ABA Is Required for *Leptosphaeria maculans* Resistance via ABI1- and ABI4-Dependent Signaling

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Absciscic acid (ABA) is a defense hormone with influence on callose-dependent and -independent resistance against *Leptosphaeria maculans* acting in the *RLM1*_{Col} pathway. ABA-deficient and -insensitive mutants in *Ler*-0 background (*aba1-3* and *abi1-1*) displayed susceptibility to *L. maculans*, along with a significantly decreased level of callose depositions, whereas *abi2-1* and *abi3-1* remained resistant, together with the *abi5-1* mutant of Ws-0 background. Suppressor mutants of *abi1-1* confirmed that the *L. maculans*-susceptible response was due to the dominant negative nature of the *abi1-1* mutant. Highly induced camalexin levels made ABA mutants in Col-0 background (*aba2-1*, *aba3-1*, and *abi4-1*) appear resistant, but displayed enhanced susceptibility as double mutants with *pad3-1*, impaired in camalexin biosynthesis. β -Aminobutyric acid (BABA) pretreatment of *Ler*-0 contributed to an elevated level of endogenous ABA after *L. maculans* inoculation. Comparisons between (*RLM1*_{Col})*pad3* and *rlm1*_{Ler}*pad3* showed that ABA and BABA enhancement of callose deposition requires induction from *RLM1*_{Col}. *ABI1*, but not *ABI2*, was found to be involved in a feedback mechanism that modulates *RLM1*_{Col} expression. Genetic analysis showed further that this feedback occurs upstream of *ABI4* and that components downstream of *ABI4* modulate *ABI1* activity. ABA and BABA treatments of the *L. maculans*-susceptible callose synthase mutant *pmr4* showed that ABA also induces a callose-independent resistance. Similar treatments enhanced callose depositions and induced resistance to *L. maculans* in oilseed rape, and BABA-induced resistance was found to be independent of salicylic acid.

Additional keywords: *Arabidopsis*, defense, resistance gene.

Several pathways playing crucial roles in plant defense signaling are well documented, not least the roles of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Glazebrook 2005; Hammond-Kosack and Parker 2003). In comparison, not much is known about the impact on defense responses of other types of plant hormones, such as absciscic acid (ABA). ABA regulates many aspects of plant growth and development, in-

cluding embryo maturation, seed dormancy, abiotic stress responses, leaf senescence, and stomatal aperture (Zeevaert and Creelman 1988). Transcriptome analyses have unraveled more than a thousand genes that are differentially regulated by ABA, clearly demonstrating the complexity of ABA signaling (Hoth et al. 2002; Sánchez et al. 2004; Seki et al. 2002). In biotic stress-related studies, exogenous application of ABA caused enhanced pathogen susceptibility due to the suppression of SA in various plant species (Audenaert et al. 2002; McDonald and Cahill 1999; Mohr and Cahill 2003; Rezzonico et al. 1998). By analogy, ABA-deficient tomato plants were more resistant to infection by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Thaler and Bostock 2004). In addition to interference of the SA-dependent defense pathway (Audenaert et al. 2002), antagonistic interactions between ABA and JA or ET signaling have been shown to modulate defense gene expression in response to the vascular fungal pathogen *Fusarium oxysporum* (Anderson et al. 2004).

In most cases, ABA biosynthesis is repressed after pathogen challenge; however, in some cases, such as after viral infection in tobacco, it can be induced (Whenham et al. 1986). The changes in endogenous ABA levels, however, are relatively moderate when compared with the changes observed in the biosynthesis of SA, ET, or JA after pathogen challenge (Mauch-Mani and Mauch 2005). A positive role for ABA in defense against *Alternaria brassicicola* and *Plectosphaerella cucumerina* was revealed following β -aminobutyric acid (BABA) treatments (Ton and Mauch-Mani 2004). BABA-induced resistance (IR) against the two necrotrophic fungi was found to act through the priming of callose depositions and the resistance was impaired in the callose synthase mutant *pmr4-1* and the ABA-deficient and -insensitive mutants *aba1-5* and *abi4-1*. The effects seen from BABA-IR also could be obtained by application of ABA, demonstrating that the priming for callose was an ABA-dependent mechanism (Ton and Mauch-Mani 2004). BABA, on the other hand, was found to enhance the sensitivity of the plant to ABA, resulting in resistance to salinity and drought stress after BABA pretreatment (Jakab et al. 2005). Callose deposition is dependent on vesicular secretion and regulation of specific SNARE (soluble NSF attachment receptor) proteins, representing another proposed mechanism for ABA-primed callose deposition (Mauch-Mani and Mauch 2005; Ton et al. 2005). Additional positive roles for ABA in pathogen defense have been observed in interactions with an oxalate-deficient *Sclerotinia sclerotiorum* mutant, where the regulation of guard cells was found to be the major mechanism of resistance (Guimaraes and Stotz 2004). Clearly, the role of ABA in plant defense signaling is complex and also may vary

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between pathosystems. The mechanisms behind BABA-IR remain unclear; however, in addition to the ABA-dependent priming for callose depositions, BABA-induced priming of the systemic acquired resistance signaling pathway contributed to resistance to *Botrytis cinerea*, *Hyaloperonospora parasitica*, and *Pseudomonas syringae* (Zimmerli et al. 2000, 2001).

Resistance against *Leptosphaeria maculans* recently was found to be dependent on callose depositions, induced by the *Drosophila* TOLL and human interleukin-1-receptor (TIR) nucleotide binding site (NB) leucine-rich repeat (LRR) encoded resistance gene *RLM1_{Col}* (Staal et al. 2006). The *RLM1_{Col}* and *RLM2_{Ler}* loci were identified via transgressive segregation in progenies between Col-0 (*RLM1_{Col}rlm2_{Col}*) and Ler-0 (*rlm1_{Ler}RLM2_{Ler}*) and further genomic data showed that the two loci have a common evolutionary history, indicating that the two genes are paralogs and functionally redundant (Staal et al. 2006). To expand our knowledge of signaling pathways in this plant-pathogen system, we evaluated the importance of endogenous ABA levels and the role of exogenously applied BABA for resistance responses in *Arabidopsis*. The earlier established link between *RLM1_{Col}* and callose led us to investigate whether ABA acts in the *RLM1_{Col}* pathway. This was accomplished through observations of ABA induction after *L. maculans* stress, complementation of *rlm1_{Ler}rlm2* genotypes via ABA treatments, and observations of callose deficiencies in mutants impaired in endogenous ABA. Because *L. maculans* is a devastating pathogen on *Brassica* crops (Howlett et al. 2001; Salisbury et al. 1995), we translated the information obtained from *Arabidopsis* to comparative defense studies on *Brassica napus*. Our results suggest that ABA acts as a pathogen defense hormone in the *RLM1_{Col}* pathway and, in addition to enhancement of callose deposition, induces a broader array of defense responses than earlier anticipated.

