Abscisic Acid-Induced Resistance against the Brown Spot Pathogen *Cochliobolus miyabeanus* in Rice Involves MAP Kinase-Mediated Repression of Ethylene Signaling[1][C][W][OA]

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The plant hormone abscisic acid (ABA) is involved in an array of plant processes, including the regulation of gene expression during adaptive responses to various environmental cues. Apart from its well-established role in abiotic stress adaptation, emerging evidence indicates that ABA is also prominently involved in the regulation and integration of pathogen defense responses. Here, we demonstrate that exogenously administered ABA enhances basal resistance of rice (*Oryza sativa*) against the brown spot-causing ascomycete *Cochliobolus miyabeanus*. Microscopic analysis of early infection events in control and ABA-treated plants revealed that this ABA-inducible resistance (ABA-IR) is based on restriction of fungal progression in the mesophyll. We also show that ABA-IR does not rely on boosted expression of salicylic acid-, jasmonic acid -, or callose-dependent resistance mechanisms but, instead, requires a functional Gα-protein. In addition, several lines of evidence are presented suggesting that ABA steers its positive effect on brown spot resistance through antagonistic cross talk with the ethylene (ET) response pathway. Exogenous ethephon application enhances susceptibility, whereas genetic disruption of ET signaling renders plants less vulnerable to *C. miyabeanus* attack, thereby inducing a level of resistance similar to that observed on ABA-treated wild-type plants. Moreover, ABA treatment alleviates *C. miyabeanus*-induced activation of the ET reporter gene *EBP89*, while derepression of pathogen-triggered *EBP89* transcription via RNA interference-mediated knockdown of *OsMPK5*, an ABA-primed mitogen-activated protein kinase gene, compromises ABA-IR. Collectively, these data favor a model whereby exogenous ABA enhances resistance against *C. miyabeanus* at least in part by suppressing pathogen-induced ET action in an *OsMPK5*-dependent manner.

To effectively combat invasion by microbial pathogens, plants have evolved sophisticated mechanisms providing several strategic layers of constitutive and induced defenses. Preformed physical and biochemical barriers constitute the first line of defense and fend off the majority of pathogens. However, should the pathogen overcome or evade these constitutive defenses, recognition of pathogen-derived molecules by plant receptors leads to the activation of a concerted battery of defenses designed to impair further pathogen spread. These inducible defenses are regulated by the coordinated activity of an elaborate matrix of signal transduction pathways in which the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) act as key signaling molecules (Lorenzo and Solano, 2005; Grant and Lamb, 2006; Adie et al., 2007). In response to pathogen attack, plants produce a highly specific blend of SA, JA, and ET, resulting in the activation of distinct sets of defense-related genes (Koornneef and Pieterse, 2008; Bari and Jones, 2009). It is thought that this so-called signal signature, which varies greatly in quantity, timing, and composition according to the type of attacker encountered, plays a primary role in the orchestration of the plant's defense response and eventually determines the specific nature of the defense response triggered (Rojo et al., 2003; De Vos et al., 2005; Mur et al., 2006).

Over the past decade, it has become increasingly clear that a plant's resistance to attack is not brought about by the isolated activation of parallel, linear hormonal circuits but rather is the consequence of a complex regulatory network that connects the indi-

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vidual pathways, enabling each to assist or antagonize the others (Grant and Jones, 2009; Pieterse et al., 2009). In addition to differential signal signatures, such pathway cross talk provides the plant with a powerful regulatory potential to fine-tune its immune response to different types of attackers. Thus, some exceptions notwithstanding (Thaler et al., 2004; Asselbergh et al., 2007), it is commonly accepted that SA promotes resistance against pathogens with a biotrophic lifestyle, whereas JA and ET act as positive signals in the activation of defenses against necrotrophic pathogens and herbivorous insects (Thomma et al., 2001; Rojo et al., 2003; Glazebrook, 2005). Additionally, the primary mode of interaction between the SA and JA/ET signaling pathways appears to be mutual antagonism with corresponding trade-offs between biotroph resistance, on the one hand, and resistance to necrotrophic pathogens and insect herbivores, on the other hand (Bostock, 2005; Stout et al., 2006; Spoel et al., 2007). However, this is likely an oversimplified model, as synergistic actions of SA and JA/ET have been reported as well (Van Wees et al., 2000; Mur et al., 2006; Adie et al., 2007; Truman et al., 2007).

Although mechanistic explanations of antagonistic and cooperative cross talk are scarce, a number of transcription factors and effector proteins have been characterized that are critical in the circuitry controlling signal sensitivity and transduction in induced defense. For instance, SA repression of JA signaling requires the activation of proteins such as NONEXPRESSER OF PR GENES1 (NPR1) and WRKY70 that activate the expression of SA-responsive genes while repressing JA-dependent genes (Spoel et al., 2003; Li et al., 2004, 2006). Besides transcription factors, cross talk between the SA and JA signaling pathways may also be mediated by fatty acid-derived signals and/or glutaredoxin genes (Kachroo et al., 2003; Ndamukong et al., 2007). Other important effectors that contribute to differential response activation include mitogen-activated protein kinases (MAPKs). Arabidopsis MPK4 is one such kinase and has been shown to regulate SA/JA cross talk by simultaneously repressing SA biosynthesis and promoting the perception of or response to JA, thereby functioning as a molecular switch between these mutually antagonistic pathways (Brodersen et al., 2006). On the other hand, fine-tune regulation of the antagonism and cooperation between JA and ET depends on the balance of activation by both hormones of ERF1 and MYC2, two opposing transcription factors that differentially regulate divergent branches of the JA signaling pathway involved in the response to necrotrophic pathogen attack and wounding, respectively (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003, 2004).

In contrast to the overwhelming amount of information with respect to SA, JA, and ET serving as important regulators of induced disease resistance, the role of abscisic acid (ABA) in plant defense is less well understood and even controversial. Most comprehensively studied as a global regulator of abiotic stress adaptation, ABA has only recently emerged as a key determinant in the outcome of plant-pathogen interactions. In most cases, ABA behaves as a negative regulator of disease resistance. Exogenous application of ABA increases the susceptibility of various plant species to bacterial and fungal pathogens (Mohr and Cahill, 2003, 2007; Thaler et al., 2004; Achuo et al., 2006; Asselbergh et al., 2007), while disruption of ABA biosynthesis was shown to confer resistance to the necrotroph *Botrytis cinerea* (Audenaert et al., 2002) and virulent isolates of the bacterial speck pathogen *Pseudomonas syringae* pv *tomato* DC3000 in tomato (*Solanum lycopersicum*; Thaler and Bostock, 2004) and the oomycete *Hyaloperonospora parasitica* in Arabidopsis (*Arabidopsis thaliana*; Mohr and Cahill, 2003). Moreover, an intriguing study by de Torres-Zabala and coworkers (2007) revealed that *P. syringae* hijacks the ABA biosynthetic and response machinery to inflict disease in Arabidopsis, suggesting that ABA is a susceptibility factor for this bacterium. This detrimental effect of ABA on pathogen resistance is likely explained by its well-documented ability to counteract SA- and JA/ET-dependent basal defenses (Asselbergh et al., 2008; de Torres-Zabala et al., 2009; Ton et al., 2009).

In contrast, some studies describe a positive role of ABA in the activation of defense responses and pathogen resistance. For instance, ABA primes for callose accumulation and thereby enhances basal resistance in response to *Blumeria graminis* f. sp. *hordei* and activates induced resistance in response to the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004; Wiese et al., 2004; Flors et al., 2008). In the case of bacterial leaf pathogens, ABA plays a crucial role in the activation of stomatal closure that, as part of the SA-regulated innate immune system, represents a major barrier to bacterial infection (Melotto et al., 2006). Furthermore, a recent study in Arabidopsis uncovered a new role for ABA in defense against insects (Bodenhausen and Reymond, 2007). ABA thus appears to play a complex and ambivalent role in the plant's defense response, acting as either a positive or negative regulator of disease and pest resistance by interfering at multiple levels with biotic stress response pathways.

Rice (*Oryza sativa*) is the most important staple food crop in the world, only rivaled in importance by maize (*Zea mays*) and wheat (*Triticum aestivum*). However, despite its emergence as a pivotal scientific model for monocotyledonous plants, surprisingly little is known about the effector responses and hormonal signal transduction pathways underlying rice disease resistance. This is particularly true for rice brown spot disease, caused by the ascomycete *Cochliobolus miyabeanus* (anamorph: *Bipolaris oryzae*). One of the most devastating rice diseases in rain-fed ecosystems, brown spot adversely affects the yield and milling quality of the grain (Dela Paz et al., 2006). In 1942, an epidemic of the disease was one of the major factors contributing to the great Bengal famine, which reportedly claimed the lives of no less than 2 million Indians (Stuthman, 2002). Nowadays, brown spot is as prev-

alent as ever, with recent studies by Savary et al. (2000a, 2000b) showing that among the many diseases occurring in rice fields, brown spot, along with sheath blight (*Rhizoctonia solani*), accounts for the highest yield loss across all production situations in south and southeast Asia. Although the genetic and molecular bases of the rice-*C. miyabeanus* interaction are still poorly understood, it is known that, like other *Cochliobolus* species, the fungus employs a varied arsenal of phytotoxins to trigger host cell death (Xiao et al., 1991).

Here, we show that pretreatment of rice with ABA renders leaves more resistant to *C. miyabeanus* attack and present results supporting ABA-mediated repression of pathogen-induced ET action as a core resistance mechanism. In addition, we provide novel evidence regarding the role of the ABA-inducible MAPK gene *OsMPK5* as a pivotal regulator of this ABA/ET cross talk and describe how ABA might interfere with the postulated fungal manipulation of the plant.

