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Tobacco Mesophyll Protoplasts Synthesize 1,3-β-Glucanase, Chitinases, and "Osmotins" during *in Vitro* Culture

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ABSTRACT

Tobacco (*Nicotiana tabacum*) mesophyll protoplasts synthesize six basic proteins (a, a′, a_1, b, b′, and c) which are undetectable in the leaf and whose synthesis is reduced by auxin (Y Meyer, L Aspart, Y Chartier [1984] Plant Physiol 75: 1027-1033). Polypeptides a, a′, and a_1 were shown to have similar mobilities on two-dimensional electrophoresis as one 1,3-β-glucanase and two chitinases from tobacco mosaic virus-infected leaves. In immunoblotting experiments, polypeptide a was recognized by specific antibodies raised against the 1,3-β-glucanase and a′ and a_1 reacted with anti-chitinase antibodies. Similarly, b and b′ comigrated with osmotin and its neutral counterpart, two proteins characteristic of salt-adapted tobacco cells, and reacted with anti-osmotin antibodies. In addition it has been shown that 1,3-β-glucanase and chitinase activities increased at the same time as a, a′, and a_1 accumulated in cultivated protoplasts. Finally, polypeptide c was also detected in tobacco mosaic virus-infected leaves but could not be identified as any of the pathogenesis-related proteins characterized so far in tobacco. Thus, cultivated tobacco protoplasts synthesize and accumulate typical stress proteins.

We have shown previously (16,17) that tobacco mesophyll protoplasts in culture synthesize proteins that are undetectable in the leaf tissue from which protoplasts are isolated. The synthesis of six of these, which we have named a, a′, a_1 (M_r 31,000–33,000), b, b′ (M_r 26,000), and c (M_r 14,000), is greatly reduced (up to 10-fold) by the presence of auxin in the culture medium, while the synthesis of other proteins is not modified by the hormonal treatment (16,17). We have further shown (18) that these proteins represent the majority of the polypeptides secreted by protoplasts into their vacuole and that they continue to be synthesized in *in vitro* cultivated tobacco cells. More recently, the production of antibodies against proteins a′, b′ and c has allowed us to show (19) a close immunological relationship between proteins a_1 and a′ and between proteins b′ and b, respectively, while proteins a and c appeared to be completely unrelated to these two groups and to each other.

In the present paper, we describe the results of immunodetection experiments using antibodies directed against PR[1] proteins extracted and purified from TMV-infected Samsun NN tobacco leaves, as well as against a basic 26 kD protein induced in tobacco cells by culture in the presence of NaCl. The results enable us to identify five of the six major protoplast proteins whose synthesis is diminished by auxin, as two chitinases, a 1,3-β-glucanase, and two osmotins.

MATERIALS AND METHODS

Tobacco Plants and TMV Inoculation

Tobacco plants (*Nicotiana tabacum* cv Samsun NN) were grown in a greenhouse under controlled conditions. The two first fully expanded leaves at the top of 3-month-old plants were inoculated with a suspension of purified TMV (0.1 μg/mL). Plants were then incubated in a growth chamber at 22 + 1°C (16 h of photoperiod). The leaves bearing about 200 to 300 necrotic lesions were harvested 7 d after inoculation, frozen in liquid nitrogen, and stored at −80°C.

In Vitro Culture

Protoplasts were isolated from leaves of greenhouse-grown tobacco plants (*N. tabacum* var Maryland) and cultivated as previously described (15).

The rapidly growing cell suspension line 19, derived from tobacco pith by Jouanneau and Tandeau de Marsac (10), was a gift of Dr. M. Laloue (Gif-sur-Yvette, France). It was subcultured every week by 10 times dilution. Three-day-old cultures contain rapidly dividing cells, whereas 7-d-old cultures consist of nondividing cells.

This cell line was transfered to Murashige and Skoog (20) medium containing NaCl (10 g/L). In the first weeks, growth was drastically reduced. Medium was replaced every week until growth restarted. After 1 month, cells were subcultured by two times dilution every week. After 2 months the growth rate allowed dilution of the suspension four times every week. No change in the growth rate has taken place since then.

[1] Abbreviations: PR, pathogenesis-related; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; TMV, tobacco mosaic virus; 2-D, two-dimensional.

Preparation of Plant Extracts for Electrophoresis

Freshly harvested protoplasts, suspension cells, or leaf tissues were frozen in liquid nitrogen, and proteins were extracted using the phenol/water phase partition procedure (24): 2 g of frozen material was homogenized at 0 to 4°C in 10 ml of an emulsion of 50% phenol (w/v) in Tris-HCl (0.1 M, pH 8.0) containing 5% β-mercaptoethanol, using a motor-driven Potter homogenizer. After 30 min stirring on a magnetic stirrer, the mixture was centrifuged to break the emulsion (5000*g*, 10 min). The phenol phase was extracted again for 15 min with one volume of Tris-HCl (0.1 M, pH 8.0) containing 5% β-mercaptoethanol and was saturated with phenol. The phenol phase was separated by centrifugation, and proteins were precipitated by four volumes of methanol containing 0.1 M ammonium acetate at −20°C for 4 h. The precipitate was collected by centrifugation, washed six times with methanol (−20°C) containing 0.1 M ammonium acetate, and dried under reduced pressure.

Protein concentration in these extracts was determined using the Bradford (1) staining procedure.

