Induction of Pathogenesis-Related Proteins in Tobacco Leaves

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ABSTRACT

The synthesis of pathogenesis-related proteins (PR proteins), induced in tobacco leaves in response to infection with tobacco mosaic virus or treatment with salicylic acid, was studied with in vivo pulse-labeling experiments. PR proteins synthesis began after a lag phase of about 8 hours in leaf discs treated with salicylic acid and after more than 18 hours in those infected with tobacco mosaic virus. In both cases, the synthesis declined rapidly after 50 hours. The results show that the accumulation of PR proteins results from de novo synthesis and not from degradation of preexisting precursors and that the induced synthesis is transient like other stress-inducible proteins. The proteins have a half-life of at least 50 hours. The induction of these PR proteins was not inhibited by either 25 micrograms per milliliter of actinomycin D or 200 micrograms per milliliter of a-amanitin, which completely inhibited the increase of peroxidase activity in tobacco mosaic virus-infected leaf and the induction of heat shock proteins in tobacco leaf discs. These findings indicate that the induction of PR proteins is not regulated by a transcriptional step but by a translational step.

When tobacco leaves carrying N gene are infected with TMV and necrotic lesions develop in them (7), novel soluble proteins appear in the leaves (24). These proteins, called pathogenesis-related proteins (PR proteins), are host-coded proteins induced by a wide range of pathogens including viruses, bacteria, and fungi (1). Recently, we showed that these proteins are also synthesized in response to mechanical injury, osmotic stress, and some chemicals; they are considered to be stress-inducible proteins (14). However, the induction of these proteins in tobacco leaves had not been studied because of the difficulty in preparing TMV-infected leaves which synchronously form local lesions for in vivo experiments (16).

In the present work, we conducted pulse-labeling experiments of PR proteins in leaf discs treated with salicylic acid and infected with TMV and studied the induction mechanism of these proteins. Our results showed that these proteins were synthesized de novo in leaf discs and their induction was transient. We discuss the unique induction system of PR proteins exposed to stress conditions, which is mainly regulated at the translational step.

MATERIALS AND METHODS

Plant Materials. Tobacco plants (Nicotiana tabacum cv Samsun NN) were cultivated in 12.5 cm pots under natural light in a temperature controlled greenhouse (20–32°C) for 2 to 3 months after sowing.

In Vivo Labeling and Extraction of Proteins. To induce protein formation by salicylic acid treatment, discs (5 mm in diameter) from expanded leaves sterilized with 1% NaClO were incubated in 1 mM potassium salicylate solution at 20°C for various times, and then labeled in a solution of 2 mCl/ml [35S]methionine (> 1000 Ci/mol; Amersham) containing 1 mM potassium salicylate for 2 h. After labeling, the discs were homogenized in 1 ml of 84 mM citric acid–32 mM NaHPO4 buffer, pH 2.8, containing 14 mm 2-mercaptoethanol and 6 mm sodium ascorbate (pH 2.8 buffer). The homogenate was centrifuged for 15 min at 8000g, and the supernatant fluid was used as the crude protein solution. When antibiotics were applied, discs were incubated in 1 mM potassium salicylate solution containing various concentrations of cycloheximide, actinomycin D, or a-amanitin for 1 d at 20°C, and labeled in [35S]methionine as described above.

In the case of TMV infection, synchronous formation of necrotic lesions was needed, because PR proteins are induced only in a ring around the developing lesion center (23). Thus, a leaf sterilized with 1% NaClO was inoculated with TMV (100 μg/ml) and incubated for 1 d at 30°C. At this temperature, the formation of necrotic lesions is inhibited, while TMV spreads systemically without symptoms, and PR proteins are not induced (14, 22). When the leaf is transferred to 20°C, necrotic lesions begin forming at 7 h after transfer. At various times, discs containing three necrotic lesions were cut from the leaf and labeled in a solution of 2 mCl/ml [35S]methionine for 2 h. When antibiotics were to be applied to the leaf discs, the discs were inoculated with TMV and incubated at 30°C for 1 d, then transferred to a solution containing 50 μg/ml of cycloheximide, 25 μg/ml of actinomycin D or 200 μg/ml of a-amanitin and incubated at 20°C for 2 d. These leaf discs were labeled as described above.

For pulse-chase experiments, leaf discs were incubated in 1 mM potassium salicylate solution for 1 d, and then labeled in [35S]methionine for 2 h. The discs were removed from the labeling medium, rinsed well with water, and placed in chase medium containing 0.1 mM methionine. At various times, the discs were removed and homogenized in pH 2.8 buffer.