RESULTS

Exogenous ABA Treatment Induces Resistance against *C. miyabeanus* in Rice

Six rice cultivars, including four *indica* and two *japonica* lines, were screened with two *C. miyabeanus* strains, both of which were isolated from diseased rice in field plots at the International Rice Research Institute in the Philippines (Supplemental Table S1). With the exception of *japonica* cv CR203, isolate Cm988 was highly virulent on all cultivars tested, causing typical ellipsoidal light- or dark-brown lesions with a gray sporulating center, often surrounded by chlorotic tissue. On most cultivars, these susceptible-type lesions coalesced within 96 h post inoculation (hpi), killing large areas of affected leaves (Fig. 1B, no ABA treatment). By contrast, in the case of infection by strain Cm963, fungal development was restricted to a few dark-brown necrotic spots, representing a genetically resistant reaction (Ou, 1985). Owing to its differential response to Cm988 and Cm963 and its widespread use as a pathogen-susceptible control in numerous other studies, *indica* cv CO39 was chosen for further analysis.

In a first attempt to unravel the signaling network(s) orchestrating rice defense against *C. miyabeanus*, we examined the effects of various signaling molecules and so-called plant defense activators on brown spot disease development. To this end, 5-week-old CO39 seedlings were sprayed until runoff with the respective compounds and, 3 d later, inoculated with the virulent strain Cm988. Consistent with previous reports (Ahn et al., 2005), treatment with 0.1 mM JA yielded no significant protection against *C. miyabeanus* (Fig. 1A), even though this concentration is high enough to induce JA-responsive *JIOsPR10* transcription (Jwa et al., 2001). Higher concentrations of JA also failed to trigger induced resistance (data not shown), suggesting that JA is not a major signal for the activation of defenses against *C. miyabeanus*. Intriguingly, pretreatment with 0.5 mM ethephon, an ET-releasing plant growth regulator, rendered plants more vulnerable to brown spot disease compared with noninduced controls. The disease-promoting effect of ethephon strikingly contrasted with the enhanced resistance observed in response to exogenously administered ABA. Supplying plants with 0.1 mM ABA 3 d prior to inoculation induced high levels of protection, as shown by dramatic decreases in size, type, and number of brown spot lesions in ABA-supplied leaves (Fig.

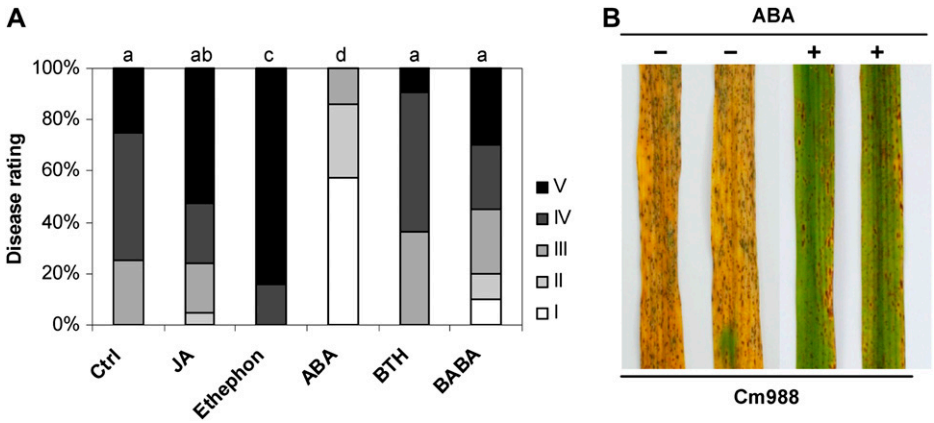

Figure 1. Exogenous application of ABA induces resistance against *C. miyabeanus* in rice. A, Effect of pretreatment with various plant defense activators and signaling molecules on subsequent infection with *C. miyabeanus*. Five-week-old CO39 plants were sprayed until runoff with 0.1 mM JA, 0.5 mM ethephon, 0.1 mM ABA, or 0.5 mM BTH or soil drenched with 0.15 mM BABA. Control (Ctrl) plants were treated with water. Three days after chemical treatment, plants were challenged with virulent *C. miyabeanus* Cm988 by spraying a conidial suspension at 1×10^4 spores mL^{-1}. Disease evaluation was performed 4 d post inoculation, using a I to V disease severity scale as described in "Materials and Methods." Different letters indicate statistically significant differences (Mann-Whitney test; $\alpha = 0.05$). B, Photographs depicting representative symptoms were taken 5 d post inoculation. Data represent one of three experiments with similar results. [See online article for color version of this figure.]

1B). On the other hand, foliar application of the synthetic SA analog benzothiadiazole (BTH; 0.5 mM) or soil-drench treatment with 150 μM β-aminobutyric acid (BABA), a nonprotein amino acid and potent elicitor of broad-spectrum disease resistance in dicot plants (Ton et al., 2005; Flors et al., 2008), resulted in a rather weak and statistically not significant reduction in disease severity compared with control plants. Collectively, these data uncover ABA as a powerful activator of induced resistance against *C. miyabeanus* and suggest that ET acts as a negative signal in the signaling circuitry underlying rice defense against this ascomycete.

ABA-Induced Resistance against *C. miyabeanus* Is Based on Restriction of Fungal Progression in the Mesophyll

To gain more insight into the nature of ABA-inducible brown spot resistance (ABA-IR), we next analyzed fungal development and cellular defense reactions in mock- and ABA-treated CO39 leaf sheaths following challenge with virulent Cm988. Regardless of ABA treatment, conidial attachment and germination occurred within 6 hpi, followed by normal hyphal growth and appressorium-mediated penetration attempts (Fig. 2A). Interestingly, at some interaction sites, invading hyphae differentiated into subcuticular finger-shaped multicell complexes (Fig. 2B), resembling the extracellular infection structures, so-called stroma, frequently formed by *Venturia inaequalis* and *Bipolaris sorokiniana* (Ortega et al., 1998; Schafer et al., 2004). Further ramification of hyphal tissue occurred predominantly but not exclusively intercellularly (Fig. 2, C and D), giving rise to a dense network that eventually penetrated all host tissue types. Epidermal and mesophyll tissue necrotization was closely associated with successful fungal infestation, whereby necrotization usually preceded fungal growth, suggesting the involvement of *C. miyabeanus*-secreted phytotoxins. Comparing control-inoculated and ABA-treated plants, we found no marked differences in the above-mentioned infection events, except for a drastic reduction of fungal spreading in the mesophyll tissue of ABA-supplied leaf sheaths. By 36 hpi, fungal spreading in control-inoculated leaves amounted to approximately 1,400 μm, corresponding to 20 to 25 mesophyll cells spanned by the fungus, as compared with 300 μm in ABA-pretreated sheath cells. Together, these observations suggest that restriction of fungal proliferation during the mesophyll-based growth phase, rather than a preinfectional, epidermis-based resistance reaction, is the cause for the reduced disease susceptibility in ABA-treated plants.

ABA-IR against *C. miyabeanus* Acts through a Callose-Independent Mechanism

Recent evidence has implicated ABA as a positive signal in priming of callose biosynthesis upon pathogen recognition, which suggests a putative mechanism explaining the role of ABA in defense activation (Ton and Mauch-Mani, 2004; Flors et al., 2008). Callose deposition is a hallmark of basal defense to attempted fungal and bacterial penetration and may serve to fortify cell walls in order to inhibit pathogen penetration of the cell. To ascertain the role of callose in the case of *C. miyabeanus*, we studied the deposition of this compound and its effects on resistance in mock- and ABA-treated leaves stained with aniline blue. Deposition of callose, visualized by an intense yellow-green fluorescence under UV light, was detectable as early as 8 hpi in epidermal control cells in close contact with the invading hyphae. This fluorescence was infrequently only present in appositions or papillae around the site of penetration, more normally being seen to encompass large multispot deposits located in the close vicinity of the periclinal and anticlinal cell walls of both infected and neighboring epidermal cells (Fig. 2E). Although no differences were evident between ABA- and control-treated plants in the onset of callose formation, ABA-induced plants tended to accumulate less callose-associated fluorescence following Cm988 challenge compared with inoculated controls (data not shown). To determine whether this altered callose formation contributed to the ABA-induced resistance, fifth and sixth stage leaves from 5-week-old CO39 plants were detached and supplied from the cut base with a solution containing 0.1 mM ABA and different concentrations of the callose inhibitor 2-deoxy-D-Glc (2-DDG; Ton and Mauch-Mani, 2004; Asselbergh et al., 2008). Two days post ABA application, the leaves were drop inoculated with a Cm988 conidial suspension, and the level of induced resistance was quantified by determining average lesion diameters at 60 hpi. Treatment with increasing concentrations of 2-DDG resulted in a partial reduction in the percentage of callose-inducing spores relative to water-treated controls, confirming the efficacy of 2-DDG to disrupt callose biosynthesis in rice. However, as shown by the results presented in Figure 3, removal of callose formation by 2-DDG treatment did not significantly affect the resistant response of ABA-supplied leaves, indicating that callose is not a critical factor in the establishment of ABA-inducible brown spot resistance.

