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Early Induction of the *Arabidopsis GSTF8* Promoter by Specific Strains of the Fungal Pathogen *Rhizoctonia solani*

Rafael Perl-Treves,[1] Rhonda C. Foley,[1] Wenqiong Chen,[2] and Karam B. Singh[1]

[1]CSIRO Plant Industry, Private Bag 5, PO Wembley, WA 6913, Australia; [2]Department of Molecular, Cell and Developmental Biology, University of California–Los Angeles, 405 Hilgard Avenue, Los Angeles 90095-1606, U.S.A.

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The *Arabidopsis* glutathione *S*-transferase *GSTF8* promoter directs root-specific responses to stress. In this study, the response of this promoter to plant infection with *Rhizoctonia solani* was investigated using a luciferase reporter system. *Arabidopsis* seedlings harboring the *GSTF8*:luciferase construct were monitored in vivo for bioluminescence following infection with *R. solani*. Although the reporter gene was induced in infected roots, the response differed markedly between *R. solani* strains and was not observed with aggressive strains that caused death of the seedlings. The three strains tested in detail progressed through typical stages of infection, but ZG1-1 induced the *GSTF8* promoter in most seedlings, ZG3 induced it in approximately 25% of seedlings, and ZG5 caused little response. Induction of specific root segments occurred early in the infection process in root regions with very limited mycelium visible. In root segments with substantial mycelium, *GSTF8* promoter activity no longer was observed. Induction by ZG1-1 also was observed in plants harboring a tetramer of the ocs element from the *GSTF8* promoter, suggesting that this element helps mediate the response. Crossing *GSTF8*:luciferase plants with plants harboring an Nah-G construct that degrades salicylic acid did not abolish the response, indicating that the *GSTF8* promoter response to *R. solani* may be mediated by signals other than salicylic acid.

Additional keywords: plant–pathogen interaction.

The soilborne fungus *Rhizoctonia solani* (Kühn) causes disease on many economically important crops throughout the world (Sneh et al. 1996). *R. solani* is a multinucleate species within the large genus *Rhizoctonia,* whose members vary widely in morphology, ecology, and pathology. Although many *Rhizoctonia* isolates are pathogenic, some may be saprophytic or even mycorrhizal. Pathogenic isolates can be described as opportunistic parasites that attack and kill seedlings; symptoms on older plants are less severe. Researchers attempted to classify and subdivide *Rhizoctonia* isolates into formae speciales based on pathogenicity on various plant taxa and morphology of cultures. Anastomosis grouping (AG), based on vegetative interactions between isolates, is an effective mean to classify strains: there are currently approximately 12 AGs (Adams 1996; Carling 1996). Strains of the same AG may still differ in their pathogenic characteristics; therefore, a more detailed subdivision was elaborated, based on activity-stained zymograms of pectic enzymes secreted from fungal cultures (Sweetingham and MacNish 1994).

R. solani infects primarily roots and stems, and the stages of plant infection have been reviewed by Keijer (1996) and Weinhold and Sinclair (1996). The initial growth over the plant is by round, nonattached hyphae. After 10 to 12 h, hyphae may become flattened and firmly attached to the plant surface. In the next stage, described as "directed growth", attached hyphae grow along anticlinal cell walls and branch at right angles. Such branches may terminate in simple infection structures (i.e., swollen tips or simple appressoria), or divide and give rise to dome-like, multilayered hyphal cushions. Penetration through the cuticle follows, aided by hydrolytic enzymes and hydrostatic pressure. Within the cortex, rapid hyphal growth occurs, which may result in the collapse of seedlings. Our understanding of the plant response to an attack by *R. solani* is limited, as is the case for many economically important necrotrophic fungal pathogens, particularly at the molecular level. In addition, the extent of specificity between *R. solani* and its host is poorly understood.

In this study, we have used a stress-responsive promoter from the *Arabidopsis* glutathione *S*-transferase *GSTF8* gene, previously called *GST6* (Chen et al. 1996), to study changes in gene expression following *R. solani* infection in *Arabidopsis* spp. Plant GSTs are a family of multifunctional enzymes that detoxify xenobiotic compounds and participate in a range of stress responses (Edwards et al. 2000; Marrs 1996). The *GSTF8* promoter is strongly inducible in seedling roots following treatment with the defense signals salicylic acid (SA) and H_2O_2 (Chen and Singh 1999). This induction is mediated in part by an ocs element (Chen and Singh 1999), a plant enhancer sequence which is important for the expression of some pathogen- and stress-responsive genes (Singh et al. 2002). Although the GSTF8 promoter responds to defense signals in roots, little is known about possible induction by biotic interactions.

Here, we investigated the response of different *GSTF8*-luciferase promoter constructs in transgenic *Arabidopsis* spp. to specific strains of *R. solani*. We used in vivo imaging to allow real-time monitoring of changes in gene expression dur-

Corresponding author: Karam B. Singh; Telephone: 61 8 93336320; Fax: 61 8 93878991; E-mail: karam.singh@csiro.au

R. Perl-Treves and R. C. Foley contributed equally to this work.

Current address of R. Perl-Treves: Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel.

Current address of W. Chen: Diversa, 4955 Directors Place, San Diego, CA 92121, U.S.A.

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ing the infection process. We set up a co-cultivation system on sterile agar plates to follow the interaction between *Arabidopsis* seedlings and *R. solani.* This system allows the continuous observation of mycelial growth, seedling development, and changes in bioluminescence produced by the *GSTF8* promoter-mediated expression of the luciferase gene. It has provided us with a good molecular handle to probe the interaction between the plant and this important pathogen. We report an inducible transcriptional response that occurs in *Arabidopsis* seedlings at an early stage of infection with *R. solani*. We also show that a construct containing a tetramer of the ocs element from the *GSTF8* gene linked to a minimal promoter shows a similar response to *R. solani* infection as the *GSTF8* promoter. We used an Nah-G line which expresses an SA-degrading enzyme (Lawton et al. 1995) to show that SA was not required for induction of the *GSTF8* promoter by *R. solani*. The specificity of the *GSTF8* promoter response to different *R. solani* strains as well as binucleated *Rhizoctonia* strains was examined. We found that the response was specific for particular strains, and that the induction of *GSTF8* promoter activity did not occur with aggressive strains which were able to cause plant death.

RESULTS

Infection of *Arabidopsis* seedlings grown on agar plates with *R. solani* strains.

Three *R. solani* strains that belong to different AGs and differ in their pathogenic characteristics initially were selected for this study (Table 1). These were ZG1-1 (belonging to AG8), ZG3 (belonging to AG11), and ZG5 (belonging to AG2-1). Wild-type seed of *Arabidopsis thaliana* ecotype Columbia or those from homozygous, T4 lines carrying *GSTF8*-derived promoter::luciferase constructs were sown aseptically on Murashige-Skoog (MS) agar plates containing 3% sucrose. At 5 to 7 days after planting, when the seedlings had two open cotyledons and a main root of 1 to 2 cm, they were infected with fungal mycelium. Infected plates were returned to the growth chamber. At 2 to 3 days after inoculation, hyphae spreading from the inoculation point became visible to the naked eye and the extent of visible growth was scored. If left to grow, the fungus would cover and overwhelm the plantlet shoot and macerate the roots between days 6 and 7. The three strains exhibited typical differences in color and density of growth, but we used plates that supported similar amounts of mycelium to compare the course of infection and the effect on *GSTF8* promoter activity between fungal strains. Plates at day 5 post inoculation are shown in Figure 1A, with extensive (stage +++) fungal growth.