RESULTS

Ler-0 mutants impaired in ABA biosynthesis and signaling are susceptible to *L. maculans*.

An assessment of *Arabidopsis* mutants to explore novel key defense factors in the *L. maculans* plant-pathogen system was

Fig. 1. Disease phenotypes on Ler-0 mutants compromised in abscisic acid (ABA) biosynthesis and signaling, 14 days postinoculation (dpi). The experiment was repeated twice, with 20 plants. **A**, Resistant phenotype on Ler-0. Susceptible phenotype on **B**, *aba1-3* and **C**, *abi1-1*. Resistant phenotype on **D**, *abi2-1*; **E**, *abi3-1*; **F**, *abi5-1*; and **G**, Ws-0. **H**, Moderately susceptible phenotype on *abi1-1R1*. Resistant phenotype on **I**, *abi1-1R2*; **J**, *abi1-1R3*; **K**, *abi1-1R4*; **L**, *abi1-1R5*; and **M**, *abi1-1R7*. **N**, Moderately susceptible phenotype on *abi1-1R4abi2-1R26*. **O**, A hypersusceptible phenotype on *lms1abi1-1*, 4 dpi.

performed. Among a number of physiologically defined *Arabidopsis* mutants, the ABA-defective mutants of Ler-0 origin, *aba1-3* and *abi1-1*, but not the insensitive mutants *abi2-1*, *abi3-1*, or *abi5-1* (Ws-0 background) were found to be susceptible to *L. maculans* (Fig. 1A through G). *ABI1* and *ABI2* are two structurally and functionally homologous genes coding for protein-phosphatase 2C (PP2C) proteins. The *abi1-1* and *abi2-1* mutations both affect an amino acid at the catalytic site, which results in a dominant phenotype (Leung et al. 1997). In order to confirm that the susceptible phenotype seen in the *abi1-1* mutant was due to the dominant negative activity, seven cognate suppressor lines (Gosti et al. 1999) were evaluated for *L. maculans* responses (Fig. 1H through N). Interestingly, only the *abi1-1R1* showed a moderate disease phenotype on some (5 of 36) leaves, whereas all other suppressor lines remained resistant. In contrast to the other suppressor lines, which show complete or near-complete loss of ABI1-1 phosphatase activity, *abi1-1R1* still shows 1.3% activity (Gosti et al. 1999) indicating that even a low ABI1-1 activity negatively influences *L. maculans* resistance. Weaker phenotypes on the remaining lines correlate with the phenotypic analyses presented by Gosti and associates (1999). Surprisingly, the *abi1-1R4abi2-1R26* double mutant showed susceptible phenotypes on a few (4 of 27) leaves, whereas *abi1-1R4* remained completely resistant. This implies that ABI1 and ABI2 may have opposite effects on *L. maculans* resistance. Expression patterns show that *ABI3* and *ABI5* are expressed only in seed, indicating that those genes have little effect on adult leaf ABA responses (Schmid et al. 2005).

Camalexin-free background confirms ABA requirement for *L. maculans* resistance in Col-0.

ABA-impaired mutants in Col-0 background (*aba2-1*, *aba3-1*, and *abi4-1*), in contrast to mutants in Ler-0 background, did not display any disease symptoms (Fig. 2A through D). Col-0 expresses approximately three times higher levels of the phytoalexin camalexin compared with Ler-0 and Ws-0, a defense component which can interfere with susceptibility assessments (Staal et al. 2006). No difference in camalexin induction between susceptible ABA mutants and resistant genotypes of Ler-0 background could be found (data not shown). In order to further evaluate the role of ABA, double mutants between the

Fig. 2. Disease phenotypes on Col-0 mutants compromised in abscisic acid (ABA) biosynthesis and signaling. Disease phenotypes against *Leptosphaeria maculans* are unveiled on mutants in Col-0 background when crossed to the camalexin-deficient mutant *pad3-1*. The experiment was repeated twice with 20 plants. Resistant phenotype on **A**, Col-0; **B**, *aba2-1*; **C**, *aba3-1*; and **D**, *abi4-1*, 14 dpi. Disease development on **E**, *aba2pad3* and **F**, *aba3pad3*, 11 days postinoculation (dpi). **G**, Clear lesion phenotype on the *abi4pad3* double mutant, 11 dpi. Susceptible phenotype on **H**, *pmr4pad3* and **I**, *rlm1_{Ler}pad3*, 11 dpi. **J**, No visible disease lesions on the *pad3-1* background genotype, 11 dpi. **K**, The mutant *pad3-1* started to display clear lesions 15 dpi.

camalexin-deficient *pad3-1* and *aba2-1*, and *aba3-1* and *abi4-1* were made. The responses of those genotypes (Fig. 2E through G) were compared with *pmr4pad3* and *rlm1_{Ler}pad3* (Fig. 2H and I), a hypersusceptible double mutant deriving from *pad3-1* (*RLM1_{Col}rlm2_{Col}*), and a Col-0 × Ler-0 line (*rlm1_{Ler}rlm2_{Col}*), which has lost the *RLM1_{Col}* allele and its putative paralog *RLM2_{Ler}* due to transgressive segregation (Staal et al. 2006). Disease severity 11 days postinoculation showed that *rlm1_{Ler}pad3* was clearly more susceptible than *pmr4pad3* (Fig. 2H and I), indicating that *RLM1*-dependent resistance is controlled by additional components independent of callose deposition. The double mutants *aba2pad3*, *aba3pad3*, and *abi4pad3* all displayed an enhanced *L. maculans* susceptibility (Fig. 2E through G), which shows that all steps in the ABA biosynthesis are important for *L. maculans* resistance. ABA-dependent resistance against *L. maculans* also is regulated by a more extensive response than enhancement of callose deposition, because the *abi4pad3* double mutant displayed a clearly more severe disease phenotype compared with *pmr4pad3*, even if the emergence of lesions was earlier in *pmr4pad3* than in *abi4pad3*. In contrast to *ABI3* and *ABI5*, expression of the Apetala2 (AP2) domain transcription factor *ABI4* can be found throughout the whole plant (Schmid et al. 2005). *ABI4* previously has been shown to be involved in both BABA-induced sterility and BABA-induced resistance to *A. brassicicola* and *Plectosphaerella cucumerina* by the priming of callose depositions (Ton and Mauch-Mani 2004; Ton et al. 2005). Interestingly, the *abi1-1* mutation, but not *abi2-1*, severely reduces *ABI4* expression (Söderman et al. 2000), which might explain the differences between the two PP2C mutants in *L. maculans* responses. The *pad3-1* background had not developed any visible disease symptoms at 11 days postinoculation (Fig. 2J) and started to show the first clear lesions at 15 days postinoculation (Fig. 2K). Thus, the single *pad3-1* mutant exposes a 4- to 5-day delay in symptom development compared with the double mutants. Furthermore, quantitative disease progression assessments in F₃ of the *aba2-1*, *aba3-1*, *abi4-1*, and *pmr4* mutants in *pad3-1* background compared with *rlm1_{Ler}pad3* and *pad3-1* confirmed partial loss of *RLM1* resistance.