To induce the formation of heat shock proteins, leaf discs were incubated at 40°C for 2 h, then transferred to 20°C and incubated in [35S]methionine for 2 h. After labeling, the discs were homogenized in 25 mM Tris-HCl buffer, pH 7.5, containing 20 mM mercaptoethanol (pH 7.5 buffer) and centrifuged as above. When antibiotics were to be applied, the discs were incubated in antibiotic solution at 40°C for 2 h and then labeled as described above.

Polyacrylamide Gel Electrophoresis. Electrophoresis on 10% polyacrylamide slab gels in nondenaturing buffer was performed according to Davis (5). Electrophoresis on 15% polyacrylamide slab gels containing 0.1% SDS was carried out by the method of Laemmli (10). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 and destained, then treated with ENHANCE (New England Nuclear) prior to drying. Fluorography was performed on dry gels using Kodak XAR-5 at ~80°C.

Peroxidase Assay. The method of assaying peroxidase activity was based on that of Kanazawa et al. (9). A leaf was inoculated with TMV and incubated at 30°C for 1 d, then transferred to
20°C and incubated in the presence or absence of antibiotics for 2 d. Next, 0.07 g of infected or healthy leaf was homogenized in 100 μl of 20 mM phosphate buffer, pH 7.0, containing 7 mg Polyclar AT (pH 7 buffer), and then centrifuged for 30 min at 10,000g. The reaction mixture, containing 0.1 M phosphate-citrate buffer, pH 5.0, 0.075% H2O2, 0.25% o-phenylene-diamine, and 10 μl of the supernatant, was incubated for 5 min at 30°C. The reaction was stopped by adding 50% NaHSO3, and the absorbance was measured at 430 nm against blanks lacking substrate.

RESULTS

Changes in the Synthesis of PR Proteins. PR proteins are produced when tobacco leaves carrying N gene respond to infection by TMV and form local lesions. However, at temperatures higher than 30°C, infected leaves carrying N gene do not show necrotic lesions and TMV multiplies systemically in leaves (18), with no PR proteins being induced under these conditions (14, 22). If these leaves are transferred to 20°C, rapid necrosis of the infected tissues develops and is accompanied by the synthesis of PR proteins. Under these conditions, many more leaf cells react synchronously to TMV than during inoculation and incubation at 20°C. Thus, we used this infection system for pulse-labeling experiments. After transfer to 20°C, there was a lag phase of more than 10 h, before the synthesis of PR proteins was detected, which then increased until 48 h and rapidly declined thereafter (Figs. 1 and 2). Under these experimental conditions, necrotic lesions began forming at 7 h after transfer to 20°C. Therefore, the synthesis of PR proteins began a few hours after the formation of necrotic lesions.

When the synthesis of PR proteins was induced by continuous treatment with 1 mM potassium salicylate, there were differences in the induction patterns among PR1a, PR1b, and PR1c. After a lag phase of about 8 h, PR1a synthesis increased almost linearly until 30 h, reached a plateau, then declined at 50 to 70 h (Figs. 3 and 4). Synthesis of PR1b and PR1c increased between 8 and 18 h and then immediately declined. The synthesis of PR1b was
as much as synthesis of PR1a in TMV-infected leaves, but in salicylate-treated leaf discs, the synthesis of PR1b was less than synthesis of PR1a. This may be due to more accumulation of PR1b in TMV-infected leaves than in salicylate-treated leaves (13).

Turnover of PR Proteins. PR proteins in cell-free extracts are resistant to digestion with proteolytic enzymes (16), suggesting that PR proteins should have a long half-life in leaf cells. Thus, we directly measured the turnover of these proteins in vivo by pulse-chase experiments (Figs. 5 and 6). Leaf discs incubated in 1 mM potassium salicylate for 1 d, in which large amounts of PR proteins were synthesized (Fig. 4), were pulse-labeled with [35S]methionine and chased with 0.1 m unlabeled methionine. Under the chase conditions, no additional label was incorporated into the total proteins (Fig. 6), indicating that the intracellular pool of methionine capable of being incorporated into proteins was small and that the chase was successful. PR1a was stable for at least 72 h and then rapidly degraded, and PR1b was stable for 40 h and degraded at 40 to 72 h. PR1c was gradually degraded until 170 h. The rapid degradation of PR1a and PR1b may have been caused by artificial senescence of the leaf discs, which became slightly yellow after 50 h and yellow and soft after 120 h.

Effect of Antibiotics on the Synthesis of PR Proteins. The synthesis of PR proteins in leaf discs treated with 1 mM potassium salicylate for 1 d was inhibited completely by 50 μg/ml cycloheximide, but not by 25 μg/ml actinomycin D or 200 μg/ml α-amamin, inhibitors of DNA-dependent RNA synthesis (Fig. 7). Interestingly, α-amanitin induced the synthesis of PR1a without salicylic acid treatment (Fig. 7, lane 12). Moreover, 500 μg/ml α-amanitin inhibited incorporation of [35S]methionine into other acid-extractable proteins, but PR1a and PR1b were synthesized under this condition (Fig. 7, lane 13).

