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Cuticular defects lead to full immunity to a major plant pathogen

Céline Chassot[1], Christiane Nawrath[2] and Jean-Pierre Métraux[1*]
[1]Département de Biologie, Université de Fribourg, Chemin du Musée 10, CH-1700 Fribourg, Switzerland, and
[2]Département de Biologie Moléculaire Végétale, Bâtiment Biophore, Quartier Sorge, Université de Lausanne, CH-1015 Lausanne, Switzerland

Summary

In addition to its role as a barrier, the cuticle is also a source of signals perceived by invading fungi. Cuticular breakdown products have been shown previously to be potent inducers of cutinase or developmental processes in fungal pathogens. Here the question was addressed as to whether plants themselves can perceive modifications of the cuticle. This was studied using *Arabidopsis thaliana* plants with altered cuticular structure. The expression of a cell wall-targeted fungal cutinase in *A. thaliana* was found to provide total immunity to *Botrytis cinerea*. The response observed in such cutinase-expressing plants is independent of signal transduction pathways involving salicylic acid, ethylene or jasmonic acid. It is accompanied by the release of a fungitoxic activity and increased expression of members of the lipid transfer protein, peroxidase and protein inhibitor gene families that provide resistance when overexpressed in wild-type plants. The same experiments were made in the *bodyguard* (*bdg*) mutant of *A. thaliana*. This mutant exhibits cuticular defects and remained free of symptoms after inoculation with *B. cinerea*. The expression of resistance was accompanied by the release of a fungitoxic activity and increased expression of the same genes as observed in cutinase-expressing plants. Structural defects of the cuticle can thus be converted into an effective multi-factorial defence, and reveal a hitherto hidden aspect of the innate immune response of plants.

Keywords: *Botrytis cinerea*, cutinase, lipid transfer proteins, peroxidase, priming, proteinase inhibitors.

Introduction

Aerial parts of land plants are covered with a cuticle made of cutin, a complex polymer rich in esterified fatty acid derivatives, associated and coated with waxes. The cuticle forms a protection against water loss, irradiation and xenobiotics, and is involved in the delimitation of organs during development (Kolattukudy, 1985; Nawrath, 2002). The importance of the cuticular layer for plant growth and development has also been evaluated experimentally by producing transgenic plants with an altered structure of the cuticle. Arabidopsis plants expressing constitutively a fungal cutinase from *Fusarium pisi f.sp.* targeted to the cell wall exhibit an altered ultrastructure of the cuticle and enhanced permeability of the cuticle to solutes. Moreover, pollen is able to germinate on leaves of cutinase-expressing (CUTE) plants but not on leaves of control plants (Sieber *et al.*, 2000). A number of developmental mutants with various changes in the structure of the cell wall and the cuticle have been described, and these serve as useful tools to study the structure–function relationship of the cuticle (Nawrath, 2006).

The cuticle is considered to constitute a physical barrier to microbial invaders through which cutinase-producing pathogens can penetrate. In addition to its role as a barrier, the cuticle is likely to be a source of signals used by invading pathogens to prepare and adjust for the colonization of their host. The production of cutinase in *Fusarium solani f.sp. pisi* is induced by cutin monomers present in the surrounding medium (Lin and Kolattukudy, 1978; Woloshuk *et al.*, 1986). A model was proposed whereby fungi sense the presence of cutin monomers on the plant surface and induce high levels of cutinase required for invasion (Kolattukudy, 1985). Cuticular components can also regulate developmental processes in pathogenic fungi. For instance, cutin monomers induce the germination and appressorium in *Magnaporthe grisea*

(Gilbert *et al.*, 1996) and formation of the appressorial tube in *Erysiphe graminis* (Francis *et al.*, 1996).

Plants can sense a variety of molecules released during interaction with pathogens. In particular, breakdown products of the plant cell wall are known to act as elicitors of defences (Boller, 1995). A hypothesis was proposed and tested suggesting that the plant might also perceive degradation products of the cuticle. Application of synthetic cutin monomers of the C18 family to barley (*Hordeum vulgare*) and rice (*Olyza sativa*) provided resistance while exhibiting no direct fungicidal effect on *Erysiphe graminis* (Schweizer *et al.*, 1994,1996). When tested in suspension-cultured potato (*Solanum tuberosum*) cells, cutin monomers induced medium alkalinization, ethylene production and accumulation of defence-related genes, indicating an effect on the activation of defence mechanisms (Schweizer *et al.*, 1996). Interestingly, the most active compound was *n*, 16-hydroxypalmitic acid ($n = 8, 9$ or 10), a major component of the potato cuticle. In etiolated and conditioned cucumber hypocotyls, cutin monomers from hydrolysates of cucumber (*Cucumis sativus*), apple (*Malus domesticus*) and tomato (*Lycopersicon esculentum*) cutin elicited H_2O_2 production (Fauth *et al.*, 1998). These experiments support the notion that plants have the potential to recognize breakdown products of the cuticle and activate defence-related mechanisms.

Further support for this notion was obtained by studying the effect of purified cutinase from *Venturia inaequalis* (or *Fusarium solani*) on spores of *Rhizoctonia solani*. Supplementing spore droplets of *R. solani* with cutinase led to a decrease in web blight symptoms on bean (*Phaseolus vulgaris*) when compared with drops containing spores alone. This surprising action of cutinase requires its catalytic lipolytic esterase activity. Unfortunately, the effect of the residues generated by the action of the cutinase has not been described. The resistance observed after cutinase treatment was found to be independent of known defence responses (Parker and Koller, 1998). This example suggests that plants might possibly detect cuticle breakdown products or alterations of their cuticle to activate defence responses.