Secondary messenger mutants reveal H₂O₂ dependency of *L. maculans* resistance.

One common denominator between pathogen responses and ABA signaling is the NADPH oxidase-dependent generation of H₂O₂ (Kwak et al. 2003; Torres et al. 2002, 2005). Evaluation of the respiratory burst NADPH-oxidase *rbohD* and *rbohF* mutants revealed only moderate susceptible responses, whereas the double mutant *rbohDF* showed a hypersusceptible response to *L. maculans* (Fig. 3A through C). Another secondary messenger important for both ABA and pathogen responses is nitric oxide (NO), which can be generated either via nitrate reductase in an H₂O₂-dependent manner or via nitric oxide synthase (Bright et al. 2006). In order to test the influence of NO, mutants in the two pathways impaired in some ABA responses were evaluated. The nitrate reductase-deficient double mutant *nia1nia2* (nitrate reductase 1 nitrate reductase 2) showed moderate susceptible phenotype, whereas the *Atnos1* (nitric oxide synthase 1) mutant remained resistant (Fig. 3D and E). In conclusion, reactive oxygen species (ROS)-generated signals and ABA play an important role in *L. maculans* responses. However, the *ibs2* (impaired in BABA-induced sterility 2) mutant impaired in BABA-induced sterility and defective in phosphoinositol signaling, which represents a pathway parallel to ABA for BABA-primed callose depositions (Ton et al. 2005), remained as resistant as the wild type (Fig. 3F).

ABA and BABA pretreatment induces resistance in *Arabidopsis* and oilseed rape.

ABA and BABA have been shown to induce resistance against different fungal pathogens in *Arabidopsis* (Jakab et al. 2001). This knowledge and the fact that some ABA mutants showed susceptibility against *L. maculans* led to experiments with ABA and BABA treatments. Application via drenching of the soil with BABA at 16 mg/liter 2 days preinoculation caused a significant induction of resistance in the susceptible *Arabidopsis* mutants *aba1-3*, *pad3-1*, *pmr4-1* *esa1*, and *pen1* (Fig. 4A through E). However, the *Arabidopsis* genotypes *abi1-1* and *rlm1_{Ler}pad3-1* and the *L. maculans*-susceptible *lms1* and *rlm1_{Ler}rlm2_{Col}* did not display any resistance due to the treatments (Fig. 4F through I) compared with the resistant wild-type Col-0 (Fig. 4J).

In order to avoid induced senescence, causing loss of potentially diseased leaves at an early stage, ABA pretreatments with 25 μM ABA 2 days before and 3 days after inoculation were performed instead of pretreatments using 80 μM ABA (Ton and Mauch-Mani 2004). The lower concentration of ABA resulted in an induction of resistance in the susceptible *Arabidopsis* mutants *aba1-3*, *pad3-1*, and *pmr4-1* (Fig. 4A through C), but also in the enhanced susceptibility mutant *esa1* and the papilla formation mutant *pen1* (Fig. 4D and E). However, as in the case of BABA pretreatment, the *Arabidopsis* mutants *abi1-1* (Fig. 4F) and the *L. maculans*-susceptible *lms1* (Fig. 4H) did not show any induced resistance. In order to see whether *lms1* and *abi1-1* reside in the same signaling pathway, as suggested by the lack of restoration by ABA and BABA, an *lms1abi1* double mutant was generated. The double mutant did show a hypersusceptible phenotype with lesions emerging already at 4 days postinduction (dpi) (Fig. 1O). However, no defects in callose deposition could be seen in the *lms1* mutant (data not shown). The epistasis between *lms1* and *abi1-1* shows that these two mutants represent two parallel pathways of central importance for *L. maculans* resistance. The additive effects seen in the *lms1abi1-1* double mutant could be analogous to previous reports on interactions between ET mutants and *abi1-1* (Fedoroff 2002), because the *lms1* mutant shows an ET-insensitive root and enhanced sensitivity to ET in the shoot (Bohman et al. 2004). The *lms1* mutant also shows glucose-insensitive germination (data not shown), a well-known cross-talk between ABA and ET (Fedoroff, 2002). The genotypes *rlm1_{Ler}pad3* (Fig. 4G) and *rlm1_{Ler}rlm2_{Col}* (Fig. 4I) both displayed partial restoration of resistance after ABA treatment, which demonstrates that ABA acts as a signal downstream of *RLM1_{Col}*.

To evaluate whether the induced resistance response found in *Arabidopsis* is effective in oilseed rape, three genotypes (the

Fig. 3. Disease phenotypes on secondary messenger mutants, 14 days postinoculation (dpi). The experiment was repeated twice with 20 plants. Susceptible phenotype on **A**, *rbohD* and **B**, *rbohF*. **C**, Hypersusceptible phenotype on *rbohDF*, 7 dpi. **D**, Susceptible phenotype on *nia1nia2*. Resistant phenotype on **E**, *Atnos1* and **F**, *ibs2*.

Fig. 4. Defense scoring after abscisic acid (ABA) and β -aminobutyric acid (BABA) pretreatments on *Arabidopsis* mutants, 21 days postinoculation (dpi). The plants were soil drenched with water and BABA at 16 mg/liter 2 days prior to infection or pretreated with 25 μ M ABA 2 days before inoculation and 3 dpi. After inoculation with *Leptosphaeria maculans*, the disease symptoms were scored every second day according to Delwiche and Williams (1979): 0 = no symptoms; 1 = lesion diameter 0.5 to 1.5 mm; 3 = dark necrotic lesions 1.5 to 3 mm; 5 = lesions 3 to 5 mm, occasional sporulation; and 7 = gray-green tissue collapse, lesions 4 to 8 mm, sporulation. The values (%) presented were based on the total number of inoculation sites. The scale on the x-axis refers to the disease scoring scale. **A**, *aba1-3*; **B**, *pad3-1*; **C**, *pmr4-1*; **D**, *esa1*; **E**, *pen1*; **F**, *abi1-1*; **G**, *rlm1^{Ler}xpad3-1*; **H**, *lms1*; **I**, *rlm1^{Ler}rlm2^{Col}*; and **J**, *Col-0*, 21 dpi. The experiment was repeated twice (10 plants and 50 to 60 inoculation sites were used for each genotype and treatment) and the standard deviation (SD) varied between 1.4 and 26.0, with a mean of 99 and 81.6, respectively.

susceptible *B. napus* cv. Hanna, *nahG* in Hanna background, and the resistant cv. Surpass 400) harboring the single dominant *L. maculans* resistance gene *LepR3* (Li and Cowling 2003) were treated with BABA and ABA as described above prior to inoculation with *L. maculans*. A significant induction of resistance in the two susceptible genotypes was observed after BABA pretreatment, demonstrating that BABA induces resistance independently of SA against *L. maculans*. ABA pretreatment also resulted in an unambiguous induction of resistance.