We also examined the extent and density of hyphal growth on MS medium lacking sucrose, because this may better reflect the situation in the soil. Under such conditions, mycelia of all three strains appeared as a very fine, transparent network that spread across the entire plate (*not shown*); whereas, on sucrose-supplemented medium, mycelia were denser and had a smaller diameter. Interestingly, strain ZG5 killed the seedlings on sucrose-less medium by day 5 post inoculation, whereas the other two strains were less aggressive and the seedlings were still alive 2 days later. In sucrose-supplemented medium, plantlets infected with all strains, including ZG5, remained alive at least until day 6. The sucrose-less medium, however, proved less convenient due to poorer seed germination and less uniform plant growth supported by such medium.

Induction of the *GSTF8* promoter by *R. solani.*

To test whether the *GSTF8* promoter is activated during a biotic interaction between *R. solani* and *Arabidopsis* spp., we infected plants containing the 791-bp *GSTF8* promoter fused to the luciferase gene (Chen and Singh 1999), henceforth called *GSTF8*::LUC, with the different *R. solani* strains. All the presented data is from a single T4 line, JC66, that was homozygous for the transgene. Luciferase-generated bioluminescence was monitored daily, as well as the extent of visible fungal growth on the plate. Plates were viewed at day 1 (approximately 20 h after inoculation), then at 24-h intervals, up to 5 or 6 days post inoculation. The experiment was repeated four times with similar results. The results of a representative experiment are shown in Table 2.

Induction of the *GSTF8* promoter varied with different *R. solani* strains. On sucrose medium, strain ZG1-1 elicited a clear and strong bioluminescence in 36 of 41 seedlings (87%). Altogether, over 100 seedlings were tested in four experiments, with over 80% of the seedlings responding. Bioluminescence was detected in a significant number of seedlings by day 3 (approximately 70 h post inoculation) and, in some cases, as early as day 2. The response peaked, in terms of bioluminescence intensity and number of responding seedlings, during days 3 to 4, and was still strong in some seedlings (but usually became weaker) on day 5. Seedlings were followed individually according to their position on the plate, and were seen to respond for varying periods of time, lasting between 1 and 3 days; specific regions of a plant typically responded for approximately 1 day. The response spread along the root from the site of inoculation into the newly growing and newly infected parts. Induction was confined to the roots and the crown region at the base of the hypocotyls, and never spread to the cotyledons or leaves, in agreement with the much higher activity of this promoter in roots (Chen and Singh 1999). Bioluminescence imaging of a ZG1-1-infected plate with six seedlings over a 4-day period are shown in Figure 1B. Similar results were seen with a second independent line (JC79), containing the *GSTF8*::LUC construct (data not shown). In noninfected plates, bioluminescence never was observed.

Strain ZG5 grew to an extent and density similar to ZG1-1, but rarely induced the *GSTF8* promoter. In five different experiments, we encountered a total of only 5 seedlings that responded of approximately 85 seedlings. For ZG3, the response was more variable: in most cases, we saw no response; yet, in a few of the experiments, such as the one shown in Table 2, part of the seedlings were bioluminescent, usually later than those infected with ZG1-1 (typically on days 5 or 6). In all, 20 of 81 seedlings (25%) responded to ZG3. In some experiments, nonresponding ZG3 and ZG5 plates were co-cultivated for longer periods, to see whether induction occurred by allowing the infection to progress further. In such cases, the fungal mycelia were more extensive and the seedlings died, but the

Table 1. Characteristics of *Rhizoctonia solani* strains used in this study (Khangura et al. 1999; Sweetingham and MacNish 1994)

Strain name	Anastomosis group	Pathogenic characteristics
ZG1-1	AG8	Bare patch and root rot diseases on legumes and cereals; pathogenic in canola
ZG3	AG11	Legume hypocotyl rot
ZG5	AG2-1	Crucifer hypocotyl rot
ZG6	AG2-1	Hypocotyl rot in canola
CZG1	CAG1	Weakly pathogenic in canola
CZG5	AGK	Pathogenic in rapeseed, weakly pathogenic in canola

GSTF8 promoter was not induced. Therefore, the difference in induction did not result from differences in the growth rates between the three strains: ZG1-1 induced a response on day 2 or 3, when the plates were significantly less infected than those harboring ZG5 and ZG3 on days 5 to 7.

We asked whether the same induction pattern occurred in seedlings grown on a sucrose-free medium, under conditions that may be closer to those in a natural soil environment. Therefore, we repeated the experiment on sucrose free MS agar medium. While the mycelial phenotype was very different from that observed on sucrose plates (see above), the response of the *GSTF8* promoter was essentially similar. Of 34 ZG1-1-infected seedlings, 12 were induced, whereas none of the ZG3- or ZG5-infected or control plants responded (Table 2). The lower rates of ZG1-1-induced response, compared with those obtained on sucrose medium, probably reflect the less favorable conditions for fungal growth on such plates, but the significant difference between the strains was maintained, as were the spatial and temporal patterns of bioluminescence in the responding seedlings.

The ocs element from the *GSTF8* promoter also is responsive to *R. solani*.

We have demonstrated previously that the ocs element, located at –423 in the *GSTF8* promoter, is responsive to SA and H_2O_2 when linked to a minimal promoter (Chen and Singh 1999). The in vivo imaging system is not able to detect the activity of the single ocs element::luciferase construct under any conditions tested. However, bioluminescence from transgenic *Arabidopsis* plants containing a tetramer of the ocs element linked to a minimal promoter can be detected. To determine whether the ocs element can respond to *R. solani* infection, we examined a homozygous, T4 transgenic line containing the 4× ocs::LUC construct following infection with ZG1-1. One-week-old seedlings (20 per treatment) were infected with ZG1-1 and the bioluminescence was monitored from day 3 to day 7 and compared with mock-infected controls. The 4× ocs tetramer is a stronger promoter than the 791-bp full-length *GSTF8* promoter and basal bioluminescence activity is observed in the uninfected plants. However, the ocs element construct was clearly induced in ZG1-1-infected plants from day 3

