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Redox-Active Pyocyanin Secreted by *Pseudomonas aeruginosa* 7NSK2 Triggers Systemic Resistance to *Magnaporthe grisea* but Enhances *Rhizoctonia solani* Susceptibility in Rice

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***Pseudomonas aeruginosa* 7NSK2 induces resistance in dicots through a synergistic interaction of the phenazine pyocyanin and the salicylic acid-derivative pyochelin. Root inoculation of the monocot model rice with 7NSK2 partially protected leaves against blast disease (*Magnaporthe grisea*) but failed to consistently reduce sheath blight (*Rhizoctonia solani*). Only mutations interfering with pyocyanin production led to a significant decrease in induced systemic resistance (ISR) to *M. grisea*, and in trans complementation for pyocyanin production restored the ability to elicit ISR. Intriguingly, pyocyanin-deficient mutants, unlike the wild type, triggered ISR against *R. solani*. Hence, bacterial pyocyanin plays a differential role in 7NSK2-mediated ISR in rice. Application of purified pyocyanin to hydroponically grown rice seedlings increased H_2O_2 levels locally on the root surface as well as a biphasic H_2O_2 generation pattern in distal leaves. Co-application of pyocyanin and the antioxidant sodium ascorbate alleviated the opposite effects of pyocyanin on rice blast and sheath blight development, suggesting that the differential effectiveness of pyocyanin with respect to 7NSK2-triggered ISR is mediated by transiently elevated H_2O_2 levels in planta. The cumulative results suggest that reactive oxygen species act as a double-edged sword in the interaction of rice with the hemibiotroph *M. grisea* and the necrotroph *R. solani*.**

Additional keywords: hypersensitive response, oxidative burst, *Pyricularia grisea*, rhizobacteria.

Rice is the most important staple food grain for more than two billion people living in the rural and urban areas of humid and subhumid Asia. Diseases are among the most important limiting factors that affect rice production, causing annual yield loss conservatively estimated at 5% (Mew et al. 2004). More than 70 diseases caused by fungi, bacteria, viruses, or nematodes have been recorded on rice (Ou 1985), among which rice blast (*Magnaporthe grisea*) and sheath blight (*Rhizoctonia solani*) are the most serious fungal constraints on high productivity.

The filamentous ascomycete *M. grisea* (T. T. Hebert) Barr (anamorph *Pyricularia grisea* (Cooke) Sacc.) is the most devastating pathogen of rice worldwide due to its widespread distribution and destructiveness (Talbot 2003). The rice–*M. grisea* interaction is a well-documented gene-for-gene system (Jia et al. 2000; Silue et al. 1992), and the fungus is a hemibiotroph because successful infection requires an initial biotrophic phase in which the pathogen forms bulbous invasive hyphae within apparently healthy plant cells (Koga 1994). Once established in the plant, the fungus switches to necrotrophic growth, killing plant cells and ramifying throughout the tissue. Rice sheath blight is caused by *R. solani* Kühn (sexual stage: *Thanetophorus cucumeris* (A. B. Frank) Donk), a soil- and waterborne fungal pathogen enjoying a very wide host range. The pathogen has a necrotrophic lifestyle and is able to produce a host-specific carbohydrate-based phytotoxin (Vidhyasekaran et al. 1997).

Resistant cultivars and application of pesticides have been used for disease control. However, the useful life span of most blast-resistant cultivars is only a few years, due to the breakdown of the resistance in face of the high pathogenic variability of the pathogen population (Song and Goodman 2001). Though partial genetic resistance to sheath blight has been reported, no major gene-governed resistance has been found so far, despite screening of more than 3,000 accessions of germ plasm worldwide (Mew et al. 2004). Because chemical means of management are often expensive, no economically viable or sustainable control measures currently are available to tackle the diseases.

Thus, there is a need to develop alternative disease control strategies providing durable, broad-spectrum resistance. Among such new strategies, induced resistance has emerged as a potential supplement in international crop protection measures. Induced resistance can be defined as the phenomenon by which plants exhibit increased levels of resistance to a broad spectrum of pathogens by prior activation of genetically programmed defense pathways. The most extensively studied type of induced resistance is systemic acquired resistance (SAR). SAR is expressed locally and systemically after a localized infection by a necrotizing pathogen and is characterized by the accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins (Durrant and Dong 2004). Colonization of roots with selected plant growth-promoting rhizobacteria (PGPR) also can lead to a type of systemic resistance, commonly denoted as induced systemic resistance (ISR) (Bakker et al. 2003; van Loon et al. 1998). Generally, the onset of ISR, unlike SAR, is not accompanied by the concomitant activation of PR genes (Van Wees et al. 1999). Instead, recent research

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revealed that ISR-expressing plants are primed to react faster to pathogen attack (Verhagen et al. 2004).

Bacterial determinants of ISR that have been identified are cell surface components, such as outer membrane lipopolysaccharides (LPS) or flagella, iron-regulated metabolites with siderophore activity, benzylamine derivatives, volatile compounds, and certain antibiotics (Bakker et al. 2003; Iavicoli et al. 2003; Meziane et al. 2005; Ongena et al. 2005; Ryu et al. 2004). In general, most rhizobacteria show redundancy in ISR-triggering traits and their effects can be complementary or additive. Moreover, the mechanisms involved in rhizobacteria-mediated ISR tend to vary among bacterial strains and pathosystems, indicating a great degree of flexibility in the molecular processes leading to ISR, which makes it difficult to derive a general model for PGPR-induced ISR.

To date, molecular biology research aimed toward understanding induced resistance mechanisms has focused mainly on dicotyledoneous model plant species such as *Arabidopsis thaliana* and tobacco. Conversely, in the class of Monocotyledoneae, including the most important agronomic cereals, molecular information on chemically and biologically induced resistance mechanisms is largely missing (Kogel and Langen 2005). One of the most compelling examples of a rice SAR-like response is the enhanced resistance to *M. grisea* that was demonstrated in response to an infection with the nonhost pathogen *Pseudomonas syringae* pv. *syringae* (Smith and Metraux 1991). However, Reimmann and associates (1995) failed to reproduce these results, indicating that conditions for SAR are critical. Although the synthetic SA analogue benzo(1,2,3)-thiadiazole-7-carbothioc acid (BTH) has been shown to induce SAR in wheat (Görlach et al. 1996) and disease resistance in rice (Rohilla et al. 2002; Schweizer et al. 1999) and maize (Morris et al. 1998), reports about the induction of systemic resistance in monocots using beneficial microorganisms are scarce. These include one in barley, where preinoculation with the root-colonizing fungus *Piriformospora indica* induced systemic resistance to several fungal diseases (Waller et al. 2005). In rice, colonization of the rhizosphere with the PGPR strains *Pseudomonas fluorescens* PF1 and FP7 enhanced resistance against sheath blight disease (Nandakumar et al. 2001). Someya and associates (2002, 2005) reported induced resistance to rice blast and sheath blight by the antagonistic bacterium *Serratia marcescens* B2.

In the present study, we assessed the PGPR strain *P. aeruginosa* 7NSK2 for its capacity to elicit systemic resistance to *M. grisea* and *R. solani* in a rice-based model system. Previously, 7NSK2 was shown to induce resistance in several dicot plant species such as bean, tobacco, and tomato (Audenaert et al. 2002; Bigirimana and Höfte 2002; De Meyer et al. 1999a and b; De Meyer and Höfte 1997). Its ability to trigger ISR has been linked to the production of SA (Bigirimana and Höfte 2002; De Meyer et al. 1999a; De Meyer and Höfte 1997). Furthermore, 7NSK2-mediated ISR was shown to be dependent on a functional SA response in the plant because 7NSK2 no longer induced resistance in transgenic *NahG* tomato or tobacco plants which are unable to accumulate SA (Audenaert et al. 2002; De Meyer et al. 1999a). However, recent evidence strongly suggests that, at least for the wild type, SA is not the inducing bacterial compound; however, the secondary metabolites pyocyanin and the SA-derivative pyochelin produced by this strain are a prerequisite for the induction of ISR in dicot plant species (Audenaert et al. 2002). The seminal articles by Hassan and Fridovich (1979, 1980) revealed that pyocyanin, like paraquat, menadione, and other xenobiotics, can undergo redox-cycling in the presence of various reducing agents and oxygen with resultant formation of reactive oxygen species (ROS) in vitro.

Here, we demonstrate that root treatment of rice seedlings with *P. aeruginosa* 7NSK2 significantly reduces rice blast but fails to mount ISR against sheath blight and provide evidence that this differential effectiveness is due to the production of the phenazine compound pyocyanin. Furthermore, our results provide new insight into the role of ROS in the interaction of rice with hemibiotrophic and necrotrophic pathogens.

RESULTS

P. aeruginosa 7NSK2 triggers ISR in rice to *M. grisea* but not to *R. solani.*

We first tested whether root colonization by *P. aeruginosa* 7NSK2 could be consistently obtained with the combined seed- and root-inoculation assay used in this study. The amount of bacteria recovered from root surfaces was determined 17 days after the last soil drench (i.e., 1 week after challenge inoculation). Pooled over three independent experiments, colonization of the roots was reflected by a bacterial titer of 1.43×10^5 CFU/g of root fresh weight ($\pm 3.4 \times 10^4$; $n = 12$). This population density, which is well above the threshold density of 10^5 CFU/g of root for *P. fluorescens* WCS374-mediated ISR in radish (Raaijmakers et al. 1995), was obtained consistently throughout all experiments performed in this study.

Next, we investigated whether colonization of the rhizosphere of rice seedlings with *P. aeruginosa* 7NSK2 had a protective effect against leaf blast disease, caused by the ascomycete *M. grisea*. In several preliminary experiments, *P. aeruginosa* 7NSK2 significantly reduced leaf blast symptoms, producing a resistance phenotype resembling that of genetically determined, intermediate resistance. This type of resistance is characterized by the formation of many small dark-brown spots (diameter <2 mm) 2 to 3 days after inoculation (Fig. 1B). In contrast, on nontreated control leaves, large, susceptible-type lesions (diameter 3 to 6 mm) with a gray center appeared, often surrounded by chlorotic or necrotic tissue (Fig. 1A). These susceptible-type lesions appeared no earlier than 4 days postinoculation. Because colonization of the rhizosphere by *P. aeruginosa* 7NSK2 did not completely abolish the formation of susceptible-type lesions, we quantified the disease by counting the number of susceptible-type lesions 6 to 7 days postinoculation. This method is both rapid and quantitative. In general, variation in disease severity between independent inoculation experiments, ranging from approximately 20 to 75

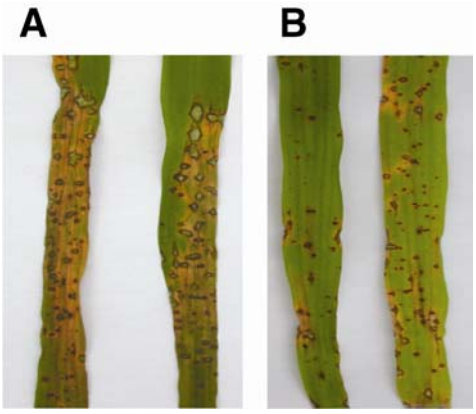

Fig. 1. Phenotype of *Pseudomonas aeruginosa* 7NSK2-mediated induced systemic resistance in rice to *Magnaporthe grisea*. **A,** Control plants were treated with water. **B,** *P. aeruginosa* 7NSK2 was grown on King's medium B (KB) and applied to rice seed, roots, and soil. Four-week-old plants (five-leaf stage) were challenge inoculated by spraying a spore suspension of virulent *M. grisea* VT5M1 at 1×10^4 spores/ml. Photographs depicting representative symptoms were taken 6 days after fungal inoculation.

lesions per control leaf, is a phenomenon inherent in the rice–*M. grisea* pathosystem (Schweizer et al. 1997). Therefore, and because no clear correlation between disease severity and the level of induced resistance could be observed, inoculation data are presented as relative infection values compared with nontreated controls. Pooled over five independent experiments, 7NSK2 reduced rice blast severity by 37.19% (±6.64; $n = 81$).