Furthermore, to assess whether BABA treatment influences ABA biosynthesis, endogenous levels of ABA were determined using gas chromatography/mass spectrometry (GC/MS) for whole-leaf analysis and enzyme-linked immunosorbent assay (ELISA) for local ABA induction analysis. BABA-treated and fungal-inoculated *Ler-0* plants demonstrated a clear enhancement (1.5 times) of ABA levels compared with the water-inoculated control 3 dpi ($P < 0.003$), which shows that ABA is pathogen induced. The water-inoculated BABA-pretreated plants displayed ABA levels comparable to *Ler-0* without BABA treatment. ELISA measurements of local ABA induction showed the same pattern with an earlier peak induction (1 dpi) after *L. maculans* or wounding (data not shown).

Transcriptional analysis reveals an enhanced SA response in susceptible ABA mutants.

Defense-related gene expression in the contrasting ABA mutants was analyzed. After inoculation, the susceptible *Ler-0* genotypes *aba1-3* and *abi1-1* expressed an enhanced *PR-1* induction, whereas the resistant mutant *abi2-1* had a *PR-1* induction comparable with the wild type (Fig. 5). *BGL2* (*PR2*) was moderately expressed in all genotypes except *abi1-1*, where a clear induction after *L. maculans* challenge could be seen. The two susceptible mutants (*aba1-3* and *abi1-1*) influenced the *PDF1.2* expression differently, where *abi1-1* had an enhanced *PDF1.2* expression; whereas *aba1-3* and *abi2-1* showed reduced *PDF1.2* expression compared with the wild type (Fig. 5). The JA-induced *VSP2* showed no difference between mock-inoculated and *L. maculans*-inoculated materials at 48 hours postinoculation (hpi). However, the wound induction of *VSP2* clearly was enhanced in *abi1-1* compared with the other genotypes (Fig. 5). No significant shift of callose synthase (*GSL5*) expression was detected in the different ABA mutants, independent of challenges, which is in agreement

Fig. 5. Expression patterns of defense-related genes in *Arabidopsis* genotypes. Northern analysis of *Ler-0*, *aba1-3*, *abi1-1*, and *abi2-1*, 48 h postinoculation with *Leptosphaeria maculans* (*Lm*) and water. All genotypes expressed the salicylic acid-responsive gene *PR-1*, and the susceptible genotypes *aba1-3* and *abi1-1* exhibited higher expression compared with the resistant genotypes *Ler-0* and *abi2-1*. Expression of the jasmonic acid and ethylene (JA/ET)-responsive gene *PDF1.2* did not correspond to susceptibility, whereas the mutants *aba1-3* and *abi2-1* had reduced *PDF1.2* gene expression. The JA-responsive gene *VSP2* did not express differently between treatments, but *abi1-1* showed enhanced *VSP2* expression compared with the other genotypes. The callose synthase *GSL5* did not vary in expression between treatments or genotypes. The experiment was repeated twice with the same expression pattern.

with data in the transcription profile database Genevestigator (Zimmermann et al. 2004). Taken together, the ABA mutants affect the expression of SA-, JA-, and JA-ET-dependent defenses after *L. maculans* challenge differently and susceptible genotypes appear to have an enhanced SA response.

Callose deposition only partially explains *L. maculans* resistance.

To evaluate whether the ABA- and BABA-induced resistance was due to altered callose deposition, aniline blue staining was performed (Gressel et al. 2002). The staining of leaves from *Arabidopsis* mutants after pretreatments revealed that both *abi1-1* and *aba1-3* had significant differences ($P \leq 0.01$) in callose densities compared with *Ler-0*, whereas the differences were minor in *abi2-1* (Fig. 6A). The analysis of callose depositions in *pad3* in Col-0 background (*(RLM1_{Col})pad3*) and *rlm1_{Ler}pad3* after ABA and BABA pretreatment revealed that callose deposition is enhanced in *RLM1_{Col}* plants, whereas callose deposition in *rlm1_{Ler}* plants is not affected by pretreatments (Fig. 6B). The dependency of *RLM1_{Col}* for callose deposition shows that ABA and BABA enhances deposition downstream of this induction. The callose synthase mutant *pmr4-1* displayed low levels of callose under all pretreatment conditions (Fig. 6B), but still exhib-

Fig. 6. Callose deposition determined via aniline blue-stained leaves of β -aminobutyric acid (BABA) and abscisic acid (ABA) pretreated *Arabidopsis* mutants. The extent of callose deposition in response to *Leptosphaeria maculans* inoculation was evaluated over time. When the first true leaves were developed, the plants were treated by soil drench with water, ABA, or BABA 2 days prior to inoculation with *L. maculans*. The staining was performed 2 days postinoculation. Box plot representation of the quantification of callose depositions in inoculated leaves for each genotype was expressed as a percentage of the average background levels (*Col-0* and *Ler-0*). The box plot was made using the 'boxplot()' function in R (R Foundation for Statistical Computing, Vienna, Austria). The middle bar represents the average value, boxes the value range of the first quartiles over and under average (50% of measured values), and the bars represent the value range of the two outer quartiles. **A**, *Ler-0*, *aba1-3*, *abi1-1*, and *abi2-1* in *Ler-0* background; **B**, *pad3*, *rlm1_{Ler}pad3*, and *pmr4-1pad3* in Col-0 background. The *pmr4* single mutant has the same callose deposition as the *pmr4pad3* double mutant. The scale on the y-axis refers to the relative callose deposition density, expressed as percentage of water-pretreated wild type. The experiment was repeated twice with 10 biological replicates.

ited a clear enhanced resistance after ABA and BABA pretreatments (Fig. 4C). This observation clearly demonstrates that ABA-induced resistance is a complex response and comprises other factors than the enhancement of callose depositions. To investigate whether SA-derived signals affect callose deposition, *pad3-1* plants were inoculated with an *L. maculans* spore solution together with 50 μ M SA. The latter is an additive which does not influence the spore germination and growth of *L. maculans*. Plants co-inoculated with SA and *L. maculans* displayed a severely reduced callose deposition compared with plants only inoculated with *L. maculans* (Fig. 6B).

