Enhanced Levels of Chalcone Synthase in Alfalfa Nodules Induced by a Fix⁻ Mutant of *Rhizobium meliloti*

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Experiments were performed to analyze the level of chalcone synthase (CHS) mRNA expression after inoculation of alfalfa roots with different Fix⁻ mutants of *Rhizobium meliloti*. Similar, low levels of CHS mRNA were detected in roots infected by the wild-type AK631, by the Fix⁻ mutant TF178, and in uninfected roots. In contrast, roots infected with another Fix⁻ mutant, AK1540, showed increased amounts of CHS mRNA at 16 and 18 days after inoculation. In nodules formed by wild-type AK631, CHS transcripts and proteins were present at low levels, and CHS mRNA was localized *in situ* in nodule cells occupied by bacteroids. Twenty-five percent of the nodules elicited by AK1540 were empty. In these empty nodules, CHS mRNA was strongly expressed 16–20 days after inoculation, with a maximum level at day 18. The CHS mRNA was localized mainly in the outermost cell layers of the empty nodules. The amount of CHS mRNA in invaded nodules elicited by AK1540 was only slightly increased or was comparable to the level in wild-type nodules. These data support the view that mutant bacteria which are unable to infect nodules can activate the plant defense response.

Additional keywords: symbiosis, ineffective nodules, *in situ* hybridization, *Medicago sativa.*

During the *Rhizobium*-legume interaction, infection of plant roots and the formation of root nodules lead to the expression of specialized plant genes (nodulins). Generally this process is not associated with induction of a plant defense response (Vance 1983). Chalcone synthase (CHS) is a key enzyme in the plant defense response in legumes (Collinge and Slusarenko 1987). Environmental stimuli including wounding, light, plant hormones, nutrient supply, and interaction with microorganisms have been shown to induce flavonoid biosynthesis and to induce CHS synthesis (Dixon and Harrison 1990).

The activity of genes encoding CHS is specifically regulated by different external stimuli or by endogenous mechanisms during plant development or tissue differentiation (Hahlbrock and Scheel 1989; Dixon and Harrison 1990). *In situ* hybridization, immunohistochemistry, and microspectrophotometry of individual cells have been used (Schmelzer *et al.* 1988) to localize CHS mRNA, CHS protein, and CHS biosynthetic products in cross sections of parsley leaves. The light-dependent, sequential occurrence of all three of these substances was restricted to epidermal cells. These results suggest that all biosynthetic steps occur in those cells in which the products accumulate.

The biosynthesis of the pterocarpan phytoalexin glyceollin downstream of CHS is induced after soybean is infected by pathogenic microorganisms (Ebel 1979) but not after being infected with *Bradyrhizobium* (Wingender *et al.* 1989). Werner *et al.* (1985) reported, however, that a Fix⁻ mutant of *Bradyrhizobium japonicum* did induce glyceollin I accumulation in soybean nodules. These invaded nodules differ from wild-type by an early loss of the peribacteroid membrane in the infected host cells.

Previously, we described mutants of *R. meliloti* blocked at different stages of nodule development (Putnoky *et al.* 1988). Morphological studies of nodules induced by these mutants and monitoring the expression of the symbiotic genes leghemoglobin and nodulin-25 from alfalfa and the *nifHD* genes from *R. meliloti* led us to classify these mutants into three groups. In nodules induced by mutants of group I, none of the monitored genes were expressed. In group II, only the plant genes were expressed, and, in group III, all three genes were transcribed.

Assuming that successful nodule formation can result only if infecting rhizobia do not elicit an effective defense reaction by the infected host and also assuming that rhizobia might have developed (a) function(s) to prevent such a defense reaction, we screened several Fix⁻ mutants of *R. meliloti* to test whether any would lead to the induction of plant defense reactions. The induction of chalcone synthase (CHS) was used as an assay for the induction of the plant defense response. In this paper we report that one particular Fix⁻ mutant was able to induce CHS mRNA to a higher level than wild-type controls.

RESULTS

In a previous paper, three groups of yet uncharacterized Fix⁻ mutants of *R. meliloti* blocked at different stages of nodule development were described (Putnoky *et al.*

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1988). Mutants of group I induced nodules where expression of late symbiotic genes of neither alfalfa (leghemoglobin and nodulin-25 genes) nor *R. meliloti* (*nifHD* genes) were detected. In group II mutants, only the nodulin genes were not transcribed, while in group III mutants all the three genes were expressed. In a preliminary assay we have screened the Fix^- mutants belonging to groups I and II for increased levels of CHS expression. Using antibodies specific for the soybean CHS protein, Western blot analysis of protein extracts from 21-day-old nodules induced by the wild-type bacterium or mutants of these two groups was performed. In nodules induced by the mutants, except strains AK1540 and TF178, the amounts of CHS protein detected did not significantly differ from those found in the nodules induced by the wild-type bacterium (data not shown). To investigate the possible activation of plant defense by Fix^- mutants of *R. meliloti* strains AK1540 and TF178 were kept for the further studies.

