Differential Expression of Chitinase and β-1,3-Glucanase Genes in Various Tissues of Potato Plants

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We have analyzed the tissue-specific expression of chitinase, β-1,3-glucanase and, for comparison, phenylalanine ammonia-lyase genes in healthy, unstressed potato (*Solanum tuberosum*) plants by using RNA-RNA hybridization and immunostaining techniques to localize the respective mRNAs and proteins *in situ*. Preparations of leaf, flower, shoot tip, node and axillary bud revealed that chitinase exhibited a distinctive and characteristic expression pattern in being confined to the epidermal cell layer, while β-1,3-glucanase and phenylalanine ammonia-lyase were present to varying extents in most tissues of these organs. These distinctive patterns of expression suggest fundamentally different roles for the two glucanohydrolases in the metabolism of unstressed plants.

Chitinases and β-1,3-glucanases are among a group of proteins which are inducible in plants in response to various forms of stress and are generally believed to serve protective functions, although the exact nature of those functions is not clear (Boller, 1987). Phenylalanine ammonia-lyase (PAL), while stress-inducible and a contributor to protective mechanisms which are comparatively well understood, is also involved in the metabolism and growth of plants under normal, unstressed conditions (Hahlbrock and Scheel, 1987; 1989). Like PAL, chitinases, β-1,3-glucanases and most other gene products categorized as defense-related are expressed constitutively at low, but detectable levels (Somssich *et al.*, 1986; Kombrink *et al.*, 1988; Trudel *et al.*, 1989). A number of possible explanations can be suggested for the constitutive expression; it could be a response to chronic levels of stress, an anticipatory defense measure, or a result of leakiness of the mechanism by which gene expression is repressed. For example, it seems reasonable to regard the expression of the protease-inhibitor feeding deterrents (Palm *et al.*, 1990) as an anticipatory defense. It remains possible, however, that other defense-related enzymes, such as chitinases and β-1,3-glucanases, fall into a category exemplified by PAL, fulfilling roles in metabolism apart from defense which require constitutive expression. While β-1,3-glucans, chitin and other polysaccharides toward which chitinase has hydrolytic activity are components of the cell walls of various plant pathogens, endogenous β-1,3-glucans are present in plants and recent work suggests that there are non-defensive roles for both β-1,3-glucanases (Castresana *et al.*, 1990) and chitinases (Lotan *et al.*, 1989; Neale *et al.*, 1990; Ori *et al.*, 1990; De Jong *et al.*, 1992). Detailed analysis of the modes of expression of the glucanohydrolase enzymes will enhance understanding of the mechanisms by which they contribute to stress resistance.

This communication describes a survey of healthy and unstressed potato organs, designed to examine the local patterns of constitutive expression of chitinase and β-1,3-glucanase genes at the

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levels of mRNA and protein. Distributions of mRNA were determined by hybridization *in situ* to labeled, antisense RNA probes and protein accumulation was detected by immunochemical localization with peroxidase-labeled second antibodies. PAL gene expression was analyzed in parallel, as a relatively well understood reference, although no detailed study has heretofore been made of constitutive PAL expression in potato, and a comparison is included of chitinase expression in healthy *vs. P. infestans* infected leaves. We have obtained the result that, while β-1,3-glucanase and PAL exhibit a general similarity in their expression patterns to the extent that they are present, with no more than minor, local variation in quantity, in all tissues of every organ examined, chitinase exhibits an unique and characteristic expression pattern in the aerial organs of healthy plants and one which differs from the pattern of chitinase expression seen in infected leaves.

Results

Leaves

The results of *in situ* RNA hybridizations and the corresponding series of peroxidase-stained immunohistochemical preparations in leaf sections are shown in Fig. 1. Comparison of spatial patterns of expression within the leaf revealed by the chitinase (Fig. 1 a and 1 e), β-1,3-glucanase (Fig. 1 b and 1 f) and PAL (Fig. 1 c and 1 g) probes shows that, for each gene, mRNA and protein are similarly localized. The most striking feature of these preparations is that chitinase mRNA and protein are strictly localized in the epidermis of the leaf, whereas β-1,3-glucanase and PAL are expressed, with somewhat variable abundance, throughout all tissue types. There appears to be a greater hybridization intensity of β-1,3-glucanase and PAL in the upper layers of the leaf tissue, but this may not be significant, since it could arise from differences in tissue or cell structure. There is no gradient evident in the distribution of the corresponding proteins.