To compare our findings from *Arabidopsis*, staining for callose deposition also was carried out on *B. napus* leaves from plants pretreated with water, ABA, and BABA 2 dpi (Fig. 7). In agreement with our observations on *RLM1*_{Col} and *rlm1*_{Ler}*rlm2* *Arabidopsis* genotypes, the resistant *B. napus* cv. Surpass 400 exhibited enhanced levels of callose deposition after pathogen challenge when compared with the susceptible cv. Hanna upon fungal inoculation (Fig. 7). However, when ABA and BABA pretreated cv. Hanna plants were analyzed, the callose levels were comparable to Surpass 400 (Fig. 7). Staining of leaves inoculated with water showed no callose deposition, demonstrating that the callose observed was exclusively pathogen induced. In order to evaluate whether the callose production was SA dependent, as described by Donofrio and Delaney (2001), the *B. napus* cv. Hanna *nahG* genotype was assessed in a similar manner. No significant differences were found between *nahG* plants and the wild type, except a slightly enhanced callose deposition in *nahG* in the water-pretreated samples (Fig. 7).

Global transcription analysis reveals differentially regulated genes between the susceptible *abi1-1* and resistant *abi2-1* mutants.

Despite the molecular similarities between *abi1-1* and *abi2-1*, the two mutants show drastic differences in *L. maculans*

Fig. 7. Callose quantification in abscisic acid (ABA)- and β -aminobutyric acid (BABA)- pretreated *Brassica napus* genotypes. Box plot representation of the quantification of callose depositions in inoculated leaves of Hanna, *nahG* in Hanna background, and Surpass 400 was expressed as a percentage of the average water-pretreated Hanna levels. When the first true leaves were developed, the plants were treated by soil drench with ABA or BABA 2 days prior to inoculation with *Leptosphaeria maculans*. The staining was performed 2 days postinoculation. The box plot was made using the 'boxplot()' function in R (R Foundation for Statistical Computing, Vienna, Austria). Middle bar represents average value, boxes the value range of the first quartiles over and under average (50% of measured values), and the bars the value range of the two outer quartiles. Boxplot of callose content measured on 8 to 10 individual leaves per treatment and genotype using the program APS assess. The scale on the y-axis refers to the relative callose deposition density expressed as a percentage of water-pretreated Hanna. The experiment was repeated twice with 10 biological replicates.

responses. In order to identify the subset of ABA responses influenced by ABI1, but not by ABI2, required for resistance a microarray comparison between the two mutants was performed after ABA pretreatment and *L. maculans* inoculation. Microarray analysis revealed 74 genes to be differentially expressed ($B > 1$, $P < 0.1$) between the resistant *abi2-1* and the susceptible *abi1-1* mutants 2 dpi. An interesting observation is that the *RLM1*-related gene At1g63870 is more highly expressed in *abi2-1*, which may indicate that ABI1 modulates *RLM1*_{Col} accumulation after *L. maculans* stress. To investigate whether this was the case, quantitative polymerase chain reaction (PCR) analyses were made on *abi1-1* and *abi2-1* array samples for the homologous TIR-NB-LRR genes in the *RLM1* locus. Assessments of most genes were inconclusive; however, At1g63750 showed a 32% higher expression ($P < 0.04$) in *abi2-1* compared with *abi1-1*. Because both *abi1-1* and *abi2-1* are of *Ler-0* background and, thus, only have a functional *RLM2* locus (Staal et al. 2006), we also compared four pools of *pad3-1* and *abi4pad3* in Col-0 background 2 dpi for evaluations of genotypes with a functional *RLM1* locus. Contrary to our expectations, At1g63870 ($P = 0.002$) and At1g64070 ($P = 0.014$) was approximately twofold upregulated in *abi4pad3* compared with *pad3*, whereas At1g63880 expression remained constant (Fig. 8). We interpret this as there being an ABI1-dependent feedback upstream of ABI4 that negatively regulates *RLM1* expression. ABI1 activity, in turn, could be feedback regulated by ABA responses downstream of ABI4 (Merlot et al. 2001; Saez et al. 2004), which would reduce ABI1-dependent repression of *RLM1* in an *abi4* background. Supporting this hypothesis, Northern blot data of *ABI1* in the four *pad3-1* and the four *abi4pad3* RNA pools revealed a 1.7-fold decrease on average ($P = 0.06$) of *ABI1* transcripts in the *abi4* background (data not shown).

DISCUSSION

Common features in pathogen responses and ABA signaling.

Previously, ABA has been associated almost exclusively with deleterious effects on plant defense responses; however, an increasing number of reports show that there are cases where ABA acts as a positive regulator of resistance (Flors et al. 2005). The present reports, however, do not show a requirement for endogenous ABA for resistance. A reduction of the

Fig. 8. Quantitative polymerase chain reaction (PCR) analysis of *RLM1*-related genes in *pad3* and *abi4pad3*. Quantitative measurements of At1g63870, At1g63880, and At1g64070 in *pad3* and *abi4pad3* determined by real-time PCR, 48 h postinoculation. The experiment was repeated twice with four biological replicates.

endogenous ABA has been reported to cause increased resistance, whereas positive influences from ABA on resistance have been found after exogenous application (Ton and Mauch-Mani 2004). In the case of *L. maculans*, endogenous ABA and a functional ABA signaling via ABI1 and ABI4 is required to maintain *RLM1*_{Col}- and *RLM2*_{Ler}-dependent resistance. The most apparently susceptible phenotype to *L. maculans* among ABA mutants was visible on the dominant negative PP2C mutant, *abi1-1*. The *abi2-1* mutation with similar effects on a homologous gene (Leung et al. 1997) lacked this distinct response, suggesting differential modes of action. Earlier transcriptional profiling has revealed that only 9% of the ABA-responsive genes are *abi1-1* independent (Hoth et al. 2002). In addition, a number of potentially important genes for plant pathogen defense were shown to be both positively and negatively regulated by ABA application in an *abi1-1*-dependent manner (Hoth et al. 2002). It has been proposed that *abi2-1* has a less severe impact on the *Arabidopsis* transcriptome compared with *abi1-1* (Xiong et al. 2002). This may be due, in part, to a repression in *abi1-1*, but not in *abi2-1*, of zeaxanthin epoxidase (*ABA1*) which is important for ABA biosynthesis. The *abi1-1* mutant also has been found to impair the activated disease resistance gene *ADRI* (coiled coil-NB-LRR), which also confers SA- and *EDS1*-dependent drought resistance (Chini et al. 2004), thus linking ABI1 activity to the NB-LRR class of pathogen resistance genes. The induction of disease response-like lesions under low humidity stress in the sensitive to low humidity 1 (*slh1*) mutant in a gene conferring resistance to *Ralstonia solanacearum* (TIR-NB-LRR-WRKY) is yet another connection between ABA responses and the activity of NB-LRR-type resistance genes (Noutoshi et al. 2005). Another lesion mimic mutant, lesion simulating disease 1 (*lsd1*), induces an *EDS1* and *PAD4*-dependent hypersensitive-response-like cell death associated with stomata regulation and photorespiration, further emphasizing the link between ABA-regulated responses and the TIR-NB-LRR resistance responses (Mateo et al. 2004).