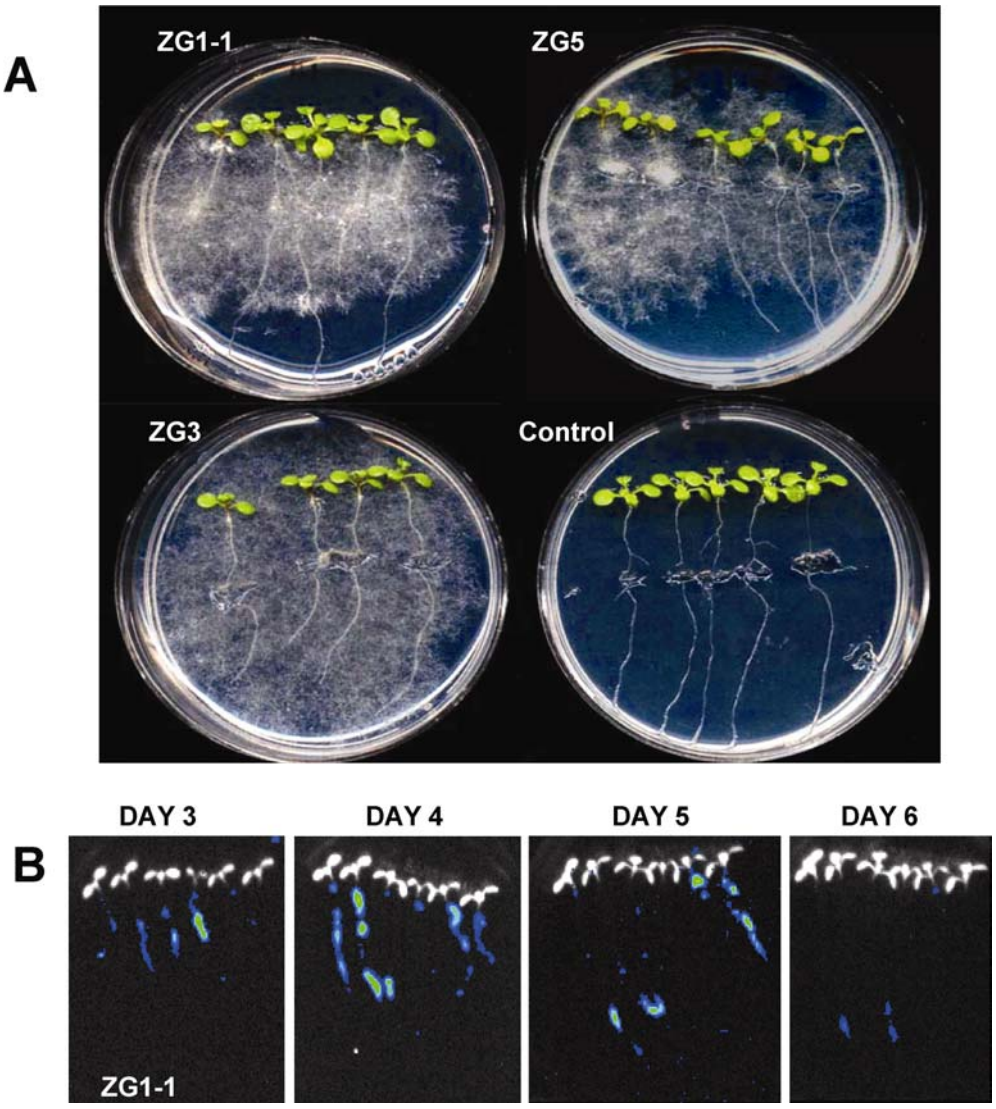

Fig. 1. *Rhizoctonia solani* infection of Arabidopsis *GSTF8*::LUC seedlings. **A,** Infection of *Arabidopsis* seedlings grown on sterile medium with *R. solani* strains. Surface-sterilized seeds were planted on Murashige and Skoog MS-sucrose plates. Six days later, they were wounded and inoculated in the middle of the rootlet with fungal mycelium (day 0). The picture was taken on day 5. **B,** In vivo luciferase assay of *Arabidopsis GSTF8*::LUC seedlings following infection with *R. solani* strain ZG1-1. The white signal comes from the cotyledons and represents the chlorophyll fluorescence which is transient and was imaged prior to the bioluminescence monitoring. Bioluminescence was monitored after 10 min in the dark, and is shown in blue. The fluorescence and bioluminescence images were superimposed in the figure.

to day 6 (Fig. 2), similar to what was observed with the full-length *GSTF8* promoter.

Microscopic examination of *Arabidopsis* roots infected with *R. solani.*

Seedlings grown on MS agar media were observed under the light microscope to examine the progress of fungal association with the plant roots and to determine whether induction of the *GSTF8* promoter was associated with a specific, visible interaction of the ZG1-1 strain with the infected root. We used aniline blue to preferentially stain fungal hyphae, whereas safranin red stained the root vascular bundle and enhanced the root meristems. Washing the root system for 2 min under running tap water removed most of the unattached mycelium. The roots, along with firmly attached hyphae, resisted this treatment. We noted that many seedlings had seemingly clean roots (Fig. 3A), with only a few fungal attachment sites: the mycelium growing over them washed off easily. When the hyphae were not washed off, we observed long runs of straight hyphae with little branching, and some loosely entangled random coils of hyphae that adhered to the root at a few sites only (Fig. 3B). Other samples displayed long stretches of firmly attached hyphae that were not removed by washing. These hyphae branched frequently, forming a typical directed-growth pattern; such patterns have been described in the literature as the stage preceding penetration and invasion of the cortex (Keijer 1996; Weinhold and Sinclair 1996). The directed-growth pattern was most frequent, and covered longer stretches, in roots infected with the ZG1-1 strain (Fig. 3C and D); it seldom was observed with the other two strains. Another difference between ZG1-1 and the other two strains involved the lateral root tips: the latter strains, while adhering less to the entire length of the root, had a preference for lateral root tips, which they attacked and eventually macerated (Fig. 3E). ZG1-1, on the other hand, exhibited an inverse preference, "probing" and adhering to the epidermal tissue of the main root and not to the tips or the younger lateral roots. On day 5, some seedlings exhibited a more advanced stage of tissue infection and maceration. This was observed most commonly with ZG3, less frequently with ZG5, and rarely with ZG1-1. On such roots, multilayered, dome-like infection cushions were observed (Fig. 3F). In the literature (Weinhold and Sinclair 1996), such structures were reported to correlate with increased aggressiveness of *R. solani* strains, whereas less aggressive strains typically produced simpler structures (i.e., swollen tips that look like primitive haustoria) (Fig. 3C). From these observations, we concluded that, in our agar plate system, infection proceeds through the typical stages of overgrowth, adherence, directed growth, and penetration, to maceration of the seedlings. It was clear, by looking at seedlings infected with ZG5 and ZG3, that these strains can progress through infection stages to tissue maceration without induction of the *GSTF8* promoter, whereas ZG1-1 elicits a response starting on day 2 or 3, before any maceration occurs.

Focusing on the ZG1-1-induced response, we asked whether extensive attachment of hyphae (directed growth) was spatially or temporally correlated with the recognition-and-induction event. For this purpose, individual seedlings infected with strain ZG1-1 were monitored for bioluminescence. Specific regions that responded were marked and immediately stained and

Table 2. Response of *GSTF8*::LUC seedlings to inoculation with *Rhizoctonia solani* strains on media differing in sucrose supplement[a]

		Total (cumulative) number of induced seedlings				
Medium, strain	**Total no. of seedlings**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Sucrose						
ZG1-1	41	5	15	32	36	36
ZG3	40	0	0	0	5	5
ZG5	40	0	0	0	0	0
Control	23	0	0	0	0	0
No sucrose						
ZG1-1	34	0	5	10	10	12
ZG3	37	0	0	0	0	0
ZG5	30	0	0	0	Dead	Dead
Control	19	0	0	0	0	0

[a] The cumulative number of seedlings exhibiting root bioluminescence on a given day post inoculation is indicated. All ZG5 seedlings died on the sugar-devoid medium.

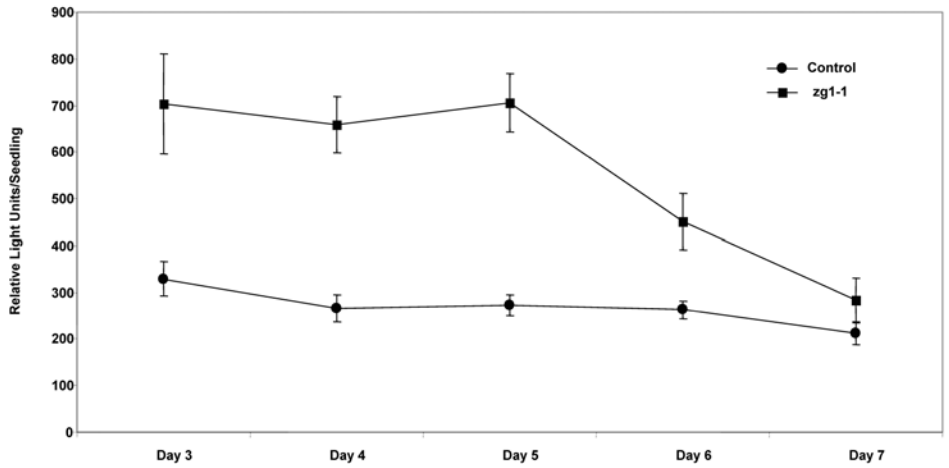

Fig. 2. *Rhizoctonia solani* infection induces the ocs element from the *GSTF8* promoter. Plants harboring the 4× ocs::LUC construct were infected with ZG1-1 and their bioluminescence monitored from day 3 to day 7 and compared with noninfected control seedlings. The average bioluminescence values, measured for 20 individual seedlings, are expressed in relative light units per seedling along with the standard errors.