Here, we describe the reaction of plants to modifications in the structure of the cuticle. We have studied the reaction of *Arabidopsis thaliana* plants that express a fungal cutinase targeted to their cell walls (CUTE plants; Sieber *et al.*, 2000) or of a mutant, *bdg*, with defects in cuticle structure and integrity (Kurdyukov *et al.*, 2006). We show that perturbations of the cuticular layer lead to full immunity to *B. cinerea*, a ubiquitous fungal pathogen that causes significant damage to many crop plants.

Results

The importance of cuticular modifications on interaction with a pathogen has been studied using CUTE plants. Surprisingly, CUTE plants were completely resistant to the virulent necrotrophic pathogen *B. cinerea*, which causes soft rot lesions on wild-type (WT) plants (Figure 1a,b), but disease symptoms after inoculation with other necrotrophic fungi (*Plectosphaerella cucumerina*, *Alternaria brassicicola* or *Sclerotinia sclerotiorum*) were unchanged (data not shown). Plants expressing the cutinase gene driven by a dexamethasone-inducible promoter (Aoyama and Chua, 1997; so-called DEX-CUTE plants) also exhibited extensive resistance (Figure 2a,b). Protection was completely lost in Arabidopsis transformed with a non-functional *F. solani* cutinase gene containing a point mutation in the catalytic site (Rogers *et al.*, 1994; data not shown). Furthermore, *A. thaliana* plants overexpressing a gene encoding a cutinase (*CUTA*) or a lipase with cutinolytic activity (*LIP1*) from *B. cinerea* also displayed full immunity to *B. cinerea* (Figure S1). Resistance was also observed in WT plants after application of purified *F. solani* cutinase (Figure 1b), the direct effect of which was harmless to *B. cinerea* when tested *in vitro* (data not shown). The complete resistance observed after cuticular modifications in CUTE plants was also observed in a recently available cuticle-defective mutant *bdg* that shares a number of morphological characteristics with CUTE plants (Kurdyukov *et al.*, 2006; Figure 1a,b).

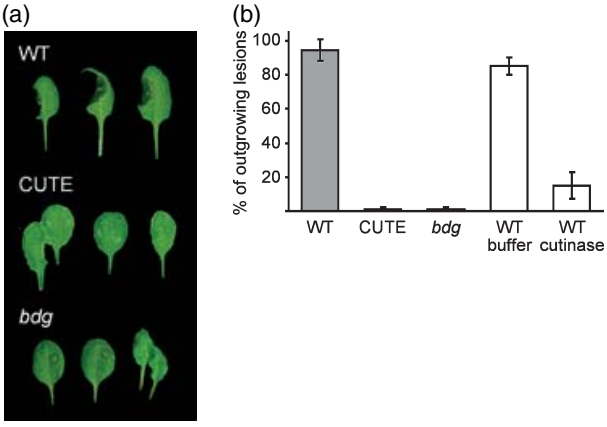


Figure 1. Effect of cuticular defects on resistance to *B. cinerea*.
(a) Appearance of WT, CUTE and *bdg* plants 3 days after inoculation with *B. cinerea*. The inoculation droplets are still visible on CUTE and *bdg* leaves.
(b) Percentage of out-growing lesions in inoculated WT, CUTE and *bdg* plants (grey bars) and WT plants treated with cutinase compared with control (white bars) 3 days after inoculation (five independent experiments, $n = 60$, means ± SD).

The development of *B. cinerea* spores deposited on *A. thaliana* leaf surfaces was monitored microscopically using various staining techniques. Hyphal growth of *B. cinerea* was inhibited after spore germination on the plant surface of CUTE plants (Figure 3a), and cross-sections through inoculation sites showed that penetration did not take place compared with WT plants (Figure 3b). The expression of fungal genes typically associated with pathogenesis, such as exopolygalacturonase, endopolygalacturonase, pectinmethylesterase, glutathione-*S*-transferase,

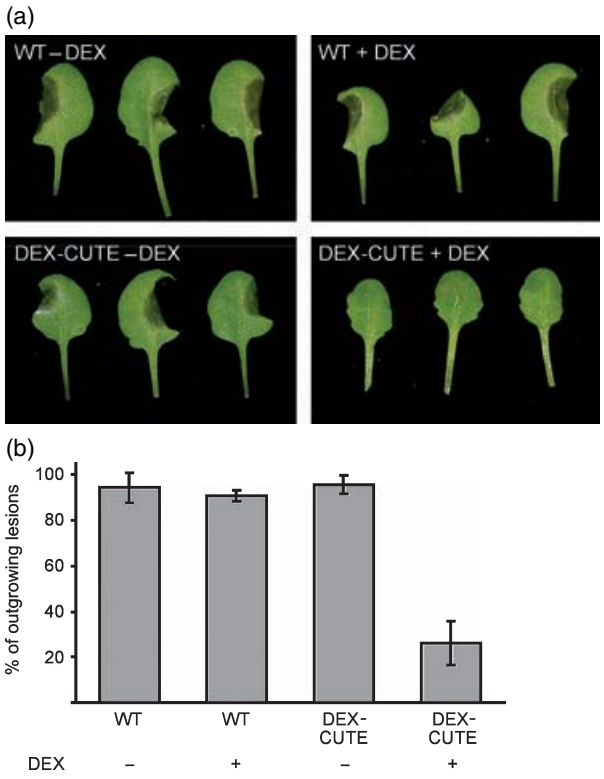


Figure 2. Effect of inducible cutinase expression on resistance to *B. cinerea*.
(a) *B. cinerea* symptoms 3 days after inoculation in DEX-induced DEX-CUTE and control plants.
(b) Percentage of out-growing lesions 3 days after inoculation in DEX-induced DEX-CUTE plants compared with controls (five independent experiments, $n = 60$, means ± SD).