The induction of ROS also might be an important connection between ABA and pathogen responses, because the formation of the secondary messenger hydrogen peroxide is dependent on superoxide via the NADPH oxidases *RbohD* and *RbohF* (Kwak et al. 2003; Torres and Dangl 2005; Torres et al. 2002). NADPH oxidases also may function as negative regulators of cell death (Torres et al. 2005), which might be the cause of the hypersusceptible phenotype observed in the *RbohDF* double mutant. Furthermore, NO is a central player in both ABA activity (Desikan et al. 2002) and the induction of SA defenses (Klessig et al. 2000). The origin of NO has been widely disputed and two pathways, either via nitrate reductase (Desikan et al. 2002) or through a nitric oxide synthase (Guo et al. 2003), based on genetic, pharmacological, and physiological evidence, have been demonstrated to be of central importance for intact ABA signaling activity. The two NO pathways appear to be activated in different contexts, where the nitrate reductase-dependent generation of NO relies on H₂O₂ from the NADPH oxidases *RbohD* and *RbohF* (Bright et al. 2006). There also may be convergences between ABA signaling events and plant defense in ROS-elicited oscillations of cytosolic Ca²⁺ (Klüsener et al. 2002). The closing of stomata, which per se can serve as entrance for some pathogens such as *L. maculans* (Chen and Howlett 1996), also may be a common denominator in plant disease resistance and ABA responses. However, regulation of stomatal closure as a mechanism of ABA-induced resistance is not a likely explanation for our infection experiments, because we caused a wound for entrance of *L. maculans* into the leaf as part of the inoculation procedure.

The positive impact of ABA on resistance to *A. brassicicola* and *P. cucumerina* was established through the requirement for ABA in SA-independent and callose-dependent cases of BABA-IR (Ton and Mauch-Mani 2004). BABA-IR against *L. maculans* in *B. napus* is clearly an SA-independent process. The slight enhancement of susceptibility observed in both water- and BABA-pretreated *B. napus* plants harboring the *nahG* gene could possibly be attributed to a detrimental effect of catechol on resistance, because catechol can induce inappropriate levels of hydrogen peroxide and probably accelerates cell death (van Wees and Glazebrook 2003), which is beneficial for *L. maculans* (Bohman et al. 2004). Our results show that ABA has a wider activity than described by Ton and Mauch-Mani (2004), because we also obtain a clear induction of resistance in a callose-deficient background.

Callose-independent ABA-induced resistance responses.

Genes differentially affected by the two PP2C isoforms are shown in microarray comparisons between *abi1-1* and *abi2-1*. Several transcription factor gene families are differentially induced in both genotypes, suggesting that control of several genes is differentially regulated by the two PP2C proteins when challenged by *L. maculans*. The documented differential regulation of *ABI4* in the two mutants (Söderman et al. 2000) is of particular interest in this context. Additional support for the differential roles of ABI1 and ABI2 is given by the recent results, which show that *abi1-1* and *abi2-1* have opposite effects on the expression of lipid-binding proteins involved in photosystem II inhibition under abiotic stress (Yang et al. 2006). In the case of *L. maculans* resistance, one major influence could be the ABI1-dependent repression of *RLM1* and *RLM1*-like genes, which could act as a feedback loop to modulate *RLM1* expression after infection. This feedback occurs upstream of ABI4, which also could explain the less severe phenotype observed on *abi4-1* compared with *abi1-1*.

Cross-talk between ABA and other pathogen resistance hormones.

Our expression analysis of resistant and susceptible genotypes of *Ler-0* background showed higher SA-dependent responses in the susceptible genotypes. Furthermore, the reduced callose deposition after co-inoculation with *L. maculans* and SA and the slightly enhanced callose levels in *nahG B. napus* compared with wild-type *B. napus* indicates that repression of SA responses may be part of the ABA-dependent resistance response. The callose synthesis gene *GSL5* did not show any alterations in any of the genotypes or differences between inoculated and control material. This indicates that callose deposition is not regulated via transcription of *GSL5*, but could be influenced by multiple downstream events such as post-translational modifications of *GSL5*, subcellular localization, substrate availability, and callose degradation via β -1,3-glucanases. ABA is known to repress both SA- and JA-dependent resistance responses (Anderson et al. 2004; Audenaert et al. 2002; McDonald and Cahill 1999; Mohr and Cahill 2003; Rezzonico et al. 1998; Thaler and Bostock 2004), which might explain why both responses are enhanced in the *abi1-1* mutant. An alternative explanation of the higher pathogenesis-related (PR) gene expression in susceptible materials is that a successful colonization of the susceptible genotypes causes a higher stress, which in turn induces higher PR gene expression. A similar interpretation of the majority of the differentially expressed genes detected on the microarray comparison between *abi1-1* and *abi2-1* also could be valid.

ABA acts downstream of *RLM1*_{Col}.

The *Arabidopsis*-*L. maculans* responses found in this study show remarkable analogy to what has been reported previously

for viral responses in tobacco. We observed an induction of ABA specifically after *L. maculans* challenge and we can induce resistance by application of ABA, in a similar manner to that seen in the tobacco–*Tobacco mosaic virus* system (Whenham et al. 1986). The same is true for the ABA-dependent repression of callases and the enhancement of callose deposition after ABA treatment (Beffa et al. 1996; Rezzonico et al. 1998). Furthermore, callose was discovered to be partially responsible for resistance to *L. maculans*, but ABA also influenced other resistance responses. Based on observations of the enhanced susceptibility on mutants impaired in ABA biosynthesis and sensitivity, we concluded that the ABA-induced resistance is linked with endogenous ABA levels, and not only via exogenous application. Together with the observation of pathogen induction of ABA and complementation of resistance by exogenous ABA application to *rlm1_{Ler}pad3* and *rlm1_{Ler}rlm2_{Col}* genotypes, it was demonstrated that ABA acts downstream of *RLM1_{Col}* (Fig. 9). BABA-IR required the function of *RLM1_{Col}* for elevated ABA biosynthesis, whereas BABA also enhances the sensitivity to ABA and affects the ABA homeostasis in the plant, presumably via a feedback loop.