observed under the microscope. We also compared regions that had not yet responded to regions where the luminescence had already decayed. In the example shown in Figure 4A and B, bioluminescence was emitted from regions of the root with very limited mycelium growth. At 1 day after *GSTF8*::LUC activity was detected on a particular root segment, strong fungal growth was observed (Fig. 4C) and, after 2 days, the fungi was observed to macerate the root (Fig. 4D). At these stages (Fig. 4C and D), *GSTF8*::LUC activity was no longer visible. From our analysis of a large number of seedlings, it seems that firm attachment and directed growth (that precede tissue invasion) occur in a particular root segment, after *GSTF8* promoter induction.

R. solani induction of the *GSTF8* promoter is SA independent.

To investigate whether the fungal induction of the *GSTF8* promoter was mediated by SA, we crossed the *GSTF8*::LUC line with Nah-G plants (Lawton et al. 1995), which express the SA-degrading enzyme salicylate hydroxylase. Strong, constitutive expression of this bacterial gene prevents pathogen-induced accumulation of SA and blocks the activation of systemically acquired resistance by exogenous SA in the transgenic plants (Gaffney et al. 1993; Lawton et al. 1995). We used reverse-transcriptase polymerase chain reaction (RT-PCR) to demonstrate that the F1 plants from the Nah-G × *GSTF8*::LUC crosses expressed the Nah-G gene (Fig. 5A). We also showed that the Nah-G gene was functioning in these F1 plants because they were susceptible to infection by an avirulent *Pseudomonas syringae* strain as opposed to *GSTF8*::LUC plants (Fig. 5B). The *P. syringae* titer on the Nah-G × *GSTF8*::LUC F1 plants was more than 100-fold greater than the titer on the *GSTF8*::LUC plants, 2 days after infection (data not shown). To examine the effect of Nah-G on the induction of the GSTF8 promoter by *R. solani,* we infected 4-day-old F1 seedlings that contained both the *GSTF8*::LUC and the Nah-G constructs with ZG1-1. *GSTF8* promoter activity was measured daily and compared with *GSTF8*::LUC wild-type plants, also infected with ZG1-1. Bioluminescence emitted from the Nah-G × *GSTF8*::LUC F1 plants did not differ significantly from *GSTF8*::LUC plants (Fig. 5C), suggesting that induction of the *GSTF8* promoter by *R. solani* does not require a SA signal.

Pathogenicity of different *R. solani* strains on *Arabidopsis* seedlings grown in soil.

The different fungal strains exhibited different aggressiveness on sucrose-free medium and elicited different responses of

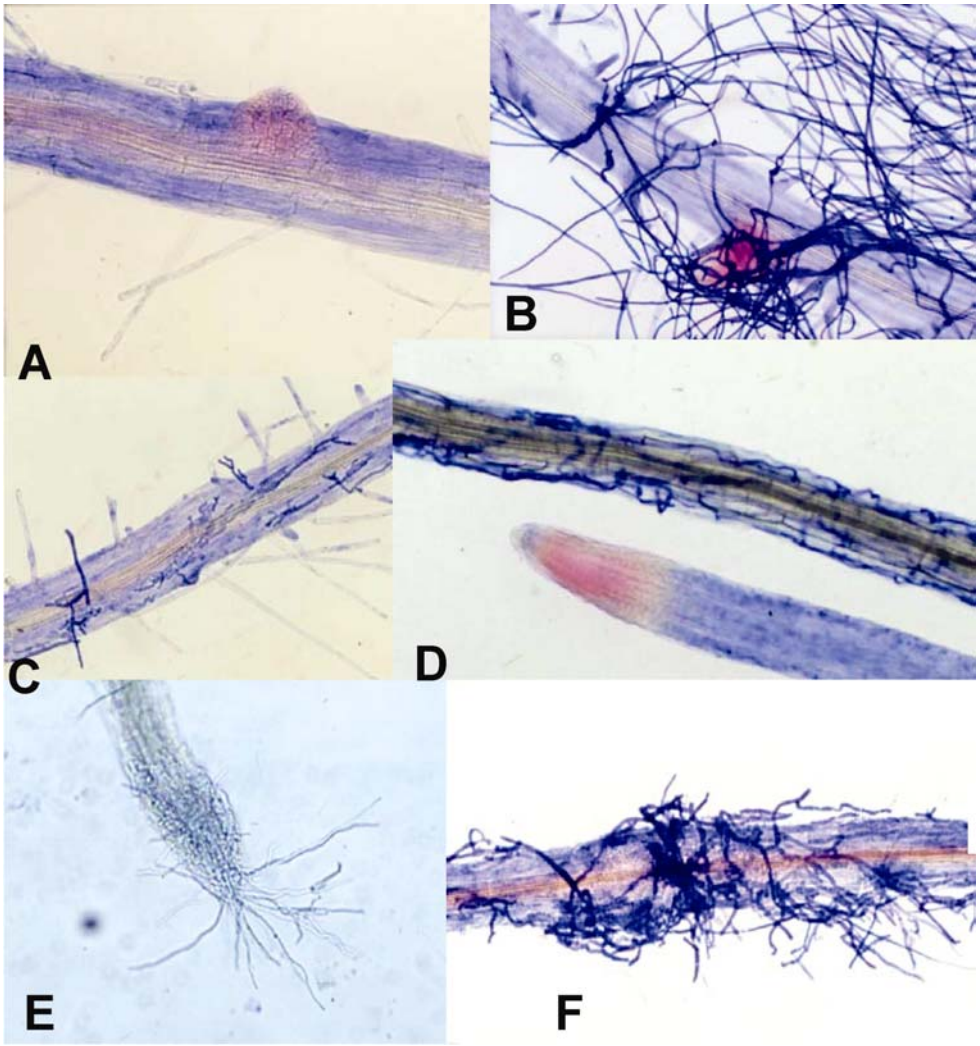

Fig. 3. Interaction of *Rhizoctonia solani* with in vitro-grown *Arabidopsis* roots. Light microscopy was done 5 days post inoculation, following washing and staining of roots with aniline blue and safranin red. **A,** Clean root from infected plate. **B,** Root overgrown by ZG5 strain mycelium (no wash), without firm attachment. **C,** Attachment of ZG1-1 hyphae to root surface; hyphae resist a 2-min wash under running water. **D,** Attachment and directed-growth pattern of ZG1-1 hyphae with frequent branching over the root. **E,** Growth of ZG5 hyphae on root tips. **F,** Formation of dome-like infection cushions and tissue maceration by ZG3 strain.

the *GSTF8* promoter; therefore, we asked whether these strains also differ in their pathogenic characteristics with soil-grown *Arabidopsis* plants. *Arabidopsis* seedlings were transplanted to soil inoculated with four millet seed that were infected with the ZG1-1, ZG3, or ZG5 *R. solani* strains, or with sterile millet seed as a control. The fungus was allowed to colonize the pots for 2 weeks before planting. We observed that the ZG5 strain caused death of the *Arabidopsis* seedlings (Fig. 6), killing up to 50% of the seedlings within 12 days. In contrast, all the seedlings survived the infection with the ZG1-1 and ZG3 strains under the conditions tested.