Influence of Exogenous ABA Treatment on Pathogenesis-Related Hydrogen Peroxide Generation

The callose independence of ABA-IR prompted us to assay for other biochemical defense responses. Tissue autofluorescence, due to the accumulation of phenolic compounds, is a key event in R protein-mediated resistance against the rice blast-causing ascomycete *Magnaporthe oryzae* (Koga, 1994). In the case of *C. miyabeanus*, however, rapid recruitment of phenolics does not appear to constitute an effective defense mechanism, as all interaction sites exhibited a strong blue light-induced autofluorescence as early as 8 hpi (Fig. 2F), irrespective of ABA treatment or the inherent level of resistance of the cultivars used (data not shown). In contrast, striking differences were

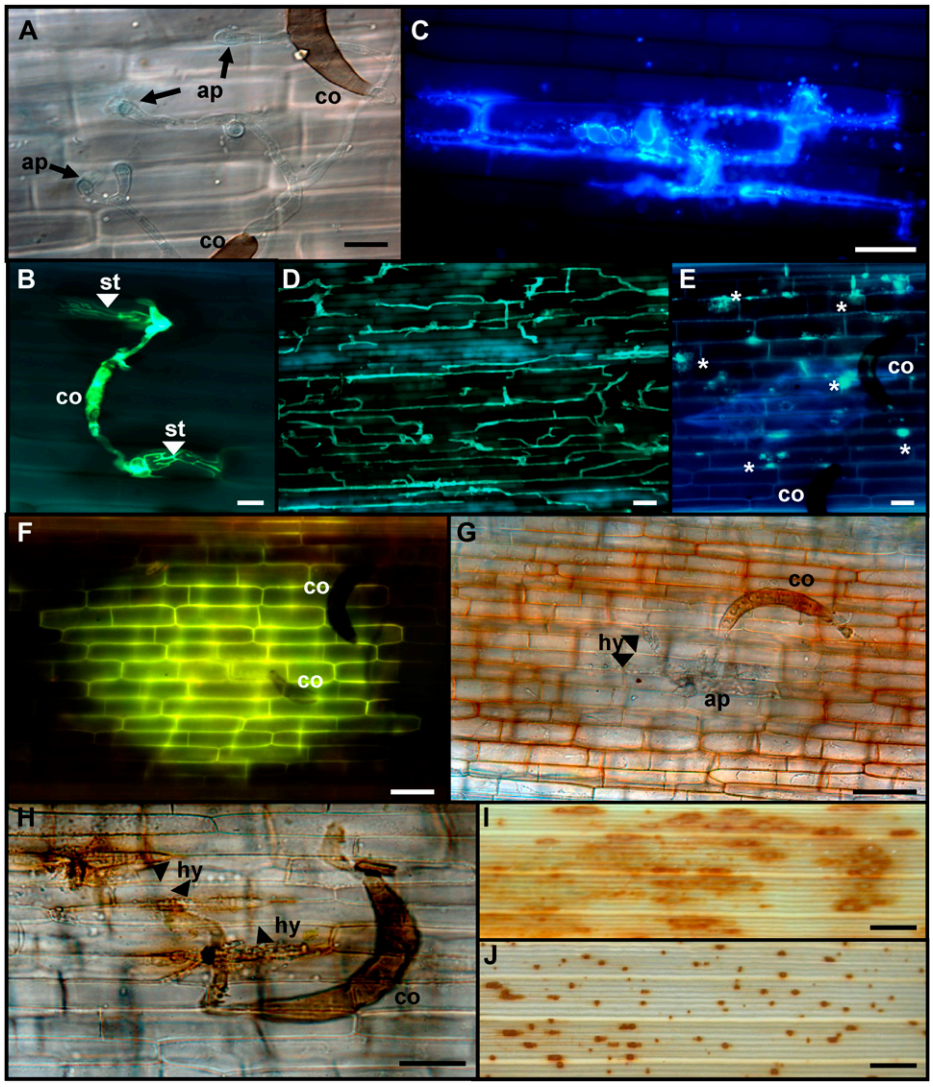

Figure 2. Microscopic analysis of the early infection events in ABA-supplemented rice sheaths inoculated with *C. miyabeanus*. Five-week-old CO39 plants were sprayed until runoff with water or 0.1 mM ABA and, 3 d later, challenged with a conidial suspension of virulent Cm988 containing 1×10^4 spores mL^{-1}. co, Conidium. A to F, Micrographs of typical infection sites in control-treated plants. Similar phenomena were observed in ABA-induced tissues save for a drastic reduction of fungal spreading in the mesophyll. A, Appressorium (ap) formation on leaf sheath epidermis (6 hpi). Bar = 20 μm. B, Following appressorium-mediated penetration, invading hyphae frequently differentiate into subcuticular stroma-like complexes (st). Fungal hyphae were stained with KOH-aniline blue and visualized under UV light excitation. Bar = 20 μm. C, Epifluorescence image of a representative epidermal cell illustrating intercellular fungal progression. Hyphae were stained with calcofluor. Bar = 50 μm. D, Extreme fungal spreading in control plants at 36 hpi. Extracellular mycelium was stained using KOH-aniline blue and analyzed under UV light excitation. Bar = 50 μm. E, Callose formation (white asterisks) at and around sites of attempted pathogen entry at 12 hpi (aniline blue stain). The dispersed pattern of callose accumulation strongly suggests the involvement of fungal toxins. Bar = 20 μm. F, Massive autofluorescence of both invaded and surrounding epidermal cells under blue light excitation (8 hpi). Bar = 50 μm. G to J, H_2O_2 accumulation in control- and ABA-treated leaf sheaths inoculated with *C. miyabeanus*. G, In control plants at 12 hpi, strong DAB staining developed in the anticlinal walls of epidermal and mesophyll cells surrounding the site of infection, whereas penetrated, hyphae (hy)-containing cells remained essentially free of DAB accumulation. H, Conversely, in ABA-treated tissue, DAB staining was exclusively detectable at sites of penetration. Bars = 50 μm. I and J, Overview images of DAB staining on infected leaf blades of control-treated (I) and ABA-treated (J) plants at 36 hpi. Bars = 2 mm.

observed when staining leaves with diaminobenzidine (DAB), a histochemical reagent for hydrogen peroxide (H_2O_2; Thordal-Christensen et al., 1997). In control plants challenged with virulent Cm988 at 12 hpi, strong DAB staining developed in the anticlinal walls of nonpenetrated epidermal and mesophyll cells surrounding the site of infection, whereas little staining was evident in infected, hyphae-containing cells (Fig. 2G). In contrast, in ABA-induced tissues, DAB accumulation was tightly restricted to the site of penetra-

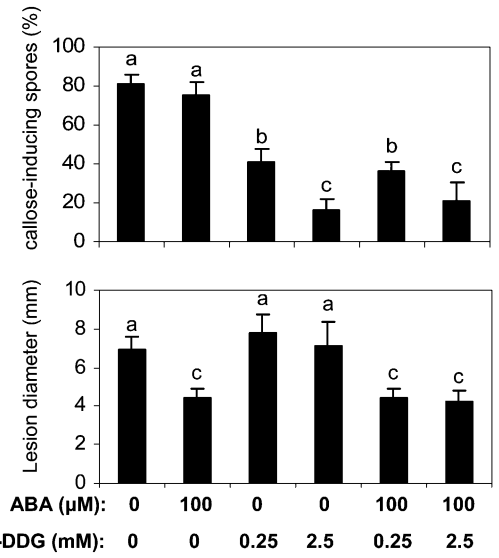

Figure 3. Effects of the callose inhibitor 2-DDG on the levels of basal and ABA-induced resistance against *C. miyabeanus* in rice. Leaves of 5-week-old CO39 plants were detached and supplied from the cut base with different concentrations of ABA and 2-DDG as described in "Materials and Methods." Forty-eight hours later, infiltrated leaves were inoculated with five 10-μL droplets of a *C. miyabeanus* Cm988 conidial suspension (5×10^4 spores mL^{-1}). Resistance was quantified by measuring lesion diameters at 60 hpi. Data shown are means ± SE of at least 19 infection sites from four different leaves. Different letters indicate statistically significant differences (Bonferroni; $\alpha = 0.05$). The experiment was repeated twice with similar results.

tion, with adjacent nonpenetrated cells being void of DAB-detectable H_2O_2 (Fig. 2H). Supplementing the DAB solution with ascorbate markedly reduced staining at the respective sites, indicating that the staining was due to H_2O_2 accumulation (data not shown). At later time points, fungal progression in the mesophyll layer resulted in an intense DAB staining dispersed throughout the inoculation site in both control and ABA-induced plants. However, while control plants developed large, dark-brown patches comprising about 30 DAB-stained mesophyll cells, in ABA-induced sheaths, DAB accumulated in discrete, small clusters with between four and eight mesophyll cells per interaction site. On the macroscopic level, this was reflected by large, DAB-soaked lesions occurring on leaves of challenged control plants (Fig. 2I; 36 hpi), as opposed to the small, pinpoint-size spots visible on ABA-treated plants (Fig. 2J; 36 hpi). Together, these data suggest a dual role of H_2O_2 in the rice-*C. miyabeanus* interaction and argue that one mechanism of ABA action is to modulate pathogenesis-related reactive oxygen species (ROS) formation.

The α-Subunit of Heterotrimeric G-Protein But Not SA Accumulation Is Required for ABA-IR against *C. miyabeanus*

Mounting evidence indicates that defense signaling is not a linear single-response event but a complex network involving a number of different signals and effectors (Koornneef and Pieterse, 2008). Therefore, to further elucidate how ABA-induced plants counteract hyphal invasion, we used several mutant and transgenic rice lines affected in hormonal and nonhormonal resistance pathways to dissect the involvement of known plant defense mechanisms. Considering the fast-growing number of reports that connect ABA signaling to the heterotrimeric G-protein complex (Pandey et al., 2009; Galvez-Valdivieso et al., 2009) and the well-described role of the G-protein α-subunit in rice pathogen defense (Suharsono et al., 2002; Komatsu et al., 2004), we first tested the effectiveness of ABA in mutant *Daikoku dwarf* plants. These so-called *d1* mutant plants, which are in the background of *japonica* cv Nipponbare, are defective in the sole α-subunit of heterotrimeric G-proteins in rice (Ashikari et al., 1999; Fujisawa et al., 1999). Consistent with the role of Gα in basal resistance against the bacterial leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* (Komatsu et al., 2004), *d1* mutants were highly susceptible to infection by virulent *C. miyabeanus* Cm988 (Fig. 4A). Furthermore, in contrast to wild-type Nipponbare plants, mutant *d1* plants failed to develop ABA-IR, indicating that Gα controls both basal and ABA-IR against *C. miyabeanus*. Although it cannot be completely excluded that the ABA-IR-minus phenotype of *d1* is due to the excessive fungal colonization in this mutant, the latter hypothesis is rather unlikely, as the use of lower inoculum densities, resulting in less severe disease symptoms, yielded comparable results (data not shown).

