Sunflower (Helianthus annuus L.) Pathogenesis-Related Proteins

Induction by Aspirin (Acetylsalicylic Acid) and Characterization

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Sunflower leaf discs floated on a solution containing aspirin (acetylsalicylic acid) produced a set of new proteins extractable at pH 5.2 and excreted into the intercellular space. More than 80% of the proteins found in the intercellular fluids of induced leaf discs have been identified as pathogenesis-related (PR) proteins by their immunological relationship with tobacco PR proteins. Members of the four major classes of PR proteins have been characterized. Sunflower PR proteins of type 1 (PR1) and of type 3 (PR3) were found to have acidic isoelectric points, whereas the induced PR protein of type 2 (PR2) had a basic isoelectric point. Members of the type 5 PR proteins (PR5), known in tobacco as thaumatin-like proteins, showed a more complex pattern. Multiple sunflower PR5 isomers of similar molecular weight but of different isoelectric points were excreted from the cells in response to the aspirin treatment. PR2 and PR3 proteins were found at very low basal levels in untreated leaves, whereas PR1 and PR5 proteins could not be detected at all in the same extracts. Glucanase and chitinase activities were always associated with PR2 and PR3 proteins in partially purified sunflower extracts. All of these data indicate that, in response to aspirin treatment, sunflower plants produce a complete set of PR proteins characterized by an apparently exclusively extracellular localization.

A number of plants can produce, in response to pathogen infection, new proteins whose synthesis leads to numerous metabolic changes. Various (bio)chemicals, such as salicylic acid (White, 1979) and ethephon (an ethylene-releasing compound) (Vera and Conejero, 1990), can also trigger the production of some of these proteins. The PR proteins belong to this family of “stress-inducible” proteins. They were first discovered in tobacco (Nicotiana tabacum) plants reacting hypersensitively to tobacco mosaic virus infection (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970), and, since, more or less closely related PR proteins have been detected in several plant species (for reviews, see Carr and Klessig, 1989; Bol and Linthorst, 1990; Bowles, 1990; Linthorst, 1991; White and Antoniw, 1991). In tobacco, four major classes of PR proteins are known: PR proteins of type 1 (Antoniw and Pierpoint, 1978), PR proteins of type 2 having a β-1,3-glucanase activity (Kauffmann et al., 1987), PR proteins of type 3 having a chitinase activity (Legrand et al., 1987), and PR proteins of type 5 (Kauffmann et al., 1990), or thaumatin-like proteins, having sequence similarities with a protein from Thaumatococcus danielli (Cornelissen et al., 1986) and with a trypsin/α-amylase inhibitor from maize (Richardson et al., 1987).

Since the work of White (1979), salicylic acid and acetylsalicylic acid (aspirin) have been known to be exogenous inducers of at least some PR proteins and of systemic acquired resistance. Recently, endogenous production of salicylic acid was demonstrated before the establishment of systemic acquired resistance in tobacco (Malamy et al., 1990) and in cucumber (Métraux et al., 1990). Salicylic acid appears, therefore, to be implicated in the transduction pathway of the stress signal.

In this paper, we describe the characterization of sunflower PR proteins induced by aspirin, which have been detected on the basis of the general properties of PR proteins and using their serological links with tobacco PR proteins. Furthermore, they are inducible by pathological conditions (J.-L. Jung and G. Hahne, unpublished data). PR proteins of the genus Helianthus have not been described previously. Members of the four major classes of tobacco PR proteins have serologically related counterparts in sunflower that have different mole wts and isoelectric points. Their localization in the leaf tissue has been characterized.

MATERIALS AND METHODS

Plant Material and Leaf Disc Experiments

Sunflower seeds (Helianthus annuus var Ha 300B) were sterilized in 3% sodium hypochlorite under vacuum for 15 min and then, after addition of a few drops of detergent, an additional 15 min under slow agitation. They were then washed in water and allowed to imbibe for 5 h. The seeds were sown in standard soil in pots and grown under controlled conditions in a growth chamber, with a light cycle (maximum 300 µE) of 16/8 h (day/night) at a temperature ranging from 18°C (night) to 23°C (day) and with 70% humidity.

