

# A permeable cuticle in *Arabidopsis* leads to a strong resistance to *Botrytis cinerea*

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The plant cuticle composed of cutin, a lipid-derived polyester, and cuticular waxes covers the aerial portions of plants and constitutes a hydrophobic extracellular matrix layer that protects plants against environmental stresses. The *botrytis-resistant 1* (*bre1*) mutant of *Arabidopsis* reveals that a permeable cuticle does not facilitate the entry of fungal pathogens in general, but surprisingly causes an arrest of invasion by *Botrytis*. *BRE1* was identified to be long-chain acyl-CoA synthetase2 (*LACS2*) that has previously been shown to be involved in cuticle development and was here found to be essential for cutin biosynthesis. *bre1/lacs2* has a five-fold reduction in dicarboxylic acids, the typical monomers of *Arabidopsis* cutin. Comparison of *bre1/lacs2* with the mutants *lacerata* and *hothead* revealed that an increased permeability of the cuticle facilitates perception of putative elicitors in potato dextrose broth, leading to the presence of antifungal compound(s) at the surface of *Arabidopsis* plants that confer resistance to *Botrytis* and *Sclerotinia*. *Arabidopsis* plants with a permeable cuticle have thus an altered perception of their environment and change their physiology accordingly.

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## Introduction

The cuticle covers the epidermal cell wall of aerial tissues forming a boundary between the plant and its environment. It represents a primary barrier minimizing water and solute loss and protecting the plants against various abiotic and biotic stresses, but may also act in a number of developmental processes, such as delimitation of organs as well as trichome and stomata formation (Goodwin and Jenks, 2005; Nawrath,

2006; Riederer, 2006). The cuticle is composed of epicuticular and intracuticular waxes and a structural polymer, cutin, that are laid down in a well-organized manner (Jeffree, 2006). Waxes are a mixture of very long-chain fatty acids and their derivatives, and in some species, triterpenes and  $\beta$ -diketones (Kunst and Samuels, 2003; Jetter *et al*, 2006). Cutin is a polyester that is, in many plant species, formed of C<sub>16</sub> and C<sub>18</sub>  $\omega$ -hydroxylated fatty acids that may carry additional hydroxy or/and epoxy groups in the mid-chain position (Kolattukudy, 2001). However, the cutin of *Arabidopsis* has an unusual composition as it consists of high amounts of C<sub>16</sub> and C<sub>18</sub> dicarboxylic acids (Bonaventure *et al*, 2004; Xiao *et al*, 2004; Franke *et al*, 2005). The large amounts of monomers only carrying carboxyl groups in the *Arabidopsis* cutin require other monomers rich in hydroxy groups in order to form polyesters. Glycerol might be such a monomer as it has recently been found to be a cutin monomer in several plant species (Graça *et al*, 2002).

Although a number of mutants potentially affected in the deposition of the cuticular polyesters have been characterized in recent years (Yephremov and Schreiber, 2005; Nawrath, 2006), changes in the amount and composition of cuticular polyesters have been determined only for a few mutants. The *Arabidopsis* mutant *attenuation of type three genes* (*att1*) having only 30% of the wild-type (WT) cuticular polyester encodes the cytochrome P450-dependent monooxygenase CYP86A2 (Xiao *et al*, 2004). The *Arabidopsis* mutant *lacerata* (*lcr*) carries a mutation in the homologue CYP86A8 and has a pleiotropic phenotype, including a permeable cuticle and a number of developmental disorders, such as organ fusions (Wellesen *et al*, 2001). Both mutants are most likely affected in cutin monomer biosynthesis as cytochrome P450s of the CYP86A type have been shown to have fatty acid hydroxylase activity (Benveniste *et al*, 1998). The organ fusion mutant *hothead* (*hth*) that exhibits a 30% reduction in dicarboxylic acids in its polyesters indicates the involvement of an oxidoreductase-dependent pathway for the formation of dicarboxylic acids in *Arabidopsis* (Krolikowski *et al*, 2003; Kurdyukov *et al*, 2006a). The extracellular  $\alpha/\beta$  hydrolase BODYGUARD (BDG) may be involved in the formation of the polyester itself (Kurdyukov *et al*, 2006b). The *bdg* mutant accumulates more ester-bound cutin monomers than WT in the outer extracellular matrix surface, but is unable to form a functional continuous cuticular membrane. A potential function of *bdg* in both hydrolysis and synthesis of the polyester has been hypothesized as a number of phenotypes of *bdg*, including structural aspects of the polyester, resemble those observed in transgenic *Arabidopsis* plants expressing a fungal cutinase (Sieber *et al*, 2000; Kurdyukov *et al*, 2006b). WAX2 and LONG-CHAIN FATTY ACID SYNTHETASE 2 (*LACS2*) are also enzymes potentially involved in cutin biosynthesis. *LACS2* is an epidermis-specific long-chain acyl-CoA synthetase, whose absence, as that of WAX2, leads to altered properties and ultrastructure of the cuticular membrane (Schnurr *et al*, 2004). The *LACS2* protein expressed in

