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Accumulation of hydroxyproline-rich glycoprotein mRNAs in response to fungal elicitor and infection

(bean/extensin/plant disease resistance/RNA blot hybridization/tomato)

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ABSTRACT **Hydroxyproline-rich glycoproteins (HRGPs) are important structural components of plant cell walls and also accumulate in response to infection as an apparent defense mechanism. Accumulation of HRGP mRNA in biologically stressed bean (*Phaseolus vulgaris* L.) cells was monitored by blot hybridization with ^{32}P-labeled tomato genomic HRGP sequences. Elicitor treatment of suspension-cultured cells caused a marked increase in hybridizable HRGP mRNA. The response was less rapid but more prolonged than that observed for mRNAs encoding enzymes of phytoalexin biosynthesis. HRGP mRNA also accumulated during race:cultivar-specific interactions between bean hypocotyls and the partially biotrophic fungus *Colletotrichum lindemuthianum*, the causal agent of anthracnose. In an incompatible interaction (host resistant) there was an early increase in HRGP mRNA correlated with expression of hypersensitive resistance; whereas, in a compatible interaction (host susceptible), marked accumulation of HRGP mRNA occurred as a delayed response at the onset of lesion formation. In both interactions, mRNA accumulation was observed in uninfected cells distant from the site of fungal inoculation, indicating intercellular transmission of an elicitation signal.**

Plants exhibit natural resistance to disease, which involves inducible defense mechanisms including accumulation of phytoalexins, deposition of lignin-like material, accumulation of cell wall hydroxyproline-rich glycoproteins (HRGPs), and increases in the activity of certain hydrolytic enzymes (1, 2). Such responses can be induced not only by infection but also by glycan, glycoprotein, and lipid elicitors present in fungal cell walls and culture filtrates and, in some cases, by structurally unrelated artificial elicitors or mechanical damage (3–5).

Resistance is an active process dependent on host RNA and protein synthesis (1, 2), and recent studies have revealed marked changes in the pattern of RNA and protein synthesis in response to biological stress (4, 6–10). At least some of these changes involve activation of genes encoding enzymes of phytoalexin biosynthesis leading to specific accumulation of the corresponding mRNAs (7, 11–18). These observations raise the question of whether expression of other plant disease resistance mechanisms also involves accumulation of specific mRNAs. In the present paper, we have used cloned genomic HRGP sequences to monitor, by RNA blot hybridization, changes in the level of HRGP mRNAs in response to biological stress.

HRGPs are major structural components of plant cell walls (19, 20). In addition to hydroxyproline (Hyp), cell wall HRGPs are rich in serine, valine, tyrosine, and lysine. Moreover, cell wall HRGPs contain a characteristic repeating pentapeptide sequence, Ser-(Hyp)$_4$. Recently, a carrot genomic clone encoding a cell wall HRGP was isolated, sequenced, and shown to contain 25 Ser-(Pro)$_4$ repeat units, the unhydroxylated precursors of the Ser-(Hyp)$_4$ repeat units, distributed throughout the 306-amino acid coding sequence (52). The carbohydrate moiety of cell wall HRGPs is composed largely of short oligoarabinosides attached *O*-glycosidically to most of the hydroxyproline residues and to a much lesser extent of galactose, which is *O*-glycosidically linked to some of the serine residues (19, 20). The accumulation of cell wall HRGP in response to infection has been observed in a number of systems and is correlated with expression of disease resistance (21–23). HRGPs may function in defense as specific agglutinins of microbial pathogens (24) and/or as structural barriers, either directly or by providing sites for lignin deposition (25).

We report here marked increases in HRGP mRNAs in elicitor-treated bean (*Phaseolus vulgaris* L.) cells and infected bean hypocotyls during race:cultivar-specific interactions with *Colletotrichum lindemuthianum*, causal agent of anthracnose. The pattern of accumulation is broadly similar to that observed for mRNAs encoding enzymes of phytoalexin biosynthesis (refs. 7, 11–13; unpublished observations) but with significant differences in detail, possibly related to the specific distinct roles these two responses have in the overall process of plant defense.