aspartic protease, cutinase and G2 protein, was monitored by quantitative PCR in *B. cinerea* during the first 48 h after inoculation. With the exception of endopolygalacturonase and aspartic protease, which were produced in lesser amounts, no major differences were observed in the expression of all other genes of *B. cinerea* growing on CUTE compared to WT leaves (Supplementary Figure S2). The inhibition of fungal growth might possibly be related to a toxic compound from the surface of leaves. This hypothesis was tested, and a fungitoxic activity was detected that diffused from the leaf surface in CUTE plants (Figure 4).

The fungitoxic substance was not active *in vitro* against the necrotrophs *Plectosphaerella cucumerina* and *Alternaria brassicicola* that are infectious on *A. thaliana*. On the other hand, the fungitoxic diffusate was active against *Monilia laxa*, a species closely related to *B. cinerea*, against which *A. thaliana* is resistant (Figure S3). Interestingly, this fungitoxic activity was also observed diffusing from surfaces of *bdg* mutants (Figure 4). The fungitoxic activity was demonstrated in both *in vitro* and *in vivo* assays on WT *A. thaliana* leaves (Figure 3). The fungitoxic activity was also active when applied together with spores of *B. cinerea* on tomato leaves (Figure S3).

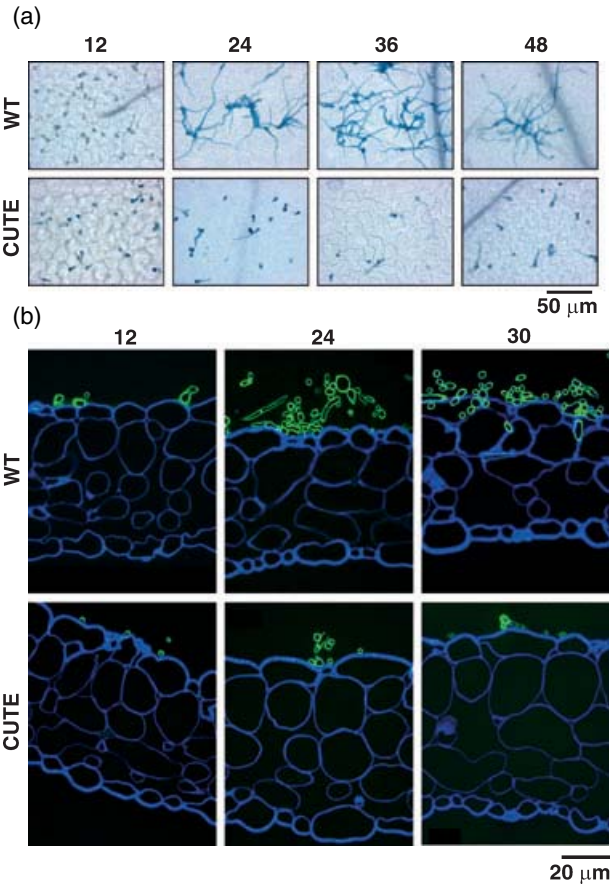


Figure 3. Growth of *B. cinerea* on WT and CUTE leaves.
(a) Trypan blue staining of inoculated WT and CUTE leaves 12, 24, 36 and 48 h after inoculation with *B. cinerea* (magnification of all pictures indicated, expect for WT, 48h where magnification is reduced by half).
(b) FITC-WGA staining of sections through inoculated WT and CUTE leaves 12, 24 and 30 h after inoculation with *B. cinerea*.

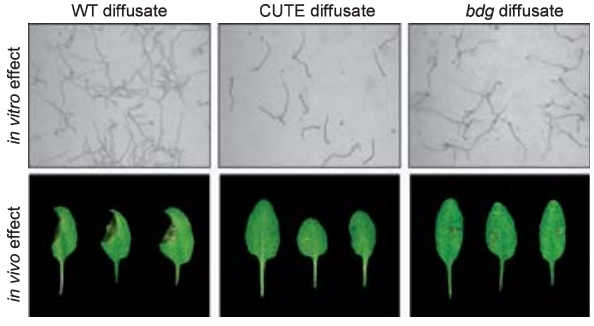


Figure 4. Fungitoxic activity diffusing from the leaf surface in plants with cuticular defects.
Upper images: *in vitro* fungitoxic activity of CUTE and *bdg* diffusates to *B. cinerea*, in comparison with WT diffusate (10 independent experiments, typical examples are shown).
Lower images: *in vivo* activity of diffusate, shown by the appearance of WT plants 3 days after inoculation with *B. cinerea* mixed with diffusates of WT, CUTE and *bdg* plants (10 independent experiments, $n = 60$).