The induction of protein synthesis by TMV infection was not inhibited by 200 μg/ml α-amanitin (Fig. 8, lane 4), but actinomycin D inhibited incorporation into almost all acid-extractable proteins. This may not be caused by inhibition of transcription by actinomycin D, but by side effects like inactivation of translation activity in leaf disc cells which had become yellow and soft after incubation in 25 μg/ml actinomycin D for 2 d. The accumulation of these PR proteins was observed in the leaves treated with 20 μg/ml actinomycin D as well as in those treated with 200 μg/ml α-amanitin.

Our findings suggest that transcription is not required for the induction of PR proteins, but there is another possibility that these antibiotics did not inhibit the synthesis of RNA in tobacco leaf discs. Thus, we examined the effects of these inhibitors on the increase in peroxidase activity which is also induced in necrotic lesions forming in TMV-infected leaves. The peroxidase activity in crude extract with a pH 7 buffer increased about 2-fold when TMV-infected leaf discs were incubated at 30°C for 40 h.
then transferred to 20°C and incubated for 2 d at 20°C (Table I). The increase in peroxidase activity was completely depressed by 200 μg/ml of α-amanitin (Table I). On the other hand, the inhibition by actinomycin D may be caused by side effects as described above.

We also examined how these inhibitors affect the incorporation of [35S]methionine into heat shock proteins in leaf discs. When leaf discs were incubated at 40°C for 2 h, at least four heat shock proteins were produced (Fig. 9). They had apparent mol

Table 1. Effects of Antibiotics on the Increase in Peroxidase Activity after TMV Infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (healthy leaves)</td>
<td>0.747</td>
<td>100a</td>
</tr>
<tr>
<td>TMV infection*</td>
<td>1.60</td>
<td>214</td>
</tr>
<tr>
<td>+Cycloheximide (50 μg/ml)</td>
<td>0.253</td>
<td>34</td>
</tr>
<tr>
<td>+Actinomycin D (25 μg/ml)</td>
<td>0.899</td>
<td>119</td>
</tr>
<tr>
<td>+α-Amanitin (200 μg/ml)</td>
<td>0.533</td>
<td>71</td>
</tr>
</tbody>
</table>

*TMV infected leaf discs were incubated as given in Figure 5 in water or each antibiotic solution, and then crude extracts of these discs with pH 7 buffer were assayed for the peroxidase activity. a Activity in TMV-infected leaf discs relative to that in healthy leaves.

wt of 82, 71, 20, and 18 kD and resembled those found in tobacco suspension cells (8). The induction of these heat shock proteins was also completely inhibited by both actinomycin D and α-amanitin, indicating that the inhibitors almost completely block the transcriptional activity of RNA polymerase II in tobacco leaves under the conditions used.

Heat shock proteins in soybean have similar mol wt (15,000–18,000) but can be resolved into more than 20 species on isoelectric focusing (11). Similar situations have been observed with PR proteins, PR1a, PR1b, and PR1c, which have similar mol wt (16,000) and similar immunological properties but different isoelectric points (13). These PR proteins are induced by stresses like mechanical injury or osmotic stress, but do not seem to be heat shock proteins because they are not induced by heat shock (Fig. 9, lane 3).

**DISCUSSION**

The induction of PR proteins had been proposed to result from de novo synthesis of these proteins, although no direct evidence has been presented (2, 23). The present work clearly shows that the PR proteins are synthesized de novo and do not arise by degradation of preexisting precursor proteins. The present work also shows that the induced synthesis was transient, like other stress proteins. The induced synthesis of these proteins occurred after lag phases in response to stresses about 8 h for salicylic acid and about 18 h for TMV infection. We note that the induced synthesis occurred at 20°C after a long lag phase and

Fig. 7. Effects of cycloheximide, actinomycin D, and α-amanitin on the synthesis of PR proteins in salicylic acid-treated leaf discs. Leaf discs were incubated for 1 d in H2O (lanes 1, 6, 8, 10, 12, and 14) or 1 mm potassium salicylate (lanes 2, 3, 4, 5, 7, 9, 11, and 13), containing cycloheximide (10 μg/ml, lane 3; and 50 μg/ml, lane 4) or actinomycin D (5 μg/ml, lanes 5 and 6; and 25 μg/ml, lanes 7 and 8) or α-amanitin (50 μg/ml, lanes 9 and 10; 200 μg/ml, lanes 11 and 12; and 500 μg/ml, lanes 13 and 14). These discs were then labeled in 2 mCi/ml [35S]methionine for 2 h as described in Figure 1.