MATERIALS AND METHODS

Isolation and Partial Characterization of a Tomato HRGP Genomic Clone. A partial *Eco*RI tomato (*Lycopersicon esculentum*; breeding line T3) genomic library packaged in Charon 4 (generously provided by R. W. Breidenbach) was screened by *in situ* plaque hybridization (26) with both a cDNA clone (pDC5) and a genomic clone (pDC5A1) for a carrot cell wall HRGP that have been described in detail elsewhere (27, 52). A restriction map of one of the tomato HRGP genomic clones (Tom 5) was determined and the region of homology to the carrot cDNA and genomic clones was elucidated by Southern blot hybridization analysis (28). A 10-kilobase (kb) *Eco*RI restriction fragment of Tom 5 was found to hybridize to the carrot HRGP sequences and was subcloned in the plasmid vector pEMBL8+. The resulting subclone, pTom 5.10, was sequenced according to the method of Maxam and Gilbert (29) to verify the presence of HRGP sequences.

Fungal Cultures and Elicitor Preparation. The source, maintenance, and growth of cultures of *C. lindemuthianum* and generation of conidia were as described (30). Elicitor was

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Abbreviations: HRGP, hydroxyproline-rich glycoprotein; kb, kilobase(s).

the high molecular weight fraction released by heat treatment of isolated mycelial cell walls (31).

Plant Material. Bean (*P. vulgaris* L. cv. Canadian Wonder) cells were grown as described, except the cultures were maintained in total darkness (32). Experiments were conducted with 7- to 10-day-old cultures, the medium of which exhibited a conductivity between 2.5 and 2.8 mho. Germination and growth of bean cv. Kievitsboon Koekoek and inoculation of hypocotyls from 8-day-old seedlings with spores of *C. lindemuthianum* races β and γ were as described (13).

Isolation of RNA. Polysomal RNA was isolated by a modification (33) of the method of Palmiter (34). Total cellular RNA was isolated from samples homogenized directly in a phenol/0.1 M Tris·HCl, pH 9.0, emulsion as described by Haffner *et al.* (35). Further purification of the phenol-extracted total cellular RNA was identical to that used for polysomal RNA.

RNA Blot Hybridization. RNA (15 μg) from bean cell cultures was denatured with formaldehyde and separated by electrophoresis on 1% agarose gels (36). RNA (5 μg) from hypocotyls was denatured with glyoxal and separated by electrophoresis on 1.2% agarose gels (36). The gels were blotted onto nitrocellulose and hybridized with ^{32}P-labeled tomato HRGP genomic sequences prepared by nick-translation (36) of the 10-kb *Eco*RI insert of pTom 5.10. Hybridizations were carried out at 42°C for 24 hr in 4× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M Na citrate, pH 6.8)/2× Denhardt's solution (1× Denhardt's solution = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.2% $NaDodSO_4$/100 μg of carrier DNA per ml/50% formamide with 10 ng of the tomato HRGP probe per ml nick-translated to a specific activity of 1–3 × 10^8 cpm/μg. After incubation, the filters were washed in 2× NaCl/Cit/0.1% $NaDodSO_4$ first at room temperature and then at 50°C until the background was acceptable for autoradiography. HRGP mRNA was quantitated by scanning densitometry of the autoradiograms calibrated with reference to internal standard HRGP mRNA samples.

RESULTS

Tom 5, a tomato genomic clone encoding a cell wall HRGP, was isolated by screening a tomato genomic library with carrot cDNA and genomic clones for cell wall HRGP (unpublished data). A single 10-kb *Eco*RI fragment of Tom 5 was hybridized to the carrot HRGP in Southern blot analysis and was subjected to restriction mapping and DNA sequence analysis (Fig. 1). The DNA sequence of a portion of this *Eco*RI fragment encoded numerous Ser-$(Pro)_4$ repeat units, which are posttranslationally modified to the characteristic cell wall HRGP sequence Ser-$(Hyp)_4$.

The 10-kb *Eco*RI tomato genomic fragment was labeled with ^{32}P and shown to hybridize to three RNA species of 1.6, 2.7, and ≈5.6 kb present in total cellular RNA isolated from elicitor-treated bean cells (Fig. 2). Identical RNA species were also detected by blot hybridization of RNA from suspension-cultured bean cells with ^{32}P-labeled cDNA and genomic carrot HRGP sequences (data not shown).