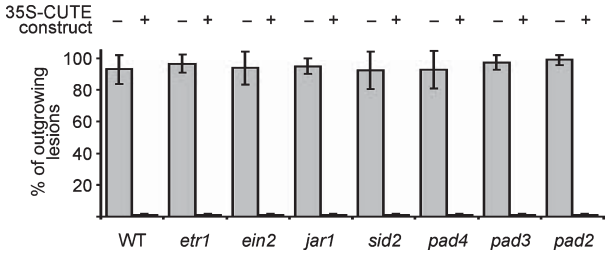


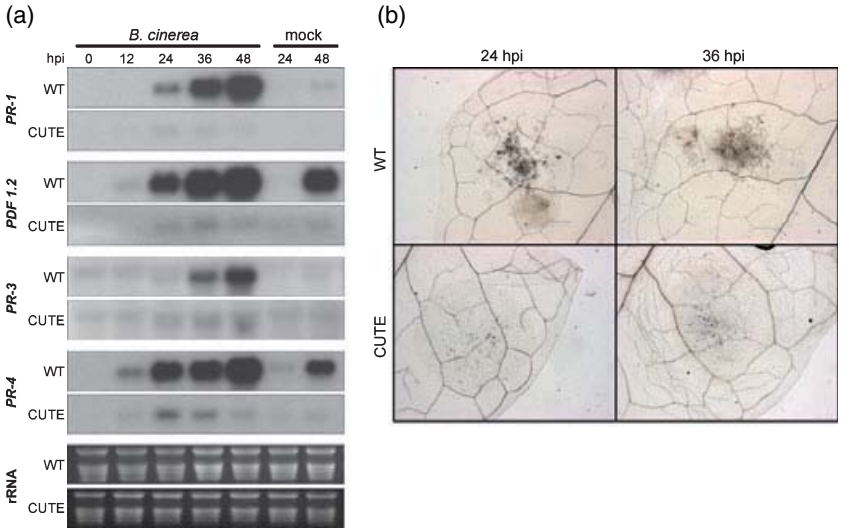
Figure 5. Resistance to *B. cinerea* in signalling mutants overexpressing cutinase.
Percentage of out-growing lesions in various defence signalling mutants overexpressing the cutinase of *F. solani* f. sp. *pisi* (35S–CUTE construct) in comparison with untransformed controls 3 days after inoculation with *B. cinerea* (three independent experiments, $n = 24$, means ± SD).

We tested the involvement of the major signalling routes commonly associated with plant defence reactions and involving the hormones salicylic acid (SA), ethylene (ET) or jasmonic acid (JA). Mutants defective in the SA (*sid2*, *pad4*; Glazebrook and Ausubel, 1994; Nawrath and Métraux, 1999), ET (*ein2*, *etr1*; Guzman and Ecker, 1990; Schaller and Bleecker, 1995) or JA (*jar1*; Staswick *et al.*, 2002) pathways were transformed with the *F. solani* cutinase under the control of a constitutive promoter. All transformants were fully resistant to *B. cinerea* compared with the original CUTE plants (Figure 5). Therefore, resistance of CUTE plants is independent of pathways involving these signals, although it cannot be excluded that some of these pathways redundantly regulate resistance in CUTE plants. Furthermore, overexpression of the *F. solani* cutinase in the *pad2* and *pad3* (Glazebrook *et al.*, 1997) mutants impaired in the production of the fungitoxic phytoalexin camalexin resulted in fully immune plants, indicating, in this case, that camalexin is not involved (Figure 5).

The expression of known defence-related genes was compared in CUTE and WT plants. CUTE plants show no constitutive expression of genes encoding pathogenesis-related proteins (PRs) or plant defensin (*PDF1.2*). No induction took place during infection with *B. cinerea*, unlike infected WT plants (Figure 6a). In addition, the production of reactive oxygen species was strongly reduced in CUTE plants (Figure 6b). In contrast, the induction of these responses in WT plants correlates with the massive development of symptoms.

Further insights in the changes associated with resistance of CUTE plants were obtained by genome-wide gene expression studies using Affymetrix® microarrays. Gene expression was analysed 12 and 30 h after inoculation with *B. cinerea* (Table S1). Interesting candidate genes were selected on the basis of a stronger induction in CUTE plants compared to WT plants after inoculation with *B. cinerea* (so-called priming). The selection of genes exhibiting this expression pattern was made using the recently published microarray software 'FiRe' (Garcion *et al.*, 2006). The search criteria were set as genes exhibiting at least a twofold induction both in WT and CUTE plants after infection. This reduced the number of candidates to about hundred genes. Genes in this group that had a higher expression level in infected CUTE plants compared with infected WT plants at either 12 or 30 h after inoculation were retained. Of these genes, candidates were chosen on the basis their conspicuous affiliation to large gene families (Table S1), resulting in the selection of 13 genes. The microarray data for these final candidates were confirmed using real-time RT-PCR (data not shown). The possible relevance for defence of the genes presented in Table S1 was tested by constitutive overexpression in WT plants. Resistance to *B. Cinerea* was observed in transformed plants overexpressing closely-related members of the lipid transfer protein (LTP) family (At4g12470, At4g12480, At4g12490; Arondel *et al.*, 2000), the class III peroxidase (PER) family (At2g37130, At5g39580, At5g64120; Tognolli *et al.*, 2002) and the prote-

Figure 6. Defence reactions in WT and CUTE plants after inoculation with *B. cinerea*.
(a) Gene expression analysis (Northern blot) of *PR-1*, *PDF1.2*, *PR-3* and *PR-4* in WT and CUTE plants 0, 12, 24, 36 and 48 h post-inoculation (hpi) with *B. cinerea*. Five micrograms of RNA per sample were loaded. The experiment was repeated twice with similar results.
(b) Production of H_2O_2 in infected WT and CUTE plants. H_2O_2 was visualized by DAB staining 24 and 36 h post-inoculation (hpi) with *B. cinerea*. The experiment was carried out on 12 plants and repeated twice. Typical examples are shown.



