resistance.

Increased PA in homogenates of inoculated tissue coincided approximately with lesion appearance (Fig. 1), and gradually attained a level 2.7 times that of homogenates of the noninoculated controls at 3 days after inoculation. Significantly increased activity of the enzyme in noninoculated upper leaf homogenates was noted 4-5 days after primary inoculation, and attained a sustained maximum activity 2.9 times greater than the controls 3 days later.

The increased PA in upper leaves coincided with the development of resistance (Fig. 1). Comparable results were obtained in other similar experiments. In some, sampling was continued to 21 days after challenge inoculation; both PA and resistance reached a maximum at about the same time, and remained at a significantly higher level in primary-inoculated plants throughout the period than those not so treated.

In other experiments, half-leaves were inoculated with TMV. PA and resistance increased concomitantly in the opposite half-leaves, beginning 3-4 days after inoculation and reaching a maximum in about 7 days.

An increase in PA paralleled development of local resistance in tests in which narrow strips 20 mm apart were inoculated with TMV inoculum (juice diluted 10^{-2}). Increased PA was detected between the inoculated strips 6 hr after lesion appearance. By 7 days,
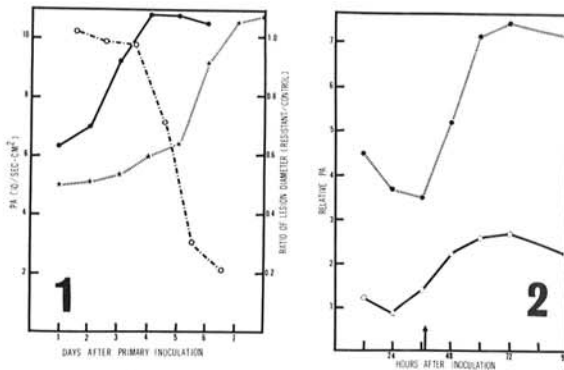
Fig. 1-2. 1) Changes in peroxidase activity (PA) associated with the induction of systemic resistance to tobacco mosaic virus (TMV) in Samsun NN tobacco. Ascending curves show PA of homogenates of TMV-inoculated (closed circles) lower leaves and of noninoculated upper leaves (black stars) of the same Samsun NN tobacco plants. PA of the control leaves (both the water-rubbed lower leaves and upper noninoculated leaves) did not increase appreciably with time, and thus data for such leaves are not shown. Development of resistance is shown by the decreasing ratio (white stars) of lesion diam in upper leaves challenge-inoculated with TMV at intervals following inoculation of lower leaves to lesion diam in challenge-inoculated upper leaves of control plants, the lower leaves of which were rubbed with water. **2)** Relative peroxidase activity (PA) of homogenates of TMV-inoculated resistant (closed circles) and nonresistant leaves (open circles) of Samsun NN tobacco; PA values are expressed relative to the PA of homogenates of noninoculated control leaves, no part of which were inoculated or rubbed. Resistance was induced by inoculation of three lower leaves of each plant with TMV 7 days before challenge inoculation of test leaves. Nonresistant control leaves were from plants of which the lower leaves had been rubbed with water.

PA in such areas was 5.7 times that of comparable samples taken from between strips brushed only with water. The area between inoculated strips, but not that between control strips, was highly resistant to TMV inoculation.

Systemic resistance was induced in a group of 35 tobacco plants by inoculation of lower leaves. Lower leaves were removed 8 days later, and the upper leaves were inoculated with potent TMV inoculum (juice, 1-100). In both primary-inoculated and control plants, PA was less in samples taken at 24 hr than in those taken at 12 hr after inoculation. This decline may have reflected a temporary condition caused by the injury resulting from inoculation. It was followed in both resistant and nonresistant leaves by increases in PA that began about the time of lesion formation and continued during the time of lesion development (Fig. 2). The PA increase was more marked in resistant leaves than in nonresistant ones, despite the subsequent development of more extensive necrosis in the latter.

The importance of increased levels of peroxidase activity in restricting movement of pathogens has been questioned (9), since markedly increased PA is detectable only in the necrogenic phase of certain host-pathogen relationships. The facts that (i) PA increase in upper leaves is a concomitant of resistance develop-

ment induced by virus infection in lower leaves; (ii) PA increase is maintained in leaves with such resistance; and (iii) PA levels that develop in resistant and nonresistant leaves are inversely correlated to the area of necrotic tissue developing in the two kinds of leaves after inoculation, lead us to suggest a direct role for peroxidase in the localization of TMV. So far, this enzyme is the only factor that has been correlated with induced resistance. No correlation was detected between induced resistance and polyphenoloxidase (1); ribonuclease (2); pectinmethylesterase (2); free amino acids and amides (1); or respiration rate (1).

The exact nature of the putative role of peroxidase still needs to be defined. We have found (*unpublished data*) orthodihydric phenols (known substrates of the enzyme in the presence of peroxide) to decrease more rapidly during lesion formation in resistant leaves than in nonresistant ones. Accordingly, it is tempting to suggest an unusually rapid accumulation of quinones at or near the site of virus synthesis as being directly and causally implicated in the enhanced hypersensitivity of resistant leaves. Until data concerning the other physiological changes associated with enhanced activity of this diversifunctional enzyme are assembled, however, these suggestions must remain speculative.

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