To test the spectrum of 7NSK2-mediated ISR in rice, we then assayed for induction of resistance against sheath blight, which ranks next to blast in causing yield losses, especially in intensified production systems. Although small protective effects were observed in single experiments, 7NSK2 proved unable to consistently reduce the length of lesions caused by the sheath blight fungus *R. solani* (data not shown).

In vitro dual culture experiments revealed a clear antagonistic potential of *P. aeruginosa* 7NSK2 to both *M. grisea* and *R. solani* (data not shown). To exclude direct antagonism between the inducing agent *P. aeruginosa* 7NSK2 and the challenging leaf pathogens *M. grisea* and *R. solani*, possible systemic plant colonization by the bacterium was checked. However, 7NSK2 and the derived mutants were never detected in sheath and leaf extracts of root-treated rice seedlings at distinct time points, indicating that bacterial plant colonization remained confined to the root zone (data not shown). The detection limit of this assay is approximately 10 CFU per stem or leaf. Given the spatial separation between the inducing bacterium (root) and the challenging pathogen (leaf or leaf sheath), the observed disease reduction can be attributed to ISR.

The phenazine compound pyocyanin is an essential determinant of 7NSK2-mediated ISR to *M. grisea* in rice.

Preliminary experiments with 7NSK2 inoculum prepared from iron-rich medium revealed that 7NSK2-triggered resistance to *M. grisea* is not dependent on the iron nutritional state of the inoculum, suggesting that siderophores such as pyochelin do not play a crucial role in ISR to *M. grisea* (data not shown). In order to identify the bacterial factors operative in triggering systemic resistance to *M. grisea*, the potency of *P. aeruginosa* 7NSK2 to induce ISR was compared with that of a collection of mutants deficient in the production of pyocyanin or pyochelin. All bacterial strains were grown routinely on iron-poor King's medium B (KB). The pyochelin-negative mutant KMPCH (also pyoverdin deficient) induced resistance to an extent similar to that induced by the wild type (Fig. 2A), hereby excluding an essential role of the siderophores pyoverdin and pyochelin in ISR in rice to *M. grisea*. Treatment with the newly generated pyocyanin-negative mutants 7NSK2-phzM and KMPCH-phzM no longer caused disease reduction, indicating the involvement of the phenazine antibiotic pyocyanin in ISR. Both mutant strains were constructed by gene replacement of the *phzM* gene, encoding an *O*-methyl transferase which is necessary for the conversion of phenazine-1-carboxylate to the pyocyanin precursor 5-methylphenazine-1-carboxylic acid betaine (Mavrodi et al. 2001). A deficiency in root colonization could be ruled out, because bacterial counts in the rhizosphere of plants inoculated with strains 7NSK2-phzM and KMPCH-phzM were similar to those of 7NSK2-treated plants (data not shown). In trans complementation of 7NSK2-phzM for pyocyanin production (strain 7NSK2-phzMc) restored the capacity to induce resistance to *M. grisea*, confirming the essential role of pyocyanin in 7NSK2-mediated ISR (Fig. 2B). Root colonization with the pyocyanin-overproducing strain 7NSK2-phz2, which is mutated in the regulatory gene *retS*, encoding a hybrid sensor kinase, yielded variable results. A significant reduction in the number of susceptible-type lesions was observed in one experiment (relative infection [RI] value = 76%), whereas treatment with 7NSK2-phz2 generated a higher infection rate (RI = 129%) in another trial. In the remaining two experiments, no statistically significant differences could be observed between control plants and plants colonized with 7NSK2-phz2

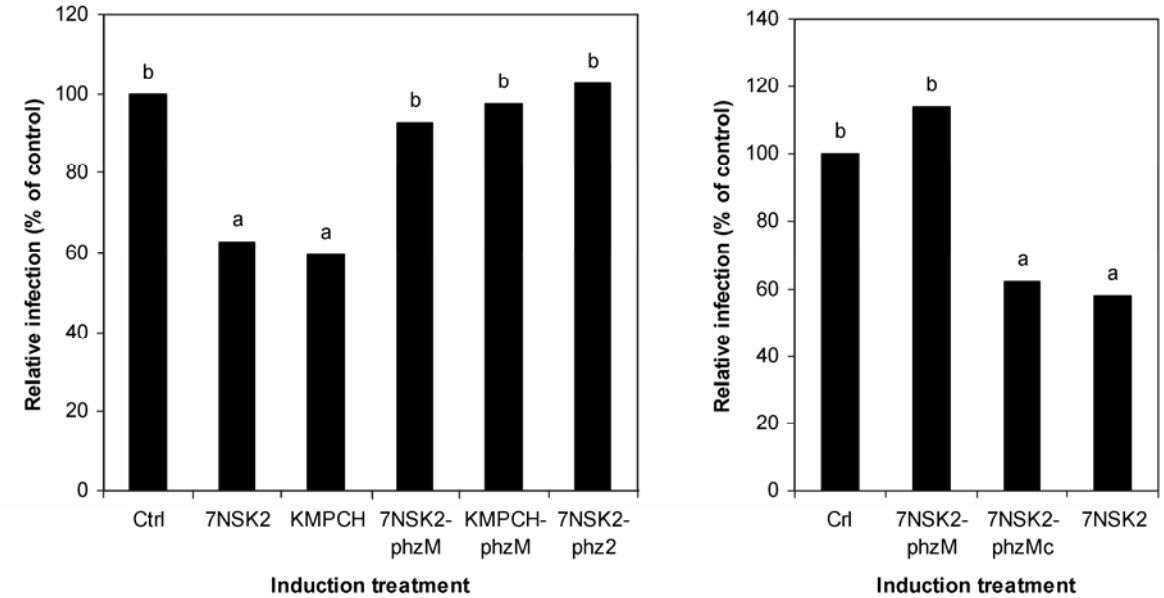

Fig. 2. Influence of root treatment with *Pseudomonas aeruginosa* 7NSK2 and various mutants on rice blast (*Magnaporthe grisea*) severity. *P. aeruginosa* 7NSK2 and derived mutants were grown on King's medium B (KB) and applied to rice seed, roots, and soil. Control plants were treated with water. Four-week-old plants (five-leaf stage) were challenge inoculated by spraying a spore suspension of virulent *M. grisea* VT5M1 at 1×10^4 spores/ml. Six days after challenge infection, disease was rated by counting the number of susceptible-type lesions per leaf 4 and expressed relative to challenged control plants. Statistical analysis was performed on pooled data, because interaction between treatment and experiment was not significant at $\alpha = 0.05$ by analysis of variance. Different letters indicate statistically significant differences between treatments according to Kruskal-Wallis followed by Mann-Whitney comparison tests ($P = 0.05$). Mutants derived from strain 7NSK2 have the following characteristics: KMPCH (pyoverdin and pyochelin deficient), 7NSK2-phzM (phzM-, nonproducing pyocyanin), KMPCH-phzM (pyoverdin and pyochelin deficient; phzM-, nonproducing pyocyanin), and 7NSK2-phzMc = strain 7NSK2-phzM complemented with functional phzM gene of 7NSK2, restoring pyocyanin production.

(RI = 92 and 106%). In vitro experiments monitoring production of pyocyanin by 7NSK2 and respective mutant strains revealed that 10^9 CFU of 7NSK2-phz2 produces approximately 25.05 μg of pyocyanin/ml of culture supernatant (±1.166; $n = 7$), thereby showing a fivefold increase compared with the wild type.

Pyocyanin-negative mutants of 7NSK2 trigger ISR in rice to *R. solani*.

In spite of its resistance-inducing potential against *M. grisea*, *P. aeruginosa* 7NSK2 proved unable to consistently mount ISR to the sheath blight fungus *R. solani* in several preliminary experiments. These data notwithstanding, we tested the same set of mutant strains as described before in a series of infection assays with *R. solani* as challenging pathogen. All strains were grown routinely on KB. Pooled over three independent experiments, neither the wild-type strain 7NSK2 nor the pyochelin-negative mutant KMPCH significantly reduced sheath blight severity (Fig. 3). However, inoculation of the rhizosphere of rice seedlings with the corresponding pyocyanin-deficient strains (7NSK2-phzM and KMPCH-phzM) resulted in significantly higher protection levels to *R. solani* compared with wild-type-treated and control plants. Conversely, no statistically significant differences could be observed between treatment with the pyocyanin-overproducing strain 7NSK2-phz2 and control plants. The inability of the pyocyanin-positive strains to mount ISR to *R. solani* was not due to insufficient root colonization of the rice seedlings, because bacterial counts in the rhizosphere of plants inoculated with the respective strains showed no marked differences (data not shown).

Pyocyanin induces resistance to *M. grisea* but enhances infection by *R. solani*.

The observation that pyocyanin-deficient mutants, unlike wild-type strains, triggered resistance to *R. solani*, whereas the same mutants lost their ability to mount ISR to *M. grisea* (Figs. 2A and 3), suggested that the secretion of pyocyanin might account for the differential effectiveness of 7NSK2-mediated ISR to the latter pathogens. Therefore, and because it has been reported before that high concentrations of purified pyocyanin can induce resistance to *Botrytis cinerea* in bean (Abeysinghe 1999), we wanted to further explore the role of bacterially produced pyocyanin in 7NSK2-mediated ISR in rice. To this purpose, we isolated pyocyanin from the pyocyanin-overproducing strain 7NSK2-phz2 using a chloroform-based extraction assay (Kanner et al. 1978) and applied the purified compound to the roots of rice seedlings. To avoid excessive immobilization of the metabolite through adsorption on soil particles or spontaneous degradation or biodegradation in soil, a soil-less gnotobiotic rice-growing system was developed. In this system, 24-day-old rice seedlings were fed hydroponically with a dilution series of pyocyanin by adding the desired concentration to the nutrient solution. In order to include a pyocyanin concentration in the experimental set-up that is equivalent to the production by *P. aeruginosa* 7NSK2, we spectrophotometrically quantified the in vitro pyocyanin production by the latter strain. Pooled over two independent experiments, 10^9 CFU of 7NSK2 produced 4.958 μg of pyocyanin/ml of culture supernatant (±0.483 μg; $n = 7$). Provided that pyocyanin production by 7NSK2 is proportional to the amount of 7NSK2, 10^5 CFU of 7NSK2 should produce approximately 0.5 ng of pyocyanin. This is similar to the amount of pyocyanin applied in a 25 pM pyocyanin solution (1,000 ml of 25 pM pyocyanin = approximately 5.2 ng of pyocyanin per tray or 0.43 ng per seedling). Thus, feeding a 25 pM pyocyanin solution in the hydroponic system resembles the production of pyocyanin in the rhizosphere of soil-grown rice plants colonized by 7NSK2. No signs of phytotoxicity were observed in leaves of plants after pyocyanin feeding at any of the concentrations tested. In the range of 25 pM to 100 nM pyocyanin, ISR to *M. grisea* was evident for all concentrations tested. However, no significant protection could be observed at 50 μM pyocyanin (Fig. 4A). Furthermore, pyocyanin did not reduce the number of virulent lesions to the same extent as treatment of the roots with 7NSK2. Taken together, these results indicate that *P. aeruginosa* 7NSK2-mediated ISR to *M. grisea* can be partially mimicked by application of pyocyanin to roots. Conversely, pyocyanin feeding favored subsequent infection by *R. solani*, irrespective of the applied concentration (Fig. 4B). Because pyocyanin is known for its antibiotic properties (Hassan and Fridovich 1980), we checked whether pyocyanin feeding leads to translocation of the compound to distal plant tissues such as leaves. However, pyocyanin remained undetected in leaves of root-fed plants using high-performance liquid chromatography (HPLC) analyses (detection limit, 13 ng/g of fresh weight). Summarized, these data suggest a dual role of pyocyanin in 7NSK2-mediated ISR and corroborate the results obtained in the ISR assays with the pyocyanin-negative mutants 7NSK2-phzM and KMPCH-phzM.