In several plant pathosystems, ABA has been shown to influence disease outcome through its effect on SA-regulated defense (Robert-Seilaniantz et al., 2007; Asselbergh et al., 2008). To investigate the SA dependence of ABA-IR against *C. miyabeanus*, wild-type Nipponbare and SA-deficient NahG transgenic plants (Yang et al., 2004) were routinely sprayed with 0.1 mM ABA and subsequently tested for expression of IR. As shown in Figure 4A, NahG plants retained the strong level of ABA-IR characteristic for wild-type plants, indicating that SA accumulation is not an essential prerequisite for ABA-induced resistance against *C. miyabeanus*. To further probe whether ABA elicits an SA-independent defense mechanism, we examined the expression of the SA-responsive genes *OsPR1b* and *PBZ1* in CO39 plants following challenge infection. Both of these *PR*-like genes are responsive to BTH treatment and have recently been shown to function in the NPR1-dependent branch of the rice SA pathway (Shimono et al., 2007). Quantitative reverse transcription (RT)-PCR analysis revealed that treatment with 0.1 mM ABA alone did not induce transcription of either gene (Fig. 4B). Moreover, at all time points investigated, pathogen-induced *OsPR1b* and *PBZ1* transcription was considerably lower in ABA-supplied plants compared with noninduced controls. Collectively, these data suggest that ABA-IR against *C. miyabeanus* does not rely on boosted expression of SA-inducible defense responses.

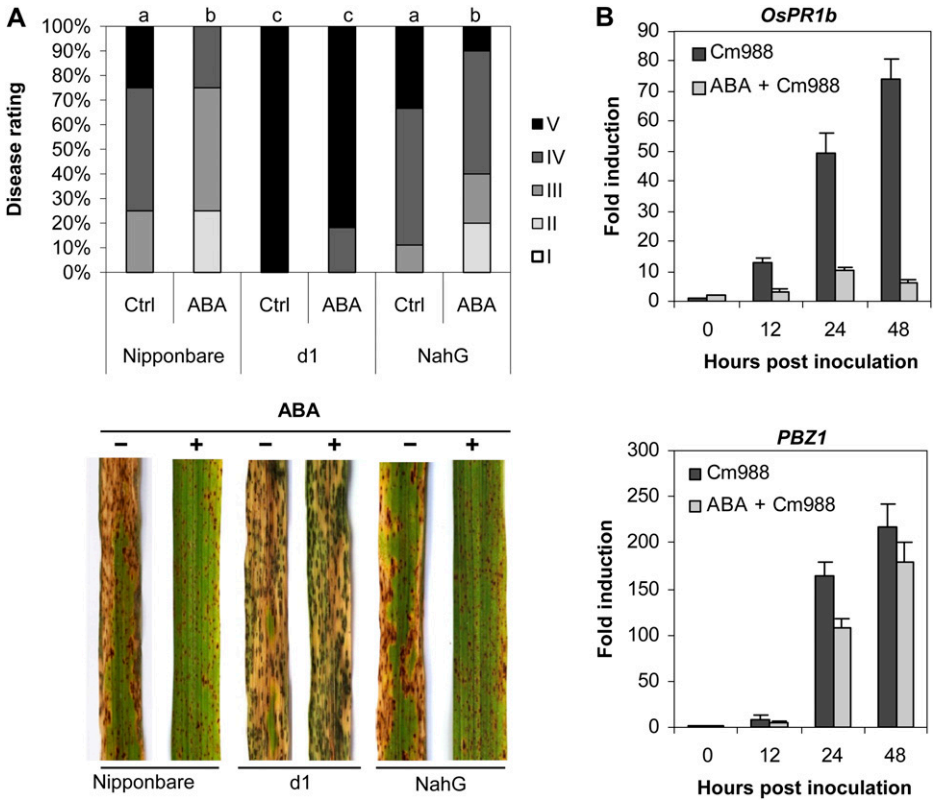

Figure 4. ABA-induced resistance against *C. miyabeanus* is independent of SA accumulation but requires the heterotrimeric G-protein α-subunit. A, Wild-type Nipponbare, mutant *d1*, and transgenic NahG plants were sprayed until runoff with water or ABA (0.1 mM) and, 3 d later, challenged with virulent *C. miyabeanus* Cm988. Disease was evaluated as described in the legend to Figure 1. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken at 5 d post inoculation. Repetition of experiments led to results very similar to those shown. Ctrl, Control. B, Effects of ABA pretreatment on transcript accumulation of *OsPR1b* and *PBZ1* in leaves of CO39 inoculated with Cm988. At the indicated time points post inoculation, fully expanded fifth and sixth leaves from five plants were harvested, converted to cDNA, and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin as an internal reference and expressed relative to the normalized expression levels in mock-treated control plants at 0 h. Data presented are means ± SD of three replicates from a representative experiment. Plants were treated and inoculated as described in Figure 1. [See online article for color version of this figure.]

The Role of JA-Dependent Defenses in ABA-IR against *C. miyabeanus*

Besides cross talk with the SA pathway, ABA has also been found to modulate JA-regulated resistance mechanisms (Anderson et al., 2004; Adie et al., 2007). To decipher the role of the JA-mediated defense pathway in ABA-IR against *C. miyabeanus*, we tested the effectiveness of ABA in the JA biosynthesis mutant *hebiba* (Riemann et al., 2003). By analogy with the results obtained in the Nipponbare and CO39 lines, treatment of wild-type Nihonmasari plants with 0.1 mM ABA resulted in a statistically significant reduction in disease severity compared with noninduced controls (Fig. 5A). Mutant *hebiba* plants, however, failed to develop resistance when induced by ABA, which could point to JA-regulated defenses being an integral part of the ABA-induced resistance machinery. However, as noninduced *hebiba* plants are much more sensitive to *C. miyabeanus* infection than wild-type plants, it is equally possible that the failure of ABA to induce resistance in *hebiba* is due to a lower efficacy of ABA in the face of the high infection pressure in this mutant. To discriminate between these possibilities, we next examined the effect of exogenous ABA application on the activity of lipoxygenase (LOX; EC 1.13.11.12), a key JA biosynthetic enzyme, in wild-type Nihonmasari leaves. Interestingly, whereas ABA pretreatment had no significant impact on the steady-state kinetics of LOX in mock-inoculated controls, it severely attenuated pathogen-induced LOX activation in Cm988-challenged leaves (Fig. 5B). Similar results were obtained when monitoring the expression of the JA-inducible defense gene *JIOsPR10* (Jwa et al., 2001) in leaves of CO39 plants upon infection with Cm988. As expected, *JIOsPR10* mRNAs accumulated to high levels in inoculated control plants, resulting in an approximately 9-fold induction relative to mock-treated plants by 48 hpi (Fig. 5C). However, *JIOsPR10* expression

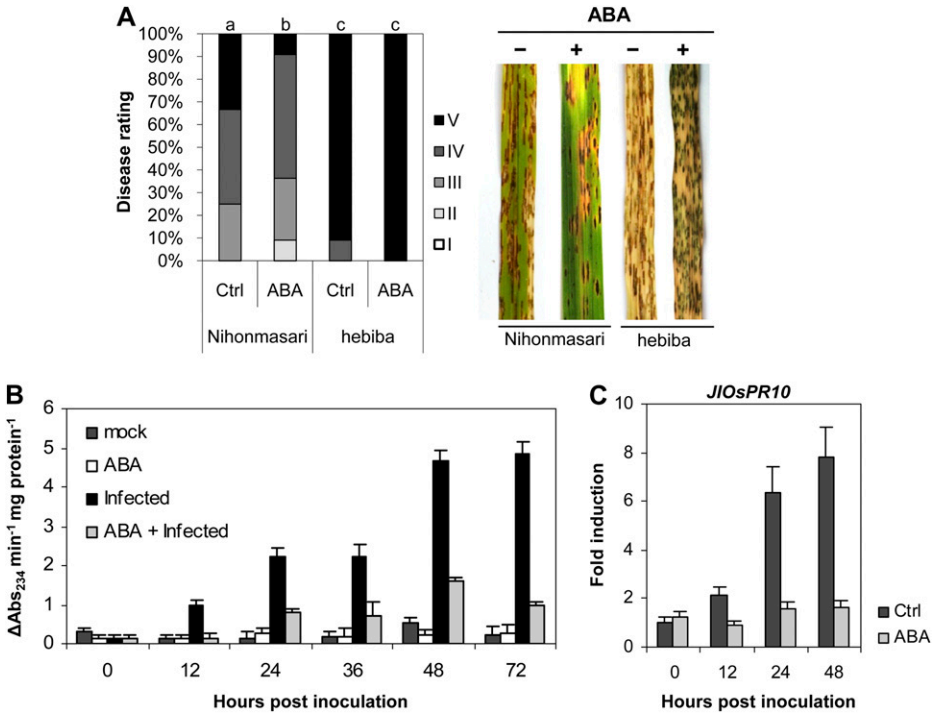

Figure 5. Role of the JA pathway in ABA-IR against *C. miyabeanus* in rice. A, ABA-IR is blocked in the JA-deficient mutant *hebiba*. Wild-type Nihonmasari and mutant *hebiba* plants were sprayed with water or ABA (0.1 mM) and, 3 d later, challenged with the virulent *C. miyabeanus* strain Cm988. Disease was evaluated as described in the legend to Figure 1. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken at 5 d post inoculation. Repetition of experiments led to results very similar to those shown. Ctrl, Control. B, Effects of ABA pretreatment on *C. miyabeanus*-induced LOX activity. LOX activity was measured at 234 nm in samples taken from the fifth and sixth stage leaves of Nihonmasari plants at different time points after inoculation. Each bar represents average data and SD from two independent experiments. C, Effects of ABA pretreatment on expression of the JA-responsive *PR* gene *JIOsPR10* in leaves of CO39 plants inoculated with *C. miyabeanus* Cm988. Data presented are means ± SD of three replicates from a representative experiment. [See online article for color version of this figure.]

was induced only slightly, if at all, in CO39 leaves pretreated with 0.1 mM ABA. Together with the inability of exogenously administered JA to cause substantial disease reduction (Fig. 1B), these results strongly suggest that JA-dependent defense mechanisms do not contribute significantly to ABA-inducible brown spot resistance.