The probes also show the presence of gene products within leaf hair structures. The results suggest that there are differences in expression between epidermal and glandular hairs. For reference, complete epidermal hair structures are visible in the PAL immunolocalization (Fig. 1 g) and, faintly, in the immunolocalization control (Fig. 1 h). The structures consist of a small number of basal cells surmounted by a cap structure. Comparison of Fig. 1 g with 1 h indicates that PAL is expressed in all parts of the structure. The hybridizations to chitinase and β-1,3-glucanase in Fig. 1 a and 1 b indicate that these two mRNAs are localized in different parts of the epidermal hair structure, chitinase being confined to the basal cells and β-1,3-glucanase to the distal cap. The chitinase immunolocalization in Fig. 1 e supports this interpretation. The protruding structure in Fig. 1 f is probably also an epidermal hair, but it is too collapsed to permit clear identification of the cell types.

In the glandular hairs, which appear as globular objects supported by stalks protruding from the epidermis of the leaf (Fig. 1 c), high autoradiographic densities are seen in PAL hybridizations. Chitinase appears not to be expressed in glandular hairs, as indicated by the absence of hybridization to the two hairs faintly visible in Fig. 1 a. A few hybridizations and peroxidase stained preparations suggest the presence of PAL and β-1,3-glucanase in glandular hairs, but the rarity of intact structures makes this interpretation uncertain.

Organs other than leaves

Other organs examined include stem node, axillary bud, shoot tip, flower, root, stolon and tuber. Although complete series of *in situ* hybridizations and immunolocalizations with all probes and including controls are not available for these organs, the results obtained for the aerial organs indicate that the three genes are expressed in them in patterns similar to those seen in leaves. Typical examples are shown in Fig. 2 and 3.

Fig. 2 shows a complete series of immunolocalizations, with antibodies to chitinase, β-1,3-glucanase and PAL and including a preimmune serum control, to sections through stems and axillary buds (a–d) and to cross-sections through flowers (e–h). Chitinase protein in stem and axillary bud is again localized predominantly in the epidermis. In flowers, chitinase is expressed primarily in the outward-facing and, to a lesser extent, in the inward-facing epidermis of the sepals. This pattern is repeated, although less conspicuously, in the petals. The approximately equivalent staining intensity of the anthers and style to that in the control in-