Previously, 75% of nodules induced by AK1540 were found to be invaded, while almost all nodules induced by TF178 were empty or bacteria were senescent, even in the infection threads (Putnoky *et al.* 1988). Further studies of the mutant nodules induced by AK1540 revealed that invaded nodules developed normally until about 14 days after infection (Fig. 1A,B). At later stages, bacteroids and invading bacteria became senescent (Fig. 1C–D).

To determine the level of CHS mRNA accumulation in nodules after infection by the two *Rhizobium* mutants, a kinetic study was carried out. For these experiments, a cloned chalcone synthase gene from soybean (Wingender *et al.* 1989) was used as a hybridization probe. DNA blot experiments shown in Figure 2 revealed a high degree of homology between the CHS1 gene of soybean and several alfalfa CHS genes. Roots of 4-day-old alfalfa seedlings infected with TF178, AK1540, or wild-type AK631 as well as uninfected roots were harvested every second day for up to 28 days. After 10 days, roots infected by these *Rhizobium* strains developed clearly visible nodules. Hybridization of total RNA samples with the soybean CHS1 probe revealed that CHS mRNA accumulation in roots infected by either the wild-type strain or by TF178 was similar to that observed in uninfected roots. In contrast, AK1540 infected roots showed a slightly increased amount of CHS mRNA 16 and 18 days after inoculation (data not shown). Since, in these experiments total RNA from roots with or without visible nodules was used (depending on the time point after infection), it was still unclear which plant cells in the root tissue were responsible for the increased level of CHS mRNA detected at days 16 and 18 after infection by AK1540.

To substantiate the observation on the increased CHS expression in nodules induced by AK1540 and to localize CHS expression in the plant tissue, *in situ* hybridization experiments with roots and with empty as well as infected nodules of different ages were performed. Some sections were selected for the determination of the level of nucleic acids retained (Fig. 3). After staining with acridine orange, the DNA showed greenish fluorescence, while the RNA appeared orange. Nodules induced by the wild-type strain AK631 (Fig. 3A) appeared bright orange, reflecting high RNA content. Most of the RNA was detected in the meristematic zone and cells invaded by the bacteroids. Nodules induced by strain AK1540 were either similar to the wild-type nodules (data not shown) or were partially invaded (Fig. 3B) or empty (Fig. 3C). In these mutant nodules the amount of RNA was unchanged in the meristematic zone in comparison to the wild-type nodules. In contrast, the level of RNA was highly reduced in the symbiotic zones. In the partially invaded nodules, only the infected plant cells of the symbiotic zone had larger amounts of RNA (Fig. 3B), while the empty, noninvaded nodules did not contain significant levels of RNA inside the nodule (Fig. 3C).

For the *in situ* hybridization experiments, 12–22 days after infection roots and nodules formed by the wild-type control (AK631) and AK1540, respectively, were harvested, fixed, and sectioned. The 1-kb *Sph*I/*Kpn*I fragment containing the exon2 from the soybean CHS1 gene (Wingender *et al.* 1989) cloned in Bluescript (Stratagene, La Jolla, CA) was transcribed in both directions to generate both sense and antisense ^{35}S-labeled RNA probes. The antisense RNA probe was used to detect the CHS mRNA in roots or nodules, whereas the sense RNA probe served to determine the level of unspecific background hybridization. As shown on Figure 4A, using 18-day-old empty nodules induced by AK1540 and hybridized with the antisense probe, the high level of CHS expression could be localized to the outermost cell layers. No specific hybridization was observed on the same nodule type with the sense probe (Fig. 4C), in nodules induced by the wild-type bacterium (Fig. 4B,D), or in roots of either uninfected or infected plants (not shown). The invaded nodules induced by AK1540 showed only slight increase of CHS accumulation in the infected region (data not shown). These results indicate that the *in situ* hybridization conditions applied were suitable to demonstrate that CHS indeed expressed specifically in the AK1540-induced empty nodules.

To follow the kinetics of CHS expression in empty nodules induced by AK1540 labeled CHS, antisense RNA was used to perform *in situ* hybridizations. As controls, wild-type nodules collected at the same time points were also analyzed (Fig. 5). Whereas no significant hybridization was observed in wild-type nodules, 16, 18, and 20 days after infections, CHS transcripts were readily detected in empty nodules induced by the AK1540 mutant (Fig. 5B–D). CHS expression reached its maximum at day 18. A comparison of more than 50 sections of each AK1540 and the wild-type control nodules revealed that the CHS signals in AK1540-induced empty nodules were about 3–10 times more abundant than in wild-type nodules. At day 14 and day 22 empty nodules formed by AK1540 did not show significantly more CHS expression than the wild-type ones (Fig. 5A,E,F,K). In all cases, the CHS signals found were mainly located in the outermost cell layers of the nodule.