We then extended the study to examine additional *Rhizoctonia* strains. We examined *R. solani* ZG6 and two binucleated *Rhizoctonia* strains; CZG1 and CZG5. ZG6 can cause significant hypocotyl rot under certain growth conditions in canola, whereas CZG1 and CZG5 were only weakly pathogenic on canola, with mild symptoms of hypocotyl, tap root, and lateral root rots (Khangura et al. 1999). We examined the ability of these new strains to both induce the *GSTF8* promoter construct and cause disease symptoms on *Arabidopsis* seedlings grown in soil. We also retested ZG1-1 and ZG5, and the combined results are presented in Figure 7. We found that none of the new *Rhizoctonia* strains tested, in contrast to ZG1-1, induced the *GSTF8* promoter. We also found that only one of the new strains, ZG6, caused disease symptoms that led to plant death, similar to what was seen with ZG5. Interestingly, ZG6 belongs to the same AG group as ZG5. These results confirm that the response of the *GSTF8* promoter to *Rhizoctonia* spp. is very strain specific and does not correlate with the aggressiveness of the *Rhizoctonia* strain on *Arabidopsis* spp.

DISCUSSION

The *GSTF8*::LUC reporter system provides an elegant tool to monitor in vivo stress responses of *Arabidopsis* seedlings. In the present study, we asked whether the promoter, reported to respond to oxidative stress and SA, also would respond to infection by the fungal pathogen *R. solani*. For this purpose, we developed a system for co-cultivation of *Arabidopsis* seedlings with the fungus, using agar plates. We periodically monitored the emitted bioluminescence, along with observation of the plant–pathogen interaction, under the microscope.

We found that *R. solani* infects *Arabidopsis* roots and, depending on the strain, this may result in the induction of the *GSTF8* promoter 2 to 3 days after inoculation. The response does not seem to be a general stress response of decaying tissue because it occurs very early in the infection process in root regions with very limited mycelium visible and has been turned off in root segments with substantial mycelium present. Moreover, all three fungal strains that were studied in detail grew and infected the seedlings, progressed through typical infection stages, and eventually macerated the seedling—but only one strain, ZG1-1, reproducibly induced the *GSTF8* promoter in the majority of the seedlings. Of the other two strains, ZG5 very rarely induced the promoter, whereas the response to ZG3 was more variable and occurred in approximately 25% of the seedlings. The differential response to the three strains suggests that infection of *Arabidopsis* spp. by *R. solani* involves at least some degree of specific recognition, initiating a cascade that leads to the induction of the *GSTF8* promoter.

Other studies have reported changes in plant defense gene expression following *R. solani* infection, with the RNA levels of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), and hydroxyproline-rich glycoprotein (HRGP) being induced in bean (Guillon et al. 2002); whereas, in rice, PAL and peroxidase activities were reported to increase (Deborah et al. 2001) as well as the RNA levels for thaumatin-like proteins (TLP) (Velazhalan et al. 1998). However, in contrast to the early induction of the *GSTF8* promoter, the increase in PAL, CHS, CHI, and HRGP RNA occurred only late in the infection process, when fully developed lesions had formed on the hypocotyls and colonization of roots was at the highest levels; a systemic activation of these genes also was observed (Guillon et al. 2002).

R. solani is a soilborne fungus that is adapted to live outside the plant and exploit suitable host plants as food sources. Differences in host-plant specificity among *R. solani* strains have

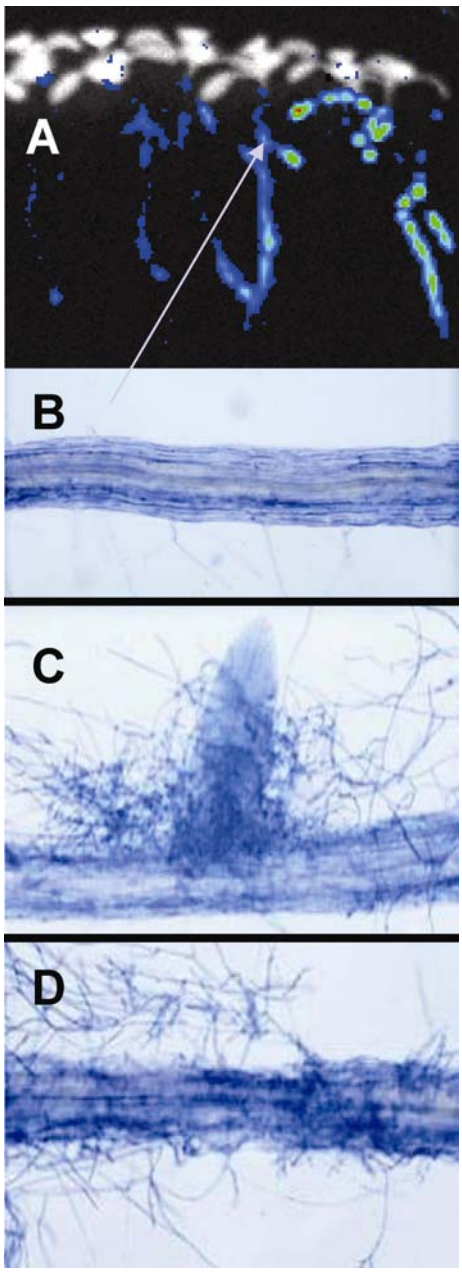

Fig. 4. Observation of *Rhizoctonia solani* ZG1-1 infection and *GSTF8*::LUC induction. **A,** In vivo luciferase assay of *Arabidopsis* GSTF8::LUC seedlings 3 days after infection with *R. solani* strain ZG1-1. Bioluminescence was monitored after 10 min in the dark, and is superimposed onto the chlorophyll fluorescent image. **B,** Light microscope observation of the region indicated by the arrow in A. **C,** Light microscope observation of a root 4 days after infection. No *GSTF8*::LUC activity was observed at this root segment at day 4; however, *GSTF8*::LUC activity was observed 1 day earlier (at day 3). **D,** Light microscope observation of a root 5 days after infection. No *GSTF8*::LUC activity was observed at this root segment at days 4 and 5; however, *GSTF8*::LUC activity was observed at day 3.

been observed (Keijer et al. 1997; Khangura et al. 1999), and this appears to be a phenomenon of relative preference rather than an absolute compatibility, typical of fungi such as rusts. Nevertheless, the basis for strain specificity is, as yet, poorly understood. A suitable host may be recognized by the growing hypha via a thigmotropic stimulus (Armentrout and Downer 1987), a host-released chemical inducer, or a nonhost chemical inhibitor (Keijer et al. 1997). Keijer and associates (1997) used an agar-plate assay similar to the one reported here to investigate strain-specificity of *R. solani* toward several hosts, including *Arabidopsis* spp. In pathogenic strain–host combinations, hyphae adhered to and then invaded the stem or root tissue; whereas, in nonpathogenic situations, the fungus overgrew the plant without adhering. Although strains generally could be classified as being more or less aggressive, and many cases of intermediate compatibility were evident, strain–host specificity was observed and generally reflected the relationship known from the field.