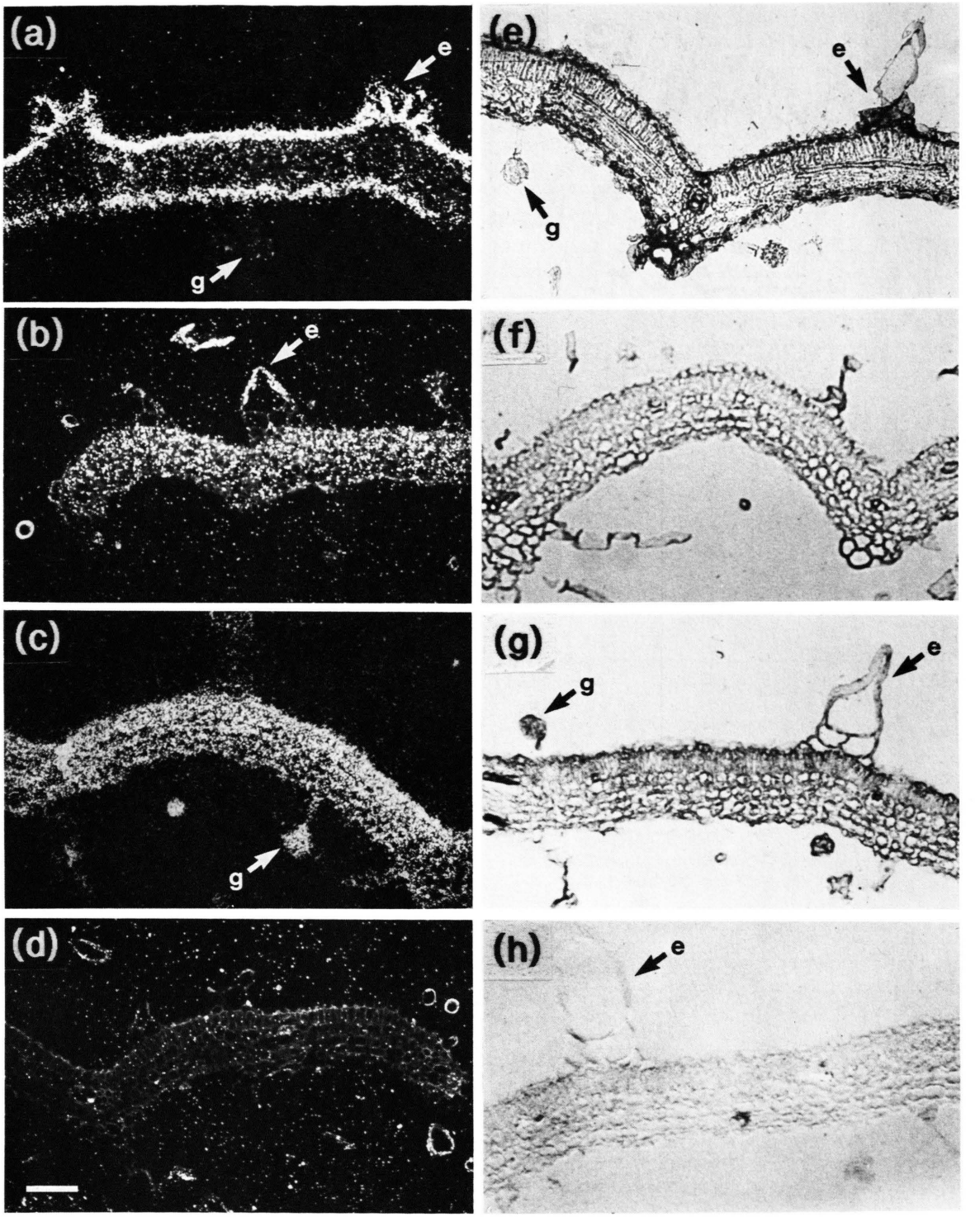

Fig. 1. Hybridization *in situ* to mRNAs (a–d) and immunohistochemical localization of proteins (e–h) in potato leaf sections. Hybridization probes were (a) chitinase antisense, (b) β-1,3-glucanase antisense, (c) PAL antisense, (d) chitinase sense RNA (control). First serum was directed against (e) chitinase, (f) glucanase, (g) PAL, or was (h) unspecific control serum. The notations on the figures "g" and "e" with arrows indicate epidermal and glandular hairs respectively. Bar: 100 μM.