Pyocyanin triggers enhanced levels of H_2O_2 in local and systemic tissue upon root treatment.

Given the fact that pyocyanin has the capacity to undergo redox cycling under aerobic conditions with resulting generation of superoxide and hydrogen peroxide in vitro (Hassan and Fridovich 1980), we asked whether pyocyanin also would be capable of producing ROS in our gnotobiotic sys-

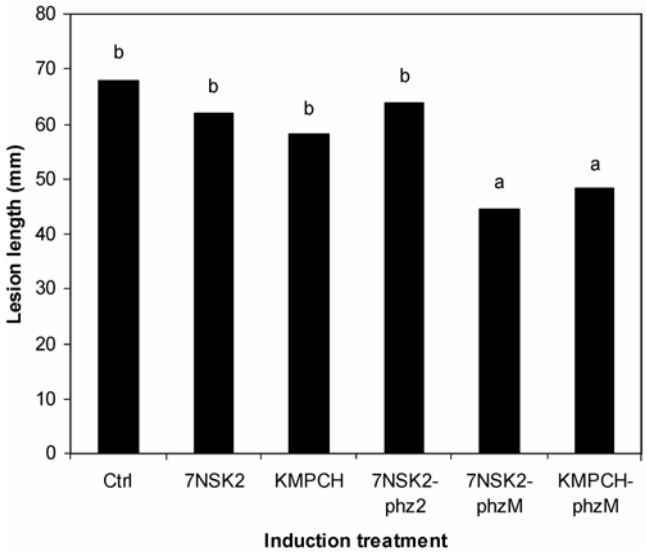

Fig. 3. Influence of root treatment with *Pseudomonas aeruginosa* 7NSK2 and various mutants on sheath blight (*Rhizoctonia solani*) severity. *P. aeruginosa* 7NSK2 and derived mutants were grown on King's medium B (KB) and applied to rice seed, roots, and soil. Control plants were treated with water. Four-week-old plants (five-leaf stage) were challenge inoculated by placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. Disease severity was assessed by measuring the length of *R. solani* lesions 4 days after challenge infection. Data presented are means from at least three independent experiments with 12 replications per treatment in each experiment. Statistical analysis was performed on pooled data, because interaction between treatment and experiment was not significant at $\alpha = 0.05$ by analysis of variance. Bars with the same letter are not significantly different by nonparametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$. Mutants derived from strain 7NSK2 have the following characteristics: KMPCH (pyoverdin and pyochelin deficient), 7NSK2-phz2 (overproducing pyocyanin), 7NSK2-phzM (phzM-, nonproducing pyocyanin), and KMPCH-phzM (pyoverdin and pyochelin deficient, phzM-, nonproducing pyocyanin).

tem. To this end, we monitored the levels of H_2O_2, which is the major and most long-living ROS, both on the roots and in the leaves of hydroponically grown rice seedlings in response to pyocyanin feeding. Detection of H_2O_2 on roots was carried out by means of an endogenous peroxidase-dependent staining procedure with 3,3′-diaminobenzidine (DAB). Roots of rice seedlings treated with 100 nM pyocyanin showed strong DAB staining compared with Hoagland-treated control roots. However, DAB staining was not observed on roots in the presence of the H_2O_2 scavenger, ascorbic acid, confirming the specificity of the staining (Fig. 5). The in planta accumulation of H_2O_2 was determined following the titanium (IV) chloride method as described by Wu and associates (1995). Inclusion of 100 nM pyocyanin in the nutrient solution revealed a transient rise in H_2O_2 levels in systemic leaves at 8 h postapplication compared with control plants, followed by decay to control levels (Fig. 6A). A second, more pronounced rise in H_2O_2 was observed at 48 h posttreatment and persisted for at least 24 h. Regulation of H_2O_2 levels in plant tissue is brought about by the coordinated activities of H_2O_2 generating and degrading enzymes; therefore, we sought to extend our analysis of the ROS-generating potential of pyocyanin in the gnotobiotic system by monitoring the level of various antioxidant enzymes in response to pyocyanin feeding. Changes in total superoxide dismutase (EC 1.15.1.1) activity were concomitant with the biphasic generation pattern of H_2O_2, reaching 1.8-fold higher levels after 48 h of incubation, suggesting that pyocyanin-induced H_2O_2 accumulates sequentially from superoxide as the primary origin (Fig. 6B). Comparative analysis of the kinetics of several H_2O_2-degrading enzymes such as catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), and guiacol peroxidase (EC 1.11.1.7) revealed distinct enzyme-specific activity patterns (Fig. 6C, D, and E). For instance, the first phase of H_2O_2 accumulation coincided with declined activities of catalase and guiacol peroxidase, whereas no significant alteration of ascorbate peroxidase activity could be observed. In addition, catalase activity showed a declining trend between 24 and 72 h of incubation, whereas both ascorbate and guiacol peroxidase activity progressively increased within this time frame, indicating a balanced interplay between H_2O_2 detoxifying enzymes in response to pyocyanin feeding. Taken together, these data clearly demonstrate the ability of bacterial pyocyanin to generate ROS on the root surface of rice seedlings as well as in systemic leaves.

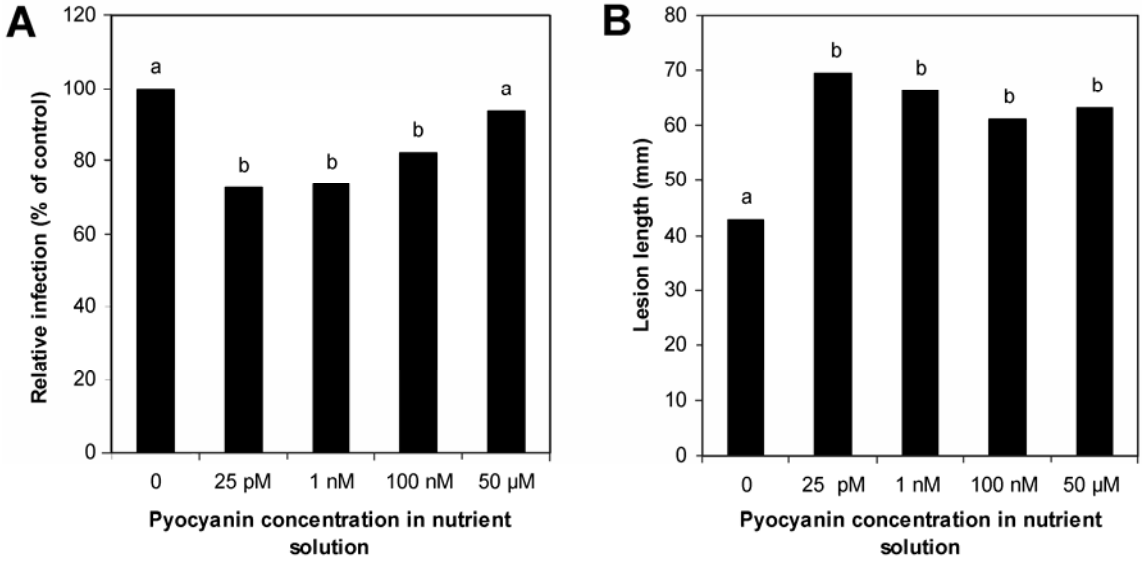

Fig. 4. Effect of adding pyocyanin to the nutrient solution on **A,** leaf blast severity and **B,** the length of *Rhizoctonia solani* lesions on hydroponically grown rice plants. Pyocyanin was purified from *Pseudomonas aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution at different concentrations; 4 days later, plants were inoculated by **A,** spraying a spore suspension of virulent *M. grisea* VT5M1 at 1×10^4 spores/ml or **B,** placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. At 6 and 4 days after pathogen inoculation, respectively, disease was rated by **A,** counting the number of susceptible-type blast lesions per leaf 4 or **B,** measuring the total length of sheath blight lesions. In the case of *M. grisea* infections, results were expressed relative to challenged control plants. The values presented are from a representative experiment that was repeated three times with similar results. Bars with the same letter are not significantly different by nonparametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$.

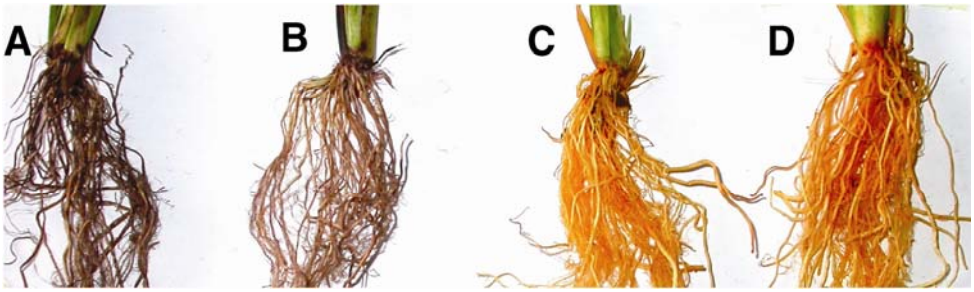

Fig. 5. Detection of pyocyanin-derived H_2O_2 by 3,3′-diaminobenzidine (DAB) staining. Roots of hydroponically grown rice seedlings were immersed for 2 h in nutrient solution **A,** with or **B,** without pyocyanin (100 nM), rinsed several times with distilled water, and subsequently incubated in DAB solution (1 mg/ml) for 12 h at room temperature. **C** and **D,** The specificity of the staining was verified by adding 10 mM ascorbic acid to the DAB solution.