To provide independent experimental support for the activation of plant defense in empty nodules induced by AK1540, we assayed the nodules for an increase of phenolic compounds and for the appearance of phytoalexinlike flavonoids. When phenolic compounds from 21-day-old nodules were analyzed by HPLC, it was found that mutant nodules induced by AK1540 contained about five times more phenolic compounds than wild-type nodules (data

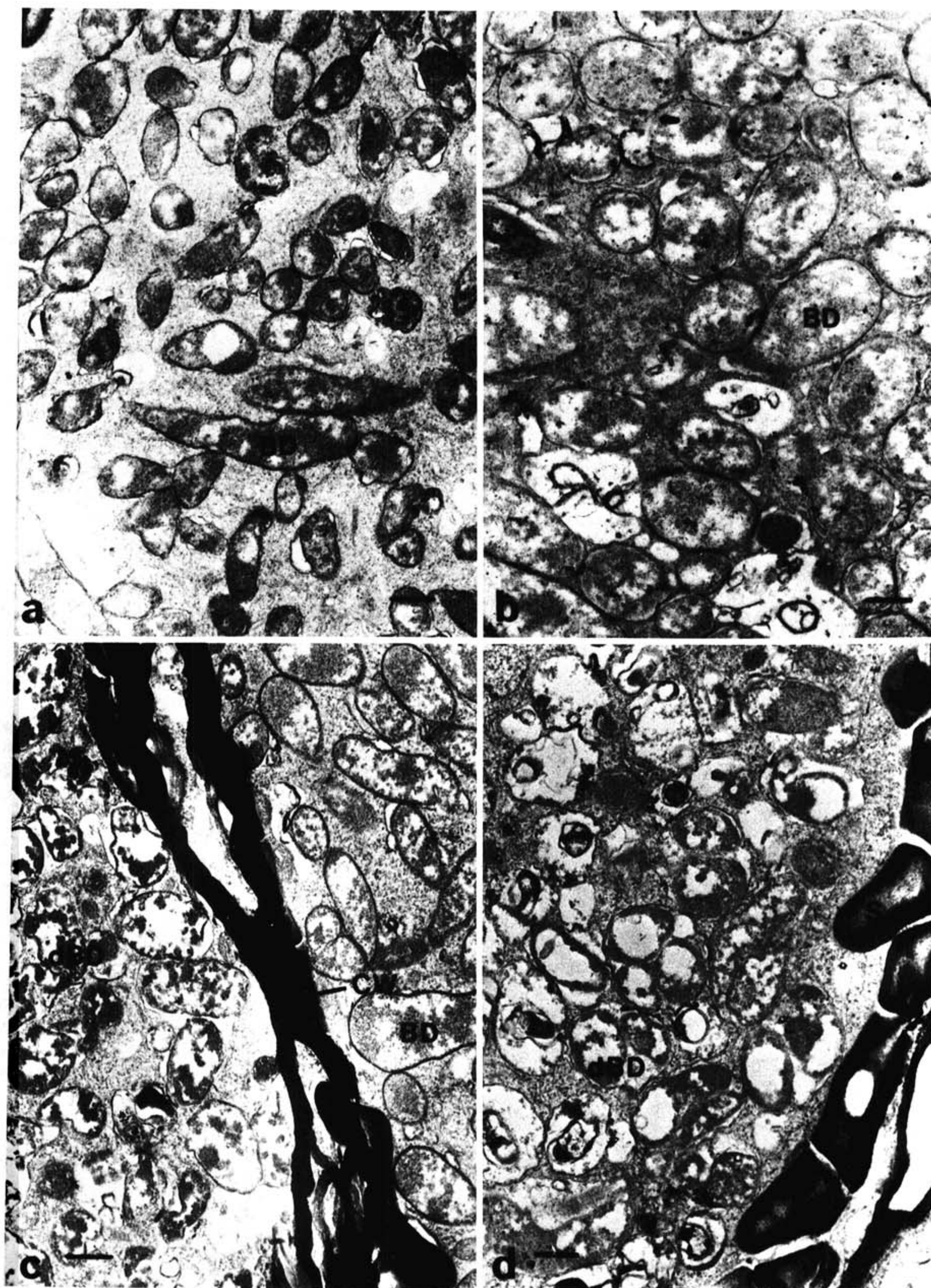

Fig. 1. Ultrastructure of nodules induced by AK1540, **A,** 12, **B,** 14, **C,** 16, and **D,** 18 days after infection. BD, bacteroid; dBD, deteriorating bacteroid; CW cell wall; S, starch. Bar, 1 μm.

not shown). However, the use of medicarpin as a reference for the induction of phytoalexin production in alfalfa (Dalkin *et al.* 1990) revealed that none of the extracts contained detectable amounts of medicarpin.

DISCUSSION

It has been well documented that CHS is a key enzyme in the defense response of legumes against pathogens (Ryder *et al.* 1987; Habereder *et al.* 1989). CHS has been shown to be induced in response to symbiotic interactions in soybean and pea nodules (Estabrook and Sengupta-Gopalan 1991; Yang *et al.* 1992). In soybean, it was shown that a special subset of CHS genes, distinct from the symbiosis-specific CHS genes, were activated in nodules formed by a Fix$^-$ strain of *B. japonicum* (Estabrook and Sengupta-Gopalan 1991).