Repression of ET Signaling Confers Enhanced Resistance against *C. miyabeanus*

The observation that ABA-IR against *C. miyabeanus* is not brought about by hyperactivation of SA- or JA-mediated defense responses prompted us to assess the involvement of the ET pathway. To this end, we quantified ABA-IR in wild-type Dongjin and *ETHYLENE INSENSITIVE2* (*OsEIN2*) antisense transgenic plants (Jun et al., 2004). In contrast to Arabidopsis, which carries a single copy of *EIN2*, rice contains two *EIN2*-like genes (*OsEIN2* and *OsEIN2.2*), with *OsEIN2* being the closest homolog of *AtEIN2*, sharing 52% similarity (Rzewuski and Sauter, 2008). Similar to its Arabidopsis counterpart, OsEIN2 is predicted to encode a positive regulator of the rice ET response (Jun et al., 2004). Accordingly, the *OsEIN2* antisense transgenics, which display a somewhat stunted phenotype, exhibit ET insensitivity and show a decreased expression of ET-responsive defense genes (Jun et al., 2004). Yet, under our experimental conditions, noninduced *OsEIN2* antisense seedlings turned out to be significantly more resistant to attack by *C. miyabeanus* than wild-type plants, indicating that ET action negatively interferes with basal resistance to *C. miyabeanus* (Fig. 6A). Moreover, while the level of resistance of noninduced *OsEIN2* transgenic plants mirrored that of ABA-treated wild-type plants, application of exogenous ABA failed to cause an additional reduction in disease severity on the *OsEIN2* transgenics, suggesting that suppression of pathogen-triggered ET signaling may be an important facet of the resistance mechanism underpinning ABA-inducible brown spot resistance. Consistent with this hypothesis, feeding detached leaves with silver thiosulfate (STS), a well-established inhibitor of ET action (Navarre and Wolpert, 1999), significantly reduced symptom development relative to water-treated controls (Fig. 6B). Moreover, coapplication of 0.1 mM ABA

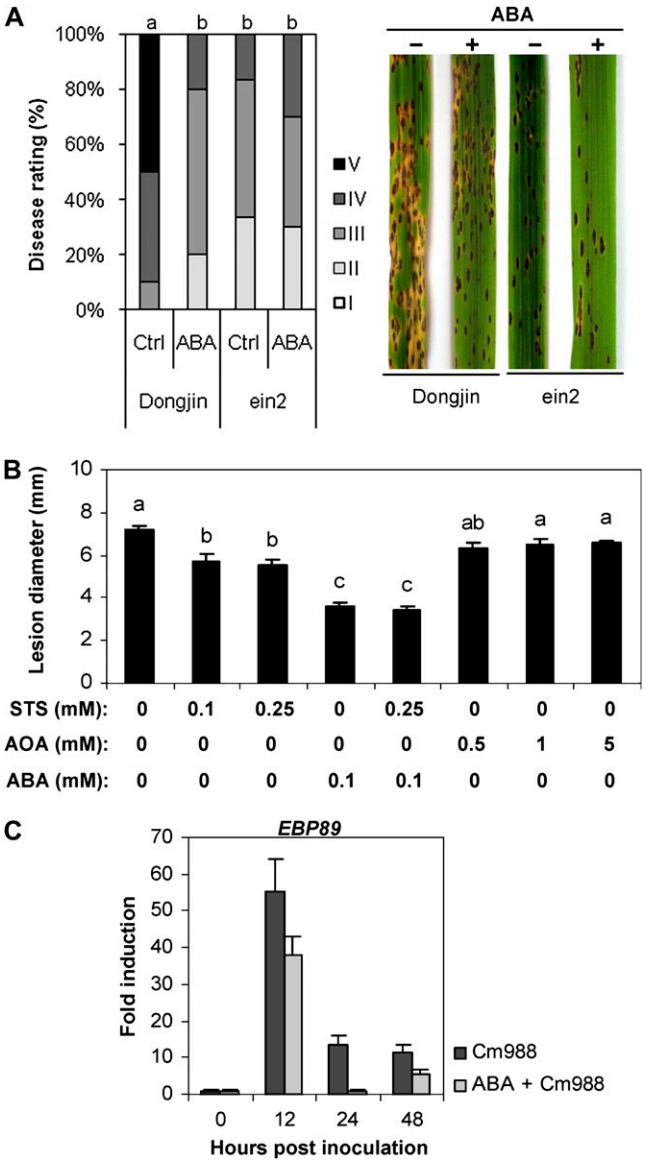

Figure 6. Involvement of the ET pathway in ABA-IR against *C. miyabeanus* in rice. A, Antisense suppression of *OsEIN2* increases brown spot resistance. Wild-type Dongjin and *OsEIN2* antisense plants were sprayed with water or ABA (0.1 mM) and, 3 d later, challenged with virulent Cm988. Disease was evaluated as described in the legend to Figure 1. Different letters indicate statistically significant differences (Mann-Whitney test; $n \geq 12$; $\alpha = 0.05$). Photographs depicting representative symptoms were taken at 5 d post inoculation. Repetition of experiments led to results very similar to those shown. Ctrl, Control. B, Effects of AOA and STS, inhibitors of ET biosynthesis and action, respectively, on *C. miyabeanus* resistance. Leaves of 5-week-old Dongjin plants were detached and supplied from the cut base with different concentrations of AOA and STS. ABA treatment, fungal inoculation, and disease evaluation were performed exactly as described in the legend to Figure 3. Values presented are means and SE of at least 19 infection sites stemming from four different leaf segments. Different letters indicate statistically significant differences (Bonferroni; $\alpha = 0.05$). C, Effects of ABA pretreatment on expression of the ET-responsive transcription factor gene *EBP89* in leaves of CO39 plants inoculated with virulent Cm988. Data presented are means ± SD of three replicates from a representative experiment. [See online article for color version of this figure.]

with 0.25 mM STS did not reveal an additive effect, further supporting our hypothesis. On the other hand, treatment with increasing concentrations of aminooxyacetic acid (AOA), a potent inhibitor of ET biosynthesis in rice (Iwai et al., 2006), yielded significantly lower protection levels, suggesting that ET action, rather than de novo ET biosynthesis, may be important for *C. miyabeanus* pathogenicity. To further test whether ABA-IR against *C. miyabeanus* is associated with a down-regulation of the ET response system, we analyzed the expression of the ET-responsive element-binding protein gene *EBP89* (Yang et al., 2002) in control and ABA-IR-expressing CO39 plants. Figure 6C shows that in control noninduced leaves, *EBP89* transcript levels accumulated rapidly, reaching a maximum 12 h after Cm988 challenge. In ABA-treated samples, *EBP89* expression likewise peaked at 12 hpi, albeit to a significantly lower extent. Furthermore, whereas in control samples *EBP89* mRNA levels were still high at 24 and 48 hpi, showing an approximately 10-fold induction over the mock control, they had decayed to near basal levels in ABA-treated samples. In conjunction with analyses of the *OsEIN2*-suppressed transgenic plants and the disease-promoting effect of exogenously administered ethephon, these results support the notion that activation of the ET pathway favors *C. miyabeanus* infection and strengthen the hypothesis that ABA-IR involves repression of *C. miyabeanus*-induced ET action.

ABA-Induced Resistance against *C. miyabeanus* Depends on the MAPK Gene *OsMPK5*

Protein kinases operate at the core of signal transduction networks, channeling information from upstream effectors to downstream cellular responses. One such kinase is the ABA-inducible MAPK OsMPK5, whose role as a critical regulator of pathogen defense and abiotic stress tolerance in rice is well documented (Xiong and Yang, 2003). In order to examine whether *OsMPK5* is also involved in ABA-IR against *C. miyabeanus*, we initially analyzed *OsMPK5* transcript levels in control noninduced and ABA-IR-expressing CO39 plants. As shown in Figure 7A, *OsMPK5* showed a potentiated expression pattern in ABA-treated plants following Cm988 challenge, indicating that ABA primes rice for enhanced *OsMPK5* transcription. To determine whether this primed *OsMPK5* response is required for ABA-IR against *C. miyabeanus*, we determined the level of ABA-inducible brown spot resistance in the *OsMPK5*-suppressed transgenic line RI3 (Xiong and Yang, 2003). This transgenic line, which exhibits constitutive expression of several *PR* genes, was generated by introducing a double-stranded RNA interference (RNAi) construct in the background of cv Nipponbare (Xiong and Yang, 2003). Although symptom development was slightly accelerated in the double-stranded RNAi transgenics, we were unable to detect any reproducible or significant differences in overall disease severity between noninduced wild-type plants and similarly treated

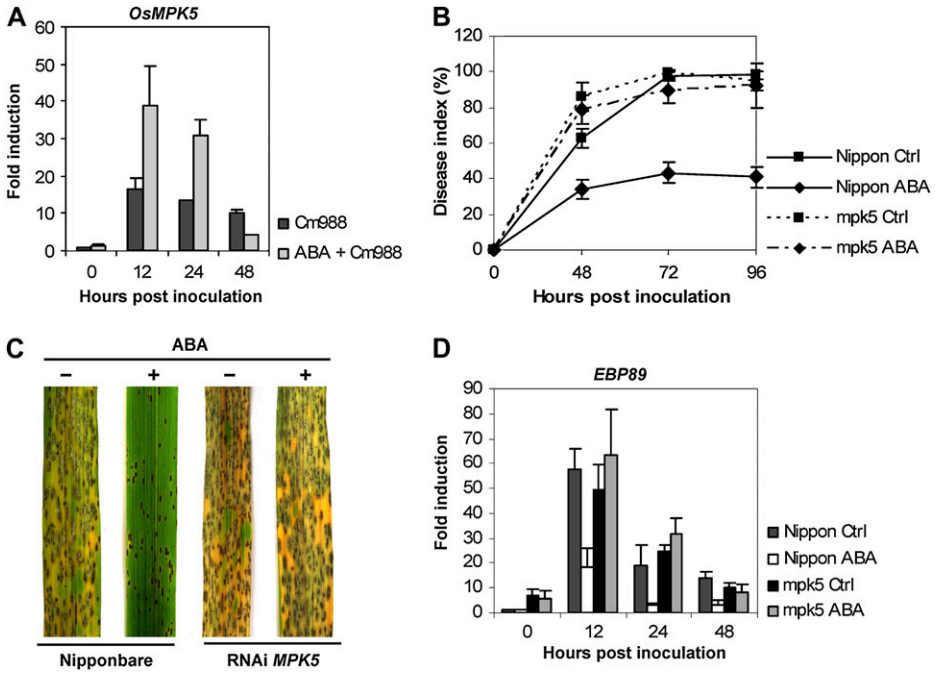

Figure 7. ABA induces resistance to *C. miyabeanus* in an *OsMPK5*-dependent manner. A, Effects of ABA pretreatment on *OsMPK5* transcript accumulation in leaves of CO39 plants inoculated with virulent *C. miyabeanus* Cm988. Data presented are means ± SD of three replicates from a representative experiment. B and C, RNAi-mediated silencing of *OsMPK5* attenuates ABA-IR. Photographs depicting representative symptoms were taken at 5 d post inoculation. Plants were treated and inoculated as described in Figure 1. D, ABA-induced repression of *C. miyabeanus*-activated ET signaling is blocked in RNAi *OsMPK5* plants. Quantitative RT-PCR analysis of *EBP89* transcription in wild-type Nipponbare and *OsMPK5*-suppressed plants was performed exactly as described in Figure 6. Data presented are means ± SD of three replicates from a representative experiment. [See online article for color version of this figure.]