The third level of true leaves of 4-week-old plants (the leaves of the fourth level were approximately 3 cm long)

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Abbreviations: IF, intercellular fluids; PR protein, pathogenesis-related protein.
were cut, and leaf discs of 1-cm diameter were prepared. Five leaf discs were floated on 25 mL of 20 mM Mops (pH 7.5) supplemented or not with 5 mM aspirin (acetylsalicylic acid; Sigma) in a 10-cm Petri dish under continuous light (70–100 μE). At various times after the beginning of the experiment, leaf discs were dried, weighed, and frozen (−20°C) before protein extraction and analysis.

**Protein Extraction and Partial Purification**

PR proteins of sunflower were extracted using a modification of standard methods (Kauffmann et al., 1987). Briefly, frozen leaf discs (approximately 0.5 g) or fresh plant material (0.5–2 g) were ground in 2 mL of extraction buffer (0.5 mM sodium acetate, pH 5.2, containing 15 mM β-mercaptoethanol). The crude extract was centrifuged in Eppendorf tubes at 13,000g for 15 min at 4°C, and the supernatant was desalted on a Sephadex G-25 column (Pharmacia) equilibrated in 20 mM sodium acetate (pH 5.2), 15 mM β-mercaptoethanol. When needed, proteins were concentrated using Centricon 10 concentrators (Amicon, Beverly, MA) or simply by evaporating one-half or two-thirds of the volume of the buffer in a Speedvac concentrator (Savant, Farmingdale, NY).

For the extraction of extracellular proteins, a modification of the in vacuo method of Parent and Asselin (1984) was used. Fresh plant material was put in a 20-mL syringe closed at the bottom with cheesecloth, immersed in a 50-mL Falcon tube filled with the extraction buffer, and infiltrated under vacuum for 5 min. The buffer was then removed, and the tube was centrifuged at 1000g for 10 min. After this centrifugation, IF was recovered in the Falcon tube. The whole procedure was performed a second time, and the two IF fractions were pooled and desalted to yield the "extracellular extract." The proteins contained in the remaining leaf tissue were then extracted by grinding to give the "intracellular extract." Contamination of the extracellular extract by intracellular proteins was checked by following the activity of α-mannosidase, a marker enzyme located in the vacuole of the cells (Boller and Kende, 1979).

For the separation of acidic, basic, and neutral proteins, desalted extracts were loaded first onto an S-Sepharose column equilibrated in 20 mM sodium acetate (pH 5.2), 15 mM β-mercaptoethanol. The acidic and neutral proteins were found in the void volume, whereas basic proteins were further eluted by increasing the ionic strength of the equilibration buffer to 500 mM with NaCl. The acidic and neutral protein extract was then dialyzed overnight against 20 mM Tris-HCl (pH 7.8) and loaded onto a Q-Sepharose column equilibrated in the same buffer. Neutral proteins were eluted in the void volume, and acidic proteins were eluted in 20 mM Tris-HCl (pH 7.8) supplemented with 500 mM NaCl. In each extract, the total protein amount was determined with the Bio-Rad protein assay kit using BSA as a standard.

**SDS-PAGE Analysis and Immunoblotting**

Electrophoresis on denaturing polyacrylamide gels was performed on slab gels by the method of Laemmli (1970) with a 5% (w/v) stacking gel and a 12.5% (w/v) resolving gel. For total protein staining, gels were first fixed in 12% (v/v) TCA for 1 h and then soaked overnight in Coomassie brilliant blue G-250 (Serva) prepared in 25% (v/v) methanol, 19% (w/v) (NH₄)₂SO₄, and 2.5% (v/v) phosphoric acid. The gels were destained in 25% (v/v) methanol. The relative quantities of the stained proteins were estimated by densitometry at 600 nm, and calculation of peak areas was performed with a Shimadzu CS 900 densitometer (Shimadzu Corp., Kyoto, Japan).

