

# Differential induction of chalcone synthase mRNA activity at the onset of phytoalexin accumulation in compatible and incompatible plant–pathogen interactions

(*Phaseolus vulgaris*/Colletotrichum lindemuthianum/plant disease resistance/phaseollin/gene expression)

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**ABSTRACT** Changes in the mRNA activity of chalcone synthase, the first enzyme of phenylpropanoid metabolism specific to flavonoid/isoflavonoid biosynthesis, have been investigated in relation to expression of the phytoalexin defense response in race-cultivar specific interactions between hypocotyls of *Phaseolus vulgaris* and the partially biotrophic fungus *Colletotrichum lindemuthianum*, causal agent of anthracnose. In an incompatible interaction (host resistant) there is an early but localized increase in chalcone synthase mRNA activity prior to the onset of accumulation of the phenylpropanoid-derived phytoalexin phaseollin and expression of hypersensitive resistance. In contrast, in a compatible interaction (host susceptible) there is no induction of mRNA activity in the early stages of infection but rather a delayed, widespread increase during attempted lesion limitation at the onset of symptom development. The data indicate that control of phytoalexin gene expression is a key early component in the defense responses of biologically stressed cells during a race-cultivar specific host–pathogen interaction.

Plant disease resistance involves not only static protection but also active defense mechanisms, prominent among which is the induced accumulation of host-synthesized phytoalexin antibiotics (1). Phytoalexins can be induced by glycan, glycoprotein, and lipid elicitors present in fungal and bacterial cell walls and culture filtrates (2) and by a variety of structurally unrelated, artificial inducers (3). Accumulation is largely a result of increased synthesis from remote precursors (4, 5) and increases in the levels of appropriate biosynthetic enzymes are observed at the onset of the defense response (6).

In cell suspension cultures of dwarf French bean (*Phaseolus vulgaris* L.) elicitor treatment causes marked but transient increases in the rate of synthesis of phenylalanine ammonia-lyase (EC 4.3.1.5), the first enzyme in general phenylpropanoid metabolism, and chalcone synthase, the first enzyme specific to the flavonoid/isoflavonoid branch pathway of phenylpropanoid biosynthesis, concomitant with the onset of accumulation of phaseollin and related isoflavonoid phytoalexins (7–10). The transient increases in enzyme synthesis reflect increases in the levels of the corresponding mRNA activities (11), which, together with changes in the apparent stability of the enzymes *in vivo*, are responsible for the marked increases in enzyme activity that control phytoalexin accumulation in response to elicitor (9, 12).

Although these and similar observations in other systems (13, 14) establish a causal relationship between specific and rapid elicitor-induced changes in the pattern of protein syn-

thesis and subsequent expression of the phytoalexin defense response in cell cultures, a major question is whether similar mechanisms operate in biologically stressed cells during attempted infection of intact plant tissue. This is of particular importance in relation to operation of the phytoalexin defense response in genetically controlled race-cultivar specific interactions, which underlie many agriculturally important plant diseases (15), since such specificity is not expressed in elicitor-treated cell cultures (16). Furthermore, spatial interactions such as the intercellular transmission of elicitation (17) and other stress signals (18) may operate differently in isolated cells compared to intact tissue.

Therefore, we have initiated a study of phytoalexin gene expression in race-cultivar specific interactions between hypocotyls of *P. vulgaris* and the partially biotrophic fungus *Colletotrichum lindemuthianum*, causal agent of anthracnose disease (19). Hypocotyls, a natural site of the disease, can be reproducibly infected, without prior mechanical damage, by surface inoculation with conidia, the natural infective propagule. Detailed physiological studies have established the biological importance of phytoalexin accumulation in this system (20), which, therefore, provides a model for biochemical analysis of disease resistance. In the present paper we report clear spatial and temporal differences in the pattern of induction of chalcone synthase mRNA activity between compatible and incompatible interactions. This indicates that control of phytoalexin gene expression is likely to be a key early component in the defense responses of biologically stressed plant cells during this race-cultivar specific host–pathogen interaction.