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*Escherichia coli* has a preference for hydroxylated fatty acids, in accordance with the idea that LACS2 plays an important role in cutin biosynthesis (Schnurr *et al*, 2004).

The proposed function of the cuticle as a diffusion barrier has been intensively studied. Intercuticular waxes seem to play an important role in sealing the cuticle and are thought to be densely packed in the spaces of the cutin polyester that serves as a scaffold (Goodwin and Jenks, 2005). This model would explain why some mutants affected in cutin deposition are strongly disturbed in their permeability barrier (Sieber *et al*, 2000; Kurdyukov *et al*, 2006b). Although the cuticle forms a diffusion barrier, molecules can diffuse through the cuticle. Lipophilic non-ionic molecules diffuse through the hydrophobic cuticle and their diffusion kinetics are different from those of charged molecules that travel in an apolar path, while H<sub>2</sub>O diffuses in both routes (Schreiber, 2005; Riederer and Friedmann, 2006; Burghardt and Riederer, 2006).

Phytopathogenic fungi often secrete cutinases during their initial contact with a plant that liberates cutin monomers serving as signals for the induction of cutinases necessary for penetration and for a number of differentiation processes in fungi. For instance, germination and appressorium formation in *Magnaporthe grisea* and formation of the appressorial tube in *Erysiphe graminis* are induced by cutin monomers (Kolattukudy *et al*, 1995; Francis *et al*, 1996; Gilbert *et al*, 1996). However, a crucial role of cutinases in the invasion of a plant was not always found. For example, deletion of the cutinases and cutinolytic lipases in the necrotrophic fungus *Botrytis cinerea* did not hinder the pathogen to enter intact plant tissues (Reis *et al*, 2005).

Very little is known about the significance of cutin for plant defence (Yephremov and Schreiber, 2005; Nawrath, 2006). The *Arabidopsis* mutant *att1* was identified by its attenuating effect on virulence gene induction in phytopathogenic bacteria and its increased disease susceptibility toward bacterial infection (Xiao *et al*, 2004). In contrast, cutinase-expressing *Arabidopsis* plants and the *bdg* mutant were found to be strongly resistant to the necrotrophic fungus *B. cinerea* because of a multifactorial defence mechanism (Chassot *et al*, 2007). This resistance phenomenon has been seen in relation to earlier findings that cutin monomers may induce defence responses in plants (reviewed in Chassot and Métraux, 2005).

A forward genetic screen in *Arabidopsis* made it possible to dissect the potential pathway linking cutin or cutin monomers to resistance to *B. cinerea*. A mutant, *botrytis resistant1* (*bre1*), displaying strong resistance to infection by *B. cinerea*, was also found to be defective in its cuticular membrane. *bre1* carries a mutation in the *LACS2* gene that was previously shown to be involved in cuticle development (Schnurr *et al*, 2004), and was here found to be important for cutin biosynthesis. The study of *bre1/lacs2* and a number of other *Arabidopsis* mutants impaired in cutin monomer biosynthesis demonstrated that the increase in permeability of the cuticular membrane directly correlates with the amount of antifungal compounds released to the plant surface and *Botrytis* resistance. An interrupted cuticular membrane allowed the diffusion of signals and effector molecules across the cuticle resulting in an arrest of infection by *Botrytis* and *Sclerotium Sclerotinia*.

## Results

### Isolation and characterization of the *bre1* mutant

A total of 13 000 plants of an M2 population of EMS-mutagenized *Arabidopsis* plants were screened in parallel for two phenotypes: resistance to *B. cinerea* and increased cuticular permeability. One mutant of nearly normal size was strongly resistant to infection with *B. cinerea* strain (BMM) and was called *bre1*. Infection sites on *bre1* leaves remained usually symptom free, in contrast to those on Col leaves (less than 2% of outgrowing lesions) (Figure 1A–C). In addition, *bre1* displays increased cuticle permeability compared with WT as determined by Calcofluor staining. Calcofluor white fluoresces when it binds to  $\beta$ -glucans, such as cellulose in the cell wall. *bre1* showed an extensive staining in all parts of the plant. In particular, the staining of cotyledons, the second rosette leaf pair, as well as most parts of the flower was distinctly different in *bre1* compared with WT plants (Figure 1F, I, and L). All phenotypes were inherited in a recessive manner.

