Selected strains of nonpathogenic rhizobacteria can reduce disease in foliar tissues through the induction of a defense state known as induced systemic resistance (ISR). Compared with the large body of information on ISR in dicotyledonous plants, little is known about the mechanisms underlying rhizobacteria-induced resistance in cereal crops. Here, we demonstrate the ability of *Pseudomonas fluorescens* WCS374r to trigger ISR in rice (*Oryza sativa*) against the leaf blast pathogen *Magnaporthe oryzae*. Using salicylic acid (SA)-nonaccumulating NahG rice, an ethylene-insensitive OsEIN2 antisense line, and the jasmonate-deficient mutant *hebiba*, we show that this WCS374r-induced resistance is regulated by an SA-independent but jasmonic acid/ethylene-modulated signal transduction pathway. Bacterial mutant analysis uncovered a pseudobactin-type siderophore as the crucial determinant responsible for ISR elicitation. Root application of WCS374r-derived pseudobactin (Psb374) primed naive leaves for accelerated expression of a pronounced multifaceted defense response, consisting of rapid recruitment of phenolic compounds at sites of pathogen entry, concerted expression of a diverse set of structural defenses, and a timely hyperinduction of hydrogen peroxide formation putatively driving cell wall fortification. Exogenous SA application alleviated this Psb374-modulated defense priming, while Psb374 pretreatment antagonized infection-induced transcription of SA-responsive PR genes, suggesting that the Psb374- and SA-modulated signaling pathways are mutually antagonistic. Interestingly, in sharp contrast to WCS374r-mediated ISR, chemical induction of blast resistance by the SA analog benzothiadiazole was independent of jasmonic acid/ethylene signaling and involved the potentiation of SA-responsive gene expression. Together, these results offer novel insights into the signaling circuitry governing induced resistance against *M. oryzae* and suggest that rice is endowed with multiple blast-effective resistance pathways.
faster and stronger expression of basal defense responses upon pathogen attack. By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express infection-induced basal defenses is called “sensitization,” “priming,” or “potentiation” (Conrath et al., 2002, 2006). In some elegant work on the costs and benefits of priming in Arabidopsis (Arabidopsis thaliana), van Hulten and associates (2006) demonstrated that the fitness costs of priming are substantially lower than those of constitutively activated defense. In addition, it was shown that the benefits of priming-mediated resistance outweigh its costs when disease occurs. Priming thus offers an elegant solution to the plant’s trade-off dilemma between disease protection and the costs involved in defense activation (Conrath et al., 2006).

The classic example of an inducible plant defense response is systemic acquired resistance (SAR). SAR is triggered by a localized infection with necrotizing microbes and is manifested throughout the plant upon secondary challenge by otherwise virulent microbes (Grant and Lamb, 2006). The onset of SAR is marked by local and systemic increases in endogenously synthesized SA and is tightly associated with the transcriptional reprogramming of a battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Ryals et al., 1996; Maleck et al., 2000; Wang et al., 2006). These PR proteins, of which some possess antimicrobial activity, serve as hallmarks of SAR in several plant species and are thought to contribute to the state of resistance attained (Van Loon et al., 2006). Transduction of the SA signal requires the function of NPR1 (also known as NIM1), a master regulatory protein that was identified in Arabidopsis through genetic screens for SAR-compromised mutants (Cao et al., 1994; Shah et al., 1997).

Although SA is central to the induction and expression of SAR, it is not the long-distance SAR signal. Instead, exciting new data implicate methyl salicylate and a lipid-derived molecule, possibly JA, as mobile signals giving rise to ISR.

Compared with the vast body of information available for dicotyledonous plants, our understanding of the molecular machinery governing induced resistance responses in monocotyledonous crops is still in its infancy. Evidence demonstrating that central components of the SAR pathway, such as NPR1, are conserved in cereals has only recently been presented (Chern et al., 2001, 2005, Shimono et al., 2007; Yuan et al., 2007). Moreover, reports on SAR- or ISR-like phenomena in monocots are scarce (Kogel and Langen, 2005). Most telling in this regard, a 17-year-old report of systemic resistance in rice (Oryza sativa) triggered by preinoculation with an hypersensitive response (HR)-eliciting, nonpathogenic Pseudomonas syringae strain remains one of the most compelling examples of a monocot SAR-like response to date (Smith and Metraux, 1991). Previously, we reported that root colonization of rice by Pseudomonas aeruginosa 7NSK2 renders foliar tissues more resistant to infection by Magnaporthe oryzae (De Vleesschauwer et al., 2006). Extensive bacterial mutant analysis and cytometric characterization of the defense responses activated in planta revealed that this 7NSK2-mediated ISR acts through secretion of the redox-active pigment pyocyanin, thereby priming systemic tissues for boosted expression of HR-like cell death upon pathogen infection.

Aiming to further dissect the induced systemic resistance response in rice, we analyzed the bacterial determinants and host defense mechanisms underpinning ISR induced by Pseudomonas fluorescens WCS374r. This gram-negative bacterium, originally isolated from the rhizosphere of potato (Solanum tuberosum), has previously been shown to suppress Fusarium wilt (Fusarium oxysporum f. sp. radicis) of radish (Raphanus sativus) and to reduce disease caused by Ralstonia solanacearum in Eucalyptus (Leeman et al., 1995; Ran et al., 2005a). Remarkably, high inoculum densities of WCS374r cultivated at 28°C failed to elicit ISR in...
Arabidopsis against *P. syringae pv tomato* (Van Wees et al., 1997), whereas low inoculum densities or inoculum cultivated at elevated temperatures induced resistance against a broad spectrum of pathogens with different parasitic habits (Ran et al., 2005b; Djavaheri, 2007). This wide range of effectiveness of WCS374r-elicited ISR (WCS374r-ISR) strongly suggests that multiple resistance responses are involved. Indeed, recent studies by Ran et al. (2005b) and Djavaheri (2007) demonstrated that WCS374r-ISR against *Turnip crinkle virus* was still functional in Arabidopsis genotypes impaired in JA- and ET-dependent signaling, whereas WCS374r-ISR against *P. syringae pv tomato* was blocked in the latter genotypes. Hence, perception of WCS374r seems to result in the activation of multiple signal transduction pathways that all add to establishing broad-spectrum WCS374r-ISR.

In this study, we demonstrate the ability of WCS374r to mount ISR in rice against the leaf blast pathogen *M. oryzae* and provide evidence that this WCS374r-mediated ISR is based on pseudobactin-mediated priming for a pronounced multifaceted cellular defense response. Furthermore, we show that WCS374r-triggered ISR functions independently of SA accumulation but, unlike benzo(thio)diarylazone (BTH)-inducible resistance, requires intact responsiveness to ET as well as a functional octadecanoid pathway.

**RESULTS**

*P. fluorescens* WCS374r Mounts ISR in Rice to *M. oryzae*

The filamentous ascomycete *M. oryzae* is the causal agent of rice blast disease, one of the most devastating of all cereal diseases and a significant threat to food security worldwide (Talbot, 2003). To determine whether *P. fluorescens* WCS374r-mediated ISR is effective against *M. oryzae*, susceptible rice plants were grown in soil containing WCS374r bacteria and subsequently challenged with the latter pathogen. As a positive control, a subset of the plants was treated with BTH, a functional SA analog and one of the most extensively studied plant defense activators in rice (Nakashita et al., 2003; Ahn et al., 2005; Shimono et al., 2007). Within 4 to 5 d after inoculation, leaves of noninduced control plants developed large, spindle-shaped lesions with a gray center (diameter > 3 mm), often surrounded by chlorotic or necrotic tissue (Fig. 1). In contrast, plants colonized by WCS374r exhibited a marked reduction in the number of these susceptible-type lesions, producing a resistance phenotype characterized by the appearance of many small (<1 mm), dark-brown necrotic spots 2 to 3 d after inoculation (Fig. 1). Pooled over four independent experiments, WCS374r pretreatment caused a 47% reduction in lesion number. Application of BTH (0.05 mM) induced an even higher level of protection, reducing the number of susceptible-type lesions by as much as 68% compared with noninduced controls (Fig. 1).