Analytical 2-D Electrophoresis, Staining, and Immunodetection

As a first dimension, nonequilibrium pH gel electrophoresis was performed in cylindrical gels (1.5 mm × 80 mm) (21). The gel contained 9.2 M urea, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate, 1% Nonidet P-40, 2% ampholines (pH 3.5–10, LKB), 4% acrylamide/bisacrylamide (from a 28.4%/1.6% mixture) polymerized using 0.02% ammonium peroxodisulfate, and 0.14% tetramethylethylenediamine. Five μL samples were loaded on the acidic part of the gels. H_3PO_4 (0.01 M) and NaOH (0.02 M) were the acidic and basic electrolytes, respectively. The voltage was increased stepwise (100 V for 30 min, 200 V for 45 min, 300 V for 45 min, and 500 V for 1 h). Gels were extracted from the tubes and immediately frozen in the denaturation buffer (0.0625 M Tris-HCl (pH 6.8), 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS). For the second dimension, SDS acrylamide gels were run in a mini Protean II dual slab cell (Bio-Rad). The solution contained 12.5% acrylamide/bisacrylamide (from a 30%/0.8% mixture), 0.1% SDS, 0.375 M Tris-HCl (pH 8.8), 0.03% ammonium peroxodisulfate, 0.04% tetramethylethylenediamine. Slab cells were 0.75 mm thick, 80 mm wide, and 60 mm in height. They were overlaid with water. After 1 h polymerization, the overlaying water was replaced by a buffered solution (0.4 M Tris-HCl (pH 8.8) and 0.1% SDS) and the gel kept overnight at room temperature. An agarose-stabilized stacking gel containing 0.8% agarose (IEF Pharmacia) 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and 5% β-mercaptoethanol was layered over the resolving gel. After thawing and 5 min equilibrium in the denaturing buffer the gel of the first dimension was directly embedded in the agarose. Running buffer contained 0.25 M Tris, 1.92 M glycine, 0.1% SDS. Migration was performed at 20 mA/gel until the bromophenol blue reached the bottom of the gel (about 1 h).

For protein staining, some of the gels were incubated for 45 min in a solution containing 15% acetic acid, 20% ethanol, and 0.5% Coomassie blue and subsequently for 2 to 18 h in 10% acetic acid.

For immunodetection, proteins were transferred from other gels onto nitrocellulose sheets in Bio-Rad blotting apparatus. Generally, we processed eight minigels together side by side in the transblotting chamber. Transfer was carried out at 60 V for 4 h in 20 mM Tris-HCl, 150 mM glycine, and 20% methanol. Wet membranes were incubated by gentle shaking at 37°C for 1 h with a solution containing 20 mM Tris-HCl, 500 mM NaCl (pH 7.5), and 3% gelatin, and then were washed four times at 37°C with the same solution without gelatin but with 0.05% Tween-20. The reaction with the first antibody was carried out for 16 h and at 37°C in 20 mM Tris-HCl, 500 mM NaCl (pH 7.5), 0.05% Tween-20, and 1% defatted milk powder. Milk powder was omitted for anti-c serum, because it drastically reduces this immunological reaction. In addition this serum shows a very specific reaction even in the absence of milk powder. We generally used 20 mL of this solution containing 1,000- to 10,000-fold diluted rabbit immunoserum directed against the different proteins. The membrane was rinsed four times with the same solution without antibody. The reaction with the second antibody was performed for 2 h at 37°C in a similar solution (20 mL of the above mentioned buffer + 7 μL of the goat anti-rabbit horseradish peroxidase conjugate, Bio-Rad). The membrane was rinsed four times with the same buffer (without antibody), then twice with the same buffer but without Tween-20. The membrane was stained in 50 mL of Tris-glycine, containing 30 μL H_2O_2 (30%) to which 10 mL of cold methanol containing 30 mg horseradish peroxidase color development reagent was added. Color development began after 5 to 15 min and was stopped after 30 min by two water washes.

Antibody Production

We have used nine different rabbit antisera. Among these, three were raised against polypeptides a′, b′, and c, extracted from tobacco callus, and purified by 2-D preparative electrophoresis (19). Five other sera used in this study were raised against PR proteins of TMV-infected *N. tabacum* Samsun NN leaves. These were proteins PR-O, PR-P, PR-1b, and the basic 1,3-β-glucanase and chitinases. These proteins had been purified to homogeneity as already described (9,11,13). We also used a serum directed against osmotin, a protein abundantly produced by tobacco cells cultivated in the presence of NaCl (27). Anti-osmotin was a gift of Dr. LaRosa (Purdue University, West Lafayette, IN).

Preparation of Protein Extracts from Cultivated Protoplasts for Chitinase and 1,3-β-Glucanase Assays

Frozen protoplast preparations were crushed with mortar and pestle in two volumes of ice-cold 0.5 M sodium acetate buffer (pH 5.2) containing 15 mM β-mercaptoethanol. The different extracts were centrifuged at 14,000*g* for 30 min, and the supernatants were desalted on a Sephadex G-25 column (1.6 × 9 cm) equilibrated with 20 mM sodium acetate buffer (pH 5.2). The protein fractions were concentrated to approximately 300 μl on Centricon 10 concentrators (Amicon). The resulting solutions were further used for protein determina-

tion, using the method of Bradford (1), and for chitinase and 1,3-β-glucanase assays.

Chitinase and 1,3-β-Glucanase Assays

Chitinase and 1,3-β-glucanase activities were measured by colorimetric assays as previously described (11,13). Four different concentrations of protein (each in duplicate) were tested for chitinase assay, and the activity was calculated for enzyme concentration approaching zero. The 1,3-β-glucanase assay was performed with two different concentrations of protein (each in duplicate).