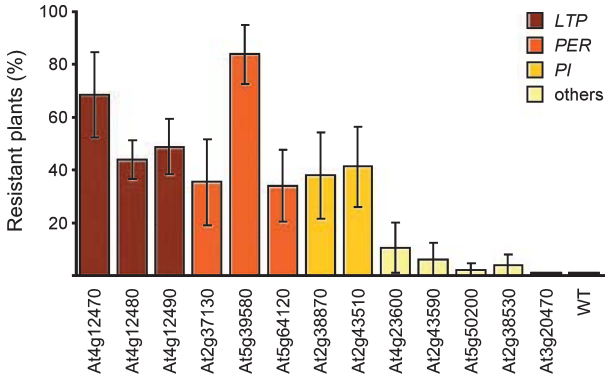


Figure 7. Effect of overexpression of candidate genes identified in microarrays on the resistance to *B. cinerea*.
Each bar represents the percentage of independent primary transformants of each selected gene (indicated by the gene locus number) exhibiting full resistance (four independent experiments, $n = 60$, means ± SD). Resistance was tested on three leaves per transformant. The resistance to *B. cinerea* conferred by overexpression of members of the *LTP*, *PER* and *PI* gene families was further confirmed in homozygous transformants of the second generation.

inase inhibitor (PI) family (At2g38870, At2g43510; Dunaevskii *et al.*, 2005; Figure 7). There was no correlation between the level of expression and the degree of resistance.

Overexpression of other candidate genes such as At4g23600 (tyrosine transaminase), At2g43590 (chitinase) or At2g50200 (wound-responsive protein 3) did not provide significant protection against *B. cinerea*, in addition to two other genes (At2g38530, At3g20470; non-specific lipid-transfer protein 2 and glycine-rich protein) with different expression patterns (Figure 7). Transformants of the second generation (T_2) were analysed for some of the transgenes that conferred resistance to *B. cinerea* in the T_1 generation (At4g12470, At5h39580 and At2g43510). Resistance was determined on 24 T_2 plants for each line. In most but not all cases, the level of overexpression of the transgene correlates with an increased resistance to *B. cinerea* (data not shown). Moreover, the cuticle-defective mutant *bdg* also showed enhanced expression of the same genes as in CUTE plants after inoculation with *B. cinerea*, providing further support that these genes are involved in the response associated with cuticular defects (Figure S4).

Discussion

Plants with cuticular defects resulting from overexpression of a fungal cutinase displayed total immunity against *B. cinerea*. This surprising observation is somewhat at odds with the notion that breaching barriers might favour the progression of an invader. Our experiments showed that the effect of cutinase is indirect, requires its full activity and does not depend on the origin of the gene. These observations corroborate very closely those carried out with the *bdg* mutant that is affected in the organized polymerization of carboxylic esters in the cuticle, and provide experimental support for the notion that plants can react to cuticular defects and activate defence responses.

The behaviour of *B. cinerea* on CUTE plants indicated that hyphal growth but not spore germination was arrested at an early stage during infection. *B. cinerea* does express several of its pathogenicity genes, for example cutinase (Figure S2), indicating recognition of the surface of CUTE plants. The host showed no signs of cellular damage, and tissue colonization did not take place. This prompted a search for the presence of a possible fungitoxic activity present in the inoculation droplets. The cutinase itself was not found to be toxic, and addition of purified cutinase to spores did not affect their germination (data not shown). An inhibitory activity diffusing from the leaf surface was detected and found to be active *in vivo* when added at the same concentration to spores of *B. cinerea* inoculated on WT *A. thaliana* or tomato plants. A certain degree of correlation could be observed between the inhibitory effect of the fungitoxic diffusate and the resistance of the plant. The direct effect against *Monilia laxa* suggests that this activity is likely to have a broader spectrum than only *B. cinerea*. A similar activity was also observed in leaf diffusates from *bdg* mutants. The chemical nature of this activity remains to be determined. This fungitoxic diffusate possibly provides a first powerful defence layer against invasion by *B. cinerea* in plants with defective cuticles.

Resistance to *B. cinerea* was shown previously to be associated mainly with the JA- and ET-signalling pathways, and with SA and camalexin being involved in local responses (Penninckx *et al.*, 1998; Thomma *et al.*, 1999; Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). Expressing the *Fusarium* cutinase gene in mutants of the SA, ET and JA signal transduction pathways tested the possible involvement of the major signalling pathways for defence. Our results rule out the SA, ET and JA signalling pathways, as well as the participation of the phytoalexin camalexin. The total resistance to *B. cinerea* observed in plants affected by cuticular defects is likely to have a different physiological basis than the resistance observed in previously published work (Ferrari *et al.*, 2003; Manfredini *et al.*, 2005), and reveals a hitherto undiscovered mode of resistance to a fungal pathogen.