Alternatively, proteins were stained with silver nitrate (Blum et al., 1987). The gels fixed in TCA were incubated three times for 20 min in 5% (v/v) methanol, soaked for 1 min in 40 mg/L of Na₂S₂O₄, washed two times in water, and incubated for 20 min in a 0.2% (w/v) AgNO₃ solution containing 0.035% (v/v) formamide. For protein coloration, the gels were washed two more times and then soaked in a solution of 3% (w/v) Na₂CO₃, 0.035% (v/v) formamide, and 40 mg/L of Na₂S₂O₄.

For immunological detection, proteins contained in the gel were electrophoretically transferred for 45 min onto a nitrocellulose (0.45-μm pore size) sheet in a buffer containing 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol (pH 7.5) at a constant current of 122 mA. The transferred proteins fixed to the nitrocellulose filter were then stained as described in detail by Jung and Hahne (1992).

Antibodies against tobacco PR proteins PR O (Kauffmann et al., 1987), PR Q (Legrand et al., 1987), and PR S (Kauffmann et al., 1990) were used at a 1:2000 dilution, and anti-PR 1b (Kauffmann, 1988) antibody was used at a 1:200 dilution.

**Enzymic Activity Assays**

**Chitinase Assay**

Chitinase activity contained in sunflower protein extracts was measured using a colorimetric assay (Boller et al., 1983; Legrand et al., 1987). Briefly, chitinase powder (Sigma), washed in water, was incubated with various volumes of the extract (1–20 μL) in 0.5 mL of 0.1 M sodium acetate (pH 5.2) on a test tube rotator, at 37°C, during various times (1–5 h). After incubation, tubes were centrifuged, and 0.3 mL of the supernatant was incubated with 20 μL of 2% (w/v) snail gut enzyme (Cytobelicase; IBF, Villeneuve la Garenne, France) to hydrolyze soluble chitin oligomers. The resulting Glc-NAc was determined as described before (Legrand et al., 1987). Chitinase activity was expressed in nkat. One nanokatal is defined as the quantity of enzyme catalyzing the formation of 1 nmol of reaction product per s.

**Glucanase Assay**

Glucanase activity was measured using laminarin (Sigma) as substrate as described by Kauffmann et al. (1987). The principle of the technique is to measure the reducing sugars formed from laminarin by the β-1,3-glucanase activity contained in the extracts using a colorimetric assay. Glucanase activity was expressed in nkat. One nanokatal is defined as the quantity of enzyme catalyzing the formation of 1 nmol of Glc equivalents per s.
**RESULTS**

Detection of PR Proteins in Sunflower Leaf Discs

The leaf disc system has been chosen to study the induction of sunflower PR proteins by aspirin for two major reasons: (a) The treatment applied to the plant material (quantity of inducer, condition of its penetration into the leaf tissue) was easy to standardize, in contrast to application by spraying or watering of entire plants, and (b) it provided the opportunity to work on homogeneous material using small batches of plants. Leaf discs showed good viability during the 7 d of the experiments in the conditions described, and only a low level of newly formed proteins was detected in control discs incubated on Mops.

We first applied a standard approach for PR protein analysis, i.e. the analysis of the total soluble protein fraction extracted at pH 5.2, produced by sunflower leaf discs incubated in the presence of 5 mM aspirin. The amount of proteins extracted in 0.5 M sodium acetate (pH 5.2) increased with time, from 0.05 mg/disc at day 0 to 0.12 mg/disc after 7 d of incubation in the presence of aspirin (in control experiments without inducer, the protein level remained stable during the incubation period). This increase of proteins extractable at pH 5.2 indicates that there was a de novo protein synthesis induced by aspirin.

Comparison of lanes 0 to 7 in Figure 1a shows that at least eight proteins were induced by aspirin during the experiment. These proteins share two major characteristics with PR proteins: high stability in acidic conditions and inducibility. They were only slightly induced in control experiments in which aspirin was absent (Fig. 1a, lane T7).