To rule out the possibility that the observed disease protection was due to direct effects of WCS374r on *M. oryzae*, possible spreading of root-inoculated bacteria to foliar tissues was assessed by plating leaf extracts from induced plants onto selective King’s medium B (KB) agar plates (King et al., 1954). However, WCS374r bacteria were never detected in leaf blades or sheaths of root-treated plants, indicating that bacterial colonization remained confined to the root zone (data not shown). In conjunction with the inability of WCS374r to inhibit growth of *M. oryzae* in dual-culture experiments (data not shown), these findings strongly suggest that the WCS374r-provoked disease suppression is not due to microbial antagonism but rather results from activation of the plant’s own defensive repertoire.

WCS374r-Triggered ISR to *M. oryzae* Is Independent of SA Accumulation But Requires Intact Responsiveness to ET as Well as a Functional Octadecanoid Pathway

To unravel the signaling circuitry governing WCS374r-mediated ISR to *M. oryzae*, bioassays were performed with transgenic and mutant rice lines impaired in various structural components of known defense pathways. As shown in Figure 2, SA-deficient NahG plants (Yang et al., 2004) and the corresponding wild-type line Nipponbare were equally responsive to WCS374r-mediated ISR, suggesting that WCS374r elicits ISR in rice either by activating the SA pathway downstream of SA or by functioning independently of SA. NahG plants also developed wild-type levels of protection against *M. oryzae* in response to treatment.
with BTH, indicating that SA accumulation is not a prerequisite for the expression of BTH-inducible blast resistance. To investigate whether JA and/or ET play a role in WCS374r-mediated ISR, we tested the effectiveness of WCS374r in the ET-insensitive OsEIN2-suppressed transgenic line 471 (Jun et al., 2004) and the JA-deficient mutant hebiba, which is impaired in an as yet unidentified step of the octadecadoid pathway (Riemann et al., 2003; Sineshchekov et al., 2004). In contrast to the respective wild-type lines Dongyin and Nihonmasari, both 471 and hebiba were blocked in their ability to develop WCS374r-mediated ISR, whereas chemical induction of blast resistance by BTH resulted in levels of induced resistance comparable to those observed in the wild type. The impaired ISR response of 471 and hebiba was not due to insufficient root colonization, since WCS374r colonized the rhizosphere of the different rice genotypes to comparable levels (5.4 ± 0.7 log colony-forming units [cfu] g⁻¹). Together, these results suggest that WCS374r-mediated ISR against M. oryzae is independent of SA accumulation but, unlike BTH-inducible blast resistance, requires the operation of an ET/JA-regulated signaling pathway.

Involvement of Iron-Regulated Metabolites in the Elicitation of ISR by WCS374r

Several lines of evidence corroborate a major role for iron-regulated bacterial metabolites in WCS374r-mediated ISR in dicotyledonous plants (Leeman et al., 1996; Ran et al., 2005a). To address whether WCS374r mounts ISR to M. oryzae in a similar manner, we first compared the ISR-triggering capacity of inoculum cultivated on iron-rich Luria-Bertani (LB) medium to that of inoculum prepared from iron-limited KB. Figure 3A shows that, in contrast to WCS374r prepared from KB, LB-grown bacteria failed to significantly reduce disease severity. Because LB- and KB-grown bacteria colonized rice to a similar extent (data not shown), the observed difference in ISR is likely due to the different iron nutritional state of both inocula. At inoculation, LB-grown inoculum had an internal iron pool visible in the red color of the bacterial pellet, whereas an internal iron pool was not observed for KB-grown WCS374r (data not shown). Although it cannot be excluded that differences in medium composition other than iron content might have contributed to the impaired ISR-triggering capacity of LB-derived inoculum, these observations strongly suggest the involvement of iron-regulated metabolites in the elicitation of WCS374r-mediated ISR.

In order to identify such iron-regulated bacterial traits operative in triggering ISR, we compared the potential of WCS374r to induce resistance with that of a collection of mutants deficient in the production of the siderophores pseudobactin, pseudomonine, and/or SA. All bacterial strains were routinely grown on iron-poor KB. As shown in Figure 3B, the pseudomonine-deficient mutant 4A1 induced ISR to an extent similar to that obtained after treatment with the wild-type strain, indicating that pseudomonine is not essential for WCS374r to induce resistance (Fig. 3B). Conversely, treatment with the pseudobactin-negative mutant 374-02, the pseudobactin and pseudomonine double negative mutant AT12, or the triple negative mutant BT1 no longer caused disease suppression, suggesting a pivotal role for pseudobactin in WCS374r-mediated ISR to M. oryzae. However, pseudobactin alone appeared to be insufficient for the onset of ISR, since we...
failed to observe any statistically significant differences in disease severity between treatment with the pseudobactin-positive but pseudomonine- and SA-deficient mutant 4B1 and control plants. A deficiency in root colonization could be ruled out, because bacterial counts in the rhizosphere of plants inoculated with the respective mutants were similar to those of WCS374r-treated plants (data not shown). Based on these results, we initially assumed pseudobactin to act in concert with SA in the elicitation of ISR. To test this hypothesis, we next examined the effect of inoculating roots with a mix of the ISR-deficient strains 4B1 (SA², Psb) and AT12 (SA⁺, Psb⁻), where Psb = pseudobactin. Alternatively, plants colonized by 4B1 were complemented with a 1 nM SA solution, a concentration equivalent to the in vitro SA production of 10⁵ cfu of WCS374r. However, none of these combination treatments was able to restore ISR, making the involvement of SA and pseudobactin in ISR by WCS374r rather questionable (Fig. 3).

SA Attenuates Pseudobactin-Induced Resistance against M. oryzae

To shed more light on the role of SA and pseudobactin in WCS374r-mediated ISR, we isolated pseudobactin from stationary phase cultures of WCS374r as described before (Meziane et al., 2005) and applied the purified compound, alone or in combination with SA, to the roots of hydroponically grown rice seedlings. As a positive control, plants were treated with BTH. As shown in Figure 4, purified pseudobactin applied at a concentration of 70 μg per root system increased resistance against M. oryzae by as much as 88%, this being similar to the level of protection induced by 0.05 mM BTH. Application of 12 μg of pseudobactin per root system was slightly less effective, as evidenced by a 67% decrease in the number of susceptible-type blast lesions. Intriguingly, hydroponic feeding of a physiologically relevant 1 nM SA solution had no marked effect on disease development, whereas coapplication of 1 nM SA and 70 μg of pseudobactin alleviated the pseudobactin-conferred protection. While indicating that pseudobactin alone suffices for full induction of WCS374r-mediated ISR to M. oryzae, these findings suggest negative cross talk in the direction of SA damping pseudobactin action.