## MATERIALS AND METHODS

**Plant and Fungal Material.** Seeds of *P. vulgaris* cv. Kievitsboon Koekoek were germinated as described and grown at 25°C and 85% relative humidity under fluorescent light with a 16-hr photoperiod (21). After 7 days, fully extended hypocotyls were excised 10 mm from the base and 20 mm below the cotyledons. The ends were sealed with molten paraffin wax and the hypocotyls were incubated horizontally in humidified boxes at 16°C under the same light conditions.

*C. lindemuthianum* races  $\beta$  and  $\gamma$  were maintained and sporulating cultures were generated by UV irradiation, as described (21). Spores obtained after 6–8 days were suspended in distilled water, washed twice by centrifugation at  $800 \times g_{av}$  for 3 min, and finally resuspended in distilled water at a concentration of  $5 \times 10^5$  spores per ml. Drops (5  $\mu$ l) of this suspension were applied at 20-mm intervals along the

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upper surface of the hypocotyls. Lesion development was monitored visually and microscopically. Control hypocotyls were treated with sterile water in the absence of spores and incubated separately, as above.

At appropriate intervals, 40–50 infected hypocotyls were collected, the terminal 5-mm portions were removed, and the remaining material was excised by scalpel to yield collections of tissue from different regions of the hypocotyl in relation to the initial sites of spore inoculation. Thus, the hypocotyls were bisected longitudinally in a horizontal plane and the upper half was then divided into two series of equal portions comprising the tissue immediately underlying the initial sites of inoculation (site 1) and the residual portion of tissue between sites of inoculation (site 2), respectively. The lower half of the hypocotyl represented site 3 and control hypocotyls after removal of the wounded terminal portions represented site 4. Typically, at each time point between 150 and 210 samples of each type of site were collected, giving about 4 g of fresh weight of site 1 and site 2 material and 7 g of fresh weight of site 3 and site 4 material. Harvested tissue was frozen in liquid N<sub>2</sub> and stored at -70°C until required for enzyme assay or mRNA preparation.

**Enzyme Extraction and Assay.** Enzyme was extracted by gently homogenizing 1 g of tissue in 2.0 ml of 0.1 M potassium phosphate (pH 8.0) containing 1.4 mM 2-mercaptoethanol. Cellular debris were removed by centrifugation of the extract for 20 min at 5000 × *g*<sub>av</sub> and chalcone synthase activity was determined with a radioactive assay procedure (22) as described (10). One unit of enzyme activity (1 kat) is defined as the amount of enzyme required for the formation of 1 mol of product in 1 s under the assay conditions.

**Protein Synthesis *in Vitro*.** RNA was assayed spectrophotometrically at 260 nm. Polysomal RNA was isolated by a modification (23) of the method of Palmiter (24). The yield of RNA was between 50 and 120 μg/g of fresh weight of tissue and the *A*<sub>260</sub>/*A*<sub>280</sub> ratio varied from 1.6 to 1.8.

Isolated polysomal RNA was translated *in vitro* in the presence of [<sup>35</sup>S]methionine by using an mRNA-dependent rabbit reticulocyte lysate translation system (25) and incorporation of [<sup>35</sup>S]methionine into total protein was measured as described (11). Polysomal RNA that efficiently directed protein synthesis *in vitro* could be obtained from infected hypocotyls even with tissue at late stages of lesion development when it displayed extensive discoloration with much dead tissue. The characteristics of *in vitro* translation were very similar to those previously observed for polysomal RNA from suspension-cultured cells, although optimal [<sup>35</sup>S]methionine incorporation was observed with zero exogenous Mg<sup>2+</sup> rather than 0.5 mM (11). The size range of these polypeptide products was very similar to that obtained with polysomal RNA from elicitor-treated suspension cultured cells or from labeling such cells *in vivo* with [<sup>35</sup>S]methionine, although the molecular weight distributions of polypeptides within this range were not fully identical (data not shown). This general correlation between the patterns of protein synthesis *in vivo* and *in vitro*, taken together with the absence of low-molecular-weight products of translation *in vitro*, confirmed that the polysomal RNA preparations from infected hypocotyls were not extensively degraded during isolation and further suggested accurate translation of exogenous *P. vulgaris* hypocotyl mRNA by the reticulocyte lysate preparation.