![Figure 1](link)
The availability of antibodies directed against the four major classes of tobacco PR proteins, namely, type 1 (Kauffmann, 1988), type 2 (β-1,3-glucanases) (Kauffmann et al., 1987), type 3 (chitinases) (Legrand et al., 1987), and type 5 (thiamatin-like) (Kauffmann et al., 1990) proteins allowed us to detect among the aspirin-induced sunflower proteins some that are serologically related to tobacco PR proteins (Fig. 1b).

All of the four major classes of tobacco PR proteins had counterparts induced in sunflower by aspirin that can also be identified on silver nitrate-stained gels (Fig. 1a). We can, therefore, propose a nomenclature for sunflower PR proteins that describes their immunological link with the classes of tobacco PR proteins: (a) In class 1, we detected one 17-kD peptide. This PR1 protein of sunflower was recognized by an antibody directed against the tobacco PR1b protein, appeared 4 d after the aspirin treatment (Fig. 1a), and has never been found in untreated plants. (b) PR2 proteins of sunflower have an apparent molecular mass of 40 kD and are recognized by an antibody directed against the tobacco PR O protein (a β-1,3-glucanase). They appeared earlier than the PRI protein and were detectable on western blots as well as on silver nitrate-stained gels as early as 2 d after the onset of the experiment (Fig. 1a, type 2). When gels were deliberately overloaded, these proteins were detected by the anti-PR O antibody as poorly expressed proteins in control extracts. (c) At least two proteins of class 3 (PR3) of sunflower were recognized and were named PR3a (29 kD) and PR3b (37 kD). They are both serologically linked to the tobacco PR Q protein. Their kinetics of appearance were very close to that of PR2 proteins. They were also present in untreated plants but in very small quantities, just at the limit of detection of western blot analysis. Some additional forms, migrating between these two molecular masses and appearing in the late phase of the kinetic study, were observed only in crude extracts and are considered to be migration artifacts. They were generally not found in partially purified extracts. (d) PR5 proteins of sunflower migrated as a diffuse band in the presence of β-mercaptoethanol in the loading buffer, with a molecular mass of approximately 20 kD. When β-mercaptoethanol was omitted, PR5 migrated as multiple higher molecular mass bands (between 40 and 80 kD) (Fig. 2), certainly reflecting the existence of multimeric forms of these sunflower PR proteins. Both forms are recognized by an antisera specific for the tobacco PR S protein, an acidic member of class 5. They were induced at a detectable level after 2 to 3 d of incubation with aspirin and were never found in untreated plants.

Antibodies specific for basic glucanases, basic chitinases, and osmotin of tobacco detected, in similar experimental conditions, the same sunflower PR proteins as sunflower PR2, PR3 a and b, and PR5. The assays for β-1,3-glucanase and chitinase activities showed a strong and coordinated increase with time in the presence of aspirin (Fig. 3). The endogenous levels of β-1,3-glucanase and chitinase activities, in L3 leaves of untreated plants, were <10 and 1 nkat/g fresh weight, respectively. Upon incubation with aspirin for 7 d, these basal levels increased by 20-fold for the β-1,3-glucanases and by 15-fold for chitinases. This increase is consistent with the appearance of PR2 and PR3, a and b, detected on western blots (Fig. 1b).

**Characterization and Partial Purification of the Sunflower PR Proteins**

In tobacco, PR proteins are known to be strictly compartmentalized. Acidic isoforms are located in the extracellular spaces of the tissues, whereas basic isoforms are intracellular and found in the vacuoles (Carr et al., 1987; Van Den Bulcke et al., 1989; Grosset et al., 1990; Keefe et al., 1990). The situation is less well defined for other plant species, and basic PR protein isoforms, such as basic chitinases and β-1,3-glucanases, have also been detected in the extracellular spaces of potato (Kombrink et al., 1988).