Figure 3. Influence of root treatment with P. fluorescens WCS374r and various mutants on rice blast (M. oryzae) severity. Unless otherwise stated, WCS374r and derived mutants were grown on KB and applied to rice roots and soil. Plants were challenged inoculated when 4 weeks old (five-leaf stage). For details on M. oryzae bioassays, see legend to Figure 1. A, Influence of the iron nutritional state of the bacterial inoculum on the level of induced resistance imparted by WCS374r. Bacteria were grown on iron-poor KB or iron-rich LB medium prior to inoculation. For comparison with chemically induced blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d before challenge. B, Quantification of ISR against M. oryzae triggered by WCS374r and various mutant strains. Mutants derived from WCS374r have the following characteristics: 02 (Psb⁻, Psm⁺, SA⁺), 4A1 (Psb⁺, Psm⁻, SA⁺), AT12 (Psb⁻, Psm⁺, SA⁺), 4B1 (Psb⁺, Psm⁺, SA⁺), and BT1 (Psb⁻, Psm⁻, SA⁻), where Psb = pseudobactin and Psm = pseudomonine. C, Effect of complementing the SA-deficient mutant strain 4B1 for SA production on the level of induced protection against M. oryzae. SA (1 nM) was applied as a soil drench 3 d before challenge. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests (α = 0.05, n > 24). Data presented are from representative experiments that were repeated at least twice with comparable results.
Pseudobactin Primed Rice for Enhanced Pathogenesis-Related Hydrogen Peroxide Formation

Production of reactive oxygen species during the oxidative burst is one of the most peculiar defense responses in plant-pathogen interactions; therefore, we next compared pathogenesis-related hydrogen peroxide (H$_2$O$_2$) generation using 3,3’-diaminobenzidine (DAB) staining. In this endogenous peroxidase-dependent assay, reddish-brown precipitates are deposited at the sites of H$_2$O$_2$ accumulation (Thordal-Christensen et al., 1997). Consistent differences between treatments were seen from 24 hpi onward. At this time, approximately one-fourth of all Psb374- or BTH-treated epidermal cells adjacent to fungal appressoria showed a local brownish staining of the anticlinal walls, whereas little staining was evident in the sheaths of control plants (data not shown). Importantly, the anticlinal cell walls and weakly enhanced vesicular activity. Epidermal sites in which the invasive hyphae were confined to the primary penetrated cell due to expression of the so-called whole plant-specific resistance (WPSR; Koga et al., 2004), a type of age-related resistance characterized by the occurrence of large, brownish granules in the cytoplasm, were scored as type C. Infection type D likewise comprised single-cell infection sites but was associated with intense browning of the anticlinal cell walls and the occurrence of round and tubular vesicles in the cytoplasm. Epidermal cells classified as type E displayed a remarkable interaction phenotype in which fungal growth was curtailed shortly after penetration by means of infection hyphae-encasing tubers, the nature of which is still elusive, as staining with phloroglucinol provided no compelling evidence for the involvement of lignin-derived deposits. Finally, type F represented a HR-like reaction, as evidenced by dense granulation of the cytoplasm and a bright autofluorescence of the epidermal cell walls. An overview of the temporal changes in the frequency of the various cellular reaction types is presented in Figure 5B. At 36 hpi, control plants almost exclusively displayed type A reactions (up to 92% of all interactions). A decrease in type A reaction from 36 to 48 hpi was accompanied by a drastic increase in the frequency of appressorial sites exhibiting a type B reaction, reaching a level of 55% by 48 hpi. BTH-induced resistance, on the other hand, was characterized by a high frequency of interaction sites with attacked cells expressing HR-related type F reaction (70% of all interactions), resulting in abrupt arrest of fungal proliferation. Most conspicuously, Psb374-supplied plants showed a strikingly different profile of effector responses in that they did not develop any HR-like responses but rather mounted type D and type E reactions, accounting for 33% and 50% of all interactions by 48 hpi, respectively. Together, these observations suggest that Psb374 primes rice for a diverse set of HR-independent cellular defenses.

Histochemical Analysis of Pseudobactin-Induced Resistance against M. oryzae

Pseudobactin Primed Rice for a Diverse Set of HR-Independent Cellular Responses

To further decipher the role of pseudobactin in WCS374r-conferring resistance in rice, we investigated the cytological and biochemical alterations associated with fungal restriction in pseudobactin-induced plants using the intact leaf sheath method developed by Koga et al. (2004). Contrary to leaf blades, leaf sheath tissue is relatively flat and optically clear, which facilitates live cell imaging, while the use of intact leaf sheaths allows the expression of numerous partial resistance responses, consistent with the continuous array of defenses. Rice sheaths of control plants and plants in which the roots were treated with either the purified pseudobactin of WCS374r (Psb374) or BTH (0.05 mM) were inoculated with a M. oryzae conidial suspension and sampled at 18, 24, 36, and 48 h postinoculation (hpi). Notably, microscopic assessment revealed no significant differences in the number of successful penetrations among treatments, indicating that both BTH- and Psb374-induced resistance are unlikely to impede prepenetration development by M. oryzae (data not shown). Starting at 36 hpi, epidermal cells were found to respond to fungal ingress through the development of various cellular reactions, which we grouped into six categories designated A to F (Fig. 5A).

Type A represented infection sites showing successful fungal invasion in the absence of any obvious host response. Type B reactions, on the other hand, were characterized by a pale yellow or brown discoloration of the anticlinal cell walls and weakly enhanced vesicular activity. Epidermal sites in which the invasive hyphae were confined to the primary penetrated cell due to expression of the so-called whole plant-specific resistance (WPSR; Koga et al., 2004), a type of age-related resistance characterized by the occurrence of large, brownish granules in the cytoplasm, were scored as type C. Infection type D likewise comprised single-cell infection sites but was associated with intense browning of the anticlinal cell walls and the occurrence of round and tubular vesicles in the cytoplasm. Epidermal cells classified as type E displayed a remarkable interaction phenotype in which fungal growth was curtailed shortly after penetration by means of infection hyphae-encasing tubers, the nature of which is still elusive, as staining with phloroglucinol provided no compelling evidence for the involvement of lignin-derived deposits. Finally, type F represented a HR-like reaction, as evidenced by dense granulation of the cytoplasm and a bright autofluorescence of the epidermal cell walls. An overview of the temporal changes in the frequency of the various cellular reaction types is presented in Figure 5B. At 36 hpi, control plants almost exclusively displayed type A reactions (up to 92% of all interactions). A decrease in type A reaction from 36 to 48 hpi was accompanied by a drastic increase in the frequency of appressorial sites exhibiting a type B reaction, reaching a level of 55% by 48 hpi. BTH-induced resistance, on the other hand, was characterized by a high frequency of interaction sites with attacked cells expressing HR-related type F reaction (70% of all interactions), resulting in abrupt arrest of fungal proliferation. Most conspicuously, Psb374-supplied plants showed a strikingly different profile of effector responses in that they did not develop any HR-like responses but rather mounted type D and type E reactions, accounting for 33% and 50% of all interactions by 48 hpi, respectively. Together, these observations suggest that Psb374 primes rice for a diverse set of HR-independent cellular defenses.