Fig. 9. Model for the role of abscisic acid (ABA) and β -aminobutyric acid (BABA) in *Leptosphaeria maculans* resistance. ABA is induced by pathogen recognition, which is enhanced by BABA pretreatment. BABA also activates defense responses downstream of ABA and enhances ABA sensitivity, but BABA cannot complement the absence of *RLM1* and *RLM2*. That BABA acts both upstream and downstream of ABA indicates that it affects a feedback loop regulating ABA homeostasis. However, both ABA and BABA responses are blocked in *abi1-1* (this mutant has a constitutive *ABI1*-dependent repression of ABA responses) and *abi4* mutants. Callose deposition is enhanced by ABA, but requires an *RLM1* signal because exogenously applied ABA is unable to complement lack of *RLM1* in callose deposition. In addition to callose deposition, ABA induces a hitherto unknown resistance factor which can partially complement the absence of *RLM1*. *ABI1* is required for a pathogen-induced accumulation of *RLM1*, but mutations in *ABI4* give opposite effects, suggesting that the feedback occurs upstream of *ABI4* and that components downstream of *ABI4* modulate *ABI1* activity.

When ABA-insensitive mutants were assessed, only *abi1-1* and *abi4-1* displayed susceptibility, whereas the other mutants (*abi2-1*, *abi3-1*, and *abi5-1*) remained resistant. This defines ABA-induced resistance to *L. maculans* as a subset of the ABA responses regulated via *ABI1* and *ABI4* and strongly suggests the presence of an unknown key resistance factor. A future challenge is to detect *ABI4*-induced genes and *ABI4* binding promoters to further dissect this response.

MATERIALS AND METHODS

Plant materials and culture conditions.

Mutants of *Arabidopsis* used in this study have been described previously: *aba1-3* (Koornneef et al. 1982); *aba2-1* and *aba3-2* (Léon-Kloosterziel et al. 1996); *abi1-1*, *abi2-1*, and *abi3-1* (Koornneef et al. 1984); *abi4-1* and *abi5-1* (Finkelstein 1994); *Atmos1* (Guo et al. 2003); *esal* (Tierens et al. 2002); *ibs2* (Ton et al. 2005); *lms1* (Bohman et al. 2004); *nialnia2* (Desikan et al. 2002); *pad3-1* (Glazebrook and Ausubel 1994); *pen1* (Sanderfoot et al. 2000); *pmr4-1* (Vogel and Somerville 2000); *rbohD*, *rbohF*, and *rbohDF* (Torres et al. 2002); and *rlm1_{Ler}rlm2_{Col}*, *rlm1_{Ler}pad3-1*, and *Ler-rlm2_{Cvi}* (Staal et al. 2006). An *lms1abi1* double mutant was generated for epistasis evaluations of the mechanisms for ABA- and BABA-insensitive susceptible phenotypes. Seven *abi1-1* suppressor lines, *abi1-1R1*, *abi1-1R2*, *abi1-1R3*, *abi1-1R4*, *abi1-1R5*, *abi1-1R4abi2-1R6*, and *abi1-1R7* (Gosti et al. 1999), were used to confirm the dominant-negative role of *abi1-1* for *L. maculans* responses. Columbia (Col-0), Landsberg erecta (Ler-0), and Wassilewskija (Ws-0) were used as controls in the experiments. The *B. napus* (L.) cv. Hanna, susceptible to *L. maculans* (Sjödin and Glimelius 1988), and Surpass 400, resistant to *L. maculans* (Li and Cowling 2003), were included for comparative studies.

The *Arabidopsis* material was cultured on sterilized soil for 8 h of light at 22°C and 16 h of darkness at 18°C, with 65% humidity in growth chambers. The light intensity was 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The humidity was raised to 100% the first 48 h after fungal inoculation to facilitate fungal growth. *B. napus* was cultured in the greenhouse under 16 h of light.

Generation of transgenic *B. napus*.

Hypocotyls of *B. napus* cv. Hanna were transformed with a binary construct harboring the *nahG* gene (Delaney et al. 1994). The transformation procedure was as described by Schröder and associates (1994) using streptomycin at 50 mg/liter in the selection step. Out of 24 primary transformants, lines with a single T-DNA insert were selected based on segregation on streptomycin plates and Southern and Northern blotting.

Inoculation of plant material.

The responses to *L. maculans* were determined on *Arabidopsis* rosette leaves, at the five- to eight-leaf stage, by inoculation with conidia using the puncture leaf test as described by Sjödin and Glimelius (1988). In all, 20 plants of each genotype were used and the experiment was repeated twice. The *B. napus* plants were inoculated on both the cotyledons and the adult leaves at plant stage BBCH 12 (Lancashire et al. 1991). The fungal isolates used were PHW 1245 and PHW 950:14 (Leroy), assigned to the race AV1-2-4-5-6-7-8 and AV1-2-3-5-6-9 respectively (Balesdent et al. 2005). *Arabidopsis* and *B. napus* plants were scored 10 to 21 dpi using the scoring scale of Delwiche and Williams (1979), where 0 = no symptoms; 1 = lesion diameter 0.5 to 1.5 mm; 3 = dark necrotic lesions, 1.5 to 3 mm; 5 = lesions 3 to 5 mm, occasional sporulation; 7 = gray-green tissue collapse, lesions 4 to 8 mm, sporulation; and 9 = rapid tissue collapse, accompanied of profuse sporulation in large lesions (more than 5 mm).

Chemical treatments prior inoculation.

Solutions containing 25 μM ABA (Sigma Aldrich, Steinheim, Germany) or BABA at 16 mg/liter (Sigma Aldrich) were applied to the soil in each pot to obtain an optimal distribution in the plant leaves (Jakab et al. 2001). BABA treatments of the plant material were performed 2 days preinoculation. Studies on ABA-induced resistance on *Arabidopsis* and *B. napus* were made using a 25- μM ABA (Sigma Aldrich) treatment 2 days preinoculation and 3 dpi. Controls were treated with an equal volume of water.

RNA isolation.

Leaves from inoculated plants were collected 48 h after inoculation. The plant material was ground in liquid nitrogen and total RNA was prepared using a Qiagen Plant minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Probes and hybridization.

Total RNA (6 μg) was denatured and transferred onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). The *PR-1* and *PDF1.2* genes used as probes were described previously by Uknes and associates (1992) and Penninckx and associates (1996), respectively. The *GSL5* (At4g03550) probe was generated from *Arabidopsis* genomic DNA using primers TCTTGGTCCTCCGGTTTTCT and TCGCCTTTTGATTCTTCCC and the *VSP2* (At5g24770) probe was amplified from *Arabidopsis* DNA using the primers TCTCACTTTCACTTCTCTTGCTC and ATAAGCCTCTTTG CACACGGTT. The primers were designed from CDS sequences in the AGI database on domains with as low a sequence similarity to other transcripts as possible. The *ABI1* probe was amplified using the caps markers described by Leung and associates (1997). The PCR cycle was 95°C for 60 s; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 72°C for 10 min for final extension. The probes were labeled and hybridized as previously described by Bohman and associates (2004). For normalization of gel loading, Elongation factor 1- α (*EF1- α*) was used as hybridization control (Norman-Setterblad et al. 2000). Determination of differential expression between genotypes or treatments within a comparison set was made based on a one-tailed *t*-test.