Elicitor treatment caused a marked and prolonged accumulation of HRGP mRNA from relatively low basal levels in unelicited cells (Fig. 2). Increases in the 2.7-kb species were first observed after a lag of ≈4 hr. There was a rapid increase in hybridizable mRNA between 6 and 12 hr after elicitor treatment, after which the mRNA remained at high levels. Accumulation of the 1.6- and 5.6-kb HRGP mRNA species followed a similar pattern, although at each time point these forms were considerably less abundant than the 2.7-kb species. Accumulation of HRGP mRNAs in the polysomal RNA fraction followed a similar time course (Fig. 2*C*).

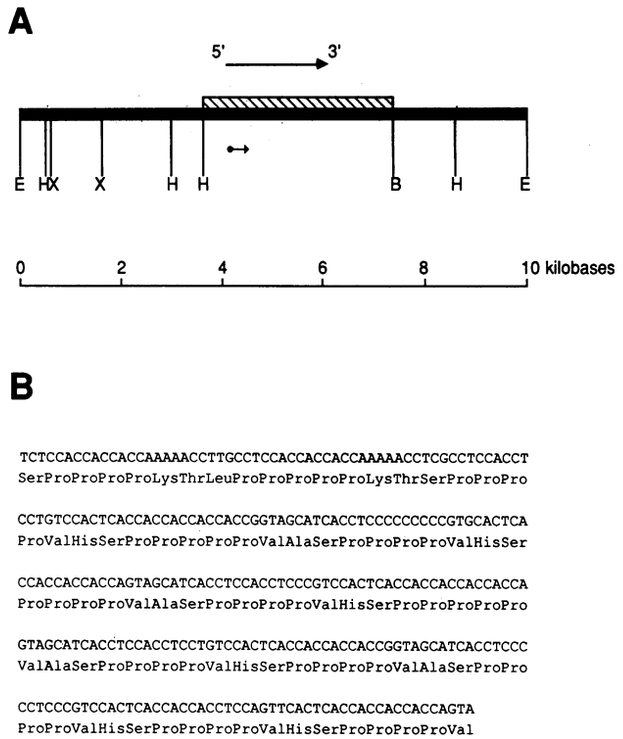

FIG. 1. Restriction map (*A*) and partial DNA sequence (*B*) of the 10-kb *Eco*RI fragment of the tomato genomic HRGP clone Tom 5. The region of hybridization of this restriction fragment to carrot cDNA and genomic HRGP sequences is indicated by ▨. The direction of transcription is as shown, and the region of DNA sequence analysis is indicated by ↔. The DNA was cut and labeled at an internal *Hin*fI site and read in a 5′ to 3′ direction off the coding strand. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba* I.

Moreover, there was no appreciable increase in the level of these HRGP mRNAs in mock-treated unelicited control cell cultures (Fig. 2*A*).

Changes in the level of HRGP mRNA were also measured during race:cultivar-specific interactions between hypocotyls of bean cv. Kievitsboon Koekoek and physiological races of *C. lindemuthianum*. RNA was isolated from tissue directly underlying the site of spore inoculation (site 1), from tissue laterally adjacent to the infected tissue (site 2), and from tissue beneath sites 1 and 2 (site 3) (Fig. 3).

In the incompatible interaction (host-resistant) after application of spores of *C. lindemuthianum* race β to the unwounded hypocotyl surface, there is a period of 50–60 hr during which the spores germinate and the fungus penetrates the cuticle. At this stage, when the developing hyphae first come in contact with the underlying host epidermal cells, the presence of the fungus is rapidly detected, leading to a hypersensitive response and restriction of further fungal growth (37). In the present study, we have observed an early increase in hybridizable HRGP mRNA in total cellular RNA samples isolated from tissue including, and immediately adjacent to, the sites of spore inoculation (site 1). In contrast to elicitor-treated cells, the levels of both the 1.6- and 2.7-kb species were markedly increased (Fig. 3). The 5.6-kb species was not detected. Accumulation was first observed ≈52 hr after inoculation, and during the expression of hypersensitive resistance, the level of hybridizable HRGP mRNA remained between 10- and 20-fold above that in equivalent uninfected control hypocotyls (Fig. 4). Accumulation of HRGP mRNA was also observed in RNA isolated from sites 2 and 3 with a maximum 5- to 6-fold increase over the level in equivalent uninfected hypocotyls.