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tantly, ascorbate treatment of inoculated leaf sheaths abolished staining at the respective sites, confirming the specificity of the staining for H$_2$O$_2$ accumulation. Local DAB staining of anticlinal cell walls disappeared within 36 hpi, when the fungus had started to develop branched, bulbous invading hyphae. From this time onward, different patterns of DAB staining could be distinguished (depicted at 48 hpi in Fig. 6A). Interestingly, both the susceptibility-related infection type A, in which fungal hyphae vigorously invaded living tissue, and the Psb374-specified infection type D, characterized by liginotuber-like structures encasing invasive hyphae, remained essentially free of DAB accumulation; these reactions we designated DAB type I and type II, respectively. Conversely, in some cases, H$_2$O$_2$, accumulated in the primary invaded cell following spread of the invading hyphae into neighboring cells (type III). Restriction of hyphal growth to the initially invaded cell was associated with variable patterns of DAB staining. In some cases, cells were filled with numerous DAB-positive vesicle-like bodies targeted to the invading hyphae (type IV), whereas in WPSR- and HR-expressing cells, H$_2$O$_2$ typically accumulated within the characteristic cytoplasmic aggregation (types V and VI). Finally, in a limited number of cases, abrupt arrest of fungal ingress coincided with

Figure 5. Quantitative cytological analysis of cellular responses in leaf sheath epidermal cells of control (Ctrl), BTH-pretreated, and pseudobactin-pretreated rice plants infected with M. oryzae. Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of P. fluorescens WCS374r (Psb374; 70 μg per plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of M. oryzae. A, Single-cell interaction phenotypes were grouped into six categories, designated A to F. Micrographs depict representative examples: category A, vigorous invasion of living tissues in the absence of visible host responses; category B, occurrence of cytoplasmic vesicles and slight browning of the anticlinal walls of the first-invaded epidermal cell following fungal invasion of neighboring cells; category C, epidermal cells expressing so-called WPSR (Koga et al., 2004) as indicated by the presence of large orange-brown granules in the cytoplasm; category D, restriction of fungal development to the first-invaded epidermal cell associated with intense browning of anticlinal epidermal cell walls and enhanced vesicular activity; category E, development of invading hyphae-embedding tubules confers prompt fungal arrest in Psb374-induced epidermal cells; category F, BTH-specified HR-like reaction characterized by dense cytoplasmic granulation. Ap, Appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Bars = 20 μm. B, Frequency distribution of the above-mentioned interaction phenotypes at 36 and 48 hpi. Each bar represents the mean of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at $\alpha = 0.05$. 


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massive H₂O₂ accumulation in the entire cell, beginning as early as 30 hpi (type VII). A comparative kinetic analysis of H₂O₂ formation revealed that by 36 hpi, approximately 75% of all interaction sites in both control and Psb374-treated tissue lacked any DAB-detectable H₂O₂ (Fig. 6B). However, whereas in control cells the absence of H₂O₂ accumulation at this time point was related to successful fungal colonization (this image) and the intense reddish-brown coloration due to DAB staining of H₂O₂ (images III–VII); III, DAB accumulation in a primary epidermal cell following fungal invasion of adjacent cells; IV, accumulation of DAB-positive vesicle-like bodies in the vicinity of the invasive hyphae; V, WPSR-expressing cells (for details, see legend to Fig. 5) filled with DAB-stained granules; VI, DAB-positive cytoplasmic granules in BTH-treated hypersensitively reacting cells; VII, whole-cell DAB staining. Ap, Appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Bars = 20 μm.

B, Frequency distribution of the above-mentioned DAB staining patterns at 36 and 48 hpi. Each bar represents the mean of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at α = 0.05.

whereas in control plants, the number of types I and III reactions decreased very slowly at a rate corresponding to an increase in the number of type V reactions. Compared with the well-restricted H₂O₂ production in Psb374-supplied sheath cells, BTH-triggered HR was associated with a massive oxidative burst (type VI) beginning as early as 30 hpi, suggesting that the mechanism(s) by which BTH boosts pathogen-triggered H₂O₂ generation may be different from Psb374-conditioned priming. Starting at 52 hpi, a strong accumulation of H₂O₂ was found in control mesophyll cells that appeared to collapse, whereas in Psb374- and BTH-treated plants, DAB staining in the mesophyll tissue was only rarely observed (data not shown). However, at these late infection stages, massive H₂O₂ accumulation most likely reflects deregulated cell physiology and overtaxed antioxidative capacities, rather than a controlled defense response that restricts cellular accessibility for M. oryzae. Taken together, these results indicate that Psb374-mediated resistance...
against *M. oryzae* involves a timely, highly localized, and well-restricted production of H$_2$O$_2$ in the epidermis.

**Pseudobactin-Induced Resistance against *M. oryzae* Is Associated with Priming for Accelerated Cell Wall Fortification**

Because H$_2$O$_2$ is often used as a substrate for peroxidase-dependent cross-linking of cell wall polymers, different staining procedures were performed to visualize changes in the cell wall. Cross-linking of cell wall proteins was detected with Coomassie Brilliant Blue subsequent to protein denaturation and free protein removal (Mellersh et al., 2002), whereas safranin-O was used to detect the peroxidative incorporation of phenolic compounds in the cell wall, a fortification mechanism important during lignification and suberization (Lucena et al., 2003). As shown in Figure 7, cell wall modification was more abundant and appeared earlier in Psb374- and BTH-treated plants than in the control plants; starting from 24 hpi, the anticlinal walls of Psb374- or BTH-induced epidermal cells showed intense safranin staining, whereas in control plants, staining was weak and only detectable in limited zones of the anticlinal walls of a few colonized cells from 36 hpi onward (Fig. 7A). Likewise, protein cross-linking was seldom detected prior to 48 hpi in control plants, whereas in Psb374-supplied or BTH-induced cells, it was evident in the anticlinal and/or periclinal walls of most interaction sites at 36 hpi (Fig. 7B). Similar results were obtained when assaying for autofluorescence, the early occurrence of which is considered a hallmark of rice defense against *M. oryzae* (Rodrigues et al., 2005). Although autofluorescence was detectable as early as 18 hpi regardless of the treatment, from this time onward, the frequency of autofluorescent appressorial sites increased much more.

**Figure 7.** Quantitative cytological analysis of cell wall modifications in control (Ctrl), BTH-pretreated, and pseudobactin-pretreated rice plants challenged with *M. oryzae*. Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 μg per plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. A and B, Priming of pathogen-induced cell wall reinforcements in Psb374-treated plants. Peroxidative incorporation of phenolics compound and protein cross-linking were visualized with safranin-O (red-pink; A) and Coomassie Brilliant Blue (dark blue; B), respectively. C, Left, Representative epifluorescence images of control and Psb374-supplied epidermal cells at 24 hpi (blue light excitation). Right, Psb374 and BTH prime rice for accelerated deposition of autofluorescent phenolics at sites of attempted pathogen entry. Asterisks indicate statistically significant differences compared with the noninduced control treatment. Each bar represents the mean and so of six replications stemming from three plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars = 20 μm.
more rapidly in Psb374- or BTH-treated plants than in nontreated control plants, indicating that both inducers prime rice for augmented deposition of phenolic compounds at sites of attempted pathogen entry (Fig. 7C). Conceivably, enrichment of the host cell wall with phenolics contributes to the elaboration of permeability barriers that prevent pathogen spread and enzymatic cell wall degradation.

Exogenous SA Abrogates Psb374-Induced Priming

The observation that Psb374-pretreated plants exhibited potentiated expression of multiple cellular defense responses suggested that priming for enhanced basal defense might constitute a crucial facet of the Psb374-induced resistance response. To test this hypothesis, we next examined the effect of exogenous SA application on the manifestation of Psb374-induced priming. As illustrated in Table I, coapplication of 1 nM SA with 70 μg of Psb374 significantly decreased the frequency of Psb374-specified type E reactions (i.e. infection-blocking tubules). Adding SA to the Psb374 solution also perturbed the early occurrence of DAB staining and autofluorescence in Psb374-treated tissues and alleviated Psb374-primed protein cross-linking and cell wall fortification. Along with the suppressive effect of coapplied SA on the level of Psb374-induced protection against M. oryzae (Fig. 4), these results indicate that Psb374-triggered ISR is based on priming for enhanced expression of an attacker-induced multifaceted cellular defense program.