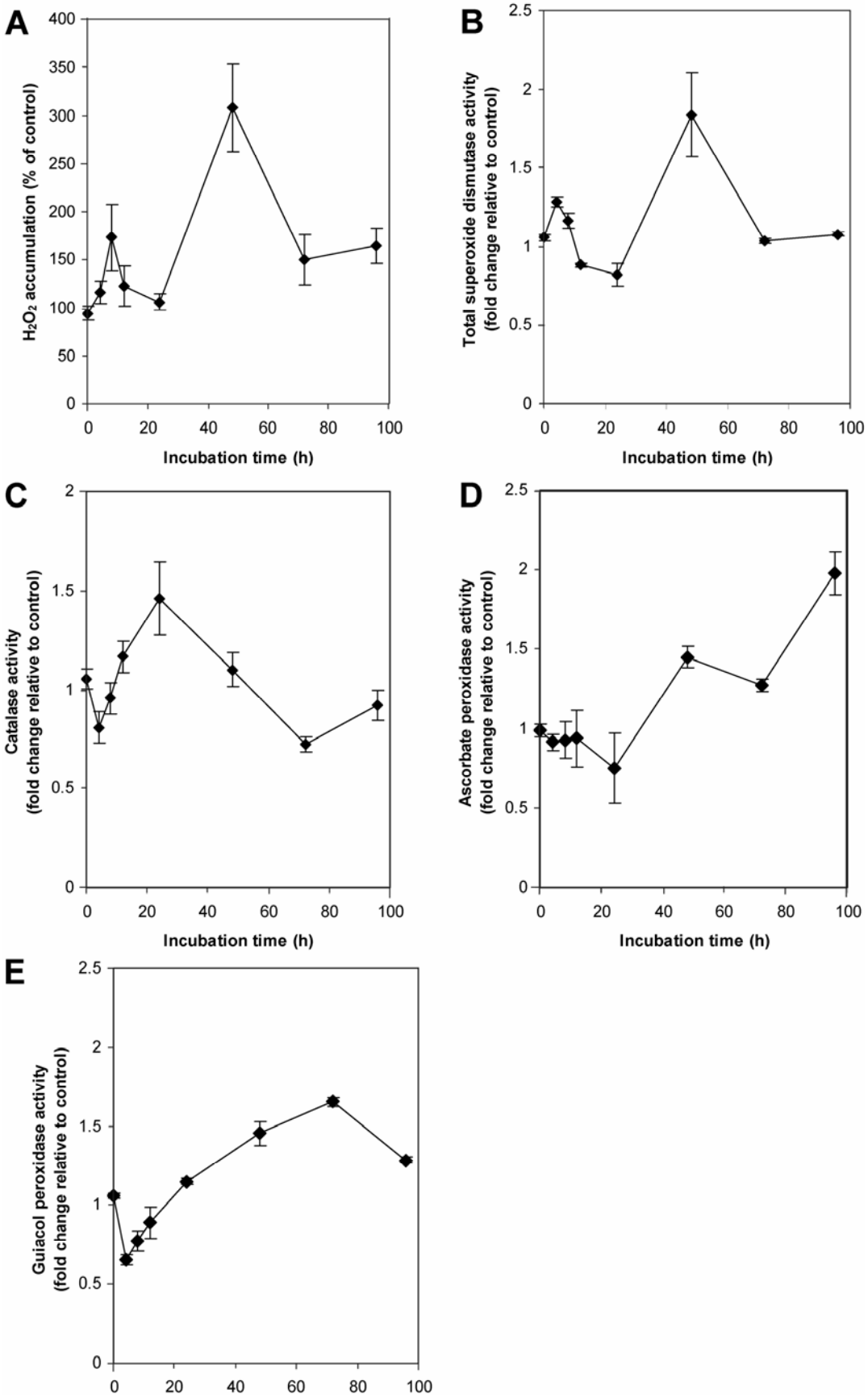

Fig. 6. Effect of pyocyanin on **A,** H_2O_2 levels and the activities of **B,** superoxide dismutase **C,** catalase **D,** ascorbate, and **E,** guiacol peroxidase in the fourth leaf of hydroponically grown rice seedlings. Pyocyanin was purified from *Pseudomonas aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution to a concentration of 100 nM. Data are means (± standard error) of four replicates of a representative experiment. Each replicate consisted of one sample pooled from six individual plants. Two series of independent experiments were carried out giving reproducible results.

Ascorbic acid attenuates the *M. grisea* resistance–inducing and *R. solani* infection–promoting potential of pyocyanin.

The observation that application of purified pyocyanin to the roots of hydroponically grown rice seedlings triggers enhanced levels of H_2O_2 and antioxidant enzymes in distal leaves prompted us to test whether ROS generated by pyocyanin in planta account for the dual role of the latter compound in 7NSK2-mediated ISR. To this end, we investigated the effect of adding ascorbate, which is one of the major natural quenching agents, to the pyocyanin solution on the subsequent challenge with *M. grisea* and *R. solani*. Co-application of 50 μM sodium ascorbate and 100 nM pyocyanin attenuated the pyocyanin-triggered resistance to *M. grisea* (Fig. 7). Similarly, addition of 50 μM ascorbate to the pyocyanin feeding solution alleviated the stimulation of *R. solani* infection by pyocyanin. At this concentration, ascorbate itself had no detectable effect on disease development. However, application of higher concentrations of ascorbate (2.5 mM to 10 mM) to the roots reduced sheath blight severity (data not shown).

Cytological comparison of fungal infection and host cellular reactions between control and pyocyanin-treated plants.

To further elucidate the mechanisms of pyocyanin-mediated ISR, cytological studies combining differential interference contrast (DIC) and incident fluorescence microscopy were conducted. To establish compatibility, it appears important for *M. grisea* to keep the invaded epidermal cell of a susceptible rice line alive in the early stages of infection before switching to necrotrophic growth (Koga 1994). Because the evidence placing ROS as central signals in the elicitation of certain types of cell death is compelling (Van Breusegem and Dat 2006), we first investigated whether pyocyanin feeding provokes cell death before challenge infection. However, pyocyanin treatment by itself did not cause any cell death in local or in systemic tissue (data not shown). Nevertheless, pyocyanin-treated plants expressed potentiated hypersensitive response (HR)-like cell death in response to infection with *M. grisea*. In control plants, fungal hyphae grew vigorously within penetrated epidermal cells (Fig. 8A). In pyocyanin-treated plants, 43.6% (±8.7%; $n = 200$) of attacked epidermal cells reacted to fungal ingress through the development of HR-like cell death, as indicated by the granulation of the cytoplasm and a bright autofluorescence of epidermal cell walls (Fig. 8B and E). These reactions were not observed in control plants up to 36 hours after inoculation (hai) (Fig. 8A and D). Addition of ascorbate to the pyocyanin feeding solution attenuated the abovementioned effects (Fig. 8C), whereas ascorbate feeding by itself did not significantly interfere with the infection process (data not shown).

In both control and pyocyanin-treated plants, germinated sclerotia of *R. solani* colonized the inner surface of the leaf sheath within 12 h of inoculation. Penetration of the sheath surface was observed 24 hai regardless of pyocyanin treatment. The most frequent penetration was by hyphal tips (Fig. 8G and H), although other infection structures, such as lobate appressoria (Fig. 8I) and infection cushions (Fig. 8J), also were observed. Hyphal tips or infection pegs produced from lobate appressoria either penetrated directly into the epidermis or first colonized subcuticularly before entering epidermal cells. Colonization of epidermal and mesophyll cells occurred both inter- and intracellularly, and often was associated with intense browning of penetrated and neighboring cells. In control plants, discrete groups of epidermal cells showing intense browning commonly were observed 5 to 15 cell layers ahead of fungal invasion, presumably due to secretion of phytotoxins by *R. solani* (Fig. 8K). In contrast, in sheaths of pyocyanin-treated plants, enlarged zones of dying cells preceding fungal colonization frequently were observed (Fig. 8L). Ascorbate largely abrogated this pyocyanin-provoked runaway cell death in response to challenge with *R. solani* (data not shown).

DISCUSSION

In this study, we analyzed *P. aeruginosa* 7NSK2-mediated ISR in the monocot model plant rice against leaf blast (*M. grisea*) and sheath blight (*R. solani*). In a standardized assay, root treatment with *P. aeruginosa* 7NSK2 effectively protected

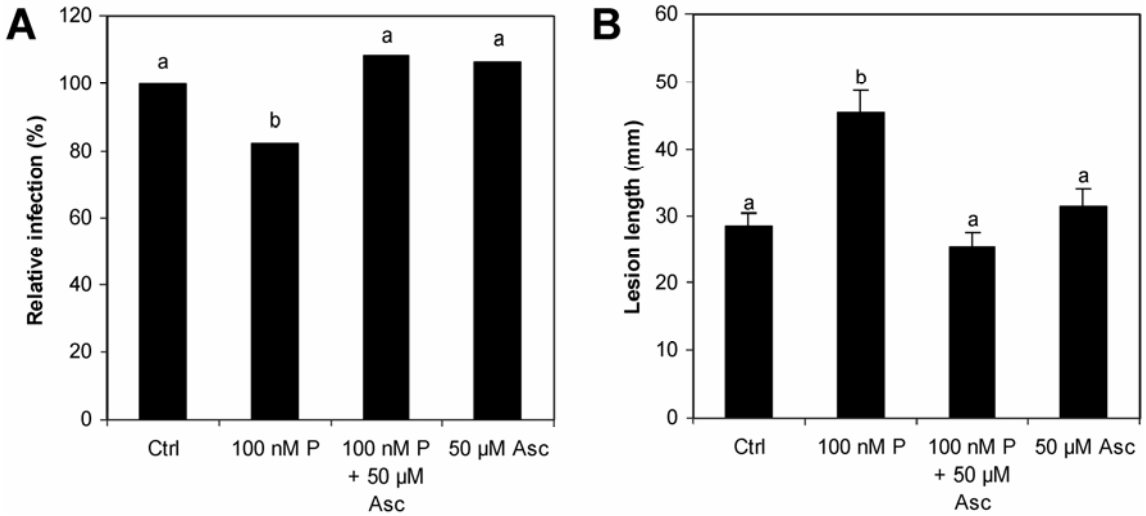

Fig. 7. Effect of adding ascorbate (Asc) to the pyocyanin feeding solution on **A,** the blast resistance-inducing potential and **B,** the *Rhizoctonia solani* infection-promoting ability of pyocyanin. Pyocyanin (P) was purified from *Pseudomonas aeruginosa* 7NSK2-phz2 cultures and added to the half-strength Hoagland nutrient solution containing 50 μM sodium ascorbate (Asc); 4 days later, plants were inoculated by **A,** spraying a spore suspension of virulent *Magnaporthe grisea* VT5M1 at 1×10^4 spores/ml or **B,** placing a 1-cm toothpick colonized by *R. solani* inside the sheath of the second youngest fully developed leaf. At 6 and 4 days after pathogen inoculation, respectively, disease was rated by **A,** counting the number of susceptible-type blast lesions per leaf 4 or **B,** measuring the total length of sheath blight lesions. In the case of *M. grisea* infections, results were expressed as relative infection values compared with control plants. The values presented are from representative experiments that were repeated three times with similar results. Bars with the same letter are not significantly different by **A,** nonparametric Kruskal-Wallis and Mann-Whitney comparisons at $P = 0.05$ or **B,** Fisher's least significant difference test ($\alpha = 0.05$).

rice against blast but failed to consistently reduce sheath blight severity. Because inducing bacteria and challenging pathogens remained spatially separated throughout the experiment, antagonism by direct interactions could be ruled out, demonstrating that 7NSK2-induced protection is plant mediated. ISR against *M. grisea* was manifested phenotypically by a reduction in the number of susceptible-type blast lesions (Fig. 1), thereby resembling the resistance phenotype of quantitative trait loci-governed partial resistance (Zahirul et al. 2005).

Recent evidence by Audenaert and associates (2002) suggests that, whereas ISR elicited by *P. aeruginosa* 7NSK2 in dicots requires the SA signaling pathway, the bacterial trigger of ISR is the combination of the SA-derived siderophore pyochelin and the phenazine pyocyanin, rather than SA itself. In view of pharmacological studies demonstrating that ferripyochelin-catalyzed hydroxyl generation from pyocyanin-derived O_2^-/H_2O_2 contributes to microvasculature injury, which occurs as a consequence of pulmonary infections with *P. aeruginosa* (Britigan et al. 1992, 1997), the authors proposed that the generation of hydroxyl radicals by the Fe–pyochelin–pyocyanin interaction might constitute the basis of 7NSK2-mediated ISR. In this work, however, we found no evidence for the involvement of iron-regulated pyochelin in 7NSK2-mediated ISR in rice. This pyochelin independency of 7NSK2-triggered ISR was borne out by the observation that bacterial inoculum prepared from iron-rich medium was as effective as inoculum prepared from iron-poor KB in controlling rice blast disease, and was confirmed further by the ISR-inducing potential of the pyochelin-negative mutant KMPCH (also pyoverdin deficient) (Fig. 2A). At inoculation, 7NSK2 grown on iron-rich medium had an internal iron pool that was visible in the red color of the bacterial pellet, whereas an internal iron pool was not observed for KB-grown 7NSK2, because siderophore-mediated iron acquisition is strictly regulated. These observations make a role for iron-regulated metabolites of *P. aeruginosa* in ISR to *M. grisea* highly unlikely.

