The Role of Cytoplasmic Mycolaminaran in Inhibiting Initial Viral Infection of Certain *Nicotiana* species

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ABSTRACT

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The effect of mycolaminaran, a β-1,3-glucan purified from the cytoplasm of *Phytophthora megasperma*, on initial infection by tobacco mosaic virus of *Nicotiana* species was tested. When mixed at 62–1,000 μg/ml with TMV inoculum and applied to *Nicotiana tabacum* 'Xanthi-nc', mycolaminaran reduced necrotic local lesion numbers by as much as 98%. In contrast, laminaran, a mannose-containing glucan derived from an alga, reduced infection by only 40–75%. Mycolaminaran did not reduce initial viral infection of *Nicotiana rustica*. This established that the polysaccharide inhibited infection but did not inactivate the virus. Mycolaminaran inhibited infection by TMV RNA as well as by TMV virions, indicating that viral infection was blocked at a stage after uncoating. When applied 4 h after inoculation, however, mycolaminaran did not reduce the number of viral lesions. Therefore, the affected stage was early in infection. The glucan also inhibited TMV infection in *N. tabacum* 'Xanthi' that lacked genes for necrotic reaction with TMV, establishing that such genes were not required for the observed inhibition. We found no evidence, however, for any systemic resistance induced by mycolaminaran. These results are consistent with the hypothesis that mycolaminaran specifically and rapidly induces a general resistance in certain plants to local viral infection on leaf surfaces treated with the glucan.

Many fungi produce compounds that inhibit plant virus infection (9,21) by inducing resistance in host plants (2,5,6). For example, cell walls of *Phytophthora infestans* contain a polysaccharide that, when mixed at 100 μg/ml with potato virus X before inoculation to tobacco, induces resistance to viral infection (7,18,22). The characteristics of the polysaccharide from *P. infestans* are similar to those of mycolaminaran, a water-soluble and branched β-1,3-linked D-glucan with an average degree of polymerization of approximately 36 glucose units (20). Mycolaminaran, in turn, resembles laminaran, a glucan produced notably by the alga *Laminaria*.

Recently, Kopp et al (11) showed that a glucan preparation (14,15) from *Phytophthora megasperma* Drechs. f. sp. *glycinea* that contained a highly-branched β-D-glucan at concentrations as low as 0.1 μg/ml protects four species of *Nicotiana* from infection by several single-stranded RNA viruses. The glucan inhibits infection by viral RNA as well as by virions and fails to block lesion formation if applied more than 10 h after a leaf has been inoculated with the virus. They concluded that the resistance results from a previously undescribed mechanism of plant defense.

We report that soluble mycolaminaran from the cytoplasm of *P. megasperma* induced resistance in three *Nicotiana* species to tobacco mosaic virus infection. This induced resistance is independent of necrosis genes, functions against both viral particles and RNA, but is not similarly induced by laminaran, nor is it induced in *N. rustica*.

MATERIALS AND METHODS

Viruses. Common strains of TMV and of southern bean mosaic virus (SBMV) were obtained from R. W. Fulton. A strain of sunn-hemp mosaic virus that causes necrotic lesions on pinto bean (*Phaseolus vulgaris* L.) is described elsewhere (23) and is referred to as SHMV-n. TMV and SHMV-n were purified as described by Sherwood and Fulton (16), and SBMV was purified as described by Hull (8). TMV RNA was prepared by the water-saturated phenol method of Ralph and Berquist (13) as modified by Sherwood and Fulton (16). Inoculations were made on corundum-dusted leaves with cheesecloth pads dipped in inoculum of virus or with RNA suspended in 30 mM sodium phosphate buffer, pH 8. Plants were grown in commercial soilless mix in 10-cm pots in the greenhouse and were fertilized weekly.

Description of tests. The effect of mycolaminaran on viral infection was tested in two ways. In the first method, powdered mycolaminaran was weighed, dissolved in buffer at twice the desired final concentration, and mixed with an equal volume of virus at twice the desired final concentration (usually 1× = 0.3 μg/ml). Control inoculum was made by mixing equal volumes of the 2× virus and buffer. Amended inoculum was applied to one half of a leaf, and control inoculum was applied to the opposite half-leaf. Infectivity was measured by counting the number of local lesions per half-leaf 4–7 days after inoculation. Only tests in which the average number of lesions per half-leaf on controls exceeded 20 are included here. Data are paired, however, and therefore are presented as averages and standard errors of ratios of lesions from amended inoculum divided by lesions from the opposite half-leaf.