Resistance of CUTE plants was not found to correlate with the induction of genes coding for various PR proteins (Figure 5a). In particular, plant defensin, a marker associated with the JA and ET signalling pathways, was not induced. This lack of correlation is consistent with the SA-, JA- and ET-independent CUTE-induced resistance to *B. cinerea.* It provides strong support for a powerful mechanism of resistance to *B. cinerea* that is independent of resistance mechanisms previously described for *B. cinerea*. The production of reactive oxygen species (ROS) is one of the earliest plant responses to pathogens, that can activate many plant defences including cell death (Apel and Hirt, 2004). *B. cinerea* can take advantage of ROS production

(Govrin and Levine, 2000). The resistant CUTE plants produced reduced amounts of ROS compared to infected WT plants at similar time points after inoculation (Figure 5b). The reduced accumulation of ROS may create an unfavourable environment for the development of *B. cinerea.* However, low levels of ROS might also result from reduced tissue damage by *B. cinerea.*

Using genome-wide expression analysis, we further explored the reactions of the host, and focused on genes belonging to the *LPT*, *PER* and *PI* families that were conspicuously primed in inoculated CUTE and *bdg* plants. A considerable increase in the expression of the selected genes was observed in CUTE and *bdg* plants after mock inoculation, and was further enhanced in the presence of *B. cinerea*. Generally, LTPs are known to be associated with the formation of the cutin and suberin layers and inhibition of fungal growth (Segura *et al.*, 1993; Blein *et al.*, 2002; Heredia, 2003). For example, heterologous expression of an LTP from pepper (*Capsicum anuum*) in Arabidopsis was recently reported to increase the resistance to *B. cinerea* (Jung *et al.*, 2005). The biochemical function of the three *LTP* genes identified remains unknown, but our data provide experimental support for their involvement in resistance to *B. cinerea* in Arabidopsis. Members of the class III *PER* gene family encode proteins involved in cell-wall lignification and cross-linking, H_2O_2 generation or detoxification, and responses to wounding and pathogens (Tognolli *et al.*, 2002). PER activity was associated with cross-linking of phenolic acids at infection sites of *Botrytis allii* in onion (*Allium cepa*) cell walls (McLusky *et al.*, 1999). In bean leaves, aggressive isolates of *B. cinerea* suppressed the PER activity compared with non-virulent isolates, supporting a role for PER in plant resistance as scavengers of harmful active oxygen species (Von Tiedemann, 1997). Our results are in agreement with these observations and provide a new biological function for a group of hitherto undescribed *PER* genes in plants in relation to *B. cinerea* resistance. *B. cinerea* secretes aspartic proteases during the early stages of infection that are likely to play a primordial role in pathogenesis (Movahedi and Heale, 1990; ten Have *et al.*, 2004). Proteinase inhibitors produced in many plants, including trypsin inhibitors, can inhibit *B. cinerea* proteases *in vitro* (Dunaevskii *et al.*, 2005), and overexpression of proteinase inhibitors from *Nicotiana alata* in tobacco (*Nicotiana tabacum*) was recently shown to protect against *B. cinerea* (Charity *et al.*, 2005). The data presented here corroborate these findings and support a role for *PI* genes in the defence of Arabidopsis against *B. cinerea*. Taken together, our data support the view that the combined action of the products of the *LTP*, *PER* and *PI* genes contributes to the resistance of cuticle-defective plants, possibly forming a second protective shield against infection with *B. cinerea*.

Thus, defective cuticles such as those in CUTE or *bdg* plants might provide cues for the plant and help mount an effective defence towards *B. cinerea*. It has yet to be determined whether this effect is based on the perception of cuticle monomers/breakdown products or other reasons, such as the increased permeability of the cuticle that was shown in both CUTE and *bdg* plants (Sieber *et al.*, 2000; Kurdyukov *et al.*, 2006). Both situations require an active cutinase for the effect to occur. The former hypothesis, for which there is no experimental evidence at this point, would require a detailed analysis of changes in soluble cuticle products, their concentration *in situ* and their biological activity. Once available, the responses of the plant could be tested after ectopic application of such inducers. A permeable cuticle, as proposed in the latter hypothesis, might improve the diffusion of elicitors released from the pathogen or the plant, allowing a faster induction of resistance mechanisms. It may also increase the diffusion of anti-microbial compounds from the cell wall or plant cell to the leaf surface. Our current work is also directed at chemical description of the anti-microbial compound(s) diffusing from the cuticle or the cell wall into the inoculation droplets.

The question remains as to why the cutinase produced by the pathogen during infection does not induce resistance. The rapidly developing *B. cinerea* can clearly overcome the effect of its cutinase on the host. This would be in agreement with the observation that a *B. cinerea* strain mutated in the *cutinase A* and *lipase 1* genes is as virulent as the WT strain (Reis *et al.*, 2005). The localized cuticle breakdown might not be extensive enough to allow an optimal release or diffusion of the fungitoxic activity, either from a pre-formed or induced source. Interestingly, the genes selected from the microarray analyses were also induced in WT plants after *B. cinerea* inoculation, albeit to a lower level, indicating that they might be part of an attempted but insufficient defence response against the virulent pathogen. It cannot be excluded that *B. cinerea* can suppress the defences described here, thus minimizing their effect.

In summary, *A. thaliana* plants displaying cuticular defects induce changes leading to complete resistance to *B. cinerea*. Immunity is likely to result from a multi-factorial defence that is independent of SA, ET and JA, and involves an effective diffusible fungitoxic activity. In addition, the expression of genes such as those of the *LTP*, *PER* and *PI* families, which are naturally induced by *B. cinerea* in WT plants, is strongly enhanced in plants with cuticular defects. The resistance syndrome presented here is a striking case where a species normally susceptible to a necrotrophic fungus establishes full immunity. These results increase our knowledge on plant defences and highlight a mechanism that might possibly be exploited to protect plants against this ubiquitous fungal pathogen.