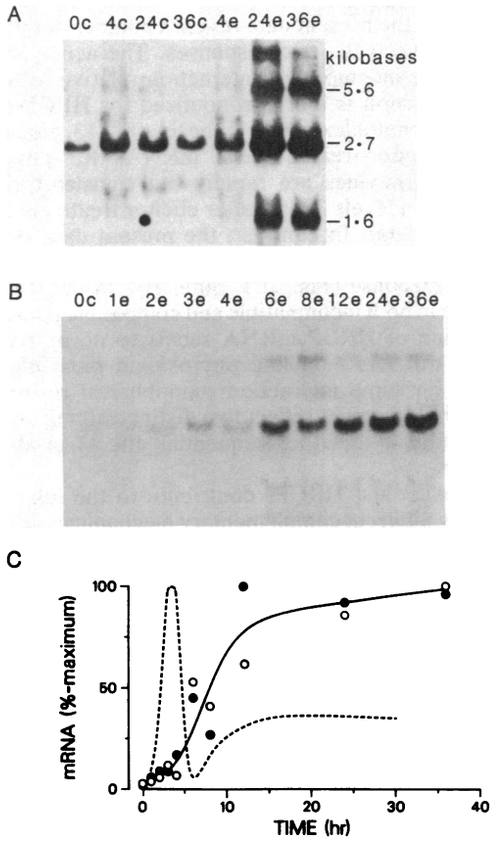

FIG. 2. Accumulation of HRGP mRNA in elicitor-treated bean cells. RNA was blot hybridized with ^{32}P-labeled tomato HRGP genomic sequences. (*A* and *B*) Total cellular RNA isolated from cells at the times (in hours) indicated after elicitor treatment (e) or from equivalent mock-treated unelicited control cells (c). (*C*) Kinetics of elicitor-induced accumulation of the HRGP mRNA species size 2.7 kb in total cellular RNA (○) and polysomal RNA fractions (●). Dotted line denotes the kinetics previously observed (12) for the accumulation of mRNA encoding the phytoalexin biosynthetic enzyme chalcone synthase in the same set of RNAs.

In the compatible interaction (host-susceptible) with *C. lindemuthianum* race γ, the infected cells remain alive and the fungus undergoes substantial biotrophic growth. Subsequently, extensive host cell death occurs and spreading anthracnose lesions develop (37). Marked accumulation of HRGP mRNA occurred somewhat later in the compatible interaction compared to the incompatible interaction and was correlated with the onset of lesion formation (Fig. 4). The 2.7-kb species was the predominant form with only relatively moderate increases in the 1.6-kb species and weak but detectable increases in the 5.6-kb species (Fig. 3). Accumulation of HRGP mRNA occurred slightly earlier in directly infected tissue at the site of spore inoculation (site 1), but there was also significant accumulation in sites 2 and 3. Maximum accumulation of HRGP mRNA in the incompatible interaction (site 1, 93 hr) was ≈80% of the maximum level attained in the compatible interaction (site 1, 168 hr).

DISCUSSION

Marked accumulation of HRGP mRNA in response to biological stress is consistent with previous studies demonstrating the accumulation of cell wall HRGPs in plant tissues infected with fungi (21, 22). Furthermore, elicitor stimulation of HRGP synthesis in melon and soybean hypocotyls and accumulation of cell wall hydroxyproline in elicitor-treated bean cells have recently been reported (38, 39). The pattern