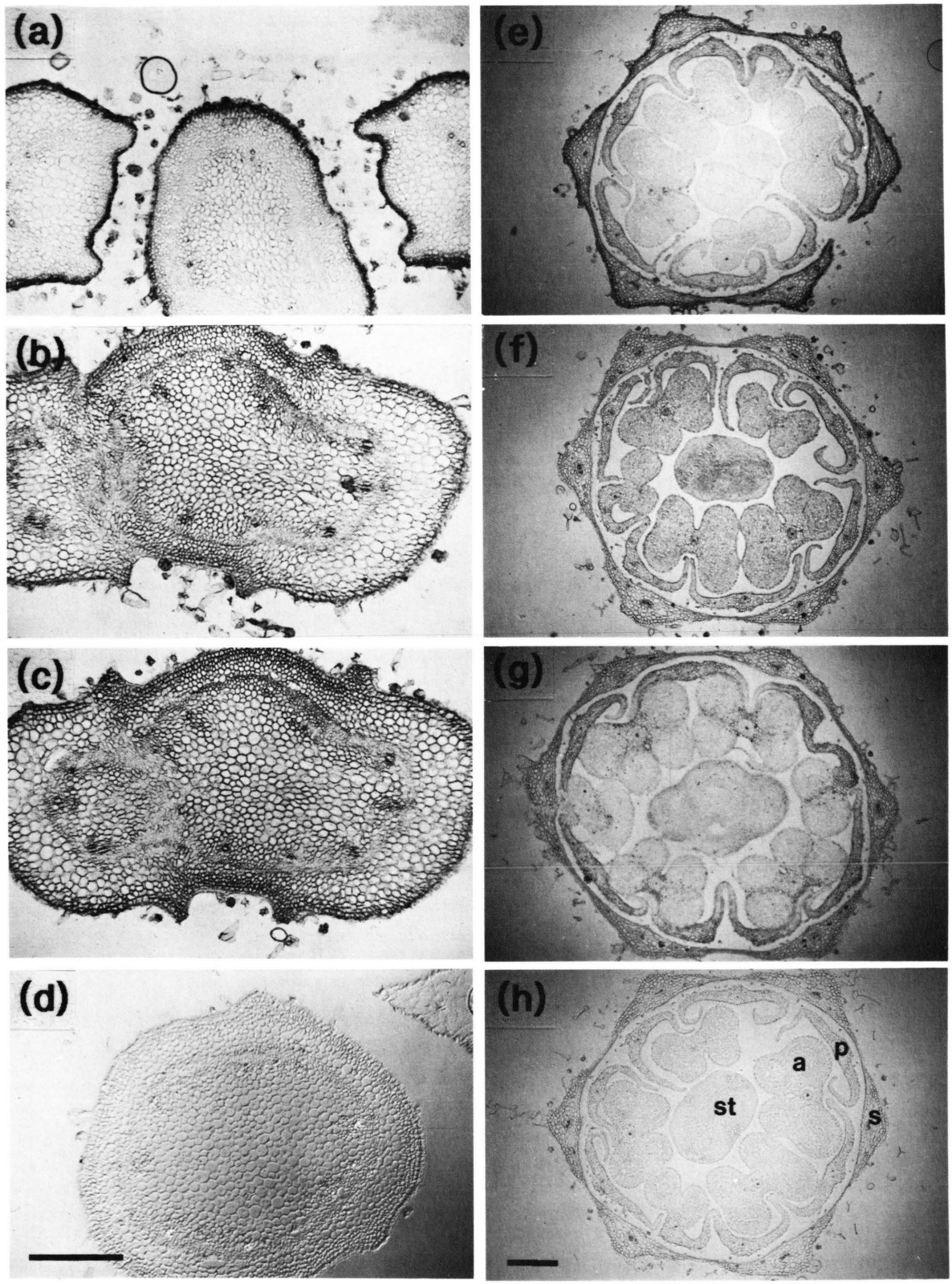

Fig. 2. Immunolocalization of proteins in cross-sections through stems (a–d) and through flowers (e–h). In (b) and (c) the sections are taken through a node and shows the shoots fused to the main stem. In (a) the section is just above this level and the developing shoots are separated from the stem. First serum in both series was specific for (a and e) chitinase, (b and f) β-1,3-glucanase, (c and g) PAL, or was (f and h) unspecific control serum. Abbreviations: a, anther; p, petal; s, sepal; st, style. Bars: 400 μM.

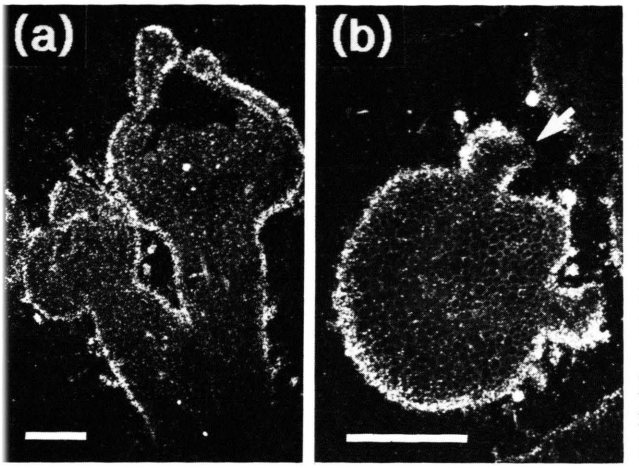

Fig. 3. Hybridization *in situ* to chitinase mRNA in meristematic tissues. Panel (a) shows a longitudinal section through a shoot tip, panel (b) a cross-section through a young shoot corresponding in position to that shown in Fig. 2a. Bars: 400 μM.

dicates that chitinase is absent from these structures. In both axillary bud and flower, β-1,3-glucanase and PAL are distributed throughout all tissues. β-1,3-Glucanase is very evenly distributed throughout all floral structures, whereas PAL is expressed to a greater extent in sepals and petals than in the anthers and style. Neither of the latter two enzymes is preferentially expressed in epidermal tissue, however.