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**Figure 2.** Different migration patterns of sunflower PR5 proteins in SDS-PAGE analysis. Protein contained in 2 mg of fresh weight of leaf discs incubated 7 d on aspirin was separated by SDS-PAGE with (+) or without (−) β-mercaptoethanol (βME) in the loading buffer, transferred to nitrocellulose, and detected by an anti-tobacco PR S antibody. In the presence of β-mercaptoethanol, sunflower PR5 proteins migrated as a diffuse 20-kD band. Higher molecular mass isoforms, between 40 and 80 kD, were detected when β-mercaptoethanol was omitted.**

**Figure 3.** Time course of aspirin-induced β-1,3-glucanase (open squares) and chitinase (filled squares) activities in sunflower leaf discs. Enzyme activities are based on the extraction of five leaf discs (0.3 g of fresh weight at the onset of treatment to 0.6 g after 1–2 d of disc imbibition). When aspirin was omitted, glucanase and chitinase activities after 7 d of incubation were <2-fold the activities of the plants before the disc preparation.
Subcellular Localization of Sunflower PR Proteins

To determine the localization of aspirin-induced sunflower PR proteins, a modification of the in vacuo method of Parent and Asselin (1984) was used. After IF was prepared, proteins were also extracted from the remaining tissue. Approximately 30% of the total extractable proteins were found in the IF (Table I), whereas only 2% of α-mannosidase activity, which was used as a marker for intracellular proteins (Boller and Kende, 1979), was detected in this extract. This indicates that the IF was not contaminated by intracellular proteins to a significant degree during the two steps of its preparation.

More than 40% of β-1,3-glucanase and chitinase activities were found in the IF, showing that these proteins were at least partially excreted from the cells. The situation was the same using SDS-PAGE analysis of total proteins as well as western blot experiments (data not shown) for all of the sunflower PR proteins detected.

We estimated the relative quantities of the proteins present in the IF by densitometry. Sunflower proteins induced by aspirin, classified as PR proteins because of their induction behavior and their serological links with tobacco PR proteins, represented almost 80% of the proteins in the IF. The major components were PR5 (45% of the total proteins in the IF) and PR2 (16%), whereas PR3, a and b (9 and 5%, respectively), and PR1 (2.5%) were detected at lower levels.

In the absence of a reliable marker for extracellular proteins, the contamination of the intracellular extract by extracellular proteins is not easily quantified (Heitz et al., 1991). The problem is of particular importance for our extracts because the spectrum of proteins detected in the two fractions is almost identical. We approached this question by preparing sunflower mesophyll protoplasts from leaf discs incubated for 7 d on 5 mM aspirin and extracting the proteins just after protoplast isolation. α-Mannosidase activity was detected in the protoplast extract, thus proving the presence of typical intracellular proteins. AgNO₃-stained gels showed a general presence of protein but no major bands corresponding to the detected PR proteins in the total extracts (see Fig. 4). Moreover, no PR proteins were detected using western blot experiments. We believe, therefore, that no extractable intracellular PR proteins have been induced by aspirin in sunflower leaf discs.

Partial Purification of Sunflower PR Proteins According to Their Isoelectric Point

Two successive steps of cation- and anion-exchange chromatography of IF resulted in a clear copurification of the sunflower PR proteins and of the enzymic activities assumed to belong to PR2 and PR3. Approximately 50% of the proteins were found in the acidic fraction, 30% in the basic fraction, and 20% in the neutral fraction (Fig. 5). Chitinase activity was detected only in the acidic fraction, and 95% of the glucanase activity was found in the basic fraction (Fig. 5). On western blots, PR2 proteins were found mainly in the basic fraction, except for two faintly stained bands of the same molecular mass that could be recognized in the neutral and acidic fractions (Fig. 6b, type 2). On the Coomassie blue-stained gel, the acidic and neutral isoforms of PR2 were close to the detection limit. Both PR3 proteins are acidic and were clearly detected by staining as well as by western blot. It is

Table I. Distribution of protein content and enzymic activities between intra- and extracellular extracts

IF was isolated from sunflower leaf discs incubated on aspirin for 7 d. Proteins contained in the remaining tissue were extracted separately. Total protein, glucanase activity, chitinase activity, and α-mannosidase activity have been determined and are expressed on a fresh weight basis.