Endogenous ABA measurement of whole leaves using GC/MS.

Arabidopsis Ler-0 plants were treated with BABA by drenching the soil 2 days preinoculation (Ton and Mauch-Mani 2004). Plants were harvested before inoculation as well as 1, 3, and 5 dpi and frozen in liquid nitrogen. The frozen plant material was ground in liquid nitrogen and 20 to 26 mg was taken for analysis. Each measurement was repeated with three biological replicates on pooled material from five different plants. ABA was extracted with 80% methanol and 0.02% (wt/vol) sodium diethyldithiocarbamate as antioxidant, and 2 ng of deuterium-labeled ABA was used as the internal standard. The extraction was done in a MixerMill for 3 min (30 Hz) followed by continuous shaking for 1 h at 4°C. After evaporation in a Speed Vac concentrator, the samples were dissolved in 500 μl of 1% acetic acid. The pH was adjusted to approximately 3 and the samples were applied to a C8 (EC) column (Isolute, Mid Glamorgan, U.K.) preconditioned with 2 ml of 100% methanol and 2 ml of 1% acetic acid. The column was washed with 2 ml of 1% acetic acid and the sample eluted with 2 ml of 80% methanol in 1% acetic acid. After reduction to dryness, the sample was treated as described by Edlund and associates (1995) and analyzed in a GC-mass spectrometer (Quadrex, New Haven, CT, U.S.A.)

with high resolution MS ($R = 5,000$, m/z 190.0630 for ABA-Me-TMS and m/z 194.0877 for 2H4-ABA-Me-TMS).

Measurement

of local endogenous ABA levels using ELISA.

Ler-0 plants were evaluated for local ABA levels after water or BABA pretreatment. Plants were harvested before inoculation as well as 1, 3, and 5 dpi. A 1-cm transverse strip comprising the two inoculation sites of each leaf was cut out and the material was frozen immediately in liquid nitrogen. The frozen plant material was ground in liquid nitrogen and 24.5 to 28.2 mg was taken for analysis. Each measurement was repeated with three biological replicates on pooled material from three different plants. ABA was extracted with 80% methanol. The extraction was done in a MixerMill for 3 min (30 Hz). After evaporation in a Speed Vac concentrator, the samples were dissolved in 100 μl of 80% methanol and analyzed with a Phytodetekt ABA Enzyme Immunoassay Test Kit (Agdia, Elkhart, IN, U.S.A.) in an ELISA reader (Benchmark; Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's instructions. A generalized linear model determined a linear relationship ($R^2 = 0.985$) between percent binding ($[\text{sample optical density at } 405 \text{ nm (OD}_{405}) - \text{non-specific binding OD}_{405}]/[100\% \text{ binding control OD}_{405} - \text{non-specific binding OD}_{405}] \times 100\%$ and $\ln([\text{ABA}(\text{pg})])$ in the range between 2.48 and 87.5% binding or between 0.0032 and 2 pg of (–) ABA. All samples were found in the linear range.

Staining procedures.

Leaves from *Arabidopsis* and *B. napus* were collected 2 dpi as well as when visible symptoms had developed and stained with aniline blue as described previously by Gressel and associates (2002). The presence of callose deposition was detected with a Nikon Microphot-FXA microscope under UV light and a UV2A filter (330- to 380-nm emission). The staining was repeated twice with two independent inoculation events using five to eight biological replicates of each genotype.

Callose quantification.

TIFF pictures were taken under identical conditions, 8 to 10 aniline blue-stained leaves per genotype, and were analyzed in APS assess software (L. Lamari, University of Manitoba, Canada). Individual quantifications were based on the relative area covered by callose depositions within 0.15 mm of the inoculation site after background adjustments. The mean and median values for each inoculation site were used as estimators of callose deposition for each leaf sample and compared using a one-tailed *t* test.

Microarray hybridizations.

The genetic identity of the *abi1-1* and *abi2-1* mutants was confirmed with CAPS markers as described by Leung and associates (1997) prior to microarray comparisons. Differential gene expression in pooled ABA pretreated *abi1-1* and *abi2-1* mutants inoculated with *L. maculans* was assessed 48 hpi. RNA was isolated using the RNeasy kit (Qiagen GmbH). The cDNA synthesis, slide hybridization, and washings were performed as described in the protocols "RNA amplification and labeling of RNA probes" and "Microarray hybridization-RNA probes" from the Meyerowitz lab at Caltech (Pasadena, CA, U.S.A.). Labeled cDNA was hybridized to CATMA 25k cDNA arrays. The experiment was done using three biological replicates and one of the replicates was dye swapped. The CATMA arrays were analyzed with GenePix 4000B (Axon Instruments, Amersham Pharmacia Biotech).

Analysis of expression data.

Array-by-array examinations were made by the arbitrary twofold expression difference determination, after filtering and normalizations with the *kth* package in R (R Foundation for Statistical Computing, Vienna, Austria) in accordance to the settings presented in the *kth* package tutorial. Simultaneous analysis on all three arrays was performed with a B-test within the same program package to identify significantly differentially expressed genes under the same filtering and normalization conditions. Data were uploaded in accordance to the MIAME standard to BASE for worldwide public access.

Real time PCR.

Quantitative measurements of At1g63750 (forward primer: 5'-AGCTTTGACTGAGTGAGCAAT-3', reverse primer: 5'-TTCTTGATCATGTTTCGCTTCAT-3'), At1g63870 (forward primer: 5'-AAGAACCTTGGAAACACAAGATTT-3', reverse primer: 5'-TGATATGCCGGACATAGCTTTA-3'), At1g63880 (forward primer: 5'-CAATGAACTTGTGCACGTAAGA-3', reverse primer: 5'-TCGATCATTATAGCTTCATTGTCC-3'), and At1g64070 (forward primer: 5'-AGATAGCCGGCTCGAGTACC-3', reverse primer: 5'-GAACCTTCCAAATTTAGCTTC TTG-3') were done on pooled material from six individual plants 48 hpi, using four biological replicates. The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 50 s at 64°C. Genomic DNA was amplified using 3 µM primers, 5 ng of template DNA per 25 µl of reaction together with 12.5 µl of Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, U.S.A.) on a ABI 7000 thermocycler (Applied Biosystems).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Arabidopsis Information Resource AGI database:
www.arabidopsis.org
 Caltech protocols: www.its.caltech.edu/~plantlab/html/protocols.html
www.catma.org
 KTH (Royal Institute of Technology, Stockholm) microarray resource
 center website: www.biotech.kth.se/molbio/microarray
 The Linnaeus Centre for Bioinformatics (BASE) database: base.lcb.uu.se/

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