Fig. 3 shows *in situ* hybridizations with chitinase antisence probe to a longitudinal section through a shoot tip (a) and to a cross-section through the stem of a young shoot (b). The section in the latter *in situ* hybridization corresponds in position to those in the immunolocalizations to stems of axillary buds shown in Fig. 2a. Localization of chitinase mRNA in the epidermis is again clearly evident in both tissue types. Chitinase mRNA is absent, however, from the tunica layer of the apical meristem and from the cells at the tips of the protruding structures marked by arrows in Fig. 3b. These cells represent the margins of the developing leaf and chitinase expression was diminished at the margins of mature leaves as well (data not shown).

Expression patterns of chitinase, β-1,3-glucanase and PAL in stolon, tuber and root tissues (data not shown) do not convincingly fit the pattern seen in aerial tissues. Differences seen with different probes were primarily ones of intensity, which are difficult to control between preparations in both *in situ* hybridization and peroxidase staining, and therefore do not alone provide a valid basis for comparison. Immunolocalizations in roots were difficult to interpret, perhaps because of high levels of endogenous peroxidase activity in this organ. In tuber epidermis, high levels of mRNA hybridization and peroxidase staining were present in all preparations, including controls, and were therefore not interpretable.

The patterns of expression shown in leaves of healthy, unstressed plants contrast with those seen in infected plants. As an example of the latter, Fig. 4 shows *in situ* hybridization with chitinase sense and antisense RNA and immunolocalization with antichitinase serum to leaves infected with *Phytophthora infestans*. In contrast to the characteristic constitutive expression pattern, it is clear in infected leaves that chitinase expression is homogeneously distributed through mesophyll and epidermis in the area around infection sites. Similar distributions of PAL and β-1,3-glucanase mRNAs were seen around fungal infection sites in potato leaves (Schröder *et al.*, 1992) and a number of other defense-related genes, including PAL, were found to be similarly expressed in fungus-infected parsley leaves (Schmelzer *et al.*, 1989). Another observation that can be made in these preparations, tentative because chitinase expression has been examined in only small numbers of infected leaves, is an apparent decrease in the characteristic pattern of constitutive epidermal expression, which becomes evident 12 h and later after infection, as expression becomes uniformly distributed around the infection site.

Discussion

The results described here consistently show, in aerial organs, high levels of constitutive chitinase gene expression confined strictly to the epidermis, whereas constitutive expression of β-1,3-glucanase and PAL is distributed, with only moderate local variation, through all tissues of every organ examined. It was not possible to reach firm conclusions about expression of these genes in terrestrial organs, except to say that there is no clear localization of chitinase to epidermal cells (data not shown).