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<th>Intracellular Proteins</th>
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<td>Glucanase activity</td>
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<td>Chitinase activity</td>
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<tr>
<td>Mannosidase activity</td>
<td>20 nkat/g fresh wt</td>
<td>98</td>
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* Variation of A₄₉₅ per min and per g of fresh weight.
species often share strong serological relationships. Linthorst, 1991; Ward et al., 1991). We have chosen acetyl-
of compounds on different plant species (White et al., 1987; cyclic acid, and benzoic acid on tobacco PR proteins (White, first report of the action of aspirin (acetylsalicylic acid), sali-
mimic the hypersensitive reaction in sunflower. Since the beginning of our work, accumulation of salicylic acid during the hypersensitive reaction in tobacco (Malamy et al., 1990; Yalpani et al., 1991) or in cucumber (Métraux et al., 1990) has been described. A salicylic acid-binding protein has been partially purified (Chen and Klessig, 1991). Salicylic acid has, therefore, been proposed as an in vivo messenger of the stress signal (Malamy et al., 1990; Métraux et al., 1990; Ward et al., 1991). In response to treatment with aspirin, sunflower plants produced proteins that possess all of the general properties of the PR proteins. The four major classes of tobacco PR proteins have serologically related counterparts in sunflower, although at different molecular masses and isoelectric points. We detected acidic (PR1, PR3, and PR5) and basic proteins (PR2 and PR5) in sunflower leaf discs.

All of these proteins are excreted in the intercellular spaces. The possibility that other PR proteins accumulate inside the cells appears unlikely because no PR proteins were detected in protoplasts isolated from leaf discs that produced PR proteins. We cannot totally exclude the possibility that only a subpopulation of cells has been liberated from the tissue during protoplast preparation due to some changes that might have occurred in the cell wall composition during the incubation with aspirin. Conclusive proof of the localization of PR protein will only be provided by immunological in situ

interesting to note that, although PR3a is present at a higher concentration in the IF than PR3b, it is less well recognized by the antiserum specific for the PR Q protein of tobacco. PR1 protein is also acidic, and, again, observations made on the total protein staining and on western blots are in perfect agreement (see Fig. 6, a and b, type 1). The situation seems to be more complex for the sunflower PR5. Coomassie blue staining shows a predominant band at approximately 20 kD in the acidic fraction but also some faintly stained bands in the basic and neutral fractions. The antiserum specific for the tobacco PR S recognized proteins in all three lanes (Fig. 6b, type 5). The type 5 of sunflower proteins must, therefore, be composed of multiple isoforms, serologically related, of close molecular mass and excreted into the intercellular space but of different isoelectric points. This can explain their abnormal migration when not separated, and a more detailed characterization will certainly indicate their complete purification.

DISCUSSION

Sunflower PR proteins have been detected on the basis of some characteristics shared by most PR proteins (for reviews, see Carr and Klessig, 1989; Bol and Linthorst, 1990; Bowles, 1990; Linthorst, 1991; White and Antoniw, 1991). (a) They are generally low molecular mass proteins that can be ex-
tracted with acidic buffers; (b) they are inducible and/or show a certain tissue-specific and developmental regulation of expression; (c) two classes possess known enzymic activi-
ties detectable in vitro (β-1,3-glucanases and chitinases); (d) PR proteins of the same class but belonging to different plant species often share strong serological relationships.

No biological system implying necrotic interaction between sunflower and pathogens appeared easy to use and sufficiently reliable. Therefore, we used a chemical inducer to mimic the hypersensitive reaction in sunflower. Since the first report of the action of aspirin (acetylsalicylic acid), sali-
cylic acid, and benzoic acid on tobacco PR proteins (White, 1979), numerous studies have shown the effect of this class of compounds on different plant species (White et al., 1987; Linthorst, 1991; Ward et al., 1991). We have chosen acetyl-
salicylic acid to test the induction of such proteins in sun-
flower. The use of the name "PR" proteins seems warranted because we have observed accumulation of all the described proteins in response to treatments with fungal toxins (J.-L. Jung and G. Hahne, unpublished data).