RESULTS

Comparison of Protein Patterns Obtained from Cultivated Tobacco Protoplasts and from Virus-Infected Leaves

During the first 18 h in culture (Fig. 1a), tobacco mesophyll protoplasts no longer synthesize most proteins accumulated in the leaf (Fig. 1c). However, new proteins are synthesized, in particular the basic polypeptides a, a′, a_1, b, b′, and c, whose synthesis has been shown to be reduced by auxin treatment (18). At this stage, accumulation of the newly synthesized polypeptides is low and they remain undetectable on Coomassie blue-stained gels (not shown). In contrast, after 4 d in culture they are clearly detectable on stained gels. A number of leaf proteins are still present at this time (Fig. 1c).

TMV-infected *N. tabacum* Samsun NN leaves represent a typical system of hypersensitive reaction to pathogens. It is well known that the major differences in acidic proteins between virus-infected and healthy control leaves of this cultivar are accounted for by the PR proteins (30). Recently, a purification procedure was developed that led to a better characterization of most of the Samsun NN acidic PR proteins (9) and to the isolation and characterization of PR proteins with basic isoelectric point (11,13). Comparison of Figure 1, c and d, clearly shows the appearance of these acidic and basic proteins upon virus infection. They are indicated by arrows in Figure 1d. But, surprisingly, comparison of Figure 1, b and d, revealed that most of the PR proteins with basic isoelectric point had the same 2-D electrophoresis characteristics as the major proteins induced during cultivation of mesophyll protoplasts and referred to as a, a′, a_1, b, b′, and c (18). The relationships between these latter proteins and the well-characterized PR proteins was investigated further by immunoblotting experiments.

Immunoblotting with Sera Raised against the Tobacco Callus Protein a′ and against the Leaf Protein PR-P (an Acidic Chitinase)

Among the well-characterized PR proteins of TMV-infected Samsun NN leaves is a group of four proteins that are serologically related and that all have chitinase activity (8,13). These are the two acidic proteins called PR-P and PR-Q and two basic chitinases labeled chi-32 and chi-34 because their M_r are 32,000 and 34,000, respectively (13). Figure 2d confirms that antibodies raised against PR-P showed a high affinity for the major chitinases, *i.e.* the two acidic proteins PR-P and PR-Q and the two basic chitinases chi-32 and chi-34. Two minor spots corresponding to unidentified proteins also became visible, one in the acidic and the other in the basic region of the 2-D electrophoregram. This serum showed high reactivity with proteins a′ and a_1 of protoplast extracts (Fig. 2b). In these extracts, two minor spots were also revealed with the same electrophoretic mobilities as PR-P and PR-Q from virus-infected leaves. Similar immunoblotting experiments performed with anti-PR-Q serum gave exactly the same results (data not shown).

On the other hand, antibodies directed against protein a′ (extracted from tobacco callus) showed high affinity for polypeptide a′ from protoplast extracts (Fig. 2a) but also for polypeptide a_1. No other spot was revealed by immunodetection with anti-a′ serum under our conditions. Figure 2c shows that this serum reacted strongly with two proteins of virus-infected leaves that had the same electrophoretic characteristics as proteins a′ and a_1 and as the two basic chitinases chi-32 and chi-34 of Figure 2d. It is noteworthy that the sera raised against the two acidic chitinases PR-P and PR-Q reacted strongly with the two basic chitinases chi-32 and chi-34 (13) and with the two basic proteins a′ and a_1. However, the reverse did not apply. Sera raised against chi-32 and chi-34 purified from virus-infected leaves by high performance liquid chromatography reacted with chi-32, chi-34 and with the two basic proteins a′ and a_1 (Fig. 4c) but did not react with PR-P and PR-Q. Similarly, the serum raised against the basic protein a′ purified from tobacco callus by 2-D electrophoresis (19) reacted with chi-32, chi-34 and the minor basic protein of infected leaves, but not (Fig. 2c) with the major proteins PR-P and PR-Q and the minor band in the acidic region that are present in the same extract (see Fig. 2d).

Immunoblotting with Sera Raised against an Acidic and a Basic 1,3-β-Glucanase Purified from Virus-Infected Leaves

Another group of well-characterized PR proteins of TMV-infected Samsun NN leaves consists of four proteins that are serologically related and that all have 1,3-β-glucanase activity (11). These are the three acidic proteins called PR-2, PR-N, and PR-O and a 1,3-β-glucanase of basic isoelectric point called gluc b (11). Figure 3 shows the results of immunoblotting experiments with anti-PR-O and anti-gluc b sera.

The serum directed against PR-O protein reacted strongly, as expected, with PR-2, PR-N, and PR-O and gave a weak reaction with two less acidic polypeptides of similar mol wt (Fig. 3c). In addition this antibody showed some affinity for the basic 1,3-β-glucanase (11). In the case of protoplast extracts anti-O reacted with polypeptide a and with two other less basic spots with the same molecular weight. These spots were also detectable on overloaded Coomassie blue-stained gels and also in trace amounts on gels obtained from virus-infected leaves. They appear to be variant forms of protein a and of the basic 1,3-β-glucanase, but we do not know if they are artefacts or native polypeptides.