Psb374 Antagonizes Pathogen-Induced Activation of SA-Responsive PR Genes

The results above, together with the PR gene independence of ISR in Arabidopsis (Pieterse et al., 1996), prompted us to investigate whether Psb374 pretreatment also affects PR transcript accumulation. To this end, we tested control, BTH-induced, and Psb374-supplied plants for expression of the rice PR-like genes OsPR1b and PBZ1/PR10a. Both of these genes are known to be responsive to M. oryzae infection (Kim et al., 2001) and have recently been implicated in the BTH-inducible and SA-mediated signaling branch of the rice defense network (Shimon o et al., 2007). Quantitative reverse transcription (RT)-PCR analysis revealed that neither BTH application nor Psb374 treatment alone significantly altered OsPR1b or PBZ1 mRNA accumulation at any of the time points investigated (Fig. 8A; data not shown). However, significant differences between treatments became evident when challenging with M. oryzae. In accordance with previous reports (Midoh and Iwata, 1996; Yang et al., 2004), PBZ1 transcript levels responded strongly to blast infection, showing an approximately 250-fold induction relative to mock-inoculated controls by 48 hpi (Fig. 8A). Interestingly, application of Psb374 prior to inoculation attenuated this pathogen-induced activation of PBZ1, whereas pretreatment with BTH caused a faster and stronger induction of the latter gene in comparison with the expression measured in challenged, noninduced plants (Fig. 8A). Transcript accumulation of the OsPR1b gene mirrored the profile observed for PBZ1 (Fig. 8B), suggesting that Psb374 antagonizes M. oryzae-induced transcription of SA-responsive PR genes.

**DISCUSSION**

ISR is a phenomenon whereby disease resistance against subsequent microbial infection is induced at the whole plant level in response to colonization of the roots by certain plant growth-promoting rhizobacteria. Compared with the relative wealth of information in experimentally tractable plant species such as Arabidopsis, our understanding of the molecular mechanisms underlying ISR in economically important cereal crops is still in its infancy. In this work, we have focused on the bacterial determinants and host defense responses underlying rhizobacteria-activated ISR in rice, the most important food source worldwide.

**Table 1. Influence of SA coapplication on Psb374-induced defense priming**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Control</th>
<th>SA</th>
<th>Psb374</th>
<th>SA + Psb374</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of interaction sites</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infection-blocking tubules (36 hpi)</td>
<td>Not seen</td>
<td>Not seen</td>
<td>43.3 ± 18.6b</td>
<td>7.5 ± 2.4a</td>
</tr>
<tr>
<td>DAB staining (24 hpi)</td>
<td>Not seen</td>
<td>Not seen</td>
<td>26.2 ± 8.7b</td>
<td>5.6 ± 2.1a</td>
</tr>
<tr>
<td>Autofluorescence (24 hpi)</td>
<td>2.1 ± 0.8a</td>
<td>9.4 ± 4.2b</td>
<td>66.8 ± 10.6c</td>
<td>18.6 ± 6.7b</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue staining (36 hpi)</td>
<td>24.6 ± 5.8a,b</td>
<td>19.2 ± 4.6a</td>
<td>94.2 ± 5.2c</td>
<td>32.4 ± 9.2b</td>
</tr>
<tr>
<td>Saffranin staining (36 hpi)</td>
<td>29.5 ± 10.2a</td>
<td>24.6 ± 6.8a</td>
<td>72.6 ± 8.6b</td>
<td>21.4 ± 11.3a</td>
</tr>
</tbody>
</table>

aSA (1 nM) and purified WCS374r pseudobactin (Psb374; 70 μg per root system) were applied either alone or in combination to the roots of hydroponically grown rice seedlings (6.5-leaf stage) at 3 d prior to challenge with M. oryzae. 

bInteraction phenotype E as described in legend to Figure 5. 

cPercentage of interaction sites associated with reddish-brown precipitates in the anticlinal cell wall.

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*P. fluorescens* WCS374r-Induced Systemic Resistance in Rice

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and a pivotal model for molecular genetic studies of disease resistance in monocotyledonous plants. We show that colonization of the roots of rice by the well-characterized biocontrol agent P. fluorescens WCS374r renders foliar tissues more resistant to rice blast disease, caused by the heterothallic ascomycete M. oryzae (Fig. 1). Our data also reveal that this WCS374r-ISR is not based on direct activation of basal resistance mechanisms but rather acts through pseudobactin-mediated priming for a pronounced multifaceted cellular defense program (Figs. 3–7). Moreover, we demonstrate that ISR by WCS374r requires components of the ET and JA pathways, rather than SA accumulation or enhanced PR gene expression, suggesting that rice might have evolved a disease resistance pathway similar to the classic ISR pathway in Arabidopsis (Figs. 2 and 8).

Pseudobactin: Iron-Chelating Protagonist in the Initiation of P. fluorescens WCS374r-Mediated ISR

To date, several bacterial traits have been implicated in the initiation of WCS374r-ISR, including the O-antigenic side chain of outer membrane lipopolysaccharides, SA, and the siderophore pseudobactin (Leeman et al., 1995, 1996; Ran et al., 2005a, 2005b). In this study, we initially assumed pseudobactin and SA to be corequired for the initiation of ISR against M. oryzae, based on the observation that both the pseudobactin-deficient mutant 374-02 and the pseudobactin-proficient, yet SA-negative, mutant 4B1 lost the ability to mount ISR (Fig. 3). However, contradictory results were obtained when testing the isolated bacterial compounds: purified WCS374r-derived pseudobactin (Psb374) triggered high levels of resistance against rice blast, whereas exogenous SA failed to cause any substantial disease reduction (Fig. 4), indicating that Psb374 alone suffices for induction of ISR. Moreover, coapplication of SA with Psb374 attenuated the Psb374-induced resistance, prompting the question of how WCS374r bacteria, which simultaneously produce both of these metabolites in vitro, are able to trigger ISR. One likely scenario originates from the observation that treatment with WCS374r does not induce systemic resistance in Arabidopsis against P. syringae pv tomato, whereas the application of SA does (Van Wees et al., 1997). This discrepancy suggests that SA produced by WCS374r is not exuded into the rhizosphere, which may be due to the fact that upon iron limitation WCS374r produces not only pseudobactin and SA but also pseudomonine, a siderophore containing a SA moiety (Mercado-Blanco et al., 2001). Accordingly, it is plausible that in the rhizosphere, where iron-limiting conditions tend to prevail, all WCS374r-produced SA is channeled into pseudomonine that does not antagonize pseudobactin action. Nonetheless, if pseudobactin is the crucial determinant of WCS374r-ISR against M. oryzae, a question remains concerning the ISR-minus phenotype of the pseudobactin-positive mutant 4B1. One possible explanation for these conflicting observations lies in the fact that mutant 4B1 was constructed by gene replacement of the native psmB gene. pmsB encodes a presumed isochorismate-pyruvate lyase that catalyzes the conversion of isochorismate to pyruvate and SA (Djavaheri, 2007). Interestingly, recent evidence suggests that, in line with its closest homolog in P. aeruginosa, the catalytically promiscuous SA biosynthesis protein PchB, PmsB not only possesses isochorismate-pyruvate lyase but also chorismate mutase activity (Kunzler et al., 2005). Since chorismate mutase is located at the branch point of the shikimate pathway leading to the biosynthesis of Tyr and Phe, the enzyme constitutes a key point of regulation for maintaining the correct balance of aromatic amino acids in the cell (Neuenschwander et al., 2007). Hence, it can be envisaged that a mutation in such a regulatory enzyme might have a pleiotropic effect hampering the induction of ISR. Alternatively, mutant 4B1 might simply produce too little pseudobactin in the rhizosphere to be effective at inducing resistance.