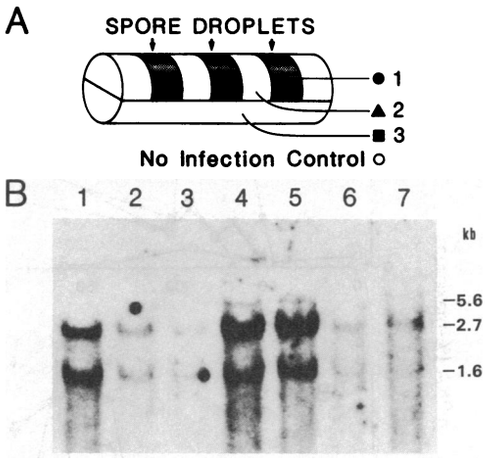

FIG. 3. Pattern of accumulation of HRGP mRNAs in hypocotyls of bean cv. Kievitsboon Koekoek during race:cultivar-specific interactions with physiological races of *C. lindemuthianum*. (*A*) Dissection of hypocotyl tissue. (*B*) Autoradiograph of RNA blot hybridized with ^{32}P-labeled HRGP genomic sequences. Lanes: 1–3, RNA from hypocotyls 79 hr after inoculation with spores of the incompatible race β; 4–6, RNA from hypocotyls 150 hr after inoculation with spores of the compatible race γ. RNA was isolated from site 1 (lanes 1 and 4), site 2 (lanes 2 and 5), site 3 (lanes 3 and 6). For comparison, lane 7 contains RNA isolated from suspension-cultured cells 7 hr after elicitor treatment.

of accumulation of HRGP mRNA is broadly similar to that previously observed for mRNAs encoding enzymes of phytoalexin biosynthesis (refs. 11–13; unpublished data),with marked increases from low basal levels in both cell cultures after elicitor treatment and infected hypocotyls during race:cultivar-specific interactions with *C. lindemuthianum*. As with the phytoalexin response, there was an early marked increase in HRGP mRNA in an incompatible interaction (host resistant) correlated with expression of hypersensitive resistance; whereas, in a compatible interaction (host susceptible), marked accumulation of HRGP mRNA occurred as a delayed widespread response at the onset of lesion formation. Hammerschmidt *et al.* have previously shown that cell wall hydroxyproline levels increase much earlier in resistant cultivars than in susceptible cultivars of cucumber infected with the fungus *Cladosporium cucumerinum* (22).

Thus, accumulation of specific mRNAs appears to underlie at least two separate and distinct defense responses during plant disease resistance. Recent studies have also demonstrated stimulation of the translatable activities of mRNAs encoding chitinase and cinnamyl alcohol dehydrogenase in biologically stressed bean cells (unpublished observations). Cinnamyl alcohol dehydrogenase is an enzyme of phenylpropanoid metabolism specific to a branch pathway for synthesis of lignin precursors (40). Chitinase has lysozymal activity (41) and may function in defense by degradation of the fungal cell wall polymer chitin (42). The overall picture emerging from these studies is that rapid selective changes in the pattern of gene expression characteristically underlie activation of plant defense responses.

As with mRNAs encoding enzymes of phytoalexin biosynthesis (ref. 13; unpublished data) there was, in both compatible and incompatible interactions, accumulation of HRGP mRNA in apparently healthy hitherto uninfected tissue distant from the initial site of fungal spore inoculation, implying intercellular transmission of an elicitation signal(s). Thus, in the compatible interaction, accumulation of mRNA in sites 2 and 3 occurs when the fungus is not present in these sites and is found only in site 1. At this stage in site 1 tissue, there is extensive cell death in hitherto uninfected cells at the periphery of the developing lesion (43). Hence, accumulation