Antibodies directed against the basic 1,3-β-glucanase extracted from TMV-infected tobacco leaves reacted strongly with the basic 1,3-β-glucanase and very weakly with the acidic 1,3-β-glucanase PR-2, PR-N, and PR-O. A similar situation

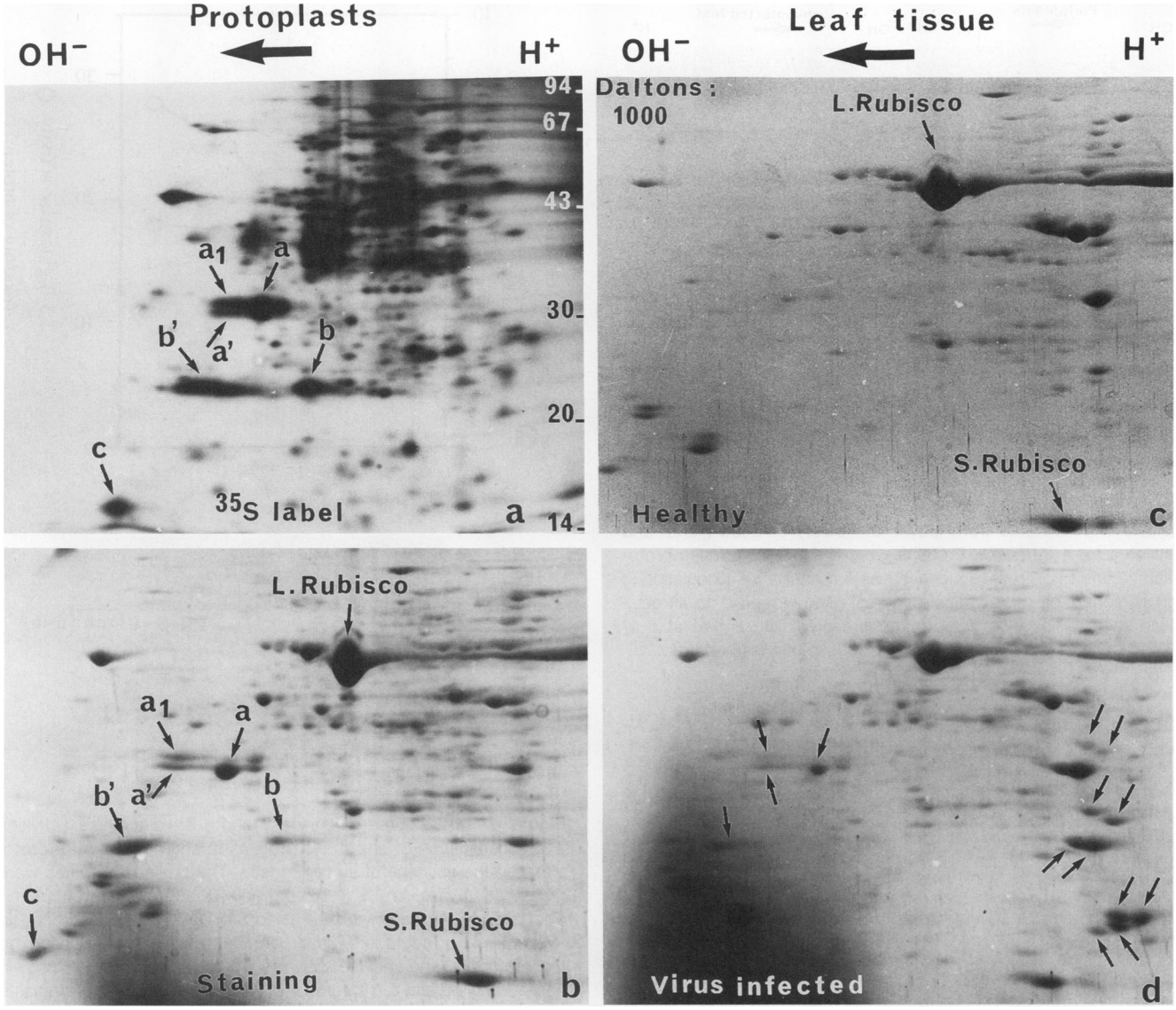

Figure 1. Protein synthesis and accumulation in tobacco protoplasts and leaves. Panel a, Freshly isolated protoplasts were labeled for 18 h with [^{35}S]methionine and the pattern of protein synthesis was analyzed by fluorography. Polypeptides a′, a_1, a, b′, b, and c are indicated on the autoradiogram. Note the absence of label incorporation in the large subunit of Rubisco. Panel b, Coomassie blue staining pattern of proteins present in protoplasts isolated from healthy leaves and cultivated for 4 d *in vitro*. Note the presence of typical leaf proteins (*i.e.* L. Rubisco and S. Rubisco) and of polypeptides a′, a_1, a, b′, b, and c. Panel c, Coomassie blue staining pattern of proteins extracted from healthy leaves. Typical leaf proteins were characterized according to their mol wt. Panel d, Coomassie blue staining pattern of proteins extracted from leaves 7 d after TMV infection. Arrows indicate the position of the major proteins induced during the hypersensitive reaction most of which are the PR proteins.

has been indicated above for the chitinases: it appears that cross-reactivity of anti-acidic hydrolases sera with the corresponding basic hydrolase(s) is significant while cross-reactivity of anti-basic hydrolases sera with the corresponding acidic hydrolases is hardly detectable. In protoplast extracts anti-gluc b serum reacted only with polypeptide a and the two variant spots (Fig. 3b).