In the second test, mycolaminaran and inoculum were separately prepared to the final concentrations and applied sequentially to leaves. Mycolaminaran was applied to one half of a leaf, and buffer was applied to the opposite half. Inoculum was then applied, using a fresh cheesecloth pad for each half-leaf to prevent the spread of mycolaminaran to control leaves. Data were expressed as described above. All trials were repeated at least twice, with a total of at least eight leaves per treatment.

In certain tests we used starch lesions processed as described by Linder et al (12) to detect TMV infection on cultivar Xanthi lacking any gene for necrosis in response to TMV infection.

Tests of systemic acquired resistance induced by mycolaminaran were performed by inoculating young plants with 500 μg/ml mycolaminaran in buffer; control plants were rubbed with buffer. After 9 days, virus was applied to newly developed leaves that had not been previously treated. The average lesions per leaf on mycolaminaran-treated plants and on controls were compared.

Isolation and purification of mycolaminaran. *P. megasperma* 695T, used as the source of mycolaminaran, was obtained from J. Hancock (Univ. Calif., Berkeley). For the isolation and purification of mycolaminaran the procedure of Faro (4) was used with modifications. Twelve grams of *P. megasperma* mycelium grown as described by Shumard et al (17) was mixed with twice the volume of 0.15 M NaCl, pH 7.2, and treated in a Waring blender for 10 min. To this mixture 24 ml of water-

saturated phenol was added and homogenized for an additional 2 min. The mixture was centrifuged at 3,500 *g* for 10 min, and the pellet was discarded. To the supernatant 0.25 volume of a 9:1 (v/v) mixture of chloroform/amyl alcohol was added, mixed thoroughly, and centrifuged at 3,500 *g* for 10 min. The solvent phase was discarded and the aqueous phase was repeatedly treated with chloroform/amyl alcohol mixture as described above until no precipitate formed at the interphase. The aqueous phase was precipitated with 2.5 volumes of cold 95% ethanol overnight at −20 C. The precipitate was sedimented by centrifugation at 3,500 *g* for 10 min, washed by resuspension and centrifugation in 15 ml of cold absolute ethanol, followed by 10 ml of ether, and dried under a stream of nitrogen.

RESULTS

The identity of the mycolaminaran was established by infrared spectra obtained by using the pressed KBr technique with laminaran (from *Laminaria digitata*; Sigma, Lot 77F-3885) as standard. Laminaran was used as the reference standard because in this polysaccharide the β-1,3 and the β-1,6 linkages are well characterized (3) and because its infrared spectrum has been shown previously to be identical to that of mycolaminaran (4). The spectra of laminaran and mycolaminaran showed an absorption peak at 890 cm^{-1} indicating β-linkages (1). In addition, the spectra showed remarkable similarity in the region between 950 and 1,200 cm^{-1}, suggesting that like laminaran, mycolaminaran contains β-1,3 linkages with β-1,6 branches (4).

Our initial purposes were 1) to confirm that mycolaminaran inhibited virus infection in plants, and 2) to compare the inhibition with that of other similar polysaccharides. We first compared the effect of the addition of one of three different carbohydrates to TMV inoculum applied to cultivar Xanthi-nc. Mycolaminaran inhibited infection approximately 4–20 times more than laminaran; glycogen did not inhibit infection significantly (Table 1). This result confirmed that mycolaminaran specifically inhibited infection of tobacco by TMV.

We then confirmed several results of Singh et al (18). The glucan at 62 μg/ml reduced TMV infection in cultivar Xanthi-nc and in *N. glutinosa* to 23 and 30% of controls, respectively, but at 500 μg/ml, mycolaminaran did not reduce TMV infection of *N. rustica*. Time course studies showed that the glucan applied at 500 μg/ml to Xanthi-nc 4 h before inoculation with TMV reduced viral infection to 28% of controls, and, when applied 7 days before inoculation, it reduced viral infection to 58% of controls. When mycolaminaran at 500 μg/ml was applied 30 and 240 min after TMV, however, treated half-leaves produced 59 and 108% as many lesions as the opposite untreated half-leaves. Mycolaminaran at 500 μg/ml did not induce detectable resistance in Xanthi-nc when the glucan was applied to the bottomside of a leaf and the virus was inoculated on the topside, even when virus was applied 24 h after the glucan (data not shown). Finally, on pinto bean, mycolaminaran at 500 μg/ml reduced infectivity of TMV and SHMV-n by only 30–50%, and did not reduce the infectivity of SBMV.