OsMPK5-silenced plants, suggesting that *OsMPK5* only plays a minor role in basal resistance against *C. miyabeanus* (Fig. 7, B and C). However, *OsMPK5* does appear to be indispensable for ABA-IR against *C. miyabeanus*, as treatment with 0.1 mM ABA resulted in a substantial reduction of disease in wild-type Nipponbare but not in *OsMPK5*-silenced plants.

The observation that expression of *OsMPK5* is necessary for ABA to induce resistance to *C. miyabeanus* prompted us to assess whether this MAPK gene also is implicated in orchestrating ABA/ET cross talk in rice. To this end, we tested wild-type Nipponbare and RNAi *OsMPK5* plants for expression of the ET reporter gene *EBP89*. In keeping with the results obtained in the CO39 background, *EBP89* expression responded strongly to pathogen infection in both wild-type and transgenic Nipponbare plants, resulting in an approximately 50-fold induction by 12 hpi. However, suppression of Cm988-induced *EBP89* expression resulting from ABA pretreatment, a typical reaction in wild-type plants, was severely attenuated in *OsMPK5*-silenced plants. In light of the results presented in Figure 6, we interpret these data to suggest that RNAi-mediated suppression of *OsMPK5* affects ABA-induced resistance against *C. miyabeanus* by blocking the antagonistic action of ABA on ET signaling.

DISCUSSION

With its relatively compact and fully sequenced genome, ease of transformation, well-developed genetics, and the availability of a dense physical map, rice is considered a model monocot system (Hsing et al., 2007; Miyao et al., 2007; Jung et al., 2008). However, although significant progress has been made in cloning rice disease resistance genes and functional genomics in general (Jung et al., 2008; Leung, 2008), little is known about the effector responses and hormonal signaling pathways operative in determining rice resistance. In this study, we have analyzed the cellular and molecular basis of rice brown spot disease, caused by the fungal pathogen *C. miyabeanus*. The data presented here offer, to our knowledge, a first insight into the myriad cellular responses that enable rice to fend off *C. miyabeanus* infection and have revealed several heretofore unknown aspects of pathway cross talk in the rice signaling circuitry. In particular, we have shown that exogenous application of ABA induces resistance against *C. miyabeanus* via a multilayered defense response involving the suppression of pathogen-induced ET action. Furthermore, our data indicate that this ABA/ET antagonism is orchestrated by the ABA-inducible MAPK gene *OsMPK5*.

In contrast to the well-established role of ABA in abiotic stress adaptation (Fujita et al., 2006), its contribution to disease resistance is less well understood and even contentious. Whereas the majority of reports have shown an inverse correlation between endogenous ABA levels and resistance to pathogens with diverse parasitic habits in several plant species (Audenaert et al., 2002; Mohr and Cahill, 2003; Asselbergh et al., 2007; de Torres-Zabala et al., 2007, 2009), others have pinpointed a positive role for this hormone in plant defense activation (Ton et al., 2005; Adie et al., 2007; Hernandez-Blanco et al., 2007). This ambivalent ABA response is also reflected in rice-pathogen interactions. For example, while our data uncover ABA as a powerful activator of resistance against *C. miyabeanus*,

Koga et al. (2004a) previously reported that exogenous ABA lowers basal resistance against the rice blast pathogen *M. oryzae*. Likewise, ABA-regulated genes were among the first to be induced in a compatible rice-blast interaction (Ribot et al., 2008). Even within the same plant-pathogen interaction, ABA may have divergent effects depending on the timing of infection (Ton et al., 2009). In Arabidopsis inoculated with *P. syringae*, for example, ABA-induced stomatal closure is a key element of preinvasion innate immunity, while ABA suppresses postinvasion disease resistance (Melotto et al., 2006; de Torres-Zabala et al., 2007, 2009). Hence, a complex picture is emerging in which ABA functions as a multicomponent regulator of different components of plant defense, with apparently divergent effects.

In line with the multiplex role of ABA in modulating plant disease resistance, a wide range of putative mechanisms underpinning ABA action have been proposed, including induction of stomatal aperture and repression of SA- and JA-regulated basal defenses (Ton et al., 2009). One of the most comprehensively studied defense responses in relation to ABA-provoked fungal resistance is the enhanced deposition of callose at sites of attempted pathogen penetration (Ton and Mauch-Mani, 2004; Flors et al., 2008). In our system, however, we found no compelling evidence for the involvement of callose, as pharmacological disruption of callose formation with the callose inhibitor 2-DDG did not significantly interfere with the resistant response of ABA-induced plants (Fig. 7). Recently, the generation of ROS emerged as an another important connection between ABA signaling and pathogen defense (Torres et al., 2006; Asselbergh et al., 2008). Most tellingly, several lines of evidence indicate that the same NADPH-dependent respiratory burst oxidase homologs are involved in H_2O_2 formation, leading to ABA-induced stomatal closure and elicitation of hypersensitive cell death in response to avirulent microbes (Torres et al., 2006). Under our experimental conditions, H_2O_2 in control plants started to accumulate from 12 hpi in the anticlinal walls of epidermal and mesophyll cells surrounding the site of infection, where it was intimately associated with spreading cell death (Fig. 2F). In ABA-treated plants, on the other hand, H_2O_2 was often present from 8 hpi specifically in the anticlinal walls of infected epidermal cells, with neighboring nonpenetrated cells remaining essentially free of H_2O_2 (Fig. 2G). Such a bimodal H_2O_2 pattern suggests that *C. miyabeanus*, in spite of its generally assumed purely necrotrophic lifestyle, might have a short biotrophic phase in the epidermis during which it is sensitive to H_2O_2-dependent defenses. This is supported by the frequent observation of living though hyphae-containing epidermal cells in the early stages of fungal infection (i.e. prior to 12 hpi; Fig. 2F; data not shown). Accordingly, although not sufficient to block *C. miyabeanus* ingress, the prompt formation of H_2O_2 in ABA-treated plants may slow down fungal invasion, allowing the plant to adequately mobilize the available biochemical and structural defenses to effectively halt the invading pathogen in the mesophyll. On the other hand, one might speculate that H_2O_2 accumulation in nonpenetrated control cells may support cell death to pave the way for *C. miyabeanus* in its necrotrophic growth stage.

To further scrutinize the underlying mechanism of ABA-inducible brown spot resistance, we attempted to gain insight into the signaling circuitry governing the induced resistance phenotype. One particularly interesting finding in this regard was the hypersusceptibility of so-called *d1* mutant plants, which are defective in the sole heterotrimeric G-protein α-subunit gene present in rice (Fujisawa et al., 1999). Interestingly, the *d1* mutation not only affected basal resistance to *C. miyabeanus* but also blocked the expression of ABA-IR, which points to a mechanistic connection between G-protein signaling at the cell surface and ABA-inducible pathogen resistance. This view is consistent with the recent discovery of the Arabidopsis Gα-subunit gene *GPA1* as a central element in ABA-dependent innate immunity in stomatal guard cells (Zhang et al., 2008). Moreover, there is ample evidence indicating that ABA signaling processes in both seeds and guard cells involve components of the heterotrimeric G-protein complex, further supporting our hypothesis (Pandey and Assmann, 2004; Fan et al., 2008; Wang et al., 2008). According to the G-protein signaling paradigm, the lack of Gα not only abolishes Gα-mediated signaling but also results in free G$\beta\gamma$, thereby possibly enhancing G$\beta\gamma$ signal output (Pandey et al., 2006). The increased disease susceptibility and lack of ABA-IR observed in *d1*, therefore, could be accounted for by either loss of the corresponding Gα-mediated signaling or by the constitutive activation of the G$\beta\gamma$ subunit.

Similar to *d1*, the JA-deficient mutant *hebiba* failed to express resistance when induced by ABA, suggesting that ABA-IR might develop coincidently with increases in endogenous JA levels (Fig. 5A). Although such a concept would be consistent with recent results in Arabidopsis supporting a model for ABA inducing JA biosynthesis in the activation of defenses against the soil-borne oomycete *Pythium irregulare* (Adie et al., 2007), it is hard to reconcile with our findings that exogenous ABA treatment alleviated *C. miyabeanus*-induced activity of the key JA biosynthetic enzyme LOX and that ABA-IR was not affected upon infiltration of the LOX inhibitors 5,8,11,14-eicosatetraynoic acid (ETYA) and salicylhydroxamate (SHAM; Fig. 5B; data not shown). Moreover, in accordance with the failure of exogenous JA to induce brown spot resistance, transcriptional analysis of the JA-inducible *JIOsPR10* gene (Jwa et al., 2001) did not reveal any primed activity of the JA defense pathway in ABA-treated plants (Figs. 1 and 5B). Therefore, it can be concluded that ABA-mediated protection against *C. miyabeanus* does not rely on the potentiation of JA-inducible defenses. In a similar vein, the failure of BTH to reduce disease severity as well as the ability

of ABA to trigger resistance in SA-deficient NahG rice also rule out a major involvement of the SA pathway.