In our study, *GSTF8* promoter induction seemed to be very strain specific and did not occur with the most aggressive *Arabidopsis* strains, ZG5 and ZG6. In contrast, the ZG1-1 and ZG3 strains, which appear to be less successful *Arabidopsis* pathogens, at least under the conditions tested in this study, induced the *GSTF8* promoter, albeit to different degrees. Binucleate isolates, including one that had been shown to be weakly pathogenic in canola (Khangura et al. 1999), neither induced

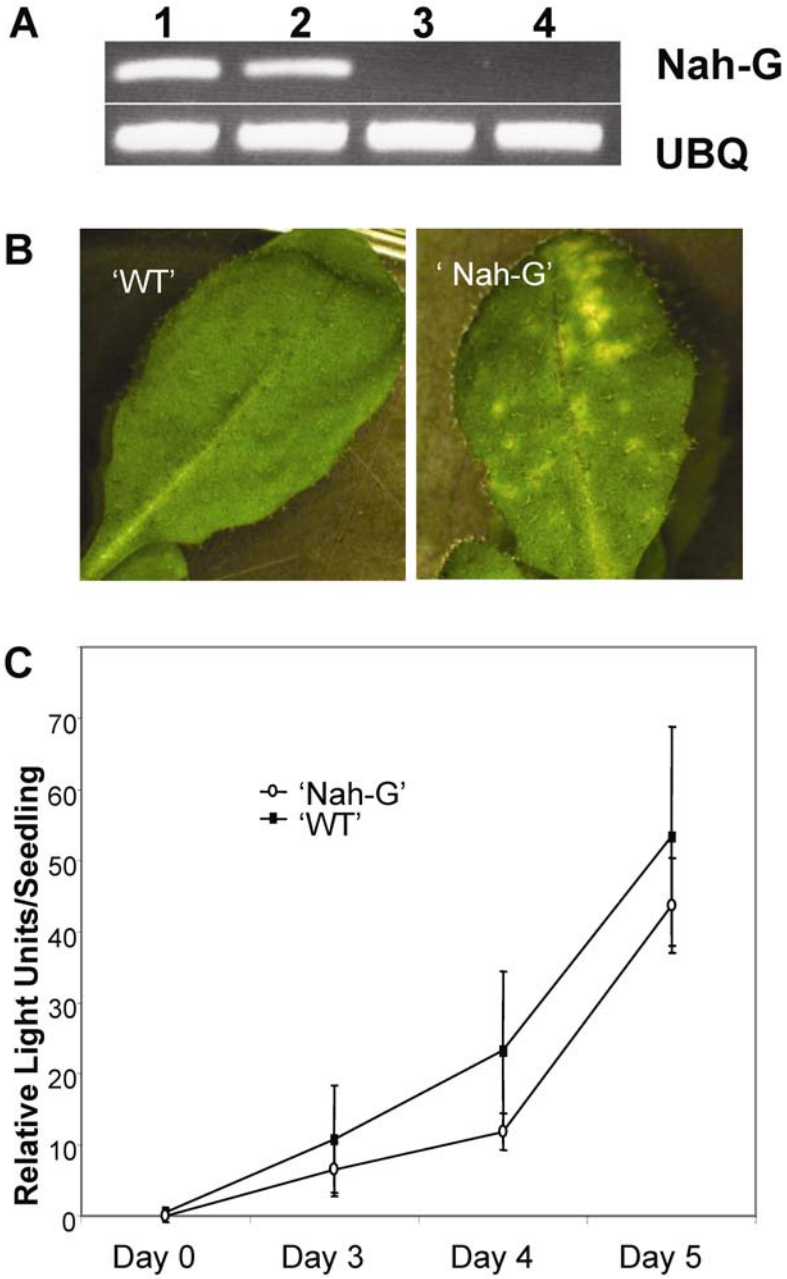

Fig. 5. Response of the *GSTF8* promoter to *Rhizoctonia solani* is salicylic acid independent. **A,** Reverse-transcriptase polymerase chain reaction analysis of Nah-G and UBQ expression in 3-week-old leaves from Nah-G × *GSTF8* F1 plants (lanes 1 and 2) and *GSTF8*::LUC plants (lanes 3 and 4). **B,** Disease symptoms on the leaves of 4-week-old *GSTF8*::LUC (WT) and Nah-G × *GSTF8* F1 plants (Nah-G), 3 days after inoculation with an avirulent strain of *Pseudomonas syringae*. **C,** *GSTF8*::LUC seedlings or F1 plants from a Nah-G × *GSTF8*::LUC cross were grown vertically for 4 days before infection with *R. solani* ZG1-1. Bioluminescence was monitored at day 0, 3, 4, and 5. The average relative bioluminescence values for *GSTF8*::LUC seedlings (WT) and F1 plants from a Nah-G × *GSTF8*::LUC cross (Nah-G) is plotted with the standard error. There is no significant difference as determined by analysis of variance of WT plants versus Nah-G plants with respect to GSTF8::LUC activity induced by *R. solani* ZG1-1.

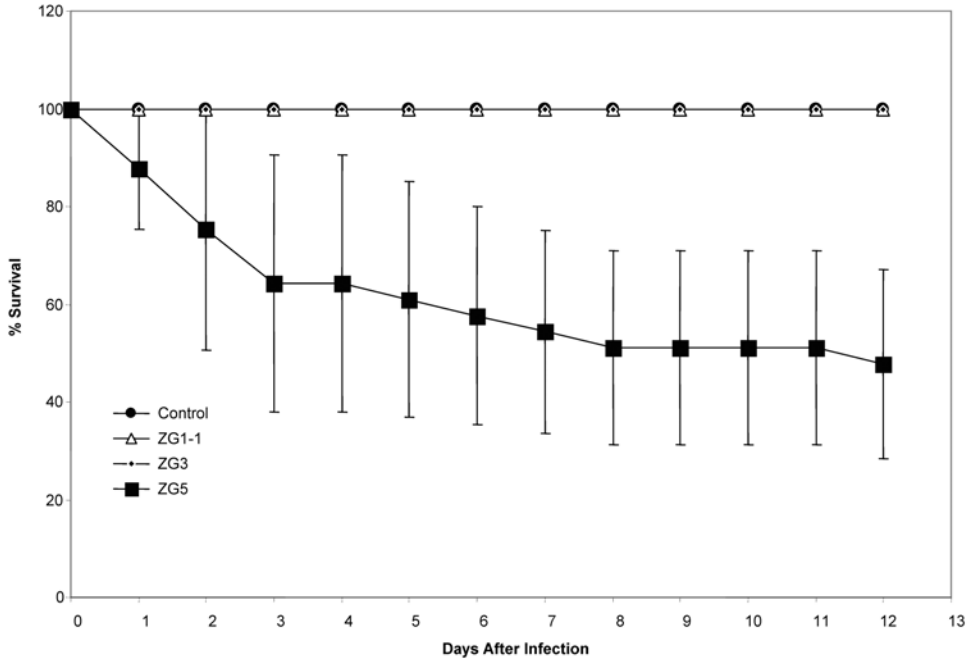

Fig. 6. Survival of *Arabidopsis* seedlings in soil infected with three different strains of *Rhizoctonia solani*. Control pots were mock-inoculated with sterile seed and seedling mortality was recorded for 12 days.