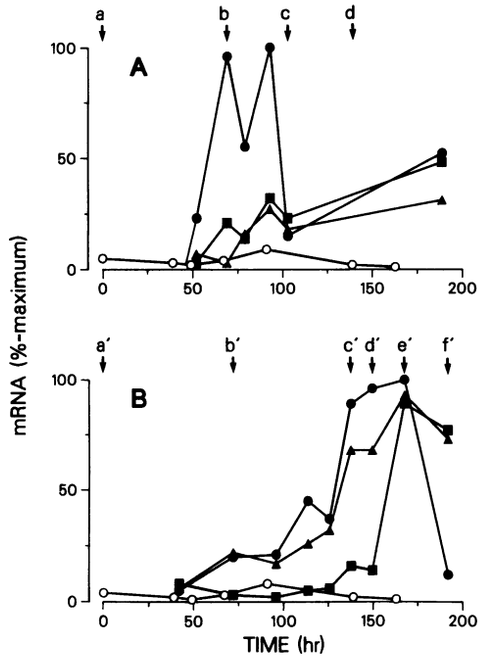

FIG. 4. Kinetics of accumulation of the HRGP mRNA 2.7-kb species in hypocotyls of bean cv. Kievitsboon Koekoek during race:cultivar-specific interactions with physiological races of *C. lindemuthianum*. (*A*) Incompatible interaction with race β. (*B*) Compatible interaction with race γ. RNA was isolated from site 1 (●), site 2 (▲), site 3 (■), and equivalent uninfected hypocotyls (○). Arrows in *A* denote events in the expression of hypersensitive resistance at site 1: a, spore inoculation; b, onset of hypersensitive flecking in a few sites; c, hypersensitive flecking apparent at most sites; d, very dense brown flecking at all sites. No visible changes occurred in sites 2 and 3 and control hypocotyls throughout the time course. Arrows in *B* denote events in lesion development at site 1: a', spore inoculation; b', no visible symptoms (cf. incompatible interaction); c', onset of symptom development at a few sites; d', pale to medium brown lesions apparent at most sites; e', onset of water soaking and development of spreading lesions; f', extensive water soaking and spreading of lesions from site 1, some browning at site 2.

of mRNA in sites 2 and 3 may reflect the mediation of endogenous elicitors (44–47) released after death of host cells and dissolution of cell walls associated with the breakdown of the biotrophic phase of fungal growth (37, 43, 48). Increases in HRGP mRNA in distant hitherto uninfected tissue may be part of an attempt to prevent further fungal spread and hence limit lesion development in the later stages of a compatible interaction (49).

Of particular interest is the observation that accumulation of HRGP mRNA in the early stages of the incompatible interaction occurs not only in site 1 but also in sites 2 and 3. Expression of hypersensitive resistance is a localized event in terms of both restriction of fungal growth to a single host cell and the occurrence of cell death and browning only in directly affected host cells (50). However, prechallenge with an avirulent pathogen can also induce systemic resistance to subsequent challenge by a normally virulent pathogen (2, 51). Accumulation of HRGP mRNA in an incompatible interaction in uninfected tissue distant from the site of localized recognition and resistance may provide a mechanism for preactivation of the defense response in hitherto uninfected tissue and/or more rapid activation of the defense response after subsequent microbial attacks and, hence, may be related to establishment and expression of induced systemic resistance.

Although biological stress causes increases in HRGP mRNAs and mRNAs encoding enzymes of phytoalexin biosynthesis, the present data reveal a number of differences in detail between the two responses. The accumulation of mRNA in the incompatible interaction relative to the compatible interaction is more pronounced for HRGP than for enzymes of phytoalexin biosynthesis (ref. 13; unpublished data). In elicitor-treated cells, mRNAs for phytoalexin biosynthetic enzymes are rapidly but transiently induced with maximum levels 3–4 hr after elicitor treatment (ref. 12; unpublished data). In contrast, the present data show that accumulation of HRGP mRNAs is a less rapid but more prolonged response (Fig. 2*C*). Similarly, in infected hypocotyl tissue in both incompatible and compatible interactions, accumulation of HRGP mRNA seems to occur somewhat later than mRNAs encoding phytoalexin biosynthetic enzymes in the same interaction (unpublished results). The different kinetics might reflect two distinct stimuli or a single stimulus leading to either sequential effects or divergent signal pathways.

Phytoalexins and HRGPs contribute to the inhibition of infection by different complementary mechanisms associated with their functional properties as toxic natural products and structural cell wall glycoproteins, respectively. Hence, differences in the accumulation of HRGP mRNAs and mRNAs encoding enzymes of phytoalexin biosynthesis may be related to specific distinct roles for these two responses in the overall process of plant defense. In this context, it is of interest that the three distinct HRGP mRNA species accumulated to different degrees in the compatible and the incompatible interactions. This polymorphism may reflect subtle regulatory and structural differences related to the specific functions of the corresponding protein products in defense.

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