Chalcone synthase was separated from other translation products by indirect immunoprecipitation with antiserum to parsley chalcone synthase and protein A-Sepharose followed by NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis (11). Chalcone synthase subunits were located by fluorography and [<sup>35</sup>S]methionine incorporation was determined as described (11). Chalcone synthase mRNA activity is expressed as the incorporation of [<sup>35</sup>S]methionine into immu-

noprecipitable chalcone synthase subunits as a percentage of incorporation into total protein.

## RESULTS

*In vitro* translation of isolated polysomal RNA allows quantitative analysis of the pattern of protein synthesis without the interpretative problems inherent in an *in vivo* labeling approach (26–29), which arise from possible changes in the uptake and compartmentalization of exogenous label as the interactions between host and fungus develop. It was shown previously that polysomal mRNA from elicitor-treated suspension-cultured cells directed the synthesis of a polypeptide with an apparent *M*<sub>r</sub> of 42,000 that was immunoprecipitable by antiserum to chalcone synthase and that was identified as authentic chalcone synthase by peptide mapping (10, 11). In the present experiments immunoprecipitable chalcone synthase synthesized *in vitro* from similar polysomal mRNA preparations was electrophoretically resolved into two subunits with apparent molecular weights differing by about 500. Polysomal mRNA from infected hypocotyls directed the synthesis of immunoprecipitable polypeptides that comigrated with these authentic chalcone synthase subunits (Fig. 1). The apparent molecular weights and the relative proportions of the two subunits were the same with RNA from tissue immediately adjacent to the site of spore inoculation (site 1), tissue more distant from the site of inoculation (sites 2 and 3), and control uninoculated tissue (site 4) in both incompatible (host resistant) and compatible (host susceptible) interactions.

In an incompatible interaction following application of spores of *C. lindemuthianum* race β to the unwounded surface of hypocotyls of *P. vulgaris* cultivar Kievitsboon Koeboek, there is a period of 50–60 hr during which the spores germinate and the fungus penetrates the cuticle (19–21). At this stage the putative surveillance mechanisms of the plant cell immediately detect the presence of the fungus and there is a hypersensitive response in the initially infected cells with marked but localized accumulation of phytoalexins and restriction of further fungal growth. Phytoalexin accumulation and flecking associated with expression of hypersensitive resistance can first be observed 60–70 hr after inoculation (Fig. 2).

In the present study we have observed an early increase in chalcone synthase mRNA activity in tissue at the sites of spore inoculation (site 1). Induction of mRNA activity was first apparent 52 hr after inoculation and remained several-fold above control values during the phase of phytoalexin accumulation and expression of hypersensitive resistance (Fig. 2). In contrast, chalcone synthase mRNA activity in sites 2 and 3 in tissue distant from the sites of spore inoculation remained at or below the low level of activity in control, uninoculated tissue (Fig. 2).

In the compatible interaction with race γ, the surveillance mechanism of the plant cells apparently does not operate, the infected cells remain alive, and the fungus is able to undergo substantial biotrophic growth (19–21). Subsequently, there is extensive host cell death and development of watery, spreading anthracnose lesions. There is marked widespread phytoalexin accumulation at this stage associated with attempted lesion limitation. In such a compatible interaction, lesion development and phytoalexin accumulation can first be detected 140–160 hr after spore inoculation and spreading of the lesion from the initial area of infection to site 2 is observed 30–40 hr later (Fig. 3).