One of the best-studied symbiotic systems is nodule formation on alfalfa by *R. meliloti* (Kondorosi *et al.* 1991). It was therefore of interest to determine if the wild-type or Fix$^-$ mutants of *R. meliloti* elicit plant defense reactions in alfalfa and if such plant defense reactions play a role in the nodulation process. In particular, we wanted to know whether the avoidance of plant defense reactions by symbiotic bacteria was an active or passive mechanism. If it resulted from an active mechanism, one would expect that mutant *Rhizobium* strains defective in this avoidance mechanism would be less efficient in establishing active nodules. Such mutants might have a Fix$^-$ phenotype (Putnoky *et al.* 1988). We therefore screened several Fix$^-$ mutants of *R. meliloti* to test whether or not their interactions with alfalfa lead to an induction of plant defense reactions (monitored by CHS expression) over and above that observed with wild-type controls.

Strains TF178 and AK1540 induce ineffective nodules on alfalfa. Eighty-six percent of the nodules induced by TF178 were found to be empty, i.e., they lacked infection threads and bacteroids; whereas only 25% of the nodules induced by AK1540 were empty, the other 75% were invaded (Putnoky *et al.* 1988). When invaded nodules of both mutants were compared, we found that more infection threads and bacteroids were present in nodules of AK1540. Furthermore, the few bacteroids found in TF178 nodules were small and senesced rapidly. These data suggest that the mutations in these two Fix$^-$ mutants interfere with nodule development at different stages. The data presented here indicate that, whereas the wild-type (AK631) and the TF178 mutant do not significantly induce the CHS gene, the AK1540 mutant does induce CHS 16–20 days after infection. These findings suggest that AK1540 may be defective in a mechanism used by *R. meliloti* to avoid the induction of a defense response in alfalfa.

The *in situ* hybridizations showed enhanced expression of CHS in the outermost cell layers of empty nodules induced by AK1540. Empty nodules contain bacteria in the outermost cell layers (Putnoky *et al.* 1988), which are likely to be responsible for this gene activation. It seems that the mutation in AK1540 affects the infection process and this might be associated with the induction of the plant defense. When bacteria succeeded in infecting the nodule, albeit ineffectively, this gene induction was weakly detectable in the infected region. At the same time, no CHS expression was detected in the uninfected parts of the roots, suggesting again the involvement of an "abnormal" infection in the induction of CHS. Interestingly, the nodules started to senesce after 14 days, at the time when, in the empty nodules, the increase in CHS expression was observed. The delay of gene expression might be explained by the relatively slow process of aborted infection. We do not know the significance of this coincidence in the timing of senescence and CHS expression.

The observation that in the invaded nodules the level of CHS was only slightly increased or was comparable to that of wild-type nodules is surprising. Perhaps successful infections suppress or decrease the "unusual" infections in the outermost cell layers and this may result in a low level of CHS expression. Nevertheless, we speculate that the high level of CHS expression depends on the presence of mutant bacteria in the peripheric regions of the nodule that are unable to carry out the normal infection process of the nodule and produce a signal leading to CHS induction.

Our results with mutant AK1540 are in contrast to the situation found in pea nodules formed by mutant strains VG2 and VG5, where the CHS was expressed at a relatively high level in the invasion zone containing bacteria released from the infection thread (Yang *et al.* 1992). The pheno-

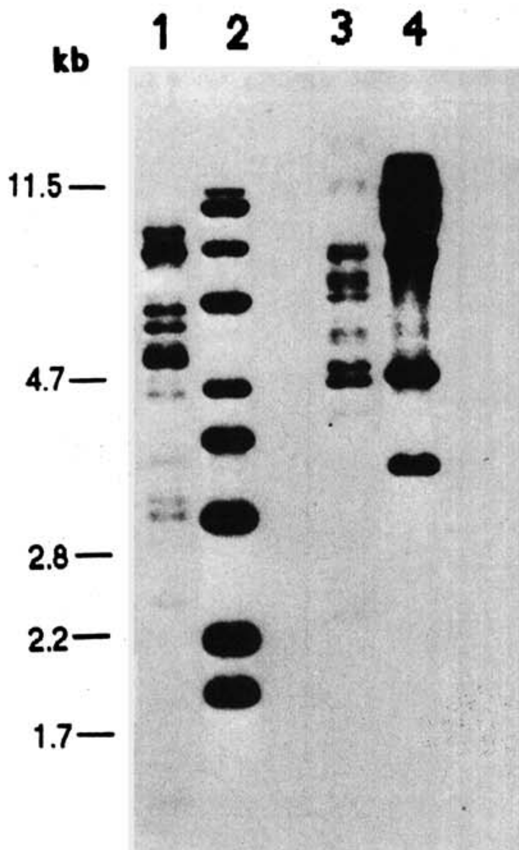

Fig. 2. Autoradiography of a Southern blot analysis of genomic DNA from alfalfa (lanes 1, 3) and soybean (lanes 2, 4). Total DNA was digested with *Hin*dIII (lanes 1, 2) or *Eco*RI (lanes 3, 4) and hybridized to the exon2 fragment of CHS1 of soybean (Wingender *et al.* 1989).

types of invaded nodules formed by mutants VG2, VG5, TF178, and AK1540 are similar. VG2 and VG5 are lipopolysaccharide (LPS) mutants, while the mutations in TF178 and AK1540 do not affect LPS (Putnoky *et al.* 1990); they are in genes with yet unidentified functions.