Induction of Chitinase and 1,3-β-Glucanase Activities during Cultivation of Mesophyll Protoplasts

Among the chitinases of TMV-infected Samsun NN leaves, the basic chi-32 and chi-34 exhibit the highest specific enzyme activities *in vitro* with chitin as substrate (13). Among the 1,3-β-glucanases there was even a much broader spectrum of specific enzyme activity *in vitro* with laminarin as the substrate (11). The basic 1,3-β-glucanase and the acidic PR-O were by far the most active. As indicated above, cultivated protoplasts apparently contain mainly the basic isoforms chi-32, chi-34 and gluc b, *i.e.*, precisely those glycanhydrolases with the higher enzyme activities *in vitro*. Indeed, accumulation of the proteins a′, a_1 (Fig. 4c) and a (Fig. 4b) during protoplast culture was accompanied by a concomitant increase in extractable chitinase and 1,3-β-glucanase activities *in vitro* (Fig. 4a). These data are in agreement with the

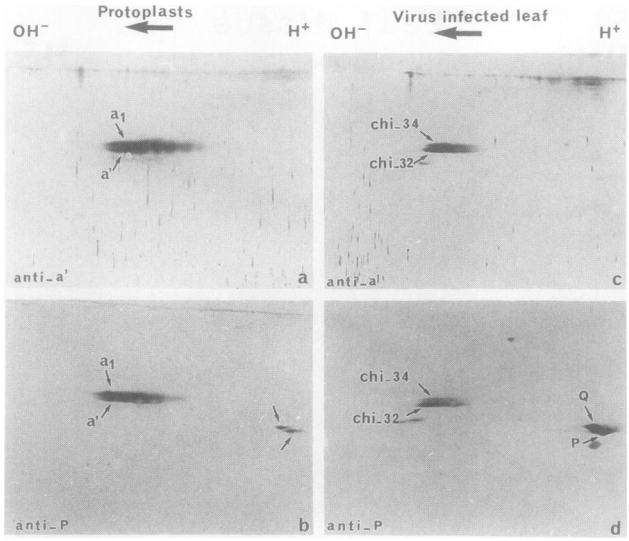

Figure 2. Immunodetection of proteins a_1, a′, and chitinases. Protoplasts were isolated from leaves of healthy tobacco plants and cultivated for 4 d *in vitro*. Proteins were isolated from these protoplasts (a and b) or from tobacco leaves 7 d after virus inoculation (c and d), separated by 2-D electrophoresis and blotted onto nitrocellulose sheets. The blots were immunoreacted with anti-a′ at a 1/1000 dilution (a and c) or anti-P at a 1/5000 dilutions (b and d).

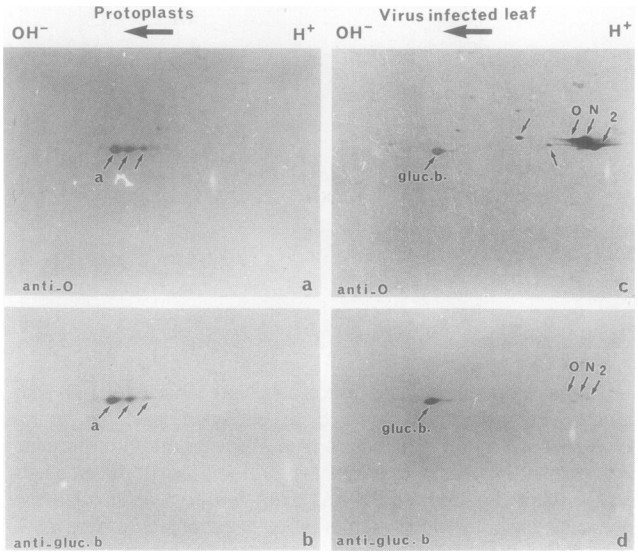

Figure 3. Immunodetection of protein a and the basic 1,3-β-glucanase. Membranes loaded with extracts of protoplasts (a and b) or with extracts of virus-infected leaves (c and d) were prepared as indicated in Figure 2 and were incubated with anti-PR-O serum at a 1/5000 dilution (a and c) or with anti-basic-1,3-β-glucanase serum at a 1/5000 dilution (b and d). The presence of variant forms of polypeptide a and of 1,3-β-glucanase is indicated by arrows. Note the absence of reactive spots in the acidic region of protoplasts extracts.

identification of proteins a′ and a_1 as chitinases and of protein a as a 1,3-β-glucanase.

Immunoblotting with Anti-b′ and Anti-Osmotin

Among proteins induced by growing tobacco cells in a NaCl containing medium, a very basic 26 kD polypeptide has