### BRE1 has a mutated LACS2 gene

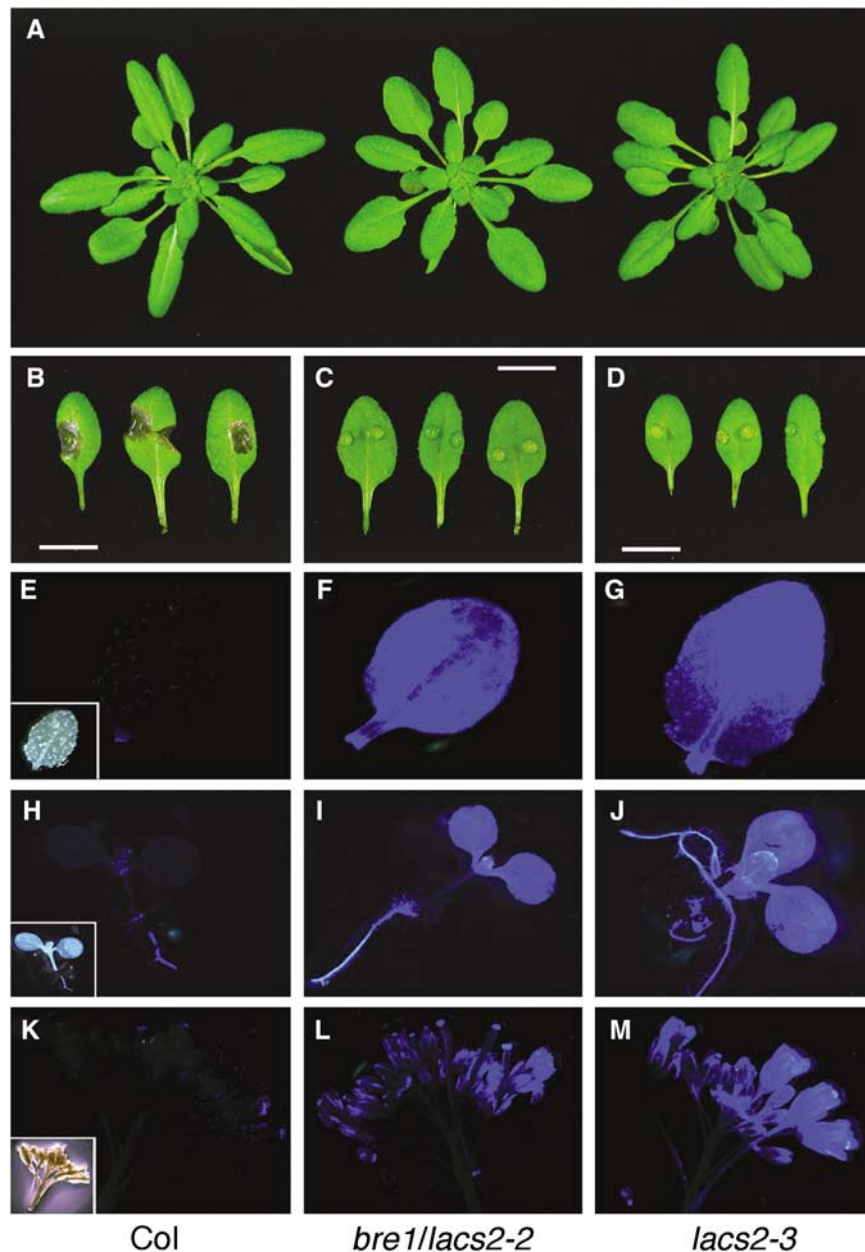
*BRE1* was mapped to the middle of chromosome 1 close to *LACS2* (Lukowitz *et al*, 2000), which has been previously identified to be important for cuticle development (Schnurr *et al*, 2004). *Arabidopsis* plants (accession Col-0) carrying a T-DNA insertion in the *LACS2* gene were obtained that were also resistant to *B. cinerea* (quantitative data shown in Figure 6B), stained strongly with Calcofluor, and had no other obvious morphological phenotypes (Figure 1A, D, J, and M) (Rosso *et al*, 2003). Complementation analysis between *bre1* and the *LACS2* T-DNA insertion line revealed that *BRE1* is indeed *LACS2*, as both mutants did not complement each other in the F1 generation (Supplementary Figure 1). We therefore named *bre1 lacs2-2* and the T-DNA insertion line *lacs2-3*.