Yang *et al.* (1992) found CHS expression in the wild-type pea nodules at the apex and the distal part of the meristem, where the cells are not infected by *Rhizobium*. Besides this basic level of expression, only the nodules formed by mutants VG2 and VG5 showed an enhanced level of CHS gene expression at sites of bacterial release into the host cells. These results were obtained by using a homologous probe of the CHS gene, but even the homologous probe did not allow a quantitation of the levels of RNA expression (Yang *et al.* 1992). In our work we used a heterologous probe of the CHS gene which is not suitable to detect either a basic level of CHS expression above background or small differences in CHS expression. However, we clearly showed CHS expression in the outermost cell layers of empty AK1540 nodules, which indicates that this expression must be relatively strong. Since in nodules formed either by TF178 or by AK1540, a few bacteria were always found in these cell layers (Putnoky *et al.* 1988), the induction of CHS seems to be specific for the mutant AK1540. Thus, the mere presence of bacteria between the nodule cells does not seem to be the only factor that leads to high level of CHS induction.

Our finding that mutant nodules formed by AK1540 contain five times more phenolic compounds than wild-type nodules is consistent with the demonstrated activation of the CHS gene(s). Because we could not discriminate between empty and invaded nodules when we prepared the extract, the concentration of these substances in empty nodules might be even higher. Monitoring of these products of the phenylpropanoid pathway (particularly to measure defense metabolites) can often be a powerful tool in the elucidation of the nature of plant responses (Graham and Graham 1991). Our analysis showed that neither the wild-type nor the mutant nodule extracts contained medicarpin, a phytoalexin often formed in alfalfa after pathogenic attack (Dalkin *et al.* 1990). Further chemical analysis of the phenolic compounds is required to demonstrate whether other phytoalexins are present in the nodules induced by AK1540.

MATERIALS AND METHODS

Strains, media, and plant growth.

Bacterial strains used were *R. meliloti* AK631 and its Fix$^-$ derivatives TF178 (Forrai *et al.* 1983) and AK1540 (Kondorosi *et al.* 1984). For the preliminary screening of increased CHS expression in Fix$^-$ mutants, strains belonging to groups I and II were used (Putnoky *et al.* 1988). Media, culture conditions, surface sterilization of seeds of *Medicago sativa* 'Nagyszenas', plant growth, and inocu-