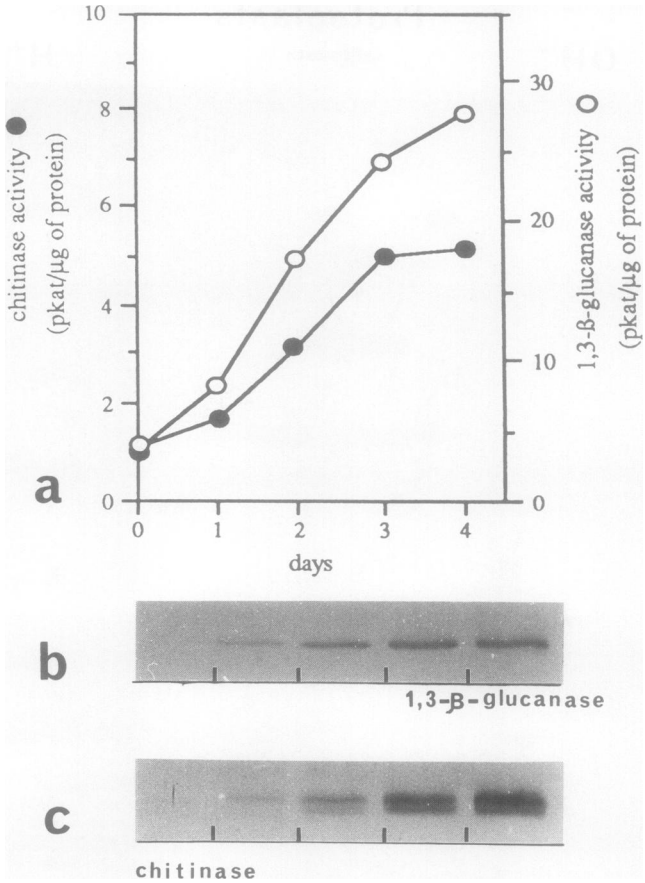

Figure 4. Time course of chitinase and 1,3-β-glucanase activities and accumulation of enzymatic proteins during cultivation of protoplasts in the absence of auxin. Panel a, The enzyme activities were measured *in vitro* on 2.5×10^6 protoplasts at each time of culture. The enzyme extracts were obtained and assayed as described in "Materials and Methods." Protein amount was evaluated according to Bradford (1) and enzyme activities are expressed on a per protein basis. Panels b and c, 10 µg total protein extracted from protoplasts after 0, 1, 2, 3, or 4 d of culture was electrophoresed under denaturing conditions, blotted onto nitrocellulose, and reacted with anti-basic-1,3-β-glucanase serum (b) or with a serum directed against a mixture of chi-32 and chi-34 (c). Only the portions of the membrane corresponding to the 30 and 35 kD region of the gels are shown.

been studied in depth (12,27,28). It has been named osmotin, although its enzymatic and cellular functions are not known. It presents sequence analogies with thaumatin, a sweet protein (27), and is consequently related to previously described acidic thaumatin-like PR proteins (3). In addition to osmotin, a neutral polypeptide immunologically related to osmotin has been described in tobacco cells (28).

Figure 5d, obtained by immunoblotting of extracts of NaCl adapted cells with a serum directed against osmotin, shows that these cells accumulate osmotin and its neutral counterpart. These polypeptides also react with antibodies directed against polypeptide b′ (Fig. 5c). On the other hand, both antibodies react with b′ and b in tobacco protoplasts (Fig. 5, a and b). Thus, b′ and osmotin have the same electrophoretic mobilities and are both recognized by two independent anti-

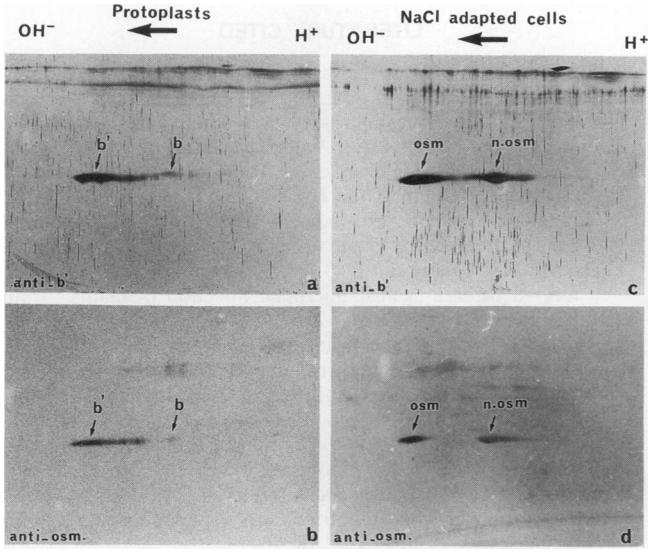

Figure 5. Immunodetection of b′, b, basic and neutral osmotins. Membranes loaded with extracts of protoplasts (a and b) or with extracts of NaCl adapted cells (c and d) were prepared as indicated in Figure 2 and were incubated with anti-b′ serum at a 1/1000 dilution (a and c) or with anti-osmotin at the 1/5000 dilution (b and d).

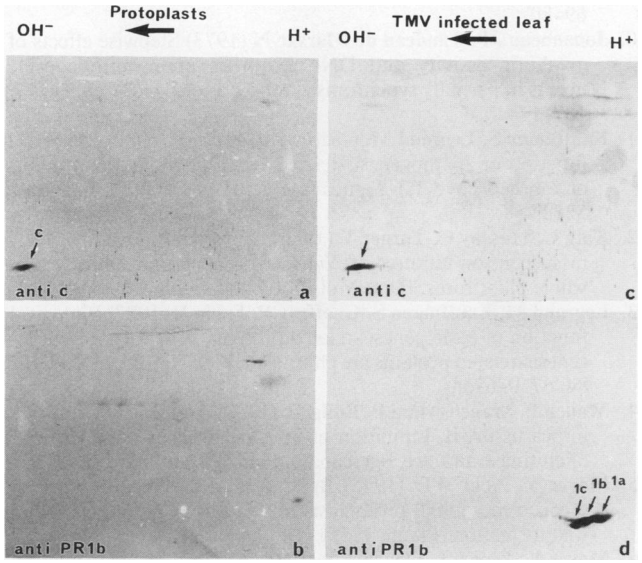

Figure 6. Immunodetection of polypeptide c and PR-1 proteins. Membranes loaded with extracts of protoplasts (a and b), or with extracts of virus-infected leaves (c and d) were prepared as indicated in Figure 2 and were incubated with anti-c serum at a 1/1000 dilution (a and c) or with anti-PR-1b serum at a 1/5000 dilution (b and d).

bodies. The same conclusion applies for b and the neutral osmotin.

Immunoblotting with Serum Raised against Polypeptide c and PR-1b Protein

We have prepared an immunoserum raised against polypeptide c purified from tobacco callus. On protoplast extracts it reacts only with polypeptide c (Fig. 6a). No reaction can be observed with healthy leaf extracts (not shown). In contrast, a polypeptide with the same electrophoretic mobility as c reacts with the anti-c serum in TMV-infected leaf extracts (Fig. 6c).