Besides interactions with SA and JA, there is overwhelming evidence that ABA modulates ET signaling (Beaudoin et al., 2000; Ghassemian et al., 2000; Tanaka et al., 2005). Although most examples of ABA-ET interactions have been described in sugar signaling (Leon and Sheen, 2003), Anderson et al. (2004) elegantly demonstrated the existence in Arabidopsis of an antagonistic ABA-ET connection that interferes with defense gene expression and disease resistance against the necrotroph *Fusarium oxysporum*. Interestingly, several pieces of evidence suggest that such negative ABA-ET cross talk also contributes to the beneficial effect of ABA on brown spot resistance. First, symptoms caused by brown spot infection were more severe on wild-type seedlings pretreated with the ET-releasing chemical ethephon, implying a negative role for ET in rice defense to *C. miyabeanus* (Fig. 1A). Second, disruption of the ET pathway, either by antisense suppression of *OsEIN2*, a central signal transducer in the rice ET pathway (Jun et al., 2004), or infiltration of STS, a well-known inhibitor of ET action, yielded significant levels of protection, often similar to those found in ABA-treated plants (Fig. 6, A and B). Moreover, foliar ABA treatment of the *OsEIN2* transgenics or coapplication of ABA with STS did not cause a further increase in resistance, suggesting that ABA specifically targets the ET pathway to condition brown spot resistance. In line with this, Bailey et al. (2009) recently found that exogenous application of ABA drastically reduces endogenous ET levels in rice. Third, transcript levels of the ET-responsive transcription factor gene *EBP89* were markedly lower in ABA-treated plants than in wild-type plants after *C. miyabeanus* attack. Fourth, RNAi suppression of the ABA-primed MAPK gene *OsMPK5* derepressed *C. miyabeanus*-activated *EBP89* transcription, a phenomenon that coincided with a loss of ABA-IR. When considered together, these results favor a model whereby ABA protects rice from *C. miyabeanus* attack at least in part by antagonizing pathogen-induced ET signaling in an *OsMPK5*-dependent manner. Implicit here is the view that *C. miyabeanus* hijacks the rice ET signaling pathway as a decoy strategy to suppress other, possibly ABA-dependent, defenses that normally serve to limit pathogen growth. In this respect, it is significant that several plant pathogens can produce ET themselves (Adie et al., 2007). For instance, some *P. syringae* pathovars have shown an ability to synthesize ET both in vitro and in planta from Met through the 2-keto-4-methylthiobutyric acid pathway (Weingart et al., 2001). This ability, together with the production of the JA mimic coronatine and auxins by the same microorganisms, is assumed to contribute to hormonal saturation and consequent circumvention of effectual defenses (Cui et al., 2005; Sreedharan et al., 2006). More recently, *Ralstonia solanacearum* has been seen to produce ET by means of the HrpG regulon (Valls et al., 2006). Sufficient to affect the plant ET response pathway, bacterial ET production is simultaneous with type 3 secretion system gene expression and contributes to the plant defense imbalance that favors pathogen infection. In light of these findings, it is not unlikely that *C. miyabeanus* may likewise synthesize ET in order to tap into the rice signaling infrastructure to interfere with host defense. Alternatively or in addition, *C. miyabeanus* may impact ET-responsive rice defenses via secretion of heretofore-specified effector proteins. Whichever mechanism is operative, manipulating plant hormone signaling and hijacking host hormonal cross talk mechanisms represent an extremely powerful virulence strategy, considering the global impact of hormone homeostasis on multiple cellular responses (de Torres-Zabala et al., 2007; Spoel and Dong, 2008).

Intriguingly, the concept that ABA-IR is based on *OsMPK5*-mediated repression of *C. miyabeanus*-induced ET action may also provide a mechanistic explanation for the aforementioned Gα-dependence of this resistance. In some interesting work using various *d1* mutant lines, Lieberherr et al. (2005) previously uncovered a pivotal role of Gα in modulating the stability and sphingolipid elicitor-induced activation of the MAPK OsMPK1, thus linking rice Gα to MAPK regulation. To our interest, these authors also demonstrated that RNAi-mediated knockdown of *OsMPK1* results in constitutive expression of *OsMPK5*, the latter being suggestive of potential cross talk and possible functional redundancy between these evolutionarily related MAPKs. In light of these findings, it is not inconceivable that the inability of *d1* to develop ABA-

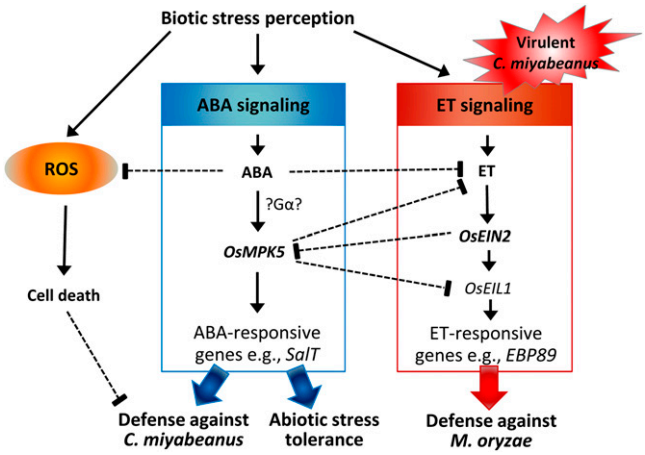

Figure 8. Model illustrating how ABA, ET, ROS, and the antagonistic interaction among the latter signaling molecules mold rice disease resistance and abiotic stress tolerance. The model is based on new data generated in this study and previous results of Bailey et al. (2009). Sharp, solid lines represent stimulatory effects, and blunted, dotted lines depict antagonistic interactions. [See online article for color version of this figure.]

IR against *C. miyabeanus* might result, at least in part, from a defect in OsMPK5 functioning.

Previously, several other studies have assessed the regulatory roles of ABA and ET in the rice defense system. An interesting picture emerges when comparing our results with *C. miyabeanus* and those obtained with the leaf blast pathogen *M. oryzae*. For instance, whereas our results clearly show that ET action negatively interferes with resistance to *C. miyabeanus*, Iwai et al. (2006) recently uncovered ET biosynthesis to be an integral component of *R* gene-mediated blast resistance, a finding that is consistent with the long-reported ability of ethephon to lower susceptibility in compatible rice-blast interactions (Singh et al., 2004). Taking these facts into account, it is tempting to speculate that ET acts as a two-faced player in the rice defense network, by alleviating stress caused by *M. oryzae* but promoting susceptibility to *C. miyabeanus*. In support of this, it was shown before that antisense suppression of *OsEIN2* not only confers ET insensitivity and heightened resistance against *C. miyabeanus* but also increases susceptibility to *M. oryzae* (De Vleesschauwer et al., 2008). Strikingly, we recently found *OsEIN2* antisense plants also to be more tolerant to cold, drought, and salinity stresses (Supplemental Fig. S1A), suggesting that *OsEIN2* positively regulates ET signaling and *M. oryzae* resistance while repressing abiotic stress-adaptive ABA responses and resistance to *C. miyabeanus*. Supportive of this notion is the observation that similar to Arabidopsis *ein2* mutants (Beaudoin et al., 2000), *OsEIN2* antisense plants are supersensitive to ABA treatment, as evidenced by reduced seed germination in the presence of ABA and hyperactivation of ABA-inducible genes such as *SalT* and *OsMPK5* (Supplemental Fig. S1, B and C). These data are particularly interesting in light of previous studies showing that RNAi suppression of *OsMPK5* not only reduces ABA sensitivity but also leads to constitutive expression of *PR* genes and enhanced resistance to *M. oryzae* (Xiong and Yang, 2003; Bailey et al., 2009). Compared with wild-type rice, *OsMPK5* suppression lines also exhibit a higher level of ET and a lower level of ABA, while recombinant OsMPK5 was recently found to directly interact with and phosphorylate rice ETHYLENE INSENSITIVE3-LIKE1, an ortholog of Arabidopsis EIN3 (Bailey et al., 2009). In conjunction with the inability of ABA to trigger brown spot resistance in an OsMPK5 RNAi line and the apparent derepression of pathogen-induced ET signaling in this ABA-insensitive background (Fig. 7), these findings draw important inferences tagging OsMPK5 and OsEIN2 as important modulators of ABA-ET cross talk in rice, with corresponding trade-offs between *C. miyabeanus* defense and abiotic stress tolerance, on the one hand, and resistance to *M. oryzae*, on the other hand. A schematic representation of these data illustrating the differential roles of ABA and ET in rice disease resistance and abiotic stress tolerance is presented in Figure 8.

CONCLUSION

In summary, we have shown that exogenous ABA treatment protects rice from infection by the brown spot pathogen *C. miyabeanus* through induction of a multilayered defense response. Besides modulating *C. miyabeanus*-induced ROS formation and requiring intact Gα signaling, we demonstrated ABA to antagonize pathogen-activated ET signaling via the MAPK gene *OsMPK5*. Moreover, the results presented here and those published previously (Xiong and Yang, 2003; Singh et al., 2004; Iwai et al., 2006; Bailey et al., 2009) highlight the fine control of rice defenses to *C. miyabeanus* and the blast pathogen *M. oryzae* through the differential engagement and balance of the ABA and ET response systems. Whether or not *C. miyabeanus* disease resistance shares substantial overlap with the signaling cascade(s) governing abiotic stress tolerance, and how Gα-mediated signaling affects ABA-IR, remain to be elucidated.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rice (*Oryza sativa*) lines used in this work included the *japonica* cv Nipponbare, the corresponding NahG (Yang et al., 2004) and RNAi *OsMPK5* transgenics (Xiong and Yang, 2003), and the *indica* cv CO39, the latter being a kind gift from the International Rice Research Institute. The Gα-deficient mutant *d1* (line DK-22), the *OsEIN2* antisense transgenic line 471 (wild type: *japonica* cv Dongjin), JA-deficient mutant *hebiba*, and the corresponding wild-type lines Nipponbare, Dongjin, and Nihonmasari, all *japonica*, were kindly provided by M. Matsuoka (Nagoya University), G. An (Yonsei University), and P. Nick (Karlsruhe University), respectively.