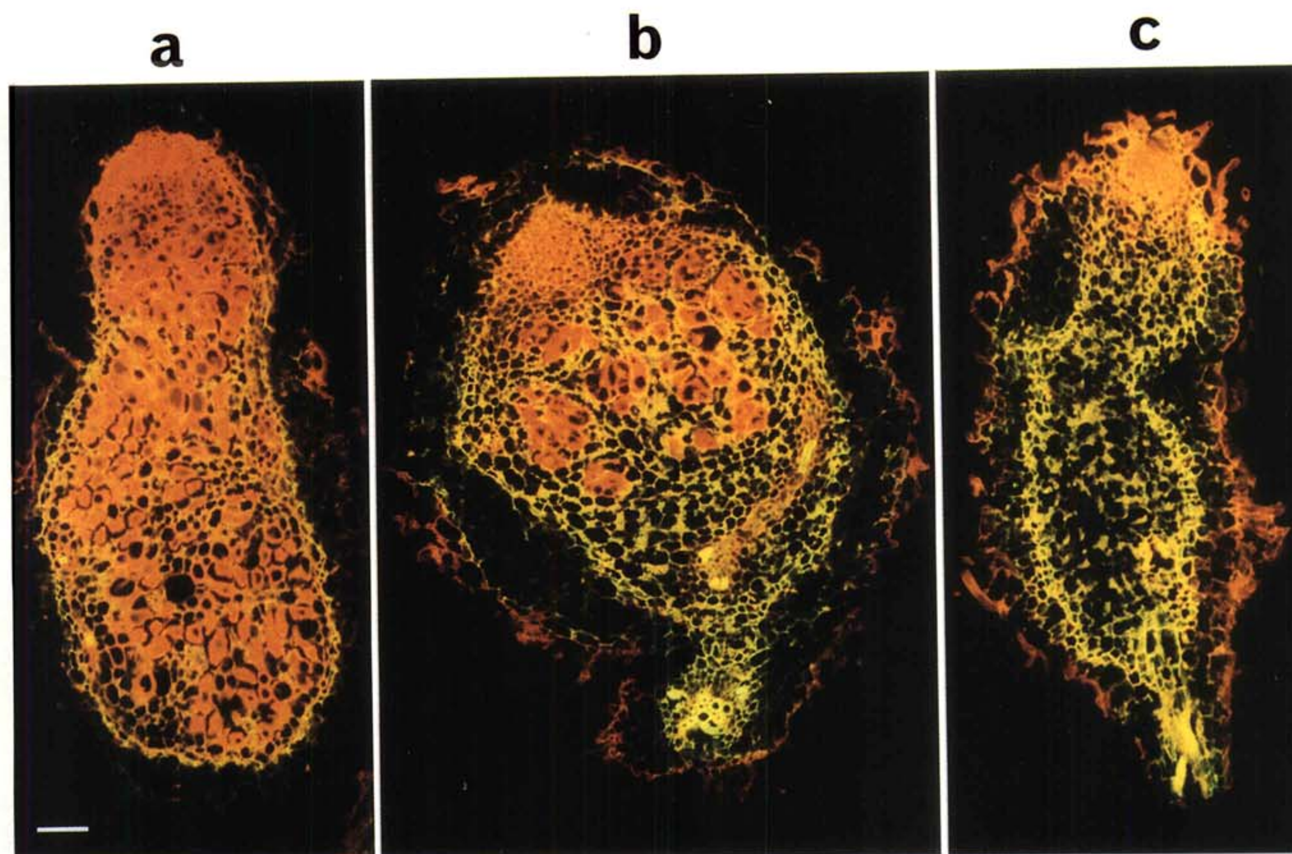

Fig. 3. Retention of RNA in longitudinal sections of alfalfa nodules after pronase treatment. The RNA shows orange fluorescence after staining with acridine orange and UV illumination. Sections of **A,** wild-type nodule and of **B,** partially invaded and **C,** empty nodules of AK1540, respectively. Bar, 100 μm.