Due to the similarity in the mol wt of polypeptide c and PR-1a, PR-1b, and PR-1c proteins, we have examined a possible immunological relationship between these polypeptides. Under our conditions a serum raised against PR-1b protein reacted with PR-1a, PR-1b, and PR-1c proteins in TMV-infected leaf extracts (Fig. 6d), but not with the basic polypeptide reacting with the anti-c serum. In the same manner, this antibody reacted (very weakly) with a few unidentified polypeptides in protoplast extracts but not with polypeptide c (Fig. 6b).

DISCUSSION

Identification of Polypeptides a, a′, a_1, b′, and b

There are several lines of evidence that polypeptides a, a′, a_1, b′, and b from cultivated tobacco protoplasts are in fact identical to major proteins already isolated from TMV-infected tobacco leaves or from tobacco calli. These lines of evidence consist of identical migrations upon 2-D electrophoresis and of identical immunological properties studied in both homologous and heterologous serological reactions with antibodies raised against proteins isolated from protoplasts, leaves, or calli.

For instance, a′ and a_1 were identified as basic chitinases. This conclusion is reinforced by the parallel increases in chitinase activity and in amounts of proteins a′ and a_1 in tobacco protoplasts during culture. In fact, two basic chitinases with the same M_r (32,000 and 34,000) and with similar pHs have been characterized in tobacco callus (25) and in TMV-infected tobacco leaves (13).

Polypeptide a showed moderate immunoreactivity with anti-PR-O (acidic, 1,3-β-glucanase) and strong reactivity with the anti-basic 1,3-β-glucanase extracted from TMV-infected tobacco leaves. In protoplasts, 1,3-β-glucanase activity increased at the same time as polypeptide a accumulated. All these data together are good evidence that polypeptide a is in fact a 1,3-β-glucanase. Indeed, a 33,000 D, basic 1,3-β-glucanase has been described in tobacco callus (6) and in TMV-infected leaves (11).

Immunoblotting of protoplast extracts with anti-osmotin showed a strong reactivity of this antibody with polypeptides b′ and b. Since osmotin and the neutral osmotin-like polypeptide have the same electrophoretic characteristics as b′ and b respectively and all four proteins have been described in tobacco, they are most probably identical. Finally, we have adapted our tobacco cell line to grow in the presence of 10 g/L NaCl. Under these growth conditions, b′ is synthesized during all growth phases and is the prevalent form.

Finally, we do not find cross-reactivity of polypeptide c with various antibodies directed against PR-proteins characterized in TMV-infected hypersensitive tobacco leaves, but polypeptide c was actually detected in the infected material.

Occurrence of Chitinase, 1,3-β-Glucanase and Osmotins in Tobacco Plants and Callus

Basic chitinases (25) and 1,3-β-glucanase (6) have been characterized in tobacco callus and osmotins accumulate in

cells cultivated *in vitro* in a NaCl containing medium (27,28). All these proteins abundantly accumulate in tobacco roots, but are not detectable or present in low amounts in noninfected, nonstressed young leaves of the plant (6,12,25,28). We have previously observed the same localization of proteins a, a′, a_1, b, b′, and c (18,19).

Synthesis Pathway and Cellular Localization

We have previously shown (16,17) that proteins a, a′, a_1, b, b′, and c are not detectable after pulse labeling of protoplasts, but only after long labeling (>1 h) or a pulse followed by a chase. This suggests that these proteins undergo a slow post-translational processing (16,17). This has recently been clearly established for 1,3-β-glucanase in a detailed study (26). The pre-pro-1,3-β-glucanase shows the same electrophoretic characteristics as the short-lived polypeptide f, which we have previously described as a potential precursor of polypeptide a (17). The synthesis pathway of chitinases and osmotins remains to be established.

In tobacco mesophyll protoplasts, proteins a′, a_1, a, b, b′, and c accumulate in the vacuole where they constitute the bulk of the proteins (18). This result is in agreement with the vacuolar localization of osmotin (27). The same cellular localization has been reported for a basic chitinase in ethylene-treated bean leaves (2), for a wound-induced carboxypeptidase inhibitor (another stress induced polypeptide) in potato leaves (7), and very recently for basic 1,3-β-glucanase in tobacco (29). This vacuolar localization contrasts with the presence of some of the acidic PR proteins in the intercellular spaces and in the cell wall of infected leaves (5,22).

CONCLUSION

As a general conclusion, tobacco protoplasts synthesize and accumulate the most abundant basic PR proteins that have been described. Among them are two chitinases and a 1,3-β-glucanase, which are believed to be defense enzymes because they are capable of attacking pathogen cell walls (14). In protoplast, and more generally in *in vitro* cultivated cells, osmotins could be involved in osmotic adjustment which is believed to occur for instance in the case of salt-adapted cells (12,27,28), but the synthesis of large amounts of defense proteins is more intriguing. The protoplasts are, indeed, isolated and cultivated in axenic conditions, *i.e.* are never in contact with pathogens. Two hypotheses can be considered. The first is that these glycanhydrolases can release molecules with signaling functions (4,23). The second possibility is that these defense proteins are induced by the isolation procedure. In fact, plant cells during pathogen infection or protoplast isolation are exposed to polysaccharides released by wounding or maceration and which can induce plant defense reactions (4,23). To obtain arguments in favor of one of these hypotheses, we are trying to establish whether a particular step in protoplast isolation is responsible for the synthesis of chitinases, 1,3-β-glucanase and osmotins in the tobacco cells.