To test whether the mycolaminaran response required a necrotic reaction, we applied mycolaminaran at 500 μg/ml to half-leaves of *N. tabacum* 'Xanthi', which lacks genes for necrosis in response to TMV but which does form discrete and countable starch lesions after inoculation with TMV. Treated half-leaves consistently produced fewer than 10% as many starch lesions as buffer-treated opposite half-leaves.

Mycolaminaran appeared to inhibit an early step in infection, one that occurred within 4 h of inoculation. To test if mycolaminaran inhibited viral uncoating, we compared the effect of the glucan on the infectivity of TMV RNA to that of intact TMV virions on both Xanthi-nc and *N. rustica*. If mycolaminaran inhibited only uncoating, then one would predict that infectivity of RNA would not be affected by the glucan (11). Mycolaminaran at 500 μg/ml was applied to one half-leaf, and buffer was applied to the opposite half-leaf. Viral inoculum, either RNA or intact virions, was immediately applied to each leaf. Mycolaminaran inhibited infection to the same degree regardless of the type of inoculum (Table 2).

It was of interest to test whether mycolaminaran affected TMV infection of upper leaves of a tobacco plant inoculated 10 days previously on lower leaves with both TMV and 500 μg/ml mycolaminaran. TMV inoculum amended with mycolaminaran at 500 μg/ml and applied to one half of a leaf produced fewer than 10% as many lesions as nonamended inoculum applied to the opposite half. Inhibition of local virus infection in upper leaves occurred regardless of previous treatment of the lower leaves of the plant with mycolaminaran and TMV, both of which induce plant defenses (10,19).

We also tested for reduced susceptibility to local infection by TMV on upper leaves of tobacco treated 9 days previously on lower leaves with 500 μg/ml mycolaminaran. In three trials with a total of 30 plants, those treated produced 78, 87, and 130% as many lesions as buffer-treated plants.

DISCUSSION

In our work, soluble mycolaminaran derived from the cytoplasm of *P. megasperma* induced resistance to TMV infection in Xanthi-nc, in *N. glutinosa*, and to a lesser extent, in pinto bean. However, the polysaccharide did not reduce infectivity of TMV on *N. rustica*; these results agree with those of Singh et al (18). The results with *N. rustica* indicate that mycolaminaran inhibits but does not inactivate TMV. This plant-specific response is consistent with an interaction between the polysaccharide and the host plant, rather than one between the polysaccharide and the virus.

Mycolaminaran was more effective than laminaran in inhibiting

TABLE 1. Number of local lesions formed on cultivar Xanthi-nc after amending tobacco mosaic virus (TMV) inoculum with three carbohydrates[a]

	Carbohydrate[b] (μg/ml)			
	1,000	500	250	125
Trial 1				
Glycogen	1.07±0.318	0.91±0.38	1.77±0.94	0.98±0.25
Laminaran	0.60±0.156	1.01±0.35	0.93±0.65	1.05±0.31
Mycolaminaran	0.02±0.027	0.05±0.03	0.01±0.00	0.05±0.04
Trial 2				
Laminaran	0.30±0.13	0.41±0.14	0.55±0.09	ND[c]
Mycolaminaran	0.03±0.005	0.02±0.00	0.08±0.04	ND
Trial 3				
Laminaran	0.24±0.073	0.38±0.10	0.64±0.09	ND
Mycolaminaran	0.06±0.011	0.02±0.00	0.05±0.03	ND

[a]Half-leaves were inoculated with 0.2 μg/ml TMV, and opposite half-leaves were inoculated with 0.2 μg/ml TMV amended with carbohydrate.
[b]Data are ratios of the number of lesions per half leaf inoculated with amended inoculum divided by the lesions on the opposite half leaf. $n = 4$ in each treatment in each trial.
[c]Not determined.

TABLE 2. Number of local lesions formed as an indicator of the ability of mycolaminaran to inhibit local infection by tobacco mosaic virus (TMV) RNA on cultivar Xanthi-nc[a]

	Xanthi-nc		*Nicotiana rustica*	
Trial	Intact virus	TMV RNA	Intact virus	TMV RNA
1	0.033 ± 0.012[b]	0.064 ± 0.017	0.98 ± 0.25	0.99 ± 0.27
2	0.033 ± 0.010	0.024 ± 0.005	0.77 ± 0.11	0.95 ± 0.29
3	0.022 ± 0.007	0.018 ± 0.005	1.06 ± 0.11	1.27 ± 0.11

[a]Mycolaminaran (500 μg/ml) was applied to one half-leaf, and buffer was applied to the opposite half-leaf. Either TMV virions (0.1 μg/ml) or TMV RNA (0.1 μg/ml) was immediately applied to the entire leaf.
[b]Data are averages of ratios of lesion numbers from half-leaves treated with mycolaminaran divided by lesion numbers for the opposite half-leaf treated with buffer. $n = 7$ for each treatment in each trial.