There was no significant increase in chalcone synthase mRNA activity above control levels during the early stages of infection equivalent to the phase in incompatible interactions of initial expression of hypersensitive resistance and increases in site 1 mRNA activity (Fig. 3). Subsequently, however, at the start of lesion development, there was

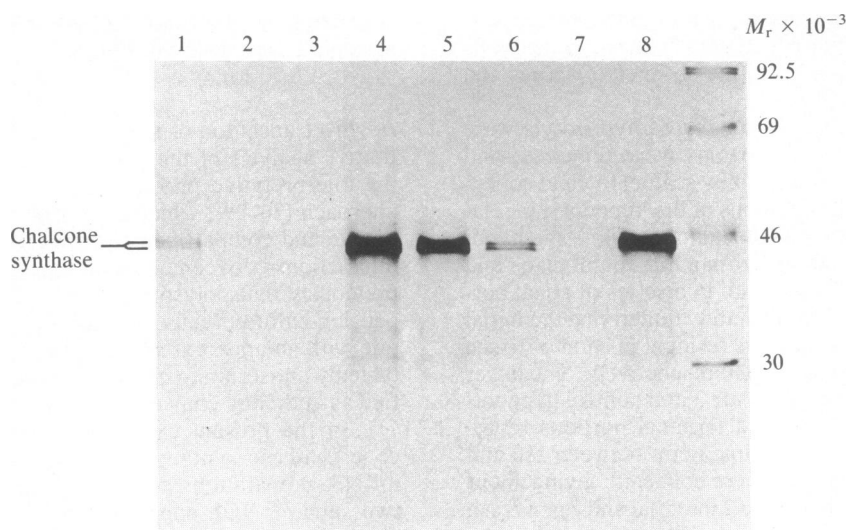


FIG. 1. Electrophoretic analysis of the [ $^{35}$ S]methionine-labeled translation products synthesized *in vitro* that were immunoprecipitated with antiserum to chalcone synthase. Lanes 1–3, polysomal RNA from hypocotyls of *P. vulgaris* 79 hr after inoculation with *C. lindemuthianum* spores of the incompatible race  $\beta$ ; lanes 4–6, polysomal RNA from hypocotyls 150 hr after inoculation with spores of the compatible race  $\gamma$ ; lane 7, polysomal RNA from uninoculated control hypocotyls; lane 8, polysomal RNA from suspension-cultured *P. vulgaris* cells 3 hr after treatment with elicitor (60  $\mu$ g of glucose equivalent/ml) heat released from cell walls of *C. lindemuthianum*. RNA was from site 1 (lanes 1 and 4), site 2 (lanes 2 and 5), site 3 (lanes 3 and 6), and site 4 (lane 7). The loading of the gel was arranged so that the amount of radioactivity in immunoprecipitable chalcone synthase subunits was proportional to the fraction of total protein synthesis devoted to chalcone synthase synthesis for each mRNA preparation. By adjusting fluorographic exposure, two chalcone synthase subunits could be resolved in each case.