Rice and Arabidopsis Share Conserved Disease Resistance Pathways

In many dicot plants, the role of SA as a global multicomponent regulator of various inducible defense responses is well established (Loake and Grant, 2007). Following pathogen infection, endogenous levels of SA and its conjugates increase dramatically, preceding the induction of PR genes and the onset of local resistance and SAR (Durrant and Dong, 2004). In
rice, however, the role of SA is still a matter of debate. Rice differs from most other plants in that it contains very high basal levels of endogenous SA that are not elevated further in response to pathogen infection, making the role of the SA signaling pathway in rice disputable (Silverman et al., 1995). A number of recent reports, however, do support an active role for a BTH-inducible and WRKY45- or NPR1-regulated SA signaling pathway in the rice defense response (Chern et al., 2001, 2005; Fitzgerald et al., 2004; Shimono et al., 2007; Yuan et al., 2007). Emerging from these studies is the view that rice, in spite of its high constitutive SA levels, has evolved a SA-mediated SAR pathway similar to that in Arabidopsis. In this study, we provide, to our knowledge, the first report of a similar phenomenon with regard to rhizobacteria-induced resistance signaling. ISR bioassays with SA-nonaccumulating NahG plants (Yang et al., 2004), the ET-insensitive OsEIN2 antisense line 471 (Jun et al., 2004), and the JA biosynthesis mutant hebiba (Riemann et al., 2003) revealed that WCS374r-mediated ISR against M. oryzae functions independently of SA but requires intact responsiveness to ET as well as a functional JA pathway (Fig. 2). In this respect, WCS374r-ISR against M. oryzae mirrors classic WCS417r-elicited ISR in Arabidopsis (Pieterse et al., 1996, 1998). Consistent with this is the finding that treatment with Psb374, which faithfully mimics WCS374r in activating ISR, does not lead to direct transcriptional activation or priming of SA-inducible PR genes, such as OsPR1b and PBZ1 (Fig. 8). In contrast to WCS374r-ISR, but similar to BTH-induced resistance in dicots (Friedrich et al., 1996; Gorlach et al., 1996), chemical induction of blast resistance by exogenous application of BTH was fully retained in 471, hebiba, and NahG rice plants and involved the potentiation of SA-inducible gene expression (Figs. 2 and 8). Taken together, these results not only reinforce the contention that rice is endowed with a BTH-inducible SAR-like resistance pathway (Shimono et al., 2007; Yuan et al., 2007) but also hint at a conserved mechanism for ISR signaling in rice and Arabidopsis. It is noteworthy, however, that unlike WCS374r-ISR, induction of systemic resistance against M. oryzae by P. aeruginosa 7NSK2 was found to be SA-dependent (D. De Vleesschauwer and M. Höfte, unpublished data), indicating that the signal transduction pathway governing rhizobacteria-mediated ISR against M. oryzae at least in part depends on the eliciting bacterium. Nonetheless, the apparent similarities between WCS374r- and WCS417r-activated ISR signaling in rice and Arabidopsis, respectively, support and further extend the earlier notion of ancient plant-inducible defense pathways that are shared between monocots and dicots (Morris et al., 1998). This notion, however, does not rule out the possibility that individual plant species may differ in the fine-tuned regulation of such conserved defense pathways. For instance, while ectopic expression of a rice NPR1 homolog induces constitutive activation of SA-responsive PR gene expression and provokes spontaneous development of a lesion mimic/cell death phenotype (Chern et al., 2005), none of these reactions is evident in NPR1-overexpressing Arabidopsis until treatment with SAR inducers or pathogen infection (Cao et al., 1998). Such species-specific regulation of conserved plant defense mechanisms may also apply to ISR-associated resistance phenomena. Indeed, whereas the impaired ISR response of JA-deficient hebiba argues that in rice WCS374r-ISR develops coincidently with increases in endogenous JA content (Fig. 2), in Arabidopsis neither induction nor expression of WCS417r-ISR was found to be associated with substantial alterations in JA biosynthesis (Pieterse et al., 2000). Instead, recent evidence indicates that elicitation of WCS417r-ISR sensitizes Arabidopsis for the perception of attacker-induced JA (Pozo et al., 2008). Hence, although rice and Arabidopsis appear to share a conserved ISR pathway, the modulation of this JA-dependent resistance conduit may be quite divergent. To our interest, the significance of elevated JA levels in mediating rice disease resistance was recently highlighted by the enhanced blast resistance of transgenic rice plants overexpressing allene oxide synthase, a key enzyme in the JA biosynthetic pathway (Mei et al., 2006).

Parallels between WCS374r-Mediated ISR and Wound-Inducible Systemic Resistance against M. oryzae

The predicted role of JA in WCS374r-ISR is reminiscent of the situation in wounded rice plants, where systemic resistance against M. oryzae is preceded by a strong and transient accumulation of nonconjugated JA in local and systemic tissues (Schweizer et al., 1998). Wound-inducible blast resistance further resembles WCS374r-ISR in that it delivers a similar level of systemic protection without the customary PR gene induction and is likewise abrogated in mutant hebiba plants (Schweizer et al., 1998; Riemann et al., 2003; D. De Vleesschauwer and M. Höfte, unpublished data). Regarding these similarities between WCS374r-ISR and wound-induced resistance, it is tempting to speculate that both phenomena are based on similar resistance mechanisms. Such a concept would also provide a mechanistic framework for the attenuation of SA-responsive PR gene expression in challenged Psb374-induced plants (Fig. 8). In some interesting work on rice responding to mechanical wounding, Lee et al. (2004) demonstrated that JA-induced depletion of endogenous SA levels constitutes an important regulatory mechanism for JA antagonism of SA signaling. In this scenario, if the establishment of WCS374r- and Psb374-mediated ISR coincides with a JA burst, the inverse correlation between endogenous JA and SA may account for the down-regulation of at least PR1b, the induction of which is considered to be a reliable marker for activation of the SA-regulated defense pathway in rice (Yuan et al., 2007). In a similar vein, antagonistic cross talk between SA and JA signaling may also explain the inhibitory effect of exogenous SA on the Psb374-provoked resistance against M. oryzae.
may constitute the in situ mechanism underpinning 
Psb374-mediated priming for enhanced defense 
between the manifestation of priming and the estab-
dependence of WCS374r-ISR, such close correlation 
Table I). In conjunction with the strict pseudobactin 
Table II.