Since the beginning of our work, accumulation of salicylic acid during the hypersensitive reaction in tobacco (Malamy et al., 1990; Yalpani et al., 1991) or in cucumber (Métraux et al., 1990) has been described. A salicylic acid-binding protein has been partially purified (Chen and Klessig, 1991). Salicylic acid has, therefore, been proposed as an in vivo messenger of the stress signal (Malamy et al., 1990; Métraux et al., 1990; Ward et al., 1991). In response to treatment with aspirin, sunflower plants produced proteins that possess all of the general properties of the PR proteins. The four major classes of tobacco PR proteins have serologically related counterparts in sunflower, although at different molecular masses and isoelectric points. We detected acidic (PR1, PR3, and PR5) and basic proteins (PR2 and PR5) in sunflower leaf discs.
detection. It is unusual that all the defense-related induced proteins should be localized in the extracellular spaces, and this might be explained by assuming that the intracellular proteins are not recognized by the antibodies raised against tobacco PR proteins. However, this hypothesis appears unlikely. Because of the results from the silver-stained gels, intracellular sunflower PR proteins would represent a very small minority, if they exist at all after aspirin induction.

Basic glucanases are accumulated in extracellular spaces of sunflower leaves following aspirin induction, which is another difference between sunflower PR proteins and tobacco PR proteins. In tobacco, basic PR proteins are accumulated in the vacuole (Van Den Bulcke et al., 1989; Grosset et al., 1990; Keefe et al., 1990). However, basic extracellular PR proteins have been described for potato (Kombrinck et al., 1988), as an example.

In leaf discs of sunflower, the maximum quantity of PR proteins was detected between the 6th and the 7th d after the onset of the treatment, but the kinetics may be slightly different on entire plants, depending on the precise position of the analyzed leaf.

β-1,3-Glucanase activity and chitinase activity were found in the same partially purified sunflower extracts as PR2 and PR3, respectively, and their kinetics of induction were very similar. Analogous to tobacco, a β-1,3-glucanase activity can, therefore, be proposed for PR2, and a chitinase activity can be proposed for PR3. The level of β-1,3-glucanase activity (expressed on a fresh weight basis) found in sunflower leaf discs incubated on aspirin was 10-fold more than the one found in hypertensively reacting tobacco plants (Kauffmann et al., 1987). In contrast, chitinase levels were comparable (Legrand et al., 1987).

The level of PR proteins in untreated sunflower plants strongly depended on their growing conditions (greenhouse, small or large culture chamber, etc., and even within one batch, individual plants behaved differently. We have determined the growth condition that produced plants with the lowest possible level of PR protein expression before the treatment. The use of the leaf disc system minimized the effect of plant-to-plant variability and, at the same time, standardized the induction treatment with aspirin. All of the proteins described in leaf discs were also observed in experiments involving whole plants.

The low level of endogenous enzymic activities detected in untreated plants and the fact that PR2 and PR3 can be detected on overloaded gels by western blot analysis implies that β-1,3-glucanases and chitinases are expressed at a basal level before the aspirin treatment. The acidic and the neutral isoforms of PR2, which are detectable after aspirin treatment at a very low level, reflect, for this class of protein, the existence of multiple isoforms. Some isoforms were only slightly induced by aspirin.

Using antibodies raised against tobacco PR proteins and one defined chemical inducer, i.e. aspirin, we have identified a number of sunflower proteins that can be considered to cover the range of the major classes of PR proteins. The totality of sunflower PR proteins can only be characterized after complete purification of the described proteins and using specific antisera raised against the pure sunflower proteins. New members of PR proteins in sunflower may be detected using these specific antisera. Furthermore, it is possible that aspirin induces only part of the sunflower PR proteins, as has been demonstrated for tobacco (Linthorst, 1991; Ward et al., 1991). Other inducers will have to be tested for a more complete description of sunflower PR proteins.

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LITERATURE CITED