Similar to 7NSK2-mediated ISR in tomato, the pyocyanin-deficient mutant 7NSK2-phzM lost the capacity to trigger ISR against rice blast. A similar phenomenon was observed for the pyocyanin and pyochelin double-negative mutant KMPCH-phzM (Fig. 2A). Because the inability of these strains to induce resistance to *M. grisea* did not result from insufficient rhizosphere populations, these data strongly suggest that pyocyanin production by *P. aeruginosa* 7NSK2 is necessary for ISR to *M. grisea* in rice. Additional support was provided by complementation experiments (Fig. 2B), as well as by the protective effect obtained upon hydroponic feeding with pure pyocyanin (Fig. 4A). Surprisingly, treatment with the pyocyanin-overproducing strain 7NSK2-phz2, which produces approximately five times more pyocyanin compared with the wild type, failed to consistently mount ISR to *M. grisea*. Provided that the pyocyanin production in vitro is an adequate indication of the capacity to produce pyocyanin in the rhizosphere by the respective strains, these results suggest that only a balanced production of pyocyanin triggers ISR to *M. grisea*. However, we found no clear dose effect for pure pyocyanin in our gnotobiotic system, at least in the physiologically relevant pico- and nanomolar range (Fig. 4A). These conflicting observations could be reconciled when considering the distinct pyocyanin application in soil-based and hydroponic assays. Contrary to the putative sustained pyocyanin production by the bacterial strains in the rhizosphere, purified pyocyanin was fed only once in the hydroponic system. In vitro studies have shown that pyocyanin has multiple deleterious effects on mammalian cells, such as inhibition of cell respiration, ciliary function, epidermal cell growth, prostacyclin release, disruption of calcium homeostasis, and inactivation of catalase and vacuolar ATPase (Lau et al. 2004). Moreover, pyocyanin induces apoptosis in neutrophils (Allen et al. 2005) and modulates the glutathione redox cycle (Muller 2002) in lung epithelial and endothelial cells. Hence, it is conceivable that sustained exposure of rice roots to substantial levels of pyocyanin, as secreted by the overproducing mutant 7NSK2-phz2, causes toxic effects that might negatively interfere with the induction of ISR. Such a concept would be consistent with previous findings by Abeysinghe (1999), who reported that only balanced doses of pyocyanin trigger resistance to *B. cinerea* in bean. Similar results were obtained by Iavicoli and associates (2003) when studying the involvement of the antibiotic 2,4-diacetylphloroglucinol in *P. fluorescens* CHAO-mediated ISR to *Peronospora parasitica* in *Arabidopsis*. On the other hand, the mutation in *phz2* is likely to have a pleiotropic effect because it results in the inactivation of the hybrid sensor kinase RetS. Although we selected this mutant as a pyocyanin hyperproducer, others demonstrated that the same mutation affects type III secretion, motility, and virulence and promotes biofilm formation (Goodman et al. 2004; Ventre et al. 2006; Zolfaghar et al. 2005). Therefore, we cannot exclude the possibility that the effect observed with 7NSK2-phz2 is not due only to pyocyanin overproduction.

One of the most peculiar events in the early phase of plant–pathogen interactions is the rapid and transient production of ROS by the plant, namely the oxidative burst. Because pyocyanin is a redox-active compound (Hassan and Fridovich 1979) and has been demonstrated before to be capable of generating ROS in an animal system (Britigan et al. 1997), we investigated whether pyocyanin production in the rhizosphere modulates the oxidative machinery of rice seedlings. By means of a combination of histochemical DAB stainings and in planta measurements of H_2O_2, we demonstrated that pyocyanin feeding of hydroponically grown rice seedlings leads to enhanced H_2O_2 levels both on the root surface of rice seedlings and, subsequently, in distal leaves (Figs. 5 and 6). The pyocyanin-elicited H_2O_2 burst in the systemic leaves adopted a biphasic generation pattern, similar to the two-phase kinetics frequently observed during an avirulent pathogen-induced oxidative burst (Levine et al. 1994; Shirasu et al. 1997). This biphasic response might indicate a capacity for multiple reiterations of pyocyanin-triggered ROS generation in order to maintain the induced state. Such a mechanism would either require signal amplification for reiteration of the pyocyanin-triggered ROS generation or, alternatively, could result from successive redox cycles of pyocyanin, because studies by Rezka and associates (2004) demonstrated that pyocyanin has the capacity to undergo redox cycling without extensive modification of the pigment's phenazine chromophore, thus leaving the pigment intact.

There is ample evidence indicating that ROS, and H_2O_2 in particular, generated in the oxidative burst perform multiple important functions in early plant defense responses. ROS are directly protective, activate phytoalexin biosynthesis, and also drive peroxidase-mediated cross-linking of proline-rich cell wall glycoproteins (Lamb and Dixon 1997). Moreover, ROS induce arrays of cellular protectant and defense genes and also cue the collapse of challenged cells (Foyer and Noctor 2005; Neill et al. 2002). In addition to these intracellular or local intercellular signal functions, some studies have highlighted the potential role for local ROS accumulation in systemic signaling leading to the establishment of SAR (Fobert and Després 2005). Elegant research by Alvarez and associates (1998) demonstrated redox changes in systemic tissues following SAR induction. These changes were observed as well-timed, transient microbursts of H_2O_2 production that were required for SAR manifestation. Taking these facts into account, we speculate that the transient enhancement of H_2O_2 levels observed in

systemic leaves of pyocyanin-treated rice seedlings might likewise function in 7NSK2-mediated ISR to *M. grisea* by low-level activation of defense responses throughout the plant, thereby contributing to the ISR-induced state. Critical to the formation of a hypothesis of transiently increased H_2O_2 levels as the central event in 7NSK2-mediated ISR in rice was the observation that inclusion of H_2O_2-quenching ascorbate into the pyocyanin-containing nutrient solution abrogated both pyocyanin-induced H_2O_2 generation and pyocyanin-triggered ISR to *M. grisea* (Figs. 5 and 7A). Although the exact nature of the quenching effect of ascorbate and, in turn, the attenuation of the ISR performance by ascorbate cannot be explained at this stage, because it is not known whether ascorbate merely has a quenching effect or might interfere further downstream of the signaling pathways leading to ISR, the involvement of H_2O_2 production by redox-active pyocyanin in relation to ISR is apparent. Hence, induction of pyocyanin-mediated H_2O_2 microbursts most likely constitutes the in situ mechanism of 7NSK2-mediated ISR against *M. grisea*. In line with this concept, there is substantial evidence demonstrating the defensive capacity of H_2O_2 in rice–*M. grisea* interactions. Induction of elevated levels of H_2O_2 in transgenic rice expressing a fungal glucose oxidase gene triggered the expression of several defense genes, cell death, and enhanced blast resistance in response to wounding and pathogen infection (Kachroo et al. 2003). Likewise, increasing endogenous levels of H_2O_2 by expression of constitutively active OsRac1, a small GTP-binding protein homologous to human Rac, triggered cell death and enhanced

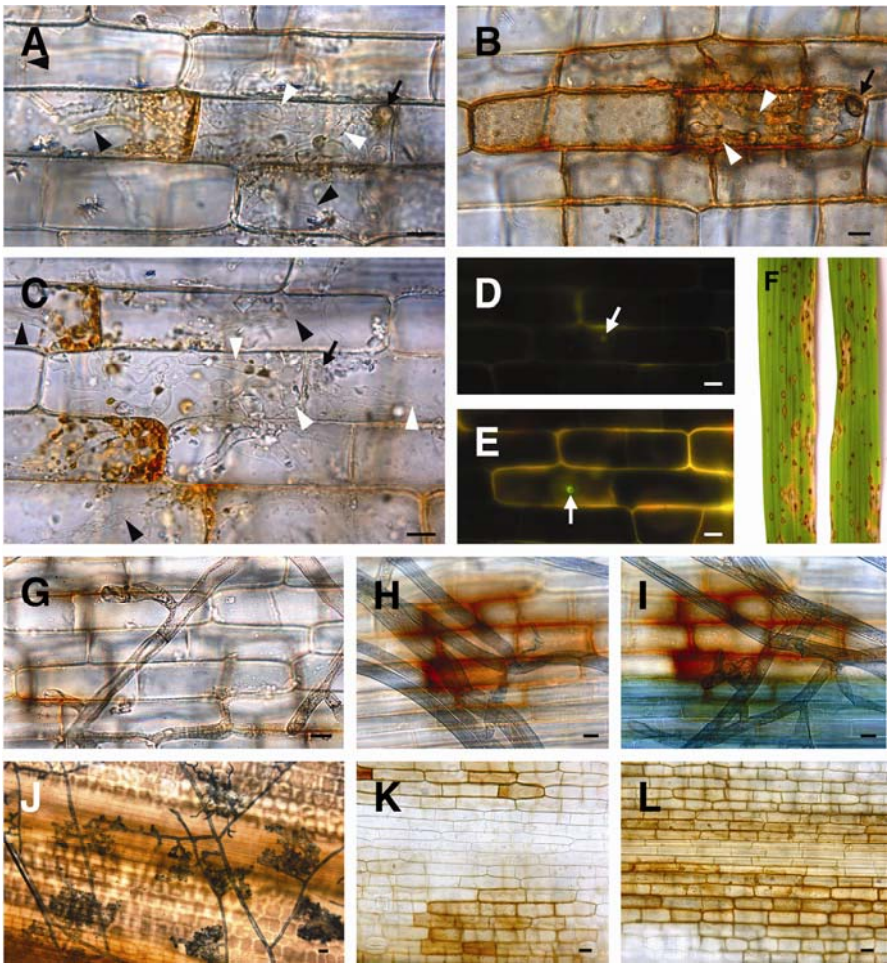

Fig. 8. Interaction phenotypes of pyocyanin-mediated cytological responses of *Oryza sativa* line CO-39 to *Magnaporthe grisea* and *Rhizoctonia solani*. **A** to **F,** Infection sites inoculated with *M. grisea*. Scale bars represent 10 µm. **A,** Vigorous invasion of living tissues in control plants. Upon penetration by a dome-shaped appressorium (black arrow), the fungus colonizes the first penetrated epidermal cell as well as neighboring cells by 36 h after infection (hai), producing primary (white arrowheads) and secondary (black arrowheads) invading hyphae. **B,** Expression of hypersensitive resistance (HR)-like cell death blocks *M. grisea* in hydroponically grown rice plants amended with 100 nM pyocyanin (36 hai). Intracellular hyphae (white arrowheads) originating from an appressorium (arrow) are restricted to the initially penetrated epidermal cell and stopped from infecting adjacent plant tissue. Note the granulation of the cytoplasm in both first invaded and neighboring epidermal cells. **C,** Addition of 50 µM ascorbate to the pyocyanin feeding solution abrogates pyocyanin-induced HR-like cell death. In consequence, fungal growth is not arrested and invading hyphae form an extensively branched mycelium both in the first invaded (white arrowheads) and surrounding epidermal cells (black arrowheads). Arrow points to the site of fungal penetration. **D,** Faint autofluorescent halo surrounding point of penetration (white arrow) as well as weak local autofluorescence in control plants under blue light excitation (24 hai). **E,** Epifluorescence image of epidermal cells of pyocyanin-fed plants responding to *M. grisea* infection (24 hai). The penetrated epidermal cell and the cell walls of three surrounding cells exhibit bright autofluorescence under blue light excitation. White arrow indicates position of the fungal appressorium. **F,** Symptoms of *M. grisea* on the fourth leaf of control plants (left, 7 days post inoculation [dpi]) and pyocyanin-treated plants (right, 7 dpi). **G** to **J,** Microphotographs of infection sites of control plants inoculated with *R. solani* (similar observations were made in pyocyanin-treated plants). **G,** Direct penetration by hyphae of *R. solani* and associated browning of epidermal cell walls at the site of contact (24 hai). Bar = 10 µm. **H,** Browning of epidermal cells underlying *R. solani* hyphae as observed by 24 hai. Fungal hyphae were stained using trypan blue dye. Bar = 10 µm. **I,** Penetration attempt by a lobate appressoria-like structure of *R. solani* and associated host cell death. Extracellular mycelium is stained with trypan blue. Bar = 10 µm. **J,** Colonization of sheath surface by hyphae and formation of infection cushions by 40 hai. Fungal mycelium was stained with trypan blue. Bar = 30 µm. **K** and **L,** Browning of nonpenetrated epidermal cells preceding fungal invasion as observed in **K,** control plants and **L,** pyocyanin-treated plants by 32 hai. Bars = 20 µm.

blast resistance in transgenic rice plants. Conversely, dominant negative OsRac1 suppressed elicitor-induced ROS production in transgenic cell cultures and, in plants, suppressed resistance gene–mediated resistance to *M. grisea* (Kawasaki et al. 2006; Ono et al. 2001). Moreover, the increased blast resistance of several rice lesion mimic mutants is linked with elevated H_2O_2 production (Takahashi et al. 1999; Ueno et al. 2003).