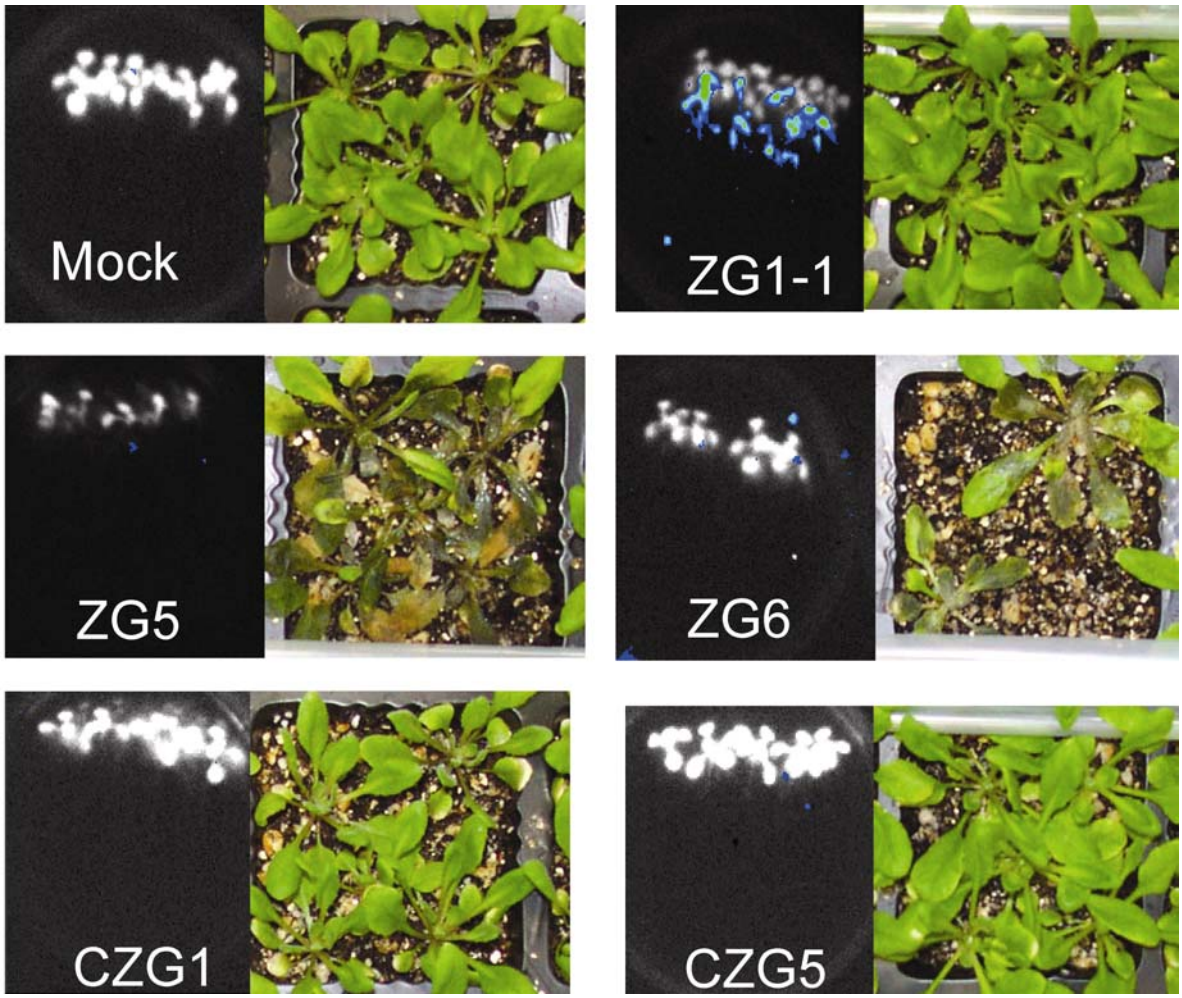

Fig. 7. Analysis of the *GSTF8* promoter response and disease symptoms in *Arabidopsis* plants infected with additional *Rhizoctonia* strains ZG6, CZG1, and CZG5 and compared with ZG1-1 and ZG5. For each strain, two images are shown. The bioluminescence (blue) and fluorescence (white) images are from the in vivo luciferase assay for 12-day-old *Arabidopsis GSTF8*::LUC seedlings (five seedlings per plate) following infection with each of the *Rhizoctonia* strains for 5 days. The second image shows the disease symptoms on 4-week-old *Arabidopsis* plants infected with the different *Rhizoctonia* strains. Each pot contained five plants. These experiments were repeated in triplicate with the same results.

the *GSTF8* promoter nor caused visible damage to *Arabidopsis* plants grown in soil. For the three strains studied in depth, we frequently observed dome-like infection cushions with ZG5 and ZG3 leading to tissue maceration, while ZG1-1, the strain that induced the *GSTF8* promoter most efficiently, seemed to persist in the directed-growth phase, adhering and probing the roots over long stretches but apparently causing less damage. This may indicate that aggressive strains such as ZG5 may escape detection by the plant defense mechanisms, or may possess an active means to repress such mechanisms. We note that dome-like infection cushions reportedly were typical of AG2-1, the AG to which ZG5 and ZG6 belong, and such structures were correlated with increased aggressiveness compared with simpler "foot-like" infection structures (Keijer 1996; Weinhold and Sinclair 1996).

In naturally occurring resistance to *R. solani,* some resistant interactions were correlated with cuticle thickness and slower progression of the pathogen within the tissue and inhibition of hydrolytic enzymes; other studies demonstrated a role for phytoalexins, calcification of cell walls, and reduction of lesion size (Weinhold and Sinclair 1996). Overexpression of pathogenesis-related genes like chitinases (Broglie et al. 1991), (Datta et al. 1999), and ribosome-inactivating protein (Maddaloni et al. 1997) resulted in enhanced resistance to *R. solani*, primarily through a delay in the development of disease symptoms, while reduced expression of lipoxygenase resulted in enhanced susceptibility (Rance et al. 1998).

It would be interesting to know whether induction of GSTs can contribute directly to plant resistance toward *R. solani*. In other words, is ZG1-1 less pathogenic on *Arabidopsis* spp. due to a protective role of GST enzymes that are induced at an early stage during the interaction with the host root? Although *GSTF8* may be only a minor component in the plethora of plant defenses that are elicited by the fungus, a few studies have correlated GSTs with protection against pathogens or abiotic stresses. The genomic locus for the potato *prp1* gene, which encodes a GST, co-locates with a locus for resistance to *Phytophthora infestans* (Hahn and Strittmatter 1994; Leonards-Schippers et al. 1994), and *prp1* expression is rapidly induced following fungal infection (Hahn and Strittmatter, 1994). Moreover, Hamid and Strange (2000) showed that GST may be the detoxifying enzyme that protects chickpea plants from solanapyrone A,B toxins produced by the blight agent, *Ascochyta rabiei,* and Takesawa and associates (2002) reported that rice that overexpressed a GST protein was tolerant to oxidative stress.

Members of the large GST gene family in plants are interesting models to study the control of stress-regulated gene expression because they are induced by a wide variety of stimuli, such as SA, pathogens, some abiotic stresses, and auxin; different family members may have either general or more specialized expression patterns (Edwards et al. 2000; Marrs 1996). Signaling pathways required for plant defense responses are complex, and even members from the same gene family that are induced by a single pathogen may require different signal molecules (Ferrari et al. 2003). SA, jasmonic acid (JA), and ethylene are hormones involved in the regulation of resistance against different pathogens. SA is a key regulator of pathogen-induced systemic acquired resistance (SAR) (Gaffney et al. 1993), whereas JA and ethylene regulate a largely distinct set of genes and are required for induced systemic resistance (ISR) (Pieterse et al. 2001). Both types of induced resistance are effective against a broad spectrum of pathogens and several lines of evidence demonstrate cross talk between the pathways (Glazebrook 2001). It appears that defense genes that are activated against necrotrophic fungi are regulated primarily by the ethylene or JA signal transduction pathways, but not by SA, whereas biotrophic pathogens are countered more efficiently by SA-controlled defense mechanisms (Thomma et al. 1998, 1999).