**WCS374r-Trigged ISR Is Based on 
Pseudobactin-Mediated Priming for a Multifaceted 
Cellular Defense Response**

In common with many other investigations (Benhamou 
et al., 1996; Ahn et al., 2002, 2007; Kim et al., 2004; 
Verhagen et al., 2004; Tjamos et al., 2005), our results 
support the view that the rhizobacteria-mediated ISR is 
not based on direct activation of defense mechanisms 
but rather results from a sensitization of the tissue to 
express basal defenses faster and/or more strongly 
upon subsequent pathogen attack. Such a priming 
effect was borne out by the observation that challenge 
inoculation of Psb374-induced plants with *M. oryzae* 
etailed the prompt expression of a pronounced mul-
tifaceted cellular defense program, comprising rapid 
recruitment of phenolic compounds at sites of attempted 
pathogen entry, elaboration of specific sheath cell 
reactions, and a timely oxidative burst putatively 
driving cell wall fortification and protein cross-linking 
(Figs. 5–7). The importance of defense priming in the 
Psb374-activated resistance mechanism was shown by 
the effect of adding SA to the Psb374 feeding solution, 
which not only counteracted the distinct Psb374-
primed cellular responses but concurrently alleviated 
Psb374-provoked resistance against *M. oryzae* (Fig. 4;
Table I). In conjunction with the strict pseudobactin 
dependence of WCS374r-ISR, such close correlation 
between the manifestation of priming and the estab-
lishment of Psb374-induced resistance suggests that 
Psb374-mediated priming for enhanced defense 
may constitute the in situ mechanism underpinning 
WCS374r-ISR against *M. oryzae*. Hence, it is not incon-
ceptible that WCS374r bacteria protect rice from *M. 
oryzae* by releasing pseudobactin-type siderophores 
into the rhizosphere, thereby inducing a prealerted 
state of defense enabling plants to respond better and 
more rapidly to subsequently inoculated pathogens. In 
line with this concept, we previously uncovered prim-
ing as a crucial facet of the resistance mechanism 
underlying *P. aeruginosa* 7NSK2-mediated ISR against 
*M. oryzae*. Feeding rice plants with the redox-active 
pigment pyocyanin, the crucial determinant of 7NSK2-
mediated ISR, resulted in enhanced attacker-induced 
HR-like cell death in naive leaves, a phenomenon 
shown to be orchestrated by reiterative H$_2$O$_2$ micro-
bursts (De Vleesschauwer et al., 2006). Interestingly, 
similar phenocopies of hypersensitively dying epider-
mal cells in the vicinity of fungal hyphae were evident 
in challenged rice plants pretreated with BTH (Fig. 5), 
suggesting that BTH and pyocyanin might feed into a 
similar resistance pathway. Psb374-elicited ISR, on the 
other hand, was not associated with HR-like cell death 
but involved the potentiation of a coordinated set of 
distinct cellular reactions, the fast manifestation of 
pathogen-blocking tubules being a prominent compo-
nent (Fig. 5). In combination with our unpublished 
findings (D. De Vleesschauwer and M. Höfte, unpub-
lished data) that WCS374r-ISR and 7NSK2-ISR differ 
in their requirement for SA, these results support the 
notion that WCS374r and 7NSK2 bacteria employ 
distinct strategies to mount ISR and suggest that rice 
is endowed with multiple, at least partly distinct, 
blast-effective resistance pathways. This conclusion is 
further supported by a large body of evidence demon-
strating minimal overlap in the gene sets activated 
by different blast resistance inducers (Midoh and 
Iwata, 1996; Schweizer et al., 1997, 1999; Nakashita 
et al., 2003; Tanabe et al., 2006). We also tested resis-
tance induced by pyocyanin or pseudobactin against 
the necrotrophic pathogens *Rhizoctonia solani* and 
Cochlodius miyabeanus. However, in both cases, no pro-
tection was obtained, suggesting that the defense 
responses triggered by the latter elicitors are not effective against necrotrophic pathogen assault (De 
Vleesschauwer et al., 2006; D. De Vleesschauwer and 
M. Höfte, unpublished data).

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### Table II. Bacterial strains used in this study with their relevant characteristics

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Characteristics</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCS374r</td>
<td>Psb', Psm', SA', spontaneous Rif' mutant of WCS374; Rif'</td>
<td>Geels and Schippers (1983)</td>
</tr>
<tr>
<td>374-02</td>
<td>Psb', Psm', SA', Tn5 transposon mutant of WCS374; Km'</td>
<td>Weisbeek et al. (1986)</td>
</tr>
<tr>
<td>4A1</td>
<td>Psb', Psm', SA', pmsA mutant of WCS374r obtained by site-directed mutagenesis; Rif', Km'</td>
<td>Djavaheri (2007)</td>
</tr>
<tr>
<td>AT12</td>
<td>Psb', Psm', SA, Tn5 transposon mutant of 4A1; Rif', Km', Tc'</td>
<td>Djavaheri (2007)</td>
</tr>
<tr>
<td>4B1</td>
<td>Psb', Psm', SA', pmsA mutant of WCS374r obtained by site-directed mutagenesis; Rif', Km'</td>
<td>Djavaheri (2007)</td>
</tr>
<tr>
<td>BT1</td>
<td>Psb', Psm', SA', Tn5 transposon mutant of 4B1; Rif', Km', Tc'</td>
<td>Djavaheri (2007)</td>
</tr>
</tbody>
</table>

Km, Kanamycin; Pch, pyochelin; Psb, pseudobactin; Psm, pseudomonine; Pvd, pyoverdine; superscript r, resistant; Rif, rifampycin; Tc, tetracycline.
CONCLUSION

In summary, we have shown that colonization of the roots of rice by pseudobactin-producing WCS374r bacteria sensitizes naïve leaves for potentiated expression of a multifaceted cellular defense response, resulting in an enhanced level of resistance against the leaf blast pathogen *M. oryzae*. Our results also provide evidence for a WCS374r-activated signaling conduit in rice similar to the classic SA-independent but JA/ET-dependent signal transduction pathway controlling rhizobacteria-mediated ISR in Arabidopsis. Furthermore, it is evident from this study that WCS374r triggers a resistance that is mechanistically different from BTH-inducible blast resistance as well as systemic resistance induced by *P. aeruginosa 7NSK2*, suggesting the coexistence of multiple pathways leading to induced resistance against *M. oryzae*. Further elucidation of the bacterial traits and dynamic host responses underpinning rhizobacteria-mediated ISR in rice will not only advance our fundamental understanding of how rice plants cope with enemies in the context of induced resistance but also may be instrumental in developing new strategies for biologically based, environmentally friendly, and durable disease control in economically important cereal crops.

MATERIALS AND METHODS

Plant Materials

The highly susceptible rice (*Oryza sativa indica*) cv CO39 was routinely used in this study. Transgenic NabG rice and its parental line, *japonica* cv Nipponbare, were a kind gift from Dr. Yinong Yang (Pennsylvania State University). Seeds of *cv Dongyin* and the transgenic line 471, expressing the *OsEIN2* antisense construct, were kindly provided by Dr. Gynheung An (Yonsei University), while JA-deficient *lehu* mutant seeds and the corresponding wild type, *japonica* cv Nihonmasari, were a kind gift from Dr. Peter Nick (Karlsruhe University). Unless otherwise noted, rice plants were grown on soil under greenhouse conditions (30°C ± 4°C, 16-h photoperiod). For seed multiplication, plants were propagated in the greenhouse and fertilized with 0.5% ammonium sulfate every 2 weeks until flowering.

Cultivation of Rhizobacteria and Pathogens

Bacterial strains used in this study are listed in Table II. *Pseudomonas fluorescens* strain WCS374r and derived mutant strains were grown for 24 to 28 h at 28°C on KB (King et al., 1954) agar plates. Bacterial cells were scraped off the plates and suspended in sterile saline (0.85% NaCl). Densities of the bacterial suspensions were adjusted to the desired concentrations based on their optical density at 620 nm.

*Magnaporthe oryzae* isolate VTSM1 (Thuan et al., 2006) was grown at 28°C on half-strength oatmeal agar (Difco). Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 7 d to induce sporulation. Conidia were harvested according to De Vleesschauwer et al. (2006), and inoculum concentration was adjusted to a final density of 1 × 10⁶ spores mL⁻¹ in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich G-6650).

Pathogen Inoculation and Disease Rating

Four-week-old rice seedlings (five-leaf stage) were challenge inoculated with *M. oryzae* isolate VTSM1 as described before (De Vleesschauwer et al., 2006). Six days after inoculation, disease was assessed by counting the number of elliptical to round-shaped lesions with a gray center indicative of sporulation of the fungus and expressed relative to nonbacterized control plants.