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LITERATURE CITED

1. **Bradford M** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254
2. **Boller T, Voegeli U** (1984) Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol **74:** 442–444
3. **Cornelissen BJC, Hooft van Huijsduijnen RAM, Bol JF** (1986) A tobacco mosaic virus-induced tobacco protein is homologous to the sweet-tasting thaumatin. Nature **321:** 531–532
4. **Darvill AG, Albersheim P** (1984) Phytoalexins and their elicitors—a defense against microbial infection in plants. Annu Rev Plant Physiol **35:** 243–275
5. **Dumas E, Lherminier J, Gianinazzi S, White RF, Antoniw JF** (1988) Immunocytochemical location of Pathogenesis-Related b1 protein induced in tobacco mosaic virus-infected or polyacrylic acid-treated tobacco plants. J Gen Virol **69:** 2687–2694
6. **Felix G, Meins JF** (1986) Developmental and hormonal regulation of 1,3-β-glucanase in tobacco. Planta **167:** 206–211
7. **Hollaender-Czytko H, Andersen JK, Ryan CA** (1985) Vacuolar localisation of wound-induced carboxypeptidase inhibitor in potato leaves. Plant Physiol **78:** 76–79
8. **Hooft van Huijsduijnen RAM, Kauffmann S, Brederode FT, Cornelissen BJC, Legrand M, Fritig B, Bol J** (1987) Homology between chitinases that are induced by TMV infection of tobacco. Plant Mol Biol **9:** 411–420
9. **Jamet E, Fritig B** (1986) Purification and characterization of 8 of the pathogenesis-related proteins in tobacco leaves reacting hypersensitively to tobacco mosaic virus. Plant Mol Biol **6:** 69–80
10. **Jouanneau JP, Tandeau de Marsac N** (1973) Stepwise effects of cytokinin activity and DNA synthesis upon mitotic cycle events in partially synchronised tobacco cells. Exp Cell Res **77:** 167–174
11. **Kauffmann S, Legrand M, Geoffroy P, Fritig P** (1987) Biological function of "pathogenesis-related" proteins: four PR-proteins of tobacco have 1,3-β-glucanase activity. EMBO J **6:** 3209–3212
12. **King G, Hussey C, Turner V** (1986) A protein induced by NaCl in suspension cultures of *Nicotiana tabacum* accumulates in whole plant roots. Plant Mol Biol **7:** 441–449
13. **Legrand M, Kauffmann S, Geoffroy P, Fritig B** (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754
14. **Mauch F, Mauch-Mani B, Boller T** (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88:** 936–942
15. **Meyer Y, Abel WO** (1975) Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. Planta **125:** 1–13
16. **Meyer Y, Aspart L, Chartier Y** (1984) Auxin-induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated in vitro. I. Characteristics of auxin-sensitive proteins. Plant Physiol **75:** 1028–1034
17. **Meyer Y, Aspart L, Chartier Y** (1984) Auxin-induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated in vitro. II. Time course and level of auxin control. Plant Physiol **75:** 1035–1040
18. **Meyer Y, Chartier Y, Alibert G** (1987) Auxin reduces the synthesis of major vacuolar proteins in tobacco mesophyll protoplast. Plant Physiol **83:** 713–718
19. **Meyer Y, Grosset J, Chartier Y, Cleyet-Marel JC** (1988) Preparation by 2-D electrophoresis of proteins for antibody production: antibodies against proteins whose synthesis is reduced by auxin in tobacco mesophyll protoplasts. Electrophoresis **9:** 704–712
20. **Murashige T, Skoog F** (1965) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol Plant **19:** 100–127
21. **O'Farrell PZ, Goodman HM, O'Farrell PH** (1977) High resolu-

tion two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12:** 1133–1142

22. **Ohashi Y, Matsuoka M** (1987) Localization of pathogenesis-related proteins in the epidermis and intercellular spaces of tobacco leaves after their induction by potassium salicylate or tobacco mosaic virus infection. Plant Cell Physiol **28:** 1227–1235
23. **Ryan CA** (1988) Oligosaccharides as recognition signals for the expression of defensive genes in plants. Biochemistry **27:** 8879–8883
24. **Schuster A, Davies E** (1983) Ribonucleic acid and protein metabolism in pea epicotyls. I. The aging process. Plant Physiol **73:** 809–816
25. **Shinshi H, Mohnen D, Meins FJ** (1987) Regulation of a plant pathogenesis-related enzyme: inhibition of chitinase and chitinase mRNA accumulation in cultured tobacco tissues by auxin and cytokinin. Proc Natl Acad Sci USA **84:** 89–93
26. **Shinshi H, Wenzler H, Neuhaus JM, Felix G, Hofsteenge J, Meins FJ** (1988) Evidence for N- and C-terminal processing of a plant defense-related enzyme: primary structure of tobacco prepro-β-1,3-glucanase. Proc Natl Acad Sci USA **85:** 5541–5545
27. **Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson HA, Pfankoch HA, Regnier FE, Bressan RA** (1987) Characterization of osmotin. A thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol **85:** 529–536
28. **Singh NK, LaRosa PC, Handa AK, Hasegawa PM, Bressan RA** (1987) Hormonal regulation of protein synthesis associated with salt tolerance in plant cells. Proc Natl Acad Sci USA **84:** 739–743
29. **Van den Bulcke, Bauw G, Castresana C, Van Montagu M, Vandekerckhove J** (1989) Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: evidence for a strictly compartmentalized plant defense system. Proc Natl Acad Sci USA **86:** 2673–2677
30. **Van Loon LC** (1985) Pathogenesis-related proteins. Plant Mol Biol **4:** 111–116