Sequence analysis revealed mutations in *LACS2-2* leading to the expression of a truncated cDNA (Supplementary Figure 2A and C). Mutations accompanied by insertion of the T-DNA in *LACS2-3* lead to an RNA-null allele (Supplementary Figure 2B and C). As *lacs2-2* and *lacs2-3* had very similar phenotypes during the initial characterization, only data for *lacs2-3* are presented in the following studies.

### The cuticle of the *lacs2* mutant

The ultrastructure of the cuticular membrane of the *lacs2-3* mutant was analyzed by transmission electron microscopy (TEM). The distinct osmium-dense cuticular membrane representing insoluble lipid-derived polymers such as cutin, visible at the epidermal outer extracellular matrix of WT plants, could not be seen at the leaf epidermis of *lacs2-3* (Figure 2A and B). Interestingly, the remaining outer epidermal extracellular matrix sometimes had a distinct laminated structure (Figure 2C). Growing plants on a large scale revealed that *lacs2-3* plants very rarely formed organ fusions (one organ fusion in less than 1% of plants). Ultrastructural analysis of such organ fusions revealed that both epidermal cell walls are directly fused without any osmium-dense layer in between them (Figure 2D).

The cuticular membrane of *lacs2-3* was not visible as an electron-opaque layer, indicating that the osmium-dense,



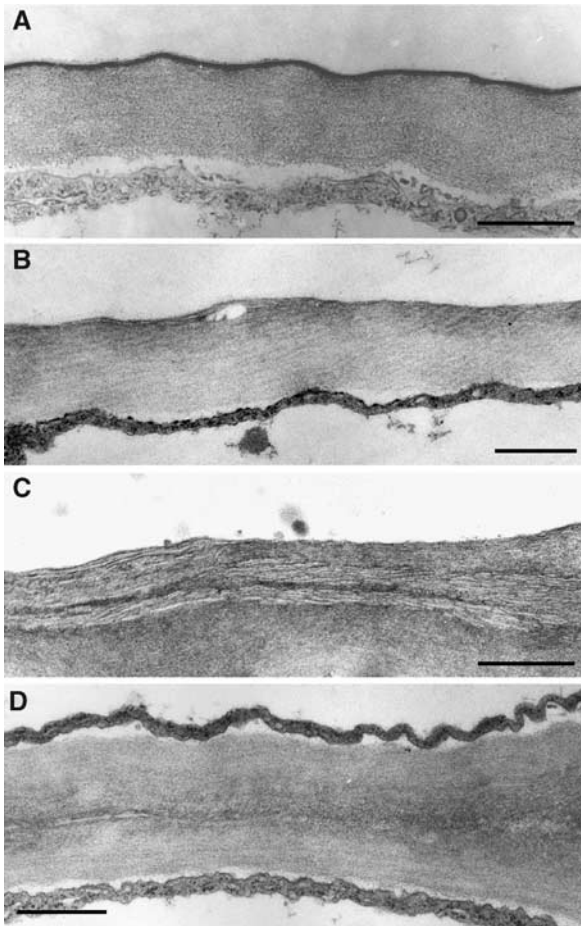
**Figure 1** Phenotypes of *bre1/lacs2-2* and *lacs2-3* mutant isolates. (A) Morphology of the rosette of Col-0, *lacs2-2*, and *lacs2-3*. (B–D) Macroscopic evaluation of symptoms 3 days after inoculation with *B. cinerea* of Col-0, *bre1/lacs2-2*, and *lacs2-3*. Scale bar: 1 cm. (E–M) Calcofluor white-stained organs of Col-0, *bre1/lacs2-2*, and *lacs2-3*, viewed under UV light. Col-0 pictures are shown with the corresponding pictures in bright-field microscopy as inset. Leaves (E–G), young seedlings (H–J), and the tip of a flowering inflorescence (K–M) are shown.

insoluble lipids might be largely missing. Thus, the amount and composition of ester-bound lipids were analyzed from plant material after complete elimination of soluble lipids. Analysis of residue-bound lipids of *lacs2-3* in comparison with Col-0 plants revealed that a number of ester-bound monomers were reduced in the *lacs2-3* mutant (Figure 3A and B). The total amount of  $\omega$ -hydroxylated fatty acids and their derivatives was reduced 4- to 5-fold in *lacs2-3* compared with Col-0 (Figure 3A). The impact of *LACS2* mutation on the amount of dicarboxylic acids, characteristic of *Arabidopsis* cutin, was particularly strong, and the amount was reduced to 15–20% of WT amount.  $\omega$ -Hydroxylated fatty acids and their dihydroxy fatty acids were reduced to less

than 50%. Thus, *lacs2* has a strongly reduced content of cuticular polyester.