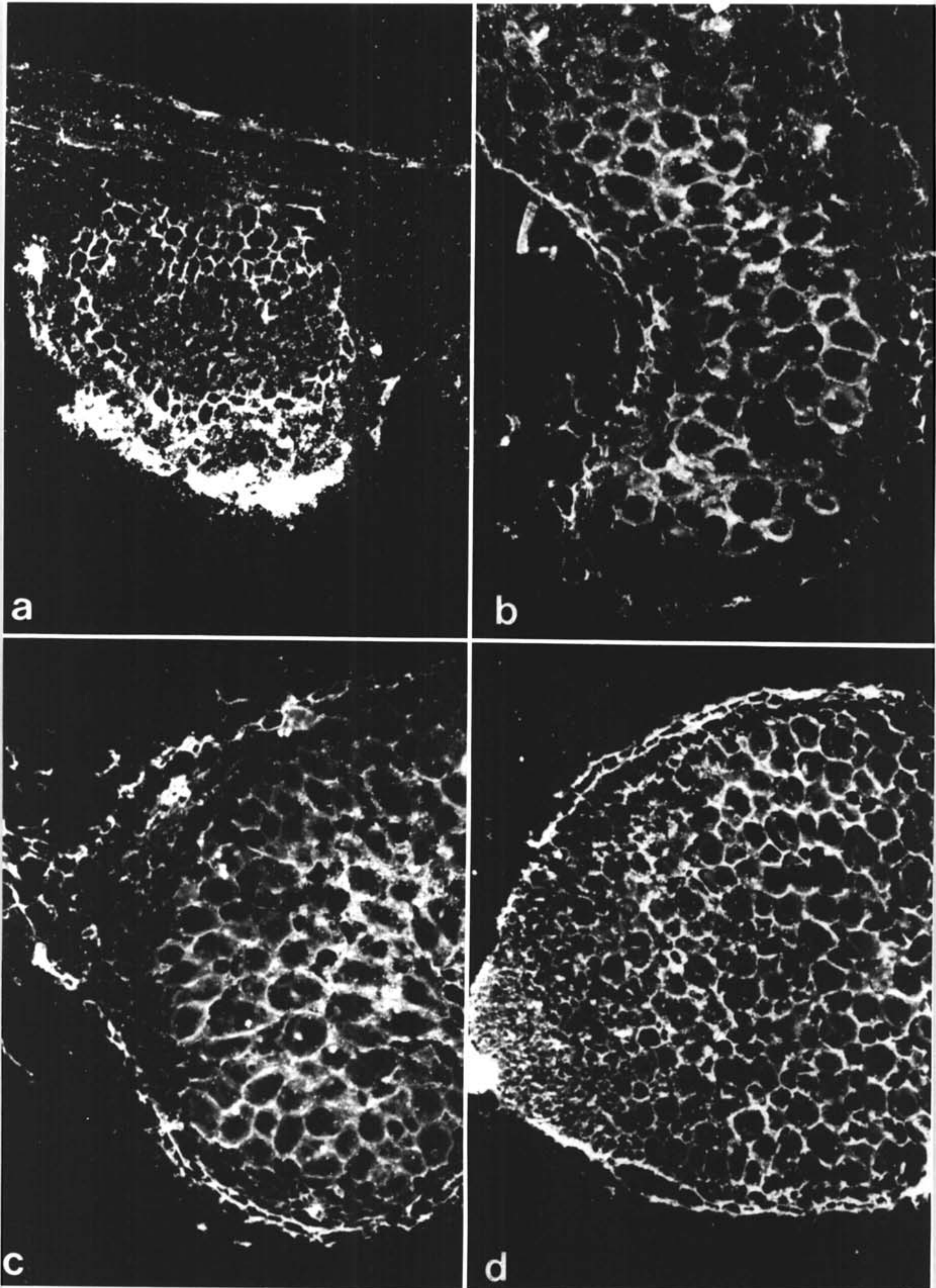

Fig. 4. Localization of CHS in sections of alfalfa nodules by *in situ* hybridization with antisense RNA and control hybridization with sense RNA. **A, C,** Eighteen-day-old nodules of AK1540 and of **B, D,** AK631 hybridized with antisense RNA (A,B) or sense RNA (C,D), respectively.

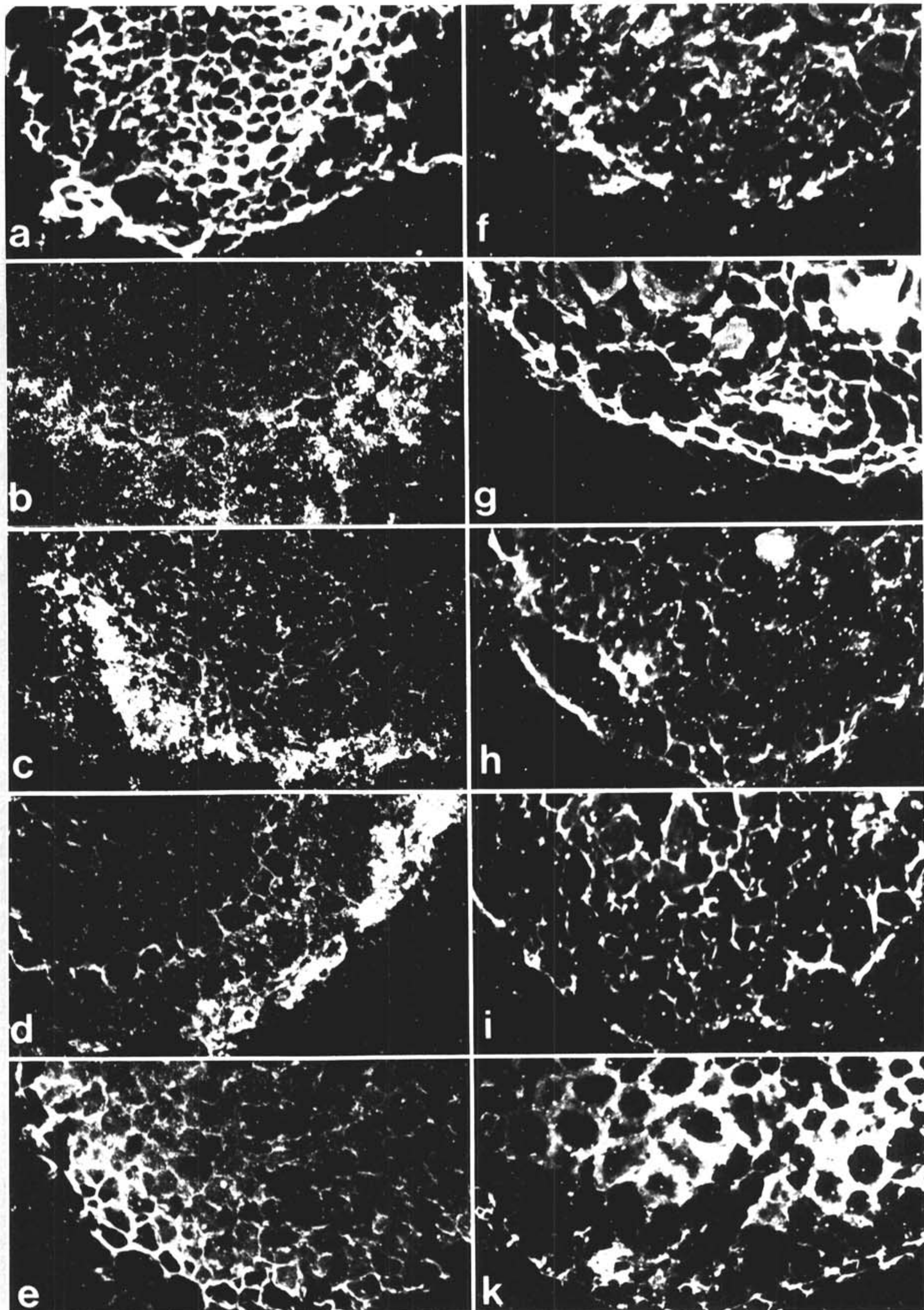

Fig. 5. Kinetics of CHS expression in nodules induced by AK1540. CHS expression was localized in longitudinal sections of alfalfa nodules by *in situ* hybridization with antisense RNA. For the better comparison of the CHS expression levels in nodules collected at different time intervals only representative parts of nodules are shown. For the orientation of the nodule region expressing CHS, see Figure 4. Empty nodules of F, AK1540 **A,** 14, **B,** 16, **C,** 18, **D,** 20, and **E,** 22 days after infection; nodules of AK631 (wild-type) **F,** 14, **G,** 16, **H,** 18, **I,** 20, and **K,** 22 days after infection.

lation of seedlings have been described previously (Putnoky and Kondorosi 1986).