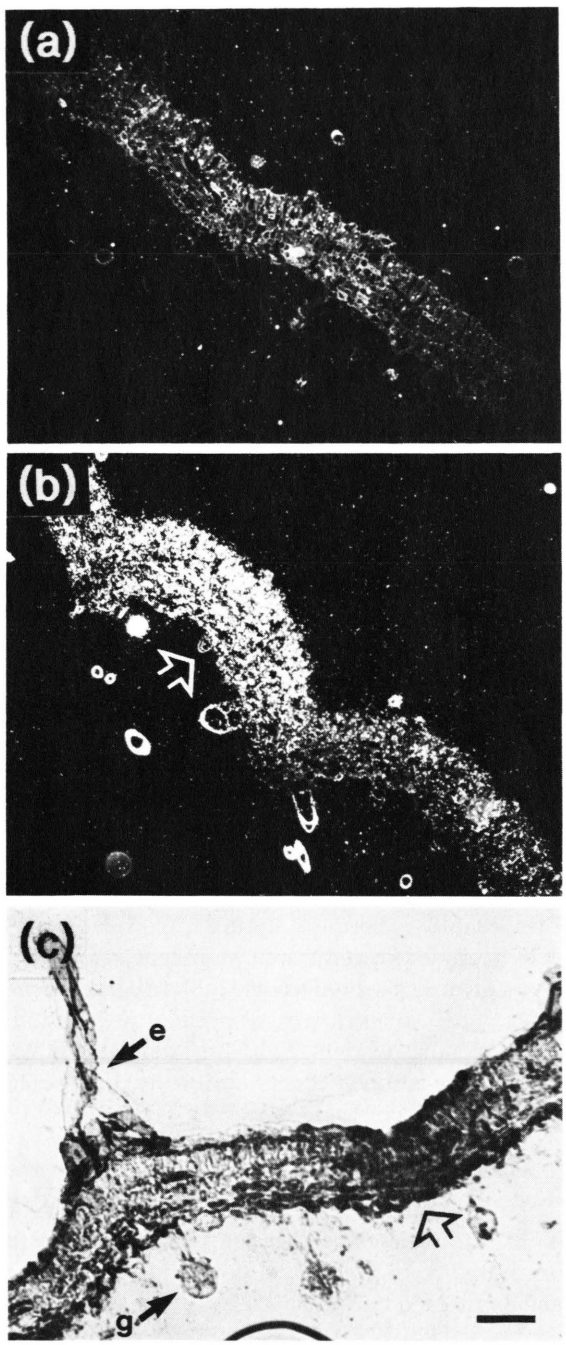

Fig. 4. *In situ* hybridization with (a) chitinase sense and (b) chitinase antisense probe and (c) immunolocalization of chitinase protein in potato leaves infected by *Phytophthora infestans*. Large arrowheads indicate the infection sites on the abaxial sides of the leaves in (b) and (c). Abbreviations: e, epidermal hair; g, glandular hair. Bar: 100 μM.

Our findings with respect to chitinase distribution in potato leaves agree with those made by Keefe *et al.* (1990) in tobacco leaves and we demonstrate further that chitinase transcripts and protein appear to be localized in the epidermis in most, perhaps all, aerial organs of the unstressed plant. Our observations in stressed leaves also correlate well with prior studies, which have shown that chitinase and β-1,3-glucanase expression become elevated and distributed throughout all leaf tissues in response to *Phytophthora* infection (Schröder *et al.*, 1992) and ethylene treatment (Keefe *et al.*, 1990). Our results with respect to constitutive β-1,3-glucanase expression in unstressed leaves differ markedly from those of Keefe *et al.* (1990) however. The latter study found β-1,3-glucanase localized in the epidermis similarly to chitinase, whereas we found no such localization, either of mRNA or of protein. The reasons for this discrepancy are not obvious, since the species are closely related and the localization probes used in the two studies were obtained from the products of similar genes, ones encoding basic, inducible isoforms of the respective proteins. The locations of the leaves taken for the localization experiments might be suspected as a contributing factor in the differences observed in β-1,3-glucanase expression patterns. We used leaves from the top of the plant down to the fourth node, whereas Keefe *et al.* (1990) used leaves from below the middle of the plant. A gradient of β-1,3-glucanase expression exists in healthy tobacco (Felix and Meins, 1986; Castresana *et al.*, 1990), with expression lowest in the upper parts of the plant. However, a similar gradient of chitinase expression exists in both plants (Shinshi *et al.*, 1987; Witte, 1991), which makes it difficult to invoke these gradients as a basis for the differences observed by ourselves and Keefe *et al.* (1990) only in β-1,3-glucanase expression.

The epidermal and glandular hair substructures of leaves represent minor exceptions to the generalizations made above about expression patterns of chitinase, β-1,3-glucanase and PAL genes in potato leaves. In the epidermal hairs, chitinase expression is localized in basal elements, whereas β-1,3-glucanase is observed only in the distal parts of the structure. PAL appears to be present in both basal and distal parts. The glandular hairs of leaves contain PAL and, perhaps, β-1,3-glucanase,

but chitinase was not detected in them. These conclusions are tentative, being based on observations of the small numbers of intact hair structures.

The observation of a loss in infected leaves of the pattern characteristic of constitutive chitinase gene expression may explain why epidermal localization of chitinase was not seen in immunolocalizations in ethylene treated bean leaves (Mauch and Staehelin, 1989). A recent paper from the same laboratory (Mauch *et al.,* 1992) indicates that β-1,3-glucanase induced by ethylene accumulates in the lower epidermis and in a few other restricted locations within leaves, however, which disagrees with the findings of Keefe *et al.* (1990), who found generalized expression after ehylene treatment in tobacco. A decrease in epidermal chitinase gene expression correlates with observations suggesting that expression of dispensable genes is repressed in infected organs. For example, the concentration of hybridizable mRNA encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (rubisco) was found to decrease greatly in potato leaves within 12 h after fungal infection or elicitor treatment (Kombrink and Hahlbrock, 1990). Decreases in rate of synthesis and activity of both large and small rubisco subunits were observed to precede the appearance of disease symptoms in fungus-infected melon leaves (Ranty *et al.,* 1987) and major decreases in amounts of extractable PAL mRNA have been observed in fungus-infected parsley leaves (Logemann and Hahlbrock, unpublished observations). The systemic decrease in constitutive gene expression has been attributed to a recruitment of materials and energy to meet the specialized metabolic demands of disease resistance (Kombrink and Hahlbrock, 1990).