Pyocyanin-negative mutants, unlike the wild-type bacterium, significantly reduced sheath blight severity (Fig. 3), whereas the same mutant strains lost the capacity to mount ISR to *M. grisea*. In concordance with these observations, pyocyanin-treated rice seedlings exhibited increased susceptibility to *R. solani* (Fig. 4B), suggesting that pyocyanin acts as a negative regulator of disease resistance responses toward *R. solani*. Similar to pyocyanin-induced ISR to *M. grisea*, addition of ascorbate to the nutrient solution alleviated pyocyanin-stimulated susceptibility to *R. solani* (Fig. 7B). Hence, the cumulative results suggest that the differential beneficial effect of pyocyanin in ISR to *M. grisea* and *R. solani* is due to its capacity to generate H_2O_2 in planta.

In keeping with our results, ROS have been thought previously to play a dual role in plant resistance to pathogens. Despite the numerous lines of evidence demonstrating the involvement of ROS in the induction of various defense reactions, including orchestration of hypersensitive cell death, which is a highly effective defense mechanism against biotrophic pathogens, their accumulation also has been reported to be involved in successful pathogenesis of necrotrophic pathogens (Glazebrook 2005; Govrin and Levine 2000; Hennin et al. 2001). Although the role of H_2O_2 in cell death induction is widely accepted (Apel and Hirt 2004; Delledonne et al. 2001), its benefit for resistance strategies varies with the type of pathosystem and host tissues. For instance, transgenic expression of animal cytoprotective antiapoptotic genes in tobacco conferred heritable resistance to several necrotrophic pathogens (Dickman et al. 2001). Similarly, the HR-deficient *Arabidopsis* mutant *dnd1* was highly resistant to the necrotrophic fungi *B. cinerea* and *Sclerotinia sclerotiorum*, whereas treatments of *A. thaliana* with pro-oxidantia, or an HR-causing *Pseudomonas syringae* strain, prior to infection with *B. cinerea* or *S. sclerotiorum* enhanced disease severity, suggesting that HR-associated cell death facilitates pathogenesis by necrotrophic pathogens (Govrin and Levine 2000). Although root treatment with pyocyanin, at least in the pico- and nanomolar range, did not induce visible cell death by itself in local or systemic tissue, a marked increase in the number of HR-expressing epidermal penetration sites was observed in response to infection with *M. grisea* (Fig. 8B). Furthermore, pyocyanin feeding, despite not interfering with the penetration process of *R. solani*, provoked intense browning of epidermal cells ahead of fungal invasion (Fig. 8L). Because ascorbate treatment inhibited these pyocyanin-mediated cellular responses (Fig. 8C), it could be reasoned that the pyocyanin-induced generation of H_2O_2 microbursts might lower the threshold for initiating programmed cell death and, in consequence, facilitate subsequent infection with *R. solani* which, as a necrotrophic pathogen, depends on host cell death as a prerequisite for successful pathogenesis.

In summary, the dual role of the phenazine antibiotic pyocyanin in *P. aeruginosa* 7NSK2-mediated ISR suggests that rice requires distinct mechanisms for defense against *M. grisea* and *R. solani*. On the one hand, root treatment with pyocyanin was effective against *M. grisea*, triggering reiterative H_2O_2 microbursts and causing rapid HR-associated cell death in response to fungal infection, which most likely leads to breakdown of the biotrophic phase of the *M. grisea* infection cycle. On the other hand, treatment with pyocyanin significantly promoted subsequent infection by the necrotrophic pathogen *R. solani* by facilitating pathogen-triggered host cell death. Hence, the oxidative burst and related HR might act as a double-edged sword in the interaction of rice with hemibiotrophic (*M. grisea*) and necrotrophic (*R. solani*) pathogens. This conclusion is substantiated with recent research by Ahn and associates (2005), demonstrating the differential beneficial effect of the HR as defense mechanism against *M. grisea* and the necrotrophic rice pathogen *Cochliobolus miyabeanus*. Considering that the effect of the oxidative burst and HR-associated cell death depends on the type of invading pathogen, the widespread cultivation of resistant blast cultivars that rely upon major resistance genes may contribute to the increase in sheath blight incidence. In this respect, our recent observation that *R. solani* colonization and sheath blight development is favored by pre-inoculation with an HR-triggering incompatible *M. grisea* isolate is of particular interest (D. De Vleesschauwer and M. Höfte, *unpublished results*) and might explain why there are no HR-triggering gene-for-gene phenomena known for *R. solani*–rice interactions. Our work underscores the importance of using appropriate innate defense mechanisms in plant breeding programs and might contribute to the development of new strategies for disease control.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacteria and plasmids used in this study are listed in Table 1. Mutant strains 7NSK2-*phzM* and KMPCH-*phzM* were constructed as described by Rabaey and associates (2005) by homologous recombination using plasmid pZM1-Gm as suicide vector. In trans complementation of 7NSK2-*phzM* for pyocyanin production was performed as described by Audenaert and associates (2002).

Assay for induced resistance.

All experiments were performed using the highly susceptible *indica* cv. CO-39 (Chen et al. 2003). Plants were grown under nonsterile greenhouse conditions (30 ± 4ºC) in potting soil (substrat no 4; Klasmann, Otrebusy, Germany). Root colonization with *P. aeruginosa* was achieved by a combined seed and soil treatment. Bacteria were grown routinely at 37ºC on KB (King et al. 1954), scraped off the plates, and suspended in sterile saline (0.85% NaCl). This suspension was diluted to the desired bacterial concentration based on optical density at 595 nm.

For seed treatment, rice seed were surface sterilized with 1% sodium hypochlorite solution for 2 min and rinsed three times in sterile distilled water. Next, seed were soaked for 10 min in a bacterial suspension of 5×10^7 CFU ml^{-1}. Rice seed then were incubated for 5 days on a wet sterile filter paper in sealed Petri dishes at 28°C. Prior to sowing, roots of germinated seed were dipped in a bacterial suspension (5×10^7 CFU ml^{-1}) for 10 min and the potting soil was mixed with bacterial inoculum to a concentration of 5×10^7 CFU g^{-1}. In control treatments, seed, roots, and soil were treated with equal volumes of sterile saline. Germinated seed were sown in perforated plastic trays (23 by 16 by 6 cm) to provide aerobic soil conditions. Ten days after sowing, an additional bacterial application was performed as a soil drench (5×10^7 CFU g^{-1}).

At the end of every assay, bacterial colonization of the roots was checked for three plants per treatment. Roots were washed to remove most of the soil, and 1 g of root was macerated in sterile demineralized water. Serial dilutions were plated on KB, amended with the appropriate antibiotics, and bacterial counts were made after incubation for 24 and 48 h at 37°C.

Evaluation of plant colonization by *P. aeruginosa* 7NSK2 and mutants.

To assess whether root colonization with *P. aeruginosa* leads to bacterial colonization of distal plant parts, leaves and stems of 20- and 35-day-old plants (end of bioassay) were checked for bacterial colonization. For four plants per treatment, leaves and stems were pooled before maceration in 1.5 ml of sterile demineralized water and plated out on KB amended with the appropriate antibiotics. Bacterial counts were made after 24 and 48 h of incubation at 37°C and the experiment was performed twice.

Pathogen inoculation and disease rating.

Magnaporthe grisea. The Vietnamese *M. grisea* isolate VT5M1 was used for all infection trials. Inoculum production and inoculation was performed exactly as described by Ninh Thuan and associates (2006). Each plant was sprayed with 1 ml of inoculum (1×10^4 spores ml^{-1} in a 0.5% gelatin solution). Six days after inoculation, disease was assessed by counting the number of susceptible-type lesions, which are defined as elliptical to round lesions characterized by a gray center indicative of sporulation of the fungus (Schweizer et al. 1997).

Rhizoctonia solani. The virulent *R. solani* isolate MAN-86 (AG-1, IA), obtained from symptomatic plants (cv. IR-50) in rice fields in the state of Karnataka (India) and provided by Dr. Sam Gnanamanickam, was used to inoculate the plants. Inoculum was obtained as described by Rodrigues and associates (2003). Inoculated plants were kept for 72 h inside the humid inoculation chambers (≥92% relative humidity) at 30 ± 4ºC and, thereafter, transferred to greenhouse conditions. At 96 h after inoculation, disease was evaluated by measuring the length of the water-soaked lesions as described by Singh and associates (2002).

Pyocyanin extraction and quantification.

The pyocyanin-overproducing strain 7NSK2-phz2 was grown for 48 h on Pseudomonas P agar medium (Difco, Le Pont de Claix, France) at 37ºC. Purification of pyocyanin was performed as described by Abeysinghe (1999). For quantification, the weight of the purified pyocyanin crystals was determined. For all experiments reported herein, pyocyanin was suspended in sterile demineralized water and filter-sterilized prior to use.

In vitro production of pyocyanin by bacterial strains.

To monitor in vitro production of pyocyanin by the distinct strains, bacteria were grown for 48 h on Pseudomonas P agar medium at 37ºC. Bacteria were scraped off the plates, suspended in sterile distilled water, and centrifuged for 10 min at 4ºC. Pyocyanin present in the supernatant was extracted twice with chloroform and determined spectrophotometrically in the presence of 0.1 M HCl (optical density at 510 nm) as described by Essar and associates (1990). The experiment was set up in six replicates and repeated twice.

Hydroponic plant growth.

For experiments in which purified pyocyanin was applied to rice seedlings, plants were grown in a hydroponic gnotobiotic system. Surface-sterilized rice seed were germinated for 5 days on wet filter paper in Petri dishes. After incubation, germinated seed were sown in perforated plastic trays (23 by 16 by 6 cm) filled with sterilized vermiculite and supplemented with half-strength Hoagland solution (Hoagland and Arnon 1938). Every 3 days, 0.5 liters of the half-strength Hoagland solution was added to each tray containing 12 seedlings. In this model, 4 days before challenge inoculation, various concentrations of pyocyanin and sodium ascorbate were applied to the plants by including the desired concentration in the nutrient solution without ethylenediaminetetraacetic acid ferric sodium salt (Acros, Geel, Belgium) to avoid possible ferric-catalyzed HO generation from pyocyanin-derived O_2^-/H_2O_2.

Histochemical detection of H_2O_2.

To assess whether pyocyanin was able to produce H_2O_2 in the gnotobiotic system, rice roots were dipped in half-strength Hoagland nutrient solution containing 1 nM pyocyanin for 2 h, rinsed thoroughly with demineralized water, and subsequently incubated for 12 h at room temperature in water with 0.01% Triton-X-100 and DAB. DAB (Sigma-Aldrich, Bornem, Belgium) polymerizes in the presence of H_2O_2 to form a brownish-red precipitate that can be visualized. The specificity of the staining was verified by adding 10 mM ascorbic acid.

In planta determination of H_2O_2.