Fig. 8. Effects of antibiotics on the synthesis of PR proteins in TMV infected leaf discs. Leaf discs were infected with TMV and incubated for 1 d at 30°C, then transferred to 20°C and incubated for 2 d in H2O, lane 1; 50 μg/ml cycloheximide, lane 2; 25 μg/ml actinomycin D, lane 3; and 200 μg/ml α-amanitin, lane 4. These discs were labeled as described in Figure 1, and crude peroxidase extracts from 1.3 mg of labeled leaf discs were applied to each lane.
which appear case direct cyclic acid proteins. induction catalase, and amanitin, these proteins was of synthesis of these proteins preceded detection of necrotic lesions, and leaf discs were indicated by arrows and an arrowhead, respectively. Positions of mol wt standards given in kD are shown on the left.

not at 30°C in TMV-infected leaf discs. We propose that the direct trigger of PR protein synthesis may not be the transfer of leaf discs from 30 to 20°C but the formation of necrotic lesions which appear at about 7 h after the transfer. Recently, Antoniw et al. (3) observed the accumulation of PR proteins in TMV-infected Xanthi-nc leaves by ELISA, and reported that the major increase of PR proteins was after lesion formation. We cannot compare directly the accumulation with the synthesis of these proteins, but the induced synthesis of these proteins occurred more rapidly (about several hours) than accumulation. In the case of the induction by salicylic acid, the accumulation of PR proteins was detected after 20 h (15). Thus, the accumulation of these proteins followed the induction of the synthesis after about 10 h. These results also confirm that the accumulation of PR proteins was caused by the induction of protein synthesis, and the synthesis of these proteins preceded about 5 to 10 h before the detection of the protein accumulation. The induction pattern of PR proteins in TMV-infected leaves is similar to the induction of some leaf enzymes induced by TMV infection, such as PAL, peroxidase, catalase, and polyphenoloxidase. In fact, these enzymes are also synthesized transiently by a hypersensitive reaction in response to infection with TMV by the formation of necrotic lesions. But the required time for the induction of PR proteins is considerably longer than that for these enzymes. For instance, the activities of PAL (21) and polyphenoloxidase (20) increase before the formation of necrotic lesions, and peroxidase synthesis (19) occurs as soon as necrotic lesions are formed. Why the induction of PR proteins is much slower than these enzymes is not clear, but it may be related to the function of the PR proteins.

The amount of PR1a synthesized due to treatment with salicylic acid was similar to that due to TMV infection, but in the case of PR1b and PR1c, more was formed in response to TMV infection (Figs. 2 and 4). These results suggest that the response of each PR protein to salicylic acid differs and the induction mechanism of salicylic acid differs from that of TMV infection. The fact that cycloheximide completely inhibited the synthesis of PR proteins confirms that these proteins are synthesized de novo on cytoplasmic ribosomes. However, neither actinomycin D nor a-amanitin, which are universally used as inhibitors of transcription, inhibited their synthesis. It is unlikely that these antibiotics had no effect on tobacco leaf discs, because they almost completely increased the induction of peroxidase activity in TMV-infected leaf discs and the induction of heat shock proteins. Thus, we assume that the untranslatable mRNAs of PR proteins are already present in healthy leaves and are changed to translatable forms by TMV infection or certain stresses like salicylic acid treatment. These findings are in agreement with the conclusion of Carr et al. (4) that PR proteins are found in the in vitro translation products of poly A-RNA from nontreated healthy Xanthi-nc plants. Similar observations have been reported for other stress proteins. For instance, synthesis of heat shock proteins in carrot somatic embryogenic cells was not inhibited by 50 μg/ml a-amanitin (17), and chick embryo cells, despite no detection of newly synthesized mRNA for the 23 kD stress proteins, showed high levels of in vivo and in vitro translational activities under stress conditions (25).

Hadjwiger and his colleagues (6, 12) reported that PAL formation in pea was induced by actinomycin D. The phenomenon of 'super induction' of PAL by actinomycin D resembles the induction of PR protein synthesis by a-amanitin. But the induction of PAL by actinomycin D was inhibited by a-amanitin and other RNA synthesis inhibitors, suggesting some involvement of transcriptional regulation. In the case of PR proteins, the induction by a-amanitin was not inhibited by actinomycin D (data not shown), showing that super induction does not occur with these proteins.

The unique translational regulation of stress-inducible proteins may be related to their function(s), which are considered to be the protection of cells against stresses (23). Cells seem to continuously produce some proteins or mRNAs of stress proteins which offer rapid protection against stresses, and the induction of these proteins may be a good system for studying translational regulation.

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