The observed distribution of chitinase expression, of course, suggests an epidermis-specific function. This is very much in agreement with the observations of De Jong *et al.* (1992), in which a role for chitinase in the formation of the protoderm of carrot embryos is strongly indicated. The pattern of *in situ* hybridization to carrot embryos with a protoderm-specific probe is, in fact, very similar to the patterns of chitinase gene expression seen here. Thus, the observation that the occurrence of chitinase is strictly confined to a single specialized cell type in aerial parts of the plant, combined with recognition that actual levels of activity in chitinase expressing cells must be much higher than would be inferred from measurements of average activity over whole organs, indicates that chitinase plays a more active role in the metabolism and development of healthy plants than current conceptions of its contribution to the defense response would suggest.

The constitutive expression patterns of β-1,3-glucanase genes differ from those of chitinase, in most tissues resembling those of PAL in being ubiquitous with local variations in intensity. PAL contributes to pathways leading to diverse end products, but perhaps its most central function is in cell wall deposition. β-1,3-Glucans also have structural functions, and subcellular localization of β-1,3-glucanase in ethylene treated bean leaves has revealed its presence in the cell wall (Mauch and Staehelin, 1989). This association may explain the similar expression patterns of the PAL and β-1,3-glucanase gene families.

Materials and Methods

Plant and fungus

The potato cultivar Datura was used throughout this work. Plants were grown in the greenhouse from tubers for approximately 4 weeks, to a height of approximately 1 m, and acclimatized under continuous light and at constant temperature for a period of 12 h prior to dissection of tissues or infection treatments. Aerial tissues used in the study were taken from the shoot apices through the fourth nodes. *Phytophthora infestans* (Mont.) de Bary race 1 was used for infections, which were done by drop inoculation of leaves with zoospores as described by Schröder *et al.* (1992) and the leaves were taken 24 h after inoculation.

Localization of gene products

Tissue fixation, paraffin embedding, sectioning, and histological preparation of sections were carried out as described by Schmelzer *et al.* (1989). Immunohistochemistry and *in situ* mRNA hybridization were carried out essentially as described by Schröder *et al.* (1992). The chitinase gene from which probes were transcribed was described by Laflamme and Roxby (1989). It is a member of the class I family of chitinase genes (Shinshi *et al.,* 1990). The potato glucanase gene and the chitinase and β-1,3-glucanase antisera used in this study were the generous gift of E. Kombrink of the Max-

Planck-Institut, Köln. The chitinase antibodies were raised against a mixture of two potato chitinases corresponding to peaks 3 and 4 from the cation-exchange column fractionation procedure described by Kombrink *et al.* (1988). These proteins have molecular weights of 33 kDa and alkaline isoelectric points. The antiserum identified proteins on western blots of the same size which are inducible by infection, elicitor treatment or ethylene and are present in both intra- and extracellular compartments (Witte, 1991). The β-1,3-glucanase cDNA sequence (E. Kombrink, personal communication) used for probe transcription is similar to the composite β-1,3-glucanase cDNA from tobacco described by Shinshi *et al.* (1988), which encodes an alkaline, vacuolar protein (Van den Bulcke *et al.*, 1989). Assuming that posttranslational modifications of this protein take place in the way proposed for the tobacco protein (Shinshi *et al.*, 1988), the potato cDNA sequence predicts a mature β-1,3-glucanase protein having molecular weight of 35 kDa and isoelectric point of 8.6. The β-1,3-glucanase antiserum was raised against a 36 kDa, basic isoform of the enzyme purified from potato (Kombrink *et al.*, 1988). Western blots on tissue fractions indicated that the protein identified by the antiserum is present in both the intracellular and extracellular compartments and that most of the β-1,3-glucanase activity in the intracellular compartment is localized in the vacuole (Schröder, 1990). The PAL probes were transcribed from a 900 bp potato PAL cDNA (Fritzemeyer *et al.*, 1987; Cuypers *et al.*, 1988). The PAL antiserum was raised against the purified parsley protein (Schröder *et al.*, 1976) and has been shown to cross-react with similar affinity to potato PAL proteins (Arabatzis, 1991).

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