The in planta accumulation of H_2O_2 was determined following the $TiCl_4$-based technique as described by Mur and associates (2005). H_2O_2 accumulation was expressed relative to values

Table 1. Bacteria and plasmids used in this study with their relevant characteristics

Strains or plasmids	Relevant characteristics[a]	Reference or source
Pseudomonas aeruginosa		
7NSK2	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, wild type	Iswandi et al. 1987
KMPCH	Pyo$^+$, Pvd$^-$, Pch$^-$, SA$^+$, chemical mutant of the pyoverdin-negative mutant MPFM1; Km^r	Höfte et al. 1993
7NSK2-phzM	Pyo$^-$, Pvd$^+$, Pch$^+$, SA$^+$, *phzM* mutant of 7NSK2, obtained by gene replacement using plasmid pZM1-Gm; Gm^r	This study
7NSK2-phzMc	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, 7NSK2-phzM containing pHZM (functional *phzM* gene on plasmid pBBR1MCS) that restores pyocyanin production, Gm^r, Cm^r	This study
7NSK2-phz2	Pyo$^+$, Pvd$^+$, Pch$^+$, SA$^+$, *retS* (PA4856) mutant of 7NSK2 obtained by miniTn*pho*A3 mutagenesis, overproduces pyocyanin, Gm^r	This study
KMPCH-phzM	Pyo$^-$, Pvd$^-$, Pch$^-$, SA$^+$, *phzM* mutant of KMPCH, obtained by gene replacement using plasmid pZM1-Gm; Gm^r	This study
Plasmids		
pBR322	Suicide vector in *Pseudomonas*, Cm^r/Cb^r/Tc^r	Bolivar 1978
pZM1-Gm	A 2,054-bp PCR-amplified fragment of primer pair 4209A-B (*phzM*) of *P. aeruginosa* PAO1, inactivated by a site-specific insertion of a 803-bp *NotI*-blunted Gm cassette, cloned in pBR322	Rabaey et al. 2005
pBBR1MCS	Broad host-range cloning vector for *Pseudomonas*, Cm^r	Kovach et al. 1994
pHZM	A 2,054-bp PCR-amplified fragment of primer pair 4209A-B (*phzM*) of 7NSK2 cloned in the *EcoRV* site of pBBR1MCS, Cm^r	Audenaert et al. 2002

[a] Pyo = pyocyanin, Pvd = pyoverdin, Pch = pyochelin, SA = salicylic acid, Km = kanamycin, Gm = gentamicin, Cm = chloramphenicol, Cb = carbenicilin, Tc = tetracycline, ^{r} = resistant, and PCR = polymerase chain reaction.

obtained in control samples. Each experiment consisted of six replicates per treatment and was repeated twice to generate the data presented.

Enzyme extraction and activity assays.

Frozen leaf samples were crushed to a fine powder in a mortar under liquid nitrogen. Soluble proteins were extracted by resuspending the powder in four volumes of 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF, 5 mM sodium ascorbate, and 5% (wt/vol) polyvinyl-polypyrrolidone. The homogenate was centrifuged at 17,000 × *g* for 10 min. The supernatant was divided into aliquots, frozen in liquid nitrogen, and stored at –80ºC for further analysis. All of the above operations were carried out at 0 to 4ºC. Activity levels of the various antioxidant enzymes (namely catalase [CAT], guiacol-dependent peroxidase [GPX], ascorbate peroxidase [APX], and superoxide dismutase [SOD]) in plant extracts were measured spectrophotometrically as described by Garcia-Limones and associates (2002).

The CAT reaction medium consisted of 50 mM sodium phosphate buffer, pH 7.0, 20 mM H_2O_2, and between 10 and 50 µl of enzyme extract. The reaction was started by adding H_2O_2 and the decrease in absorbance at 240 nm (A_{240}) (ε = 39.4 mM^{-1} cm^{-1}) produced by H_2O_2 breakdown was recorded. One CAT unit is defined as the amount of enzyme necessary to decompose H_2O_2 at 1 µmol min^{-1} under the above assay conditions.

To assay GPX activity, the reaction mixture (3.0 ml) consisted of 100 mM potassium phosphate buffer, pH 6.5, 15 mM guiacol, 0.25% (vol/vol) H_2O_2 (200 mM), and different volumes of enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of guiacol was determined by the increase in A_{470} (ε = 26.6 mM^{-1} cm^{-1}). One GPX unit is defined as the amount of enzyme that produces oxidized guiacol at 1 µmol min^{-1} under the above assay conditions.

For APX activity assays, the reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.0, 0.25 mM sodium ascorbate, 5 mM H_2O_2, and 50 µl of enzyme extract. The reaction was started by adding H_2O_2 and the oxidation of ascorbate was measured by the decrease in A_{290} (ε = 2.8 mM^{-1} cm^{-1}). One unit of APX activity is defined as the amount of enzyme that oxidizes ascorbate at 1 µmol min^{-1} under the above assay conditions.

SOD activity was determined from the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) in the presence of riboflavin. The reaction mixture (1.5 ml) consisted of 50 mM potassium phosphate buffer, pH 7.8, 0.1 mM EDTA, 13 mM methionine, 75 µM NBT, 2 µM riboflavin, and between 10 and 50 µl of enzyme extract. The reaction was started by adding riboflavin and A_{560} was recorded after 12 min of incubation at room temperature under continuous light (70W). One SOD unit was defined as the amount of enzyme that inhibits the rate of NBT reduction by 50% under the above assay conditions.

In all assays, the blank consisted of the components of the reaction mixture except for the enzyme extract, which was replaced by an equal volume of the assay buffer. In the SOD assay, the enzyme blank was taken as the 100% rate of NBT photochemical reduction. In the remaining cases, the enzyme blanks were subtracted from the assay measurements. Protein levels in enzyme extracts were determined by the Bradford method (Bradford 1976) with bovine serum albumen as a standard.

Detection of pyocyanin by HPLC analysis.

Hydroponically grown 28-day-old CO-39 seedlings were fed with a 50 µM pyocyanin solution as indicated above. At various time points postapplication, leaves were excised, grinded, and homogenized in chloroform. The soluble material was subjected to pyocyanin extraction according to Abeysinghe (1999). The extract was evaporated in vacuo to dryness and subsequently solubilized in 50% methanol. Samples were analyzed by HPLC using a Genesis C18 column as described by Rabaey and associates (2005).

Cytological investigation by using bright-field and fluorescence microscopy.

M. grisea inoculation of intact leaf sheaths and preparation of specimens for microscopy were conducted as described by Koga and associates (2004). For inoculation with *R. solani*, sheaths were opened carefully and a small piece (approximately 1 mg) of sclerotium placed inside the sheath. A few drops (100 µl) of sterile water were added to the inoculated sheath. Cytological observations were made using an Olympus model BX51 microscope (Olympus, Aartselaar, Belgium) equipped with differential interference contrast optics. The autofluorescence of epidermal cell walls or the whole-epidermal cells of each appressorial site examined was recognized by incident fluorescence microscopy (Olympus U-MWB2 GFP filter set-excitation: 450 to 480 nm, dichroic beamsplitter; 500 nm, barrier filter BA515). Images were acquired digitally (Olympus Color View II camera, Aartselaar, Belgium) and further processed with the Olympus analySIS cell^F software.

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LITERATURE CITED

Abeysinghe, S. 1999. Investigation of metabolites of the rhizobacterium *Pseudomonas aeruginosa* strain SA44 involved in plant protection. Ph.D. thesis, Vrije Universiteit, Brussels.

Ahn, I. P., Kim, S., Kang, S., Suh, S. C., and Lee, Y. H. 2005. Rice defense mechanisms against *Cochliobolus miyabeanus* and *Magnaporthe grisea* are distinct. Phytopathology 95:1248-1255.

Allen, L., Dockrell, D. H., Pattery, T., Lee, D. G., Cornelis, P., Hellewell, P. G., and Whyte, M. B. 2005. Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. J. Immunol. 174:3643-3649.

Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92:773-784.

Apel, K., and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373-399.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. 2002. Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. Mol. Plant-Microbe Interact. 15:1147-1156.

Bakker, P. A. H. M., Ran, L. X., Pieterse, C. M. J., and van Loon, L. C. 2003. Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. Can. J. Plant Pathol. 25:5-9.

Bigirimana, J., and Höfte, M. 2002. Induction of systemic resistance to *Colletotrichum lindemuthianum* in bean by a benzothiadiazole derivative and rhizobacteria. Phytoparasitica 30:159-168.

Bolivar, F. 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene 4:121-136.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantity of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Britigan, B. E., Rasmussen, G. T., and Cox, C. D. 1997. Augmentation of oxidant injury to human pulmonary epithelial cells by the *Pseudomonas*