Our results using the Nah-G cross demonstrate that *GSTF8* induction by *R. solani* ZG1-1 appears to occur via an SA-independent pathway, even though the *GSTF8* gene is inducible by exogenous SA (Chen et al. 1996), and not by ethylene treatment (Wagner et al. 2002). In the case of *Tobacco mosaic virus* (TMV), tobacco genes have been isolated whose expression in TMV-inoculated leaves is mediated via an SA-independent pathway although these genes are inducible by exogenous SA (Guo et al. 2000). Induction of the *Arabidopsis GSTF6* gene (previously called *GST1*) by avirulent *P. syringae* strains has been reported to occur independently of SA and ethylene but requires redox signaling (Grant et al. 2000). However, a recent study has reported that *GSTF6* is induced in response to an avirulent *P. syringae* strain through combined SA and ethylene signaling (Lieberherr et al. 2003). Although *GSTF8* expression is not induced following infection by either avirulent or virulent strains of *P. syringae* (L. Oñate-Sánchez and K. B. Singh, *unpublished data*), the *GSTF8* promoter does respond to H_2O_2 and auxin (Chen et al. 1999). Interestingly, *R. solani* strains produce indole 3-acetic acid (Furukawa et al. 1996), which may supply an inducing signal for the *GSTF8* promoter. The power of *Arabidopsis* molecular genetics with the many mutants and molecular tools available for analysis should allow us to further dissect the events that occur following the encounter between *R. solani* and *Arabidopsis* seedlings.

MATERIALS AND METHODS

Plant material.

The *Arabidopsis thaliana* transgenic lines contained the –783 *GSTF8* promoter fused to the luciferase reporter gene in the Columbia ecotype background as described in Chen and Singh (1999). All the data presented is from a single T4 line called JC66 that was homozygous for the transgene, but similar results were seen using a second line. The 4× ocs element promoter construct was made by ligating four copies of the *GSTF8* ocs element in front of the –58 *GSTF8* minimal promoter (Chen and Singh 1999) and a T4 line that was homozygous for the transgene was used. Wild-type plants of ecotype Columbia were used as controls. Nah-G lines in which the Nah-G gene was expressed off the constitutive Cauliflower Mosaic 35S promoter were provided by Syngenta (Greensboro, NC, U.S.A.).

Rhizoctonia strains and their maintenance.

Rhizoctonia isolates were obtained from M. Sweetingham and K. Sivasithamparam and cultured every 2 to 3 weeks onto fresh potato-dextrose agar (PDA; Sigma-Aldrich, St. Louis) plates. For soil inoculation, a millet-seed culture of each strain was grown. Moist millet seed were autoclaved twice on two consecutive days, inoculated with a week-old PDA culture, and grown at room temperature (RT) in the dark for 2 weeks, with occasional mixing. For longer-term storage, inoculated millet seed were dried overnight in a sterile laminar flow hood and kept in sealed vials at RT.

Growth of *Arabidopsis* seedlings on sterile plates.

Agar plate medium contained 1× MS salts (4.3 g/liter) (Gibco BRL, Gaithersburg, MD, U.S.A.) and 0.8% agar, with or without 3% sucrose, pH adjusted to 5.7 with 1 M KOH. Plates for the luciferase assay were supplemented with 0.5 mM luciferin (Biosynth AG, San Diego, CA, U.S.A.), added after autoclaving the medium. *Arabidopsis* seed were sterilized in 70% ethanol for 20 min, dried, and suspended in sterile 0.1% agar, and incubated for 2 to 7 days at 4°C. Approximately 6

seed were plated on 5.5-cm plates, sealed with Micropore tape, and incubated vertically in the growth room (22°C, 16-h-light and 8-h-dark photoperiod). Plants were inoculated with *R. solani* 5 to 7 days after planting.

Infection of seedlings grown in agar plates with *R. solani*.

A black line was drawn on the bottom of each plate to mark the infection sites, approximately 1 cm below the crown of the seedlings. In early experiments, plants were wounded prior to infection by squeezing the root four times with sterilized tweezers at the marked site. Control, noninoculated seedlings were similarly wounded. Plates with 1-week-old *R. solani* cultures were scraped with a sterile toothpick, and each seedling was touched three times. For each seedling, a previously untouched region in the plate was scraped, trying to provide uniform inoculation. In later experiments, we learned that wounding was unnecessary and the same infection pattern and response were observed with unwounded seedlings. Inoculated plates were resealed and incubated vertically in the growth chamber (22°C, 16-h-light and 8-h-dark photoperiod). The extent of visible growth was scored as "starting" = a few hyphae visible; + = a sparse network of hyphae of approximately 1 cm in diameter; ++ = a continuous, fine network 2 cm or more in diameter; and +++ = a dense, opaque mat of mycelium, 2 cm or more in diameter. Usually, a plate progressed from stage + to stages ++ or +++ between day 3 and day 5 after inoculation.

Bioluminescence assay.

Bioluminescence was measured in an EG & G Berthold Molecular Light Imager using a 5-min exposure after a 10-min fluorescent decay delay. Nine 5.5-cm plates were fitted per picture. The lids were kept closed to maintain sterile conditions. Plates were monitored once or twice a day. At each time point, a photograph was taken in weak room light to show the position of the seedlings; then, the camera chamber door was opened momentarily to illuminate the plants, and a photograph of chlorophyll fluorescence was taken to demonstrate seedling viability and help to locate each seedling in the plate. The bioluminescence picture was overlaid on top of the fluorescence photograph or black-and-white photo. Every day, we recorded for each plate the number of seedlings, extent of visible fungal growth, presence of contamination (other than *Rhizoctonia* spp.), death of seedlings, and number of seedlings with enhanced luciferase activity. At the end of the experiment, data were tabulated and analyzed.

Light microscope observation of *R. solani*-infected seedlings.

To monitor fungal association with *Arabidopsis* roots, seedlings were removed from agar plates and washed under running tap water for 2 min to remove nonassociated hyphae. Staining was carried out in small petri dishes with gentle shaking, and seedlings were immersed for 1 to 2 min in 0.25% safranin red, then for 1 to 2 min in aniline blue (25% lactic acid, 50% glycerol, 0.5% aniline blue), followed by 1 min of destaining (in the same solution without dye). Seedlings were examined under the microscope and photographed at ×25 to 50 magnification.

Inoculation of soil-grown plants.

Sterile soil was infected by placing four millet seed at 1 cm of depth in each pot. Fungus was left to grow for 14 days before planting. Sterile millet seed were applied to the noninoculated control pots. One-week-old *Arabidopsis* seedlings were transferred from sterile MS agar medium (Sigma-Aldrich, St. Louis) to infected pots, and grown in a growth cabinet for 12 days (22°C, 16-h-light and 8-h-dark photoperiod).

RT-PCR.

RNA extraction, cDNA synthesis, and RT-PCR were performed as described by Kang and associates (2003). Nah-G primers 5′ TGTGACCTTCTAATCGGTGCCG and 5′ CGTC GATGCCCTGGGCTCGATA and UBQ primers 5′ CTCCGG ACCAGCAGCGTCTC and 5′ AGAACACTTATTCATCAG GG were used to amplify cDNA (30 cycles) and the PCR product was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

***Pseudomonas* spp. infection.**

P. syringae pv. *tomato* DC3000, expressing the avirulence gene *avrRpt2,* was used for infection. Leaves from 4-week-old *GSTF8*::LUC plants or Nah-G × *GSTF8*::LUC F1 plants which had been grown on soil under a 12-h-light and 12-h-dark regime were infiltrated with a 10 mM $MgSO_4$ solution containing the plant pathogen *P. syringae* pv. *tomato* DC3000 (*avrRpt2*) at approximately 10^7 CFU/ml. Infiltrated leaves were harvested at day 0, 1, 2, and 3 to measure the bacteria titer.

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