Induction Treatments

Induced resistance assays were performed basically as described by De Vleesschauwer et al. (2006). Briefly, plants were grown under greenhouse conditions (30°C ± 4°C, 16-h photoperiod) in commercial potting soil (Structural; Snellbou) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for 2 min, rinsed three times with sterile, demineralized water, and incubated on wet sterile filter paper for 5 d at 28°C to germinate. Prior to sowing in perforated plastic trays (23 × 16 × 6 cm), roots of germinated seeds were dipped in bacterial suspensions (5 × 10⁶ cfu mL⁻¹) for 10 min. In addition, the bacterial inoculum was thoroughly mixed with the potting soil to a final density of 5 × 10⁶ cfu g⁻¹, and, 12 d later, applied a second time as a soil drench. In control treatments, soil and rice plants were treated with equal volumes of sterilized saline.

For chemical induction of resistance, plants were treated with BTH at 3 d prior to challenge inoculation. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was dissolved in sterilized demineralized water for use and applied as a soil drench. Control plants were treated with an equal volume of water. BTH was a kind gift from Syngenta Crop Protection.

Evaluation of Plant Colonization by *P. fluorescens* WCS374r and Mutants

Bacterial colonization of the plant roots was determined by the time the bioassays were discontinued. Roots of three plants of each treatment were rinsed to remove most of the soil, weighed, and macerated in sterile demineralized water. Serial dilutions were plated on KB agar supplemented with the appropriate antibiotics: kanamycin (25 μg mL⁻¹), tetracycline (20 μg mL⁻¹), and rifampicin (200 μg mL⁻¹). Bacterial counts were made after incubation for 24 h at 28°C. Possible spreading of root-inoculated bacteria to distal leaves was checked as stated by De Vleesschauwer et al. (2006).

Purification of Pseudobactin

Bacteria were grown in liquid standard succinate medium (Meyer and Abdallah, 1978), and pseudobactin was extracted and purified according to Meziane et al. (2005). To avoid contamination with SA or pseudomonic acid, mutant 4B1 was used.

Application of Purified Compounds

For experiments in which purified pseudobactin and/or SA were applied to rice seedlings, plants were grown in a hydroponic gnotobiotic system. Surface-sterilized rice seeds were germinated for 5 d at 28°C on wet filter paper. After incubation, germinated seeds were sown in perforated plastic trays (23 × 16 × 6 cm) filled with sterilized vermiculite and supplemented with half-strength Hoagland solution. Every 3, 0.5 L of the nutrient solution was added to each tray containing 12 seedlings. In this model, various concentrations of pseudobactin and SA were applied to the plants at 3 d before challenge by including the desired concentration in Fe-EDTA-free nutrient solution (Acros).

Visualization of Defense Responses

To gain more insight into the cytomolecular mechanisms underlying pseudobactin- and BTH-induced resistance against *M. oryzae*, intact leaf sheath assays were performed as described by Koga et al. (2004). Briefly, leaf sheaths of the sixth leaf of rice plants at the 6.5-leaf stage were peeled off with leaf blades and roots. The leaf sheath was laid horizontally on a support in plastic trays containing wet filter paper, and the hollow space enclosed by the sides of the leaf sheaths above the mid vein was filled with a conidial suspension (5 × 10⁶ conidia mL⁻¹) of *M. oryzae*. Inoculated leaf sheaths were then incubated at 25°C with a 16-h photoperiod. When ready for microscopy, the sheaths were hand trimmed to remove the sides and expose the epidermal layer above the mid vein. Lower mid vein cells were removed to produce sections three to four cell layers thick. For time-course experiments, sheath sections were generally sampled at 18, 24, 30, 36, 48, and 72 hpi, and at least six trimmed sheath tissue sections originating from different plants were used for each sampling time point.


P. fluorescens WCS374r-Induced Systemic Resistance in Rice
 Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GFP filter set; excitation at 450–480 nm, dichroic beamer splitter of 500 nm, barrier filter BA515). To detect H2O2 accumulation, staining was according to the protocol of Thordal-Christensen et al. (1997) with minor modifications. Six hours before each time point, trimmed sheath segments were vacuum infiltrated with an aqueous solution of 1 mg mL−1 DAB-HCl (pH 3.8) for 30 min. Infiltrated segments were then further incubated at room temperature in the above-mentioned DAB solution until sampling. DAB polymerizes in the presence of H2O2 and endogenous peroxidase to form a brownish-red precipitate that can be easily visualized using bright-field microscopy. Specificity of the DAB staining was verified by adding 10 mM ascorbic acid. For protein cross-linking, staining was performed as described by Meless et al. (2002). Trimmed sheath segments were submerged in 1% SDS at 80°C, stained in 0.1% Coomassie Brilliant Blue in 40% ethanol/10% acetic acid for 15 min, and subsequently rinsed in a solution of 40% ethanol/10% acetic acid. For analysis of callose deposition, trimmed sheaths were stained for 5 min in a solution containing 0.1% (w/v) aniline blue and 0.15 μM KHPo4. To visualize cell wall modifications, safranin-O staining was performed according to Lucena et al. (2003) by incubating cut sheath segments in 0.01% safranin-O in 50% ethanol for 2 min. After staining, trimmed sheath segments were mounted in 50% glycerol. Images were acquired digitally (Olympus Color View II camera) and further processed with the Olympus analySIS cell®F software.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR Analysis

Total RNA was isolated from frozen leaf tissue using the Invisorb Spin Plant RNA Mini kit (Invitek) and subsequently treated with Turbo DNase according to the manufacturer’s instructions (Ambion/Applied Biosystems). First-strand cDNA synthesis was performed by using Affinityscript reverse transcriptase and oligo(dT) primers (Stratagene/Bio-Connect), according to the manufacturer’s instructions. The following primer sequences were used: for actin (Os01g28450; similar to U89895), forward 5'-CCACGAGAATTTG-3' and reverse 5'-GCGTGGACAAAGTTTTCAACCG-3'; and for Os12g36880, similar to D83180, forward 5’-CCCTGCCGAATACGCCTAA-3’ and reverse 5’-CTCTGGACCTTCCCCATACGAGCTC-3’; for PR21 (Os12g36880; similar to D83180), forward 5’-CCCCCTGCCGAATACGCCTAA-3’ and reverse 5’-CTCTGGACCTTCCCCATACGAGCTC-3’; and for PR1b (Os1g28450; similar to U89895), forward 5’-GGCAACTTCCGGCGACAAGC-3’ and reverse 5’-CTCGGACCGGAGATGTTG-3’. For each primer pair, the optimal annealing temperatures were predetermined by gradient PCR using a Thermocycler (Bio-Rad). Furthermore, for each target, primer concentrations were optimized by performing a primer titration. Quantitative PCR amplifications were conducted on optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green Master Mix (Stratagene/Bio-Connect) to monitor double-stranded DNA synthesis. The expression of each gene was assayed in triplicate in a total volume of 25 μL including a passive reference dye (ROX) according to the manufacturer’s instructions (Stratagene). The thermal profile used consisted of an initial denaturation step at 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s. Fluorescence data were collected during the annealing stage of amplification. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer. The amount of plant RNA in each sample was normalized using actin (Os01g28450) as an internal control, and samples collected from control plants at 0 hpi were selected as a calibrator. The generated data were analyzed with the Mx3005P software (Stratagene). For all amplification plots, the optimal baseline range and threshold cycle values were calculated using the Mx3005P algorithm. Gene expression in control, BTH-treated, and pseudobactin-treated samples was expressed relative to the calibrator and as a ratio to actin expression using the measured efficiency for each gene.

ACKNOWLEDGMENTS

We gratefully thank Kris Audenaert for his support during the initial phase of the project, Bob Asselbergh for valuable help with the microscopy, and Ilse Delaere for technical assistance.

Received August 11, 2008; accepted October 17, 2008; published October 22, 2008.

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