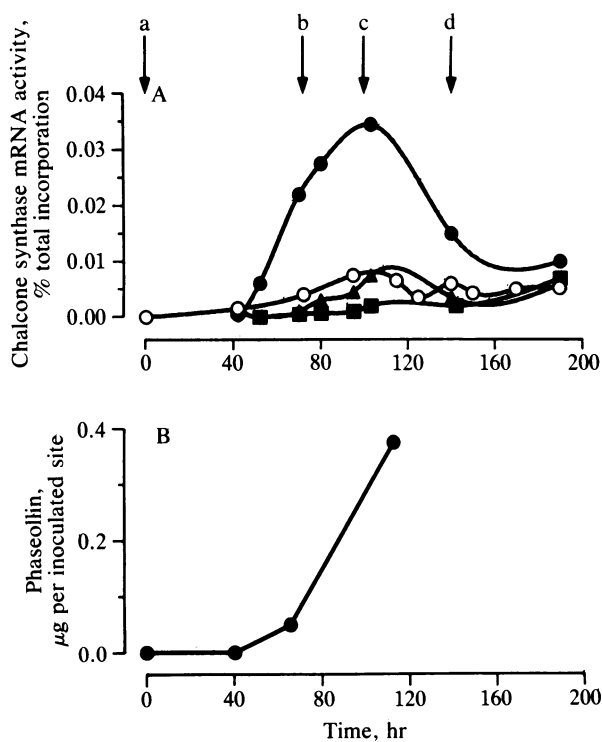


FIG. 2. Chalcone synthase mRNA activity (A) in relation to phaseollin accumulation (B) as determined (ref. 21; unpublished) and expression of hypersensitive resistance during an incompatible interaction (host resistant) between hypocotyls of *P. vulgaris* cultivar Kievitsboon Koekoek and *C. lindemuthianum* race  $\beta$ . mRNA activity was measured in directly infected tissue (site 1, ●), in tissue laterally adjacent to the infected tissue (site 2, ▲), in tissue beneath sites 1 and 2 (site 3, ■), and in equivalent control, uninoculated hypocotyls (site 4, ○). Arrows denote events in 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; and d, very dense brown flecking at all sites; no visible changes in sites 2–4 throughout the time course.

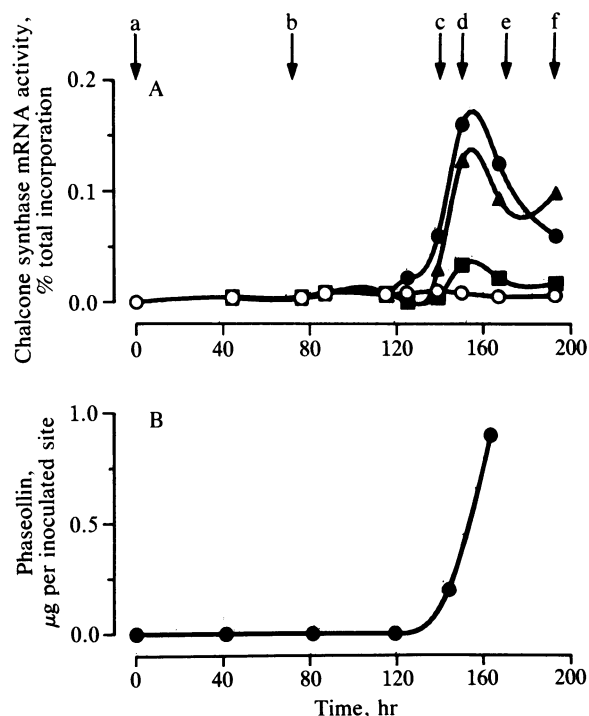


FIG. 3. Chalcone synthase mRNA activity (A) in relation to phytoalexin accumulation (B) as determined (ref. 21; unpublished) and lesion development during a compatible interaction (host susceptible) between hypocotyls of *P. vulgaris* cultivar Kievitsboon Koekoek and *C. lindemuthianum* race  $\gamma$ . mRNA activity was measured in directly infected tissue (site 1, ●), in tissue laterally adjacent to the infected tissue (site 2, ▲), in tissue beneath sites 1 and 2 (site 3, ■), and in equivalent control, uninoculated hypocotyls (site 4, ○). Arrows denote events in lesion development at site 1: a, spore inoculation; b, no visible symptoms (compare incompatible interaction); c, onset of symptom development at a few sites; d, pale to mid-brown lesions apparent at most sites; e, onset of water soaking and development of spreading lesions; and f, extensive water soaking and spreading of lesions from site 1, some browning at site 2.

marked increase in mRNA activity correlated with the onset of phytoalexin accumulation during attempted lesion limitation. Increases in mRNA activity were more pronounced and occurred slightly earlier in directly infected tissue at the site of spore inoculation. Nonetheless, in contrast to the incompatible interaction, there were significant increases in mRNA activity in sites 2 and 3 in tissue distant from the initial site of infection.

Induction of enzyme activity exhibited the same overall pattern as induction of mRNA activity with an early increase in the incompatible interaction localized to site 1 compared to a delayed but more widespread induction in the compatible interaction (Fig. 4). In the latter case, induction of mRNA activity preceded induction of enzyme activity in the respective site, whereas during the incompatible interaction mRNA and enzyme activities displayed similar induction kinetics in site 1. However, in all cases there was a strong quantitative correlation between the extent of induction of mRNA activity and enzyme activity.