Seeds were surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed three times in sterile distilled water, and germinated on wet sterile filter paper in sealed petri dishes (92% or greater relative humidity) at 28°C. Five days later, germinated seeds were grown in commercial potting soil (Universal; Snebbout) under nonsterile greenhouse conditions (30°C ± 4°C, 16-h-light/8-h-dark regime) as described previously (De Vleesschauwer et al., 2006). Plants were watered daily and fertilized with 5 g m^{-2} $(NH_4)_2SO_4$ and 10 g m^{-2} $FeSO_4{\cdot}7H_2O$ on days 8, 15, 22, and 29 after sowing. Five-week-old plants (six- to seven-leaf stage) were used for infection with *Cochliobolus miyabeanus*. For seed multiplication, plants were propagated in the greenhouse and fertilized with 0.5% ammonium sulfate every 2 weeks until flowering.

Pathogen Inoculation and Disease Rating

C. miyabeanus strains Cm988 and Cm963, obtained from the International Rice Research Institute, were grown for sporulation on potato dextrose agar at 28°C. 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 3 d to induce sporulation. Upon sporulation, conidia were harvested as described by Thuan et al. (2006) and resuspended in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich G-6650) to a final density of 1×10^4 conidia mL^{-1}. For inoculation, 5-week-old seedlings (6.5-leaf stage) were misted with conidial suspension (1 mL per plant) using an artist airbrush powered by an air compressor. Immediately following inoculation, plants were moved into a dew chamber (30°C ± 4°C, 92% or greater relative humidity) to facilitate fungal penetration and, 18 h later, transferred to greenhouse conditions (28°C ± 4°C, 16-h-light/8-h-dark regime) for disease development. Disease symptoms were scored at 4 d after inoculation, and disease ratings were expressed on the basis of diseased leaf area and lesion type using a I to V disease severity scale: I, no infection or less than 2% of leaf area infected with small brown specks less than 1 mm in diameter; II, less than 10% of leaf area infected with brown spot lesions with gray to white centers, about 1 to 3 mm in diameter; III, average of about 25% of leaf area infected with brown spot lesions with gray to white centers, about 1 to 3 mm in

diameter; IV, average of about 50% of leaf area infected with typical spindle-shaped lesions, 3 mm or longer with necrotic gray centers and water-soaked or reddish brown margins, with little or no coalescence of lesions; V, more than 75% of leaf area infected with coalescing spindle-shaped lesions. All infection trials were repeated at least twice with similar results.

Chemical and Abiotic Treatments

JA, ABA, ethephon (2-chloroethyl phosphonic acid), and BABA were purchased from Sigma. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was a gift from Syngenta Crop Protection. BTH, SA, and BABA were directly dissolved in water containing 0.02% (v/v) Tween 20, whereas ABA and JA were first dissolved in a few drops of ethanol and methanol, respectively. Equivalent volumes of both solvents were added to separate control treatments to ensure that they did not interfere with the experiments. For chemical treatment of plants, intact seedlings (6.5-leaf stage) were sprayed until near runoff with a fine mist of either compound at the indicated concentrations. Control plants were sprayed evenly with a 0.02% (v/v) Tween 20 solution only. Three days post application, chemical-treated plants were challenged with *C. miyabeanus* as described above. BABA was applied as a soil drench (16 or 30 mg L^{-1}) 1 or 2 d prior to challenge inoculation. Only soil-drench treatments were used to avoid formation of necroses observed after spraying, because such necroses might induce a systemic acquired resistance-like resistance pathway and mask the primary effect of BABA (Zimmerli et al., 2000).

Abiotic stress treatments were conducted on 4-week-old seedlings according to Xiong and Yang (2003). For cold stress, seedlings were transferred to 4°C for 5 d and then returned to greenhouse conditions for recovery. Drought stress was induced by withholding water for up to 6 d, while salt stress was triggered by immersing roots in 250 mM NaCl solution for up to 5 d. The levels of cold, drought, and salt tolerance were evaluated based on the percentage of surviving seedlings after 7 d of recovery.

ABA Germination Assays

Seeds of wild-type Dongjin and the *OsEIN2* antisense line 471 were surface sterilized with 2% sodium hypochlorite solution as described above, plated on Murashige and Skoog medium supplemented with the indicated concentrations of ABA, and incubated under laboratory conditions (21°C ± 3°C, 16-h-light photoperiod). Germination ratios were expressed as the number of germinated seeds relative to the total number of seeds plated, with the final number being normalized to the germination frequency on ABA-free medium.

Visualization of Defense Responses

To gain more insight into the cytomolecular nature of ABA-IR against *C. miyabeanus*, intact leaf sheath assays were performed as stated by Koga et al. (2004b). Briefly, leaf sheaths of the sixth leaves 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 of *C. miyabeanus* (1×10^4 conidia mL^{-1}). 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 6, 8, 10, 12, 18, 24, 36, and 72 hpi, and at least six trimmed sheath tissue sections originating from three plants were used for each sampling point. Intracellular hyphae were visualized using a modified KOH-aniline blue technique (Hood and Shew, 1996). Fresh specimens were autoclaved for 10 min at 121°C in 1 M KOH, followed by three rinses in demineralized water to remove excess stain. Rinsed specimens were next mounted on glass slides in several drops of the stain solution and examined under UV light excitation. The stain solution was prepared at last 2 h prior to use as 0.05% aniline blue dye in 0.067 M K_2HPO_4 at pH 9.0. Alternatively, hyphae were stained with 0.1% calcofluor M2R for 1 min and rinsed with demineralized water before microscopic observation. Phenolic compounds, on the other hand, were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set; excitation, 450–480 nm; dichroic beam splitter, 500 nm; barrier filter BA515). To detect H_2O_2 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 DAB-HCl (1 mg mL^{-1}, 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 H_2O_2 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 to the DAB solution. For analysis of callose deposition, trimmed sheaths were stained for 5 min in a solution containing 0.01% (w/v) aniline blue and 0.15 M K_2HPO_4. Callose-stained segments were examined using epifluorescence microscopy with UV filter (Olympus U-MWU2 filter set; excitation, 330–385 nm; DM 400 dichroic beam splitter and BA420 long-pass filter). 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.

Pharmacological Experiments

AOA, a potent inhibitor of ET biosynthesis, the LOX inhibitors ETYA and SHAM, and the callose synthase inhibitor 2-DDG were purchased from Sigma. STS, an inhibitor of ET action, was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M silver nitrate in a 4:1 ratio (Shoresh et al., 2005). All chemicals were dissolved in water at the indicated concentrations, with the exception of ETYA, which was solubilized in a few drops of ethanol prior to diluting in water (0.2% ethanol).

To evaluate the contribution of callose formation to the ABA-induced resistance, fifth and sixth stage leaves of 5-week-old rice plants were detached and placed in Falcon tubes containing 25 mL of 0.1 mM ABA. After 16 h of incubation, ABA-supplied leaves were transferred to a fresh Falcon tube containing either sterile demineralized water or 2-DDG and incubated for a further 32 h. Subsequently, detached leaves were cut into 7-cm segments, placed onto a glass slide in 14.5- × 14.5-cm petri dishes lined with moist filter paper, and drop inoculated with five 10-μL droplets of *C. miyabeanus* conidial suspension (5×10^4 conidia mL^{-1} in 0.25% gelatin). Control leaves were mock inoculated with a 0.25% (w/v) gelatin suspension. After 24 h, the droplets were removed with a laboratory tissue, and resistance was quantified by measuring lesion diameters at 60 hpi. Unlike ABA and 2-DDG, AOA and STS were applied 24 and 2 h before inoculation, respectively. In the case of ETYA and SHAM, a slightly different application method was used in that these chemicals were infiltrated in approximately 20-μL aliquots into five sites on the abaxial surface of detached leaf segments using a syringe without a needle. Approximately 8 h later, 10 μL of *C. miyabeanus* conidial suspension (5×10^4 conidia mL^{-1} in 0.25% gelatin) was drop inoculated onto the center of the infiltrated regions.

Enzyme Extraction and LOX Activity Assay

Leaf samples taken from the fifth and sixth stage leaves of approximately six to 10 plants at different time points after inoculation were crushed to a fine powder under liquid nitrogen. Soluble proteins were extracted by resuspending the powder (100 mg fresh weight) in 0.9 mL of 50 mM Na_3PO_4 buffer, pH 6.5, containing 2% polyvinylpyrrolidone, 5 mM 2-mercaptoethanol, and 0.25% Tween 20. The extracts were then incubated on ice for 30 min and centrifuged at 14,000 rpm for 10 min. The resulting supernatant was divided into aliquots, frozen in liquid nitrogen, and stored at −80°C for further analysis. LOX activity was determined at 30°C in 1 mL (final volume) of 50 mM Na_3PO_4 buffer, pH 6.5, containing 0.25% Tween 20 and 30 μL of extract supernatant. The reaction was started by adding 100 μL of 10 mM linoleic acid, and the increase in A_{234} was recorded for 10 min. LOX enzyme activity was calculated based on the slope of the linear part of the plot and expressed as ΔExt_{234} per min per mg of protein. Controls without the addition of plant extracts were recorded as described above and subtracted from the values obtained with the plant extracts. Protein levels in enzyme extracts were determined by the Bradford method (Bradford, 1976) with bovine serum albumen as a standard.

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 Turbo DNase treated according to the provided protocol (Ambion/Applied Biosystems). Before first-strand cDNA synthesis, the absence of genomic DNA was confirmed by PCR.

RNA concentration was checked before and after Turbo DNase digestion. First-strand cDNA was synthesized from 2 μg of total RNA using Affinityscript reverse transcriptase and oligo(dT) primers (Stratagene/Bio-Connect), according to the manufacturer's instructions. Nucleotide sequences of all primers are given in Supplemental Table S2. For each primer pair, the optimal annealing temperatures were predetermined by gradient PCR using a Thermocycler (Bio-Rad). Only primer pairs for which PCR efficiency varied between 90% and 110%, as determined by standard amplification curves constructed from 5-fold dilutions of cDNAs, were used for expression studies. 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 10 min, followed by 40 cycles of 95°C for 30 s, 57°C to 62°C for 60 s, and 72°C for 60 s. 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 (Os03g50890) 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 and ABA-treated samples was expressed relative to the calibrator and as a ratio to actin expression using the measured efficiency for each gene.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Characterization of *OsEIN2* antisense seedlings.

Supplemental Table S1. Virulence pattern of *C. miyabeanus* strains used in this study.

Supplemental Table S2. List of quantitative RT-PCR primers.

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