aeruginosa siderophore pyochelin. Infect. Immun. 65:1071-1076.
Britigan, B. E., Roeder, T. L., Rasmussen, G. T., Shasby, D. M., McCormick, M. L., and Cox, C. D. 1992. Interaction of the *Pseudomonas aeruginosa* secretory products pyocyanin and pyochelin generates hydroxyl radicals and causes synergistic damage to endothelial cells: implications for *Pseudomonas* associated tissue. J. Clin. Invest. 90:2187-2196.
Chen, H., Wang, S., Xing, Y., Xu, C., Hayes, P. M., and Zhang, O. 2003. Comparative analyses of genomic locations and race specificities of loci for quantitative resistance to *Pyricularia grisea* in rice and barley. Proc. Natl. Acad. Sci. U.S.A. 100:2544-2549.
Delledonne, M., Zeier, J., Marocco, A., and Lamb, C. 2001. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. U.S.A. 98:13454-13459.
De Meyer, G., and Höfte, M. 1997. Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* in bean. Phytopathology 87:588-593.
De Meyer, G., Audenaert, K., and Höfte, M. 1999a. *Pseudomonas aeruginosa* 7NSK2-induced systemic resistance in tobacco depends on *in planta* salicylic acid but is not associated with PR1a gene-expression. Eur. J. Plant Pathol. 105:513-517.
De Meyer, G., Capiau, K., Audenaert, K., Buchala, A., Métraux, J.-P., and Höfte, M. 1999b. Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. Mol. Plant-Microbe Interact. 12:450-459.
Dickman, M. B., Park, Y. K., Oltersdorf, T., Li, W., Clemente, T., and French, R. 2001. Abrogation of disease development in plants expressing antiapoptotic genes. Proc. Natl. Acad. Sci. U.S.A. 98:6957-6962.
Durrant, W. E., and Dong, X. 2004. Systemic acquired resistance. Annu. Rev. Phytopathol. 42:185-209.
Essar, D. W. Eberly, L., Hadero, A., and Strange, R. N. 1990. Identification and characterisation of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implication. J. Bacteriol. 172:884-900.
Fobert, P. R., and Després, C. 2005. Redox control of systemic acquired resistance. Curr. Opin. Plant Biol. 8:378-382.
Foyer, C. H., and Noctor, G. 2005. Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 28:1056-1071.
Garcia-Limones, C., Hervas, A., Navas-Cortes, J. A., Jiménez-Diaz, R. M., and Tena, M. 2002. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (*Cicer arietinum* L.) and *Fusarium oxysporum* f. sp. *ciceris*. Physiol. Mol. Plant Pathol. 61:325-337.
Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. 43:205-227.
Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S., and Lory, S. 2004. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. Dev. Cell 7:745-754.
Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K. H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J. 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 4:629-643.
Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. 10:757-575.
Hassan, H. M., and Fridovich, I. 1979. Intracellular production of superoxide radical and hydrogen peroxide by redox-active compounds. Arch. Biochem. Biophys. 196:385-395.
Hassan, H. M., and Fridovich, I. 1980. Mechanism of the antibiotic action of pyocyanin. J. Bacteriol. 141:156-163.
Hennin, C., Diederichsen, E., and Höfte, M. 2001. Local and systemic resistance to fungal pathogens triggered by an AVR9-mediated hypersensitive response in tomato and oilseed rape carrying the *Cf-9* resistance gene. Physiol. Mol. Plant Pathol. 59:287-295.
Hoagland, D. R., and Arnon, D. I. 1938. The water culture method for growing plants without soil. Univ. Calif. Agric. Exp. Stn. Circ. 347:1-39.
Höfte, M., Buysens, S., Koedam, N., and Cornelis, P. 1993. Zinc affects siderophore-mediated high affinity iron uptake systems in the rhizosphere *Pseudomonas aeruginosa* 7NSK2. Biometals 6:85-91.
Iavicoli, A., Boutet, E., Buchala, A., and Metraux, J. P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHAO. Mol. Plant-Microbe Interact. 16:851-858.
Iswandi, A., Bossier, P., Vandenabeele, J., and Verstraete, W. 1987. Effect of seed inoculation with the rhizopseudomonas strain 7NSK2 on the root microbiota of maize (*Zea mays*) and barley (*Hordeum vulgare*). Biol. Fertil. Soils 3:153-158.
Jia, Y., McAdams, S.A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO (Eur. Mol. Biol. Organ.) J. 19:4004-4014.
Kachroo, A., He, Z., Patkar, R., Zhu, Q., Zhong, J., Li, D., Ronald, P., Lamb, C., and Chattoo, B. B. 2003. Induction of H_2O_2 in transgenic rice leads to cell death and enhanced resistance to both bacterial and fungal pathogens. Transgenic Res. 12:577-586.
Kanner, D., Gerber, N. N., and Bartha, R. 1978. Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. J. Bacteriol. 134:690-692.
Kawasaki, T., Koita, H., Nakatsubo, T., Hasegawa, K., Wakabayashi, K., Takahashi, H., Urnemura, K., Urnezawa, T., and Shimamoto, K. 2006. Cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis, is an effector of small GTPase Rac in defense signaling in rice. Proc. Natl. Acad. Sci. U.S.A. 103:230-235.
King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
Koga, H. 1994. Hypersensitive death, autofluorescence, and ultrastructural changes in leaf sheaths of susceptible and resistant near-isogenic lines of rice (Pi-z(t)) in relation to penetration and growth of *Pyricularia oryzae*. Can. J. Bot. 72:1463-1477.
Koga, H., Dohi, K., Nakayachi, O., and Mori, M. 2004. A novel inoculation method of *Magnaporthe grisea* for cytological observation of the infection process using intact leaf sheaths of rice plants. Physiol. Mol. Plant Pathol. 64:67-72.
Kogel, K. H., and Langen, G. 2005. Induced disease resistance and gene expression in cereals. Cell. Microbiol. 7:1555-1564.
Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., and Peterson, K. M. 1994. PBBR1MCS: a broad-host-range cloning vector. Biotechniques 16:800-802.
Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:251-275.
Lau, G. W., Hassett, D. J., Ran, H., and Kong, F. 2004. The role of pyocyanin in *Pseudomonas aeruginosa* infection. Trends Mol. Med. 10:599-606.
Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593.
Mavrodi, D. V., Bonsall, R. F., Delaney, S. M., Soule, M. J., Phillips, G., and Thomashow, L. S. 2001. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 183:6454-6465.
Mew, T. W, Leung, H., Savary, S., Vera Cruz, C. M., and Leach, J. E. 2004. Looking ahead in rice disease research and management. Crit. Rev. Plant Sci. 23:103-127.
Meziane, H., Van Der Sluis, I., van Loon, L. C., Höfte, M., and Bakker, P. A. H. M. 2005. Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Mol. Plant Pathol. 6:177-185.
Morris, S. W., Vernooij, B., Titatarn, S., Starrett, M., Thomas, S., Wiltse, C. C., Frederiksen, R. A., Bhandhufalck, A., Hulbert, S., and Uknes, S. 1998. Induced resistance responses in maize. Mol. Plant-Microbe Interact. 11:643-658.
Muller, M. 2002. Pyocyanin induces oxidative stress in human endothelial cells and modulates the glutathione redox cycle. Free Radic. Biol. Med. 33:1527-1533.
Mur, L. A. J., Kenton, P., and Draper, J. 2005. In planta measurements of oxidative bursts elicited by avirulent and virulent bacterial pathogens suggests that H_2O_2 is insufficient to elicit cell death in tobacco. Plant Cell Environ. 28:548-561.
Nandakumar, R., Babu, S., Viswanathan, R., Raguchander, R., and Samiyappan, R. 2001. Induction of systemic resistance in rice against sheath blight disease by *Pseudomonas fluorescens*. Soil Biol. Biochem. 33:603-612.
Neill, S., Desikan, R., and Hancock, J. 2002. Hydrogen peroxide signaling. Curr. Opin. Plant Biol. 5:388-395.
Ninh Thuan, N. T., Bigirimana, J., Roumen, E., Van Der Straeten, D., and Höfte, M. 2006. Molecular and pathotype analysis of the rice blast fungus in North Vietnam. Eur. J. Plant Pathol. 114:381-396.
Ongena, M., Jourdan, E., Schafer, M., Kech, C., Budzikiewicz, H., Luxen, A., and Thonart, P. 2005. Isolation of an *N*-alkylated benzylamine derivative from *Pseudomonas putida* BTP1 as elicitor of induced systemic resistance in bean. Mol. Plant-Microbe Interact. 18:562-569.
Ono, E., Wong, H. L., Kawasaki, T., Hasegawa, M., Kodama, O., and Shimamoto, K. 2001. Essential role of the small GTPase Rac in disease resistance of rice. Proc. Natl. Acad. Sci. U.S.A. 98:759-764.

Ou, S. H. 1985. Rice diseases, 2nd ed. Commonwealth Mycological Institute, Kew, Surrey, U.K.

Raaijmakers, J. M., Leeman, M., Vanoorschot, M. M. P., Van Der Sluis, I., Schippers, B., and Bakker, P. A. H. M. 1995. Dose-response relationships in biological control of *Fusarium* wilt of radish by *Pseudomonas* spp. Phytopathology 85:1075-1081.

Rabaey, K., Boon, N., Höfte, M., and Verstraete, W. 2005. Microbial phenazine production enhances electron transfer in biofuel cells. Environ. Sci. Technol. 39:3401-3408.

Reimmann, C., Hofmann, C., Mauch, D., and Dudler, R. 1995. Characterization of a rice gene induced by *Pseudomonas syringae* pv. *syringae*: requirement for the bacterial *lemA* gene function. Physiol. Mol. Plant Pathol. 46:71-81.

Rezka, K. J., O'Malley, Y., McCormick, M. L., Denning, G. M., and Britigan, B. E. 2004. Oxidation of pyocyanin, a cytotoxic product from *Pseudomonas aeruginosa*, by microperoxidase 11 and hydrogen peroxide. Free Radic. Biol. Med. 36:1448-1459.

Rodrigues, F. A., Vale, F. X. R., Datnoff, L. E., Prabhu, A. S., and Korndörfer, G. H. 2003. Effect of rice growth stages and silicon on sheath blight development. Phytopathology 93:256-261.

Rohilla, R., Singh, U. S., and Singh, R. L. 2002. Mode of action of acibenzolar-*S*-methyl against sheath blight of rice caused by *Rhizoctonia solani* Kühn. Pest Manage. Sci. 58:63-69.

Ryu, C. M., Murphy, J. F., Mysore, K. S., and Kloepper, J. W. 2004. Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against *Cucumber mosaic virus* by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. Plant J. 39:381-392.

Schweizer, P., Buchala, A., and Metraux, J. P. 1997. Gene-expression patterns and levels of jasmonic acid in rice treated with the resistance inducer 2, 6-dichloroisonicotinic acid. Plant Physiol. 115:61-70.

Schweizer, P., Schlagenhauf, E., Schaffrath, U., and Dudler, R. 1999. Different patterns of host genes are induced in rice by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical inducer benzothiadiazole (BTH). Eur. J. Plant Pathol. 105:659-665.

Shirasu, K., Nakajima, H., Rajasekhar, V. K., Dixon, R. A., and Lamb, C. 1997. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell 9:261-270.

Silue, D., Notteghem, J. L., and Tharreau, D. 1992. Evidence of a gene-for-gene relationship in the *Oryza sativa-Magnaporthe grisea* pathosystem. Phytopathology 82:577-580.

Singh, A., Rohilla, R., Singh, U. S., Savary, S., Willocquet, L., and Duveiller, E. 2002. An improved inoculation technique for sheath blight of rice caused by *Rhizoctonia solani*. Can. J. Plant Pathol. 24:65-68.

Smith, J. A., and Metraux, J. P. 1991. *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. Physiol. Mol. Plant Pathol. 39:451-461.

Someya, N., Nakajima, M., Hibi, T., Yamaguchi, I., and Akutsu, K. 2002. Induced resistance to rice blast by antagonistic bacterium, *Serratia marcescens* strain B2. J. Gen. Plant Pathol. 68:177-182.

Someya, N., Nakajima, M., Watanabe, K., Hibi, T., and Akutsu, K. 2005. Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight. Biocontrol Sci. Technol. 5:105-109.

Song, F., and Goodman, R. M. 2001. Molecular biology of disease resistance in rice. Physiol. Mol. Plant Pathol. 59:1-11.

Takahashi, A., Kawasaki, T., Henmi, K., Shii, K., Kodama, O., Satoh, H., and Shimamoto, K. 1999. Lesion mimic mutants of rice with alterations in early signaling events of defense. Plant J. 17:535-545.

Talbot, N. J. 2003. On the trail of a cereal killer: Exploring the biology of *Magnaporthe grisea*. Annu. Rev. Microbiol. 57:177-202.

Ueno, M., Shibata, H., Kihara, J., Honda, Y., and Arase, S. 2003. Increased tryptophan decarboxylase and monoamine oxidase activities induce Sekiguchi lesion formation in rice infected with *Magnaporthe grisea*. Plant J. 36:215-228.

Van Breusegem, F., and Dat, J. F. 2006. Reactive oxygen species in plant cell death. Plant Physiol. 141:384-390.

van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. 1998. Systemic resistance induced by rhizosphere bacteria. Annu. Rev. Phytopathol. 36:453-483.

Van Wees, S. C. M., Luijendijk, M., Smoorenburg, I., van Loon, L. C., and Pieterse, C. M. J. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Plant Mol. Biol. 41:537-549.

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S., Bleves, S., Lazdunski, A., Lory, S., and Filloux, A. 2006. Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. Proc. Natl. Acad. Sci. U.S.A. 103:171-176.

Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H. S., van Loon, L. C., and Pieterse, C. M. J. 2004. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. 17:895-908.

Vidhyasekaran, P., Ponmalar, T., Samiyappan, R., Velazhahan, R., Vimala, R., Ramanathan, A., Paranitharan, V., and Muthukrishnan, S. 1997. Host specific toxin production by *Rhizoctonia solani*, the rice sheath blight pathogen. Phytopathology 87:1258-1263.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Fisher, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., Franken, P., and Kogel, K. H. 2005. The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc. Natl. Acad. Sci. U.S.A. 102:13386-13391.

Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., Fitzsimmons, K. C., and Shah, D. M. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-1368.

Zahirul, I., Talukder, A., McDonald, J. S., and Price, A. H. 2005. Loci controlling partial resistance to rice blast do not show marked QTL × environment interaction when plant nitrogen status alters disease severity. New Phytol. 168:455-464.

Zolfaghar, I., Angus, A. A., Kang, P. J., To, A., Evans, D. J., and Fleiszig, S. M. 2005. Mutation of *retS*, encoding a putative hybrid two-component regulatory protein in *Pseudomonas aeruginosa*, attenuates multiple virulence mechanisms. Microbes Infect. 7:1305-1316.