Experimental procedures

Plant material

Plants were grown on a pasteurized soil mix of humus/perlite (3:1) under a 12 h light/12 h dark cycle, with a night temperature of 16–18°C and a day temperature of 20–22°C (60–70% humidity). WT plants are the Arabidopsis accession Col-0, obtained from the Arabidopsis Biological Research Center (Columbus, OH, USA). The Arabidopsis mutant *bdg* that was used was *bdg-1* (Kurdyukov *et al.*, 2006); *sid2* was *sid2-1*(Nawrath and Métraux, 1999); *pad3* was *pad3-1*, *pad2* was *pad2-1* and *pad4* was *pad4-1*(Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1997); *etr1* was *etr1-1* and *ein2* was *ein2-1*(Guzman and Ecker, 1990; Schaller and Bleecker, 1995); *jar1* was *jar1-1*(Staswick *et al.*, 2002).

Inoculation with B. cinerea

B. cinerea strains B05.10 (Buttner *et al.*, 1994) and BMM (Zimmerli *et al.*, 2001) were grown on 1 × PDA (potato dextrose agar, 39 g l^{-1}; Difco www.vddusa.com/DIFCO.htm). Spores were harvested in water and filtered through glass wool to remove hyphae. Spore concentration was adjusted to 5×10^4 spores ml^{-1} in 1/4-strength PDB (potato dextrose broth, 6 g l^{-1}; Difco) for inoculation. Leaves were inoculated with 5 μl droplets of spore suspension to evaluate the symptoms. On WT Arabidopsis Col-0 plants, most inoculation sites resulted in expanding water-soaked lesions but a limited number of lesions did not spread from the inoculation site. Therefore, under our experimental conditions, the percentage of soft rot symptoms extending beyond the inoculation site (out-growing lesions) was the most adequate way to determine the level of resistance. The spore suspension was sprayed on whole plants for microarray and real-time RT-PCR experiments. Control plants were inoculated with 1/4-strength PDB. The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Both *B. cinerea* strains gave similar results for all experiments performed.

Induction with dexamethasone (DEX)

DEX (Sigma, http://www.sigmaaldrich.com/) was dissolved in ethanol at 30 mM and diluted into H_2O + 0.01% Tween-20 (Fluka www.fluka.org) to a final concentration of 0.03 mM. Two to three weeks after transplanting, DEX-CUTE and WT plants were sprayed twice a day with the DEX solution and again 4 days later. Plants were infected with *B. cinerea* 3 days after the second treatment. After spraying of DEX, trays were covered overnight with a lid. Induction of cutinase was confirmed by an enzymatic assay (Sieber *et al.*, 2000).

Staining of fungal hyphae

To visualize *B. cinerea* growth under the microscope, inoculated leaves were stained with lactophenol–trypan blue (Nawrath and Métraux, 1999). To visualize penetration of hyphae in leaves, small pieces of leaves cut around the inoculation site were fixed for 90–120 min in 2% glutaraldehyde and 0.05 M Na-cacodylate, pH 7.0. The material was washed six times for 10 min each in 0.05 M Na-cacodylate, and fixed in 1% osmium tetroxide and 0.05 M Na-cacodylate overnight at 4°C. The samples were washed, dehydrated in a graded series of acetone, and embedded by the method described by Spurr (1969). Semi-thin sections of 5 μm were fixed on glass slides by heating them to 95°C for 2 h. The resin was removed by exposing the sections to 2% NaOH in absolute ethanol for 5 min. After washing in ethanol and rehydration, the sections were immersed in H_2O_2 for 10 min. The sections were then incubated with 50 μg ml^{-1} FITC-WGA (Fluorescein isothiocyanate labelled wheat germ agglutinin; Sigma www.sigmaaldrich.com) (excitation 450–490 nm; emission 550 nm) in 10 mM phosphate buffer for 2 h. The sections were washed in distilled water, and additionally stained for 5 min with 0.1% calcofluor white. Samples were observed with a fluorescence microscope.

Visualization of H_2O_2

The presence of H_2O_2 was visualized by 3,3′-diaminobenzidine (DAB; Thordal-Christensen *et al.*, 1997) staining 24 and 36 h after inoculation with *B. cinerea*. Detached leaves were immersed in 1 mg ml^{-1} DAB-HCl, pH 3.8 (Sigma), and vacuum-infiltrated. After overnight incubation, leaves were bleached in 4 mg ml^{-1} chloralhydrate and observed with a microscope. The experiment was carried out on 32 plants and repeated twice. Typical examples are shown.

In vitro *effect of cutinase and application of cutinase to leaves*

Purified cutinase from *F. solani*, kindly provided by M. Van der Burg-Koorevaar (Unilever, Vlaardingen, The Netherlands), was diluted in 10 mM Na-acetate, pH 5.2. The *in vitro B. cinerea* growth assay was performed in a final volume of 12 μl. Nine microlitres of the cutinase solution were mixed with 3 μl of *B. cinerea* spores in PDB to a final concentration of 1000, 100 or 10 μg ml^{-1} of cutinase and 5×10^4 spores ml^{-1} in 1/4-strength PDB, and deposited on a microscope glass slide. After incubation under high-humidity conditions for approximately 14 h, fungal growth was observed under the microscope. For cutinase application on WT plants, 5 μl droplets of 100 μg ml^{-1} cutinase or buffer were deposited on leaves, and the leaves were incubated for 3 days under high humidity to prevent evaporation of the droplets. Droplets were then removed and replaced by a droplet of *B. cinerea* spores.