Isolation of plant DNA and RNA.
Nucleic acids were isolated from plant material as described by Dellaporta *et al.* (1983) for genomic DNA and as described by Chirgwin *et al.* (1979) for RNA.

Standard recombinant DNA techniques.
These were used essentially as described by Maniatis *et al.* (1982).

DNA/RNA blots and filter hybridizations.
These were carried out as described previously (Wingender *et al.* 1989), except that hybridization temperature was 37° C in the presence of 50% formamide. After hybridization, a solution of 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS was used at 60° C for the final wash.

Microscopic studies.
Light and electron microscopic studies were performed as described previously (Putnoky *et al.* 1988).

Preparation of RNA probe.
The *Sph*I/*Kpn*I fragment of the exon2 from the soybean chalcon synthase gene 1 (Wingender *et al.* 1989) was cloned into the Bluescript vector (Stratagene). T3 and T7 polymerase *in vitro* transcription was performed in the presence of ^{35}S-labeled UTP following instructions of the manufacturer (Stratagene) to obtain both sense and antisense RNA probes. Quality and size of the probes were determined by RNA blot analysis.

Preparation of nodule sections for *in situ* RNA hybridization.
Preparation of the nodule sections and *in situ* hybridization procedures were based on those described by Barker *et al.* (1988), Meyerowitz (1987), and Schmelzer *et al.* (1988). Nodules were harvested at different days after inoculation (as indicated in Fig. 5) and immediately prefixed in 2% paraformaldehyde, 1% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.0) for 2 hr on ice. Samples were washed briefly in 0.1 M sodium phosphate buffer (pH 7.0) and dehydrated by graded ethanol series (30 and 50% for 30 min each at 0° C; 70% overnight at 4° C; 85, 90, and two times 100% for 30 min each at room temperature). Ethanol was exchanged against tertiary butanol by passage of samples through mixtures of ethanol/tertiary butanol at ratios of 3:1, 1:1, and 1:3 for 40 min each at room temperature. Finally they were left in tertiary butanol for 40 min at 30° C. Embedding was done by incubating the samples in tertiary butanol (paraffin-saturated at 30° C) for 6 hr at 30° C followed by 30 min at 42° C. Subsequently they were transferred into tertiary butanol (paraffin-saturated at 42° C) and incubated overnight at 42° C with an additional 30 min at 60° C. Finally, samples were left for 3 days at 60° C in paraffin. Five- to eight-micrometer sections of the embedded nodules were spread on a droplet of water placed on poly-L-lysin-coated slides. The water was carefully removed and slides were incubated for 3 hr at 40° C on a heating plate. To remove the paraffin the slides were passed through the following solutions at room temperature: xylol for 10 min, xylol/ethanol (1:1) for 5 min, and a series of ethanol concentrations (100, 95, 80, 60, and 30%) for 5 min each. The slides were air-dried and stored at 4° C.

Pretreatment of sections.
The fixed sections were incubated for 5 min in 0.2 M phosphate buffer (pH 7.0) followed by an incubation in a freshly prepared solution of self-digested pronase (250 μg/ml in 50 mM Tris/HCl pH 7.5, 5 mM EDTA) for 10 min. Sections were postfixed as described above and dehydrated through a series of ethanol concentrations (30, 60, 80, 94, and 96%) for 2 min each, except in 80% ethanol sections, which were kept for 5 min.

Acridine orange staining, *in situ* RNA hybridization, and autoradiography.
These were performed as described previously (Schmelzer *et al.* 1988) with the following modifications in the hybridization: 1 ml of hybridization mixture contained 12.5 μg of labeled RNA probe but no denaturated DNA; the incubation temperature was 50° C. Washing conditions were three times for 1 hr each at 50° C in 1× SSC/50% formamide; 5 min at room temperature in 1× SSC; 30 min at 37° C in 10 mM Tris/HCl, pH 8.0/0.5 M NaCl/20 μg/ml RNase A; three times for 1 hr each at 50° C in 1× SSC/50% formamide; and two times each for 20 min at room temperature in 1× SSC.

Extraction of phytoalexins from nodules.
Frozen nodules were ground in liquid nitrogen. The frozen powder was extracted twice with methanol and once with chloroform. Solvents were evaporated under low pressure, and the dried compounds were dissolved in ethanol. After filtration through a 45-μm filter unit, the extract was applied to high-performance liquid chromatography (HPLC) (Waters, Millipore). Chromatography was done by using a Lichrosorp RP18 column and a steady gradient of acetonitril/1.5% phosphoric acid from 25:75 to 75:25.

Western blots.
These were performed as described by Wingender *et al.* (1989). Antibodies specific for soybean CHS protein were kindly supplied by H. Grisebach, Freiburg.

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