TMV infection in cultivar Xanthi-nc. The chemical basis of this specificity is unknown, but the mycolaminaran differs from laminaran in several ways. Mycolaminarans are branched polymers made up exclusively of glucose units with an average degree of polymerization (DP) of approximately 29–36. These glucose residues are connected to each other by β-1,3 linkages and contain one or two branches at C-6 (20). In contrast, laminaran contains D-glucose chains that terminate in a mannitol residue, and the overall DP has been reported to be 16–33 (3).

The presence of mycolaminaran on the underside of a leaf inoculated on the topside was insufficient to reduce lesion numbers. This differs from the results Gupta and Price (5) had with *T. roseum* extracts, but agrees with those of Singh et al (18). This is consistent with at least two interpretations. First, a three-way interaction between polysaccharide, virus, and host epidermis may be required. Alternatively, the mycolaminaran may induce a host signal that requires time to travel from the underside to the topside of the leaf. However, waiting 24 h between application of mycolaminaran to the underside and inoculation of the topside with TMV did not decrease virus infection. If such a signal is produced, apparently it takes more than 24 h to reach the topside of the leaf and produce an anti-viral reaction. Furthermore, Singh et al (18) showed that half-leaves infiltrated with inhibitor by using a syringe were as susceptible to potato virus X as half-leaves infiltrated with water; they concluded that the polysaccharide directly affected the epidermis of the inoculated plant.

If uncoating were the critical stage affected by mycolaminaran, then one would predict that mycolaminaran would not inhibit infection by TMV RNA, since the RNA does not need to uncoat to infect. Because mycolaminaran inhibited infection by TMV RNA on Xanthi-nc but not on *N. rustica*, we conclude that 1) uncoating is not the sole critical stage affected, and 2) the mycolaminaran preparation did not degrade the RNA.

Mycolaminaran inhibited TMV infection when the two were applied sequentially to leaves, as long as it was applied within 30 min after TMV. Mycolaminaran applied 4 h after TMV did not reduce the number of lesions per leaf; therefore, the stage in the virus infection cycle affected by mycolaminaran apparently occurs within 4 h of virus inoculation. Mycolaminaran applied before TMV, however, inhibited infection even when the time between treatment and inoculation was 7 days. The mycolaminaran may have remained stable and intact for at least a week, or it may have induced other mechanisms of inhibition that did not spread completely from a treated to an untreated half-leaf in 1 wk.

Mycolaminaran also inhibited TMV infection, as measured by starch lesions, of Xanthi tobacco lacking any genes for a necrotic response to TMV infection. This excludes any necessary role of necrosis in the mechanism of action of mycolaminaran.

Mycolaminaran inhibited TMV infection on leaves of tobacco plants that 10 days before had been infected with TMV and treated with mycolaminaran on lower leaves. This showed that it can inhibit TMV infection even on leaves that express whatever systemic acquired resistance may be induced by virus infection and mycolaminaran. Mycolaminaran itself, however, did not induce strong systemic acquired resistance in this work, as measured by lesion numbers. However, mycolaminaran apparently induces a temperature-dependent systemic resistance to TMV in tobacco (E. Buschfeld, Max-Planck-Institut, Cologne, Germany, *personal communication*).

This work shows that at least one soluble fungal glucan can elicit responses in certain plants. The differences between the resistance induced by soluble mycolaminaran and by the wall-derived glucan of Kopp et al (11) are quantitative: The latter is effective at concentrations as low as 0.1 μg/ml, compared with 62 μg/ml for the former. This may reflect differences in mechanisms of resistance, or merely differences in efficiencies of triggering the same mechanism. Both glucans induce only a mild resistance in bean to infection by TMV. Mycolaminaran fails to induce resistance in *N. rustica*, but the reaction of *N. rustica* to the glucan was not reported. Both can be applied at least 7 days before inoculation with virus and reduce lesion numbers on treated leaves; both must be applied within hours of applying the virus or no resistance is observed. Reliable comparisons of relative efficiency await direct comparison of the two glucan preparations under identical conditions of growth, especially of temperature, light, plant age, and plant variety.

We are pursuing the genetic analysis and comparative physiology of the reponse of *Nicotiana* to mycolaminaran to address questions regarding the mechanisms and the potential applications of this induced resistance to virus infection.

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