In vitro *and* in vivo *effect of leaf diffusates*

Five microlitre droplets of 1/4-strength PDB were incubated for 18 h on WT, CUTE and *bdg* leaves. Potential leaf diffusates were collected directly into these droplets. Nine microlitres of the collected diffusate solution were mixed with 3 μl of *B. cinerea* spores in H_2O to a final concentration of 5×10^4 spores ml^{-1}, and deposited on a microscope glass slide. Fungal growth was observed under the microscope after incubation under high-humidity conditions for approximately 14 h. For *in vivo* tests, WT Arabidopsis leaves were inoculated with the leaf diffusates mixed with *B. cinerea* spores. Disease symptoms were evaluated 3 days later.

Plant transformation

Arabidopsis Col-0 plants, *sid2* (Nawrath and Métraux, 1999), *ein2*, *etr1*(Guzman and Ecker, 1990; Schaller and Bleecker, 1995), *jar-1*(Staswick *et al.*, 2002), and *pad2*, *pad3* and *pad4* mutants (Glazebrook and Ausubel, 1994; Glazebrook *et al.*, 1997) were transformed with a *F. solani* cutinase as previously described (Sieber *et al.*, 2000). The presence of the 35S–CUTE construct was confirmed by the morphological phenotype typical for CUTE plants.

DEX-CUTE plants were generated using the glucocorticoid-inducible system (Aoyama and Chua, 1997). The *F. solani* cutinase gene fused with the signal sequence of tobacco chitinase A were excised from pMMB7066:SS:CUT (see Sieber *et al.*, 2000) using *Sst*I/*Xba*I,

and cohesive ends were filled in with T4 DNA polymerase (New England Biolabs www.neb.com/nebecomm/default.asp). The pTA7002 binary vector (Aoyama and Chua, 1997) was opened using the *Xho*I site, and prepared for blunt-end ligation with the SS-CUT insert. This construct was transformed in *Agrobacterium tumefaciens* pGV3101. WT plants were transformed by vacuum infiltration, and several independent homozygous lines were analysed.

The mutated cutinase gene from *F. solani* (Rogers *et al.*, 1994) was amplified from pET-16b using the oligonucleotides 5′-CUT (5′-TGCTAGCGCTGGTAGAACAACTCG-3′; *Nhe*I site underlined) and 3′-CUT (5′-TAGGTACCTCAAGCAGAACCACG-3′; *Kpn*I site underlined). The gene was cloned behind the signal sequence of tobacco chitinase A in pPMB7066:SS:CUT (see Sieber *et al.*, 2000), previously digested with *Nhe*I/*Kpn*I. The fusion construct was then cloned into the pART7/pART27 vector system (Buttner *et al.*, 1994), and WT plants were transformed as previously described (Sieber *et al.*, 2000).

Candidate genes selected from the microarray analysis were overexpressed in WT plants using the pART7/pART27 vector system (Buttner *et al.*, 1994). Gene coding sequences were amplified by PCR on cDNA from infected plants, cloned in the pGEM®-T Easy Vector (Promega; http://www.promega.com/) and excised using *Eco* RI, or using *Sac*I/*Sac*II and blunt-ended. The fragments were cloned in the pART7 vector opened with *Eco* RI or *Sma*I. and the orientation was checked by PCR using a 5′ primer annealing upstream of the insertion site (5′-ATCCCACTATCCTTCGCAA-3′) and a 3′ primer annealing in the insert. The constructs were cloned in the pART27 vector, and Arabidopsis Col-0 plants were transformed as previously described (Sieber *et al.*, 2000). Overexpression levels of the transgenes in primary transformants were confirmed using RT-PCR. Enhanced resistance to *B. cinerea* exhibited by primary transformants and overexpression of the transgenes were confirmed on 24 T_2 plants of three independent lines of the second generation for the *LTP*, *PER* and *PI* genes.

Microarray analysis

Whole-genome expression analysis using 20K Affymetrix® microarrays were carried out by the DNA Analysis Laboratory of Paradigm Genetics Inc. (Research Triangle Park, NC, USA; now ICORIA Inc. www.icoria.com). The analyses were performed at 12 and 30 h post-inoculation on WT and CUTE plant samples uninoculated, mock-inoculated or inoculated with *B. cinerea*. Genes were selected using the FiRe software (Garcion *et al.*, 2006) with the following criteria: among the genes induced by *B. cinerea* in WT and CUTE plants (>twofold), genes with greater expression in infected CUTE compared with WT plants were retained and used for the final selection of genes (induced genes with expression values <100 were eliminated). Real-time PCR confirmed the microarray data in two independent experiments. Analysis of gene expression in *bdg* and WT after inoculation with *B. cinerea* was performed by real-time RT-PCR, and expression values were normalized relative to expression of the plant *actin-2* gene.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Effect of overexpression of *B. cinerea* cutinise A (*CUTA*) or lipase 1 (*LIP1*) on resistance to *B. cinerea*

Figure S2. Expression of *B. cinerea* pathogenesis-associated genes in WT and CUTE plants

Figure S3. *In vitro and in vivo* effects of WT and CUTE diffusates on necrotrophic fungi

Figure S4. Priming of genes in the mutant *bdg* after inoculation with *B. cinerea*

Table S1. Selected candidate genes identified in microarrays.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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