### DISCUSSION

Infection of hypocotyls of *P. vulgaris* with *C. lindemuthianum* causes marked increases in the level of chalcone synthase mRNA activity with clear spatial and temporal differences in the pattern of induction between compatible and incompatible interactions. Thus, there is an early but localized increase in chalcone synthase mRNA activity in the incompatible interaction prior to the onset of phytoalexin accumulation and expression of hypersensitive resistance. In contrast, in the compatible interaction there is no induction

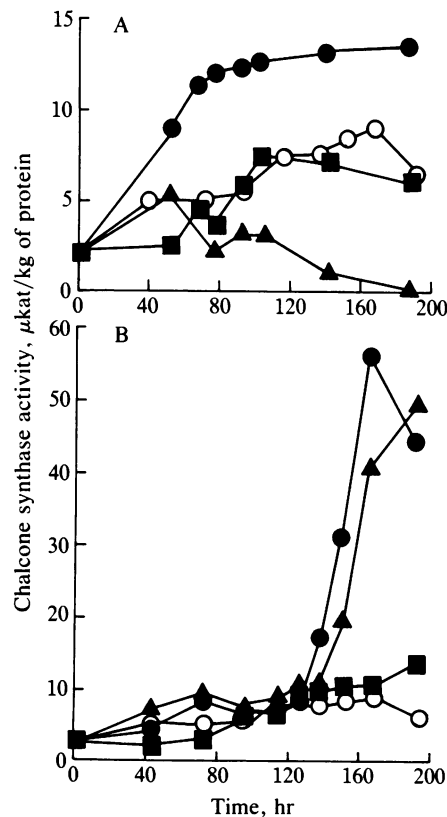


FIG. 4. Chalcone synthase enzyme activity during race-cultivar specific interactions between *P. vulgaris* cultivar Kievitsboon Koekeek and *C. lindemuthianum*. (A) Incompatible interaction (host resistant) with race β. (B) Compatible interaction (host susceptible) with race γ. Enzyme activity was measured in directly infected tissue (site 1, ●), in tissue laterally adjacent to the infected tissue (site 2, ▲), in tissue beneath sites 1 and 2 (site 3, ■), and in equivalent control, uninoculated hypocotyls (site 4, ○).

of mRNA activity in the early stages of infection but rather a delayed, widespread increase at the onset of lesion formation.

As in elicitor-treated cell suspension cultures, there is in infected hypocotyl tissue a strong correlation between stimulation of chalcone synthase mRNA activity, increased enzyme activity, and the onset of phytoalexin accumulation. The apparently weak, although early, response in the incompatible interaction reflects the fact that only a small proportion of cells (4–5%) undergoes a hypersensitive reaction (21) and phytoalexin synthesis and accumulation is limited to directly challenged cells (3, 20). If only these cells are taken into account, induction of mRNA activity and enzyme activity above control values occurs not only earlier but is also more marked than in the compatible interaction. The widespread response in the compatible interaction might reflect intercellular signal transmission by an endogenous elicitor emitted from host cells stressed by extensive fungal colonization (17, 20, 30–32). However, little is known of the events in the later stages of compatible interactions that underlie breakdown of the biotrophic phase of fungal growth that precludes lesion formation and induction of phytoalexin accumulation associated with attempted lesion limitation. Although light, temperature, and factors from other parts of the plant can affect to some extent the timing of these events in relation to fungal development, environmental factors or excision of hypocotyls do not affect the underlying distinction between incompatible and compatible interactions (20, 33).

It is concluded that induction of mRNA activities encoding enzymes of phytoalexin biosynthesis is a key component in the regulation of phytoalexin accumulation in relation to both hypersensitive resistance in an incompatible interaction and attempted lesion limitation during a compatible interaction. In particular, induction of chalcone synthase mRNA activity in the initial stages of the incompatible interaction represents an early biochemical event in a causally related sequence leading from genetically specified recognition in an intact host–pathogen system to operation of a defined, well-characterized defense response. These observations now provide the basis for elucidation of the molecular mechanisms that couple pathogen recognition to host defense responses.

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