#### **Permeability of the cuticle of *lacs2* and other cuticle mutants**

An intact cuticular membrane is important to reduce water loss from the plant under drought conditions (Goodwin and Jenks, 2005; Burghardt and Riederer, 2006). Water loss was taken as a measure of cuticular permeability and was determined in fully expanded but not yet bolting rosettes excised at the level of the hypocotyls. As shown in Figure 4, the rate of water loss from *lacs2-3* is significantly higher than that of Col-0 plants. As the stomata of both Col-0 and *lacs2-3* plants

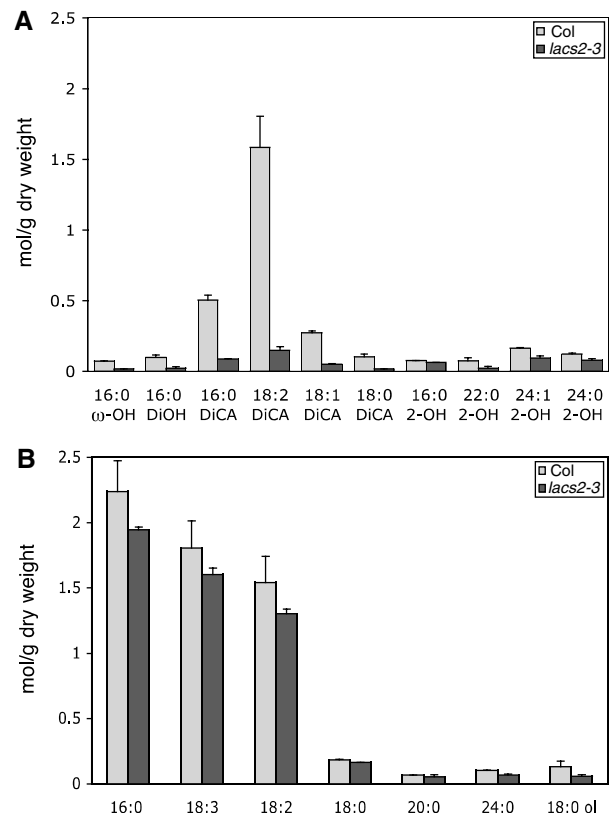


**Figure 2** Ultrastructure of the epidermal extracellular matrix of rosette leaves. Lower epidermal extracellular matrix of Col-0 (A) and *lacs2-3* (B, C) leaves. Extracellular matrix at the point of fusion between the lower epidermis of one leaf and the upper epidermal cell layer of another leaf in *lacs2-3* (D). Scale bar: 500 nm (A) and 800 nm in (B–D).

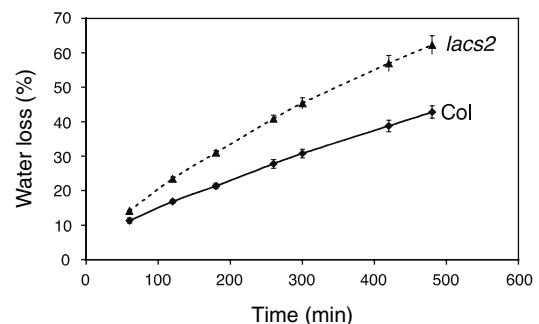
were well closed during the experiment (Supplementary Figure 3), *lacs2-3* is likely to have a higher cuticular transpiration than Col-0.

Sensitivity to xenobiotics such as herbicides also reflects the permeability of the cuticular membrane. Permeability of the upper surface of *Arabidopsis* leaves was specifically determined to avoid the problem of molecule exchange via stomata, as these openings are mostly positioned on the abaxial side of leaves. Besides WT and *lacs2-3* plants, two other mutants, *hth* and *lcr*, reported to have a permeable cuticle were included in this experiment (Lolle *et al*, 1998; Wellesen *et al*, 2001). *lacs2-3* as well as *lcr* showed severe damage at a lower concentration of BASTA<sup>®</sup> compared with *hth* and WT Col-0 plants (Table I).

Permeability of the cuticle of *lacs2* and other mutants was further assessed with toluidine blue applied in the form of droplets on the adaxial sites of the leaves (Tanaka *et al*, 2004; Kurdyukov *et al*, 2006b). As toluidine blue can be directly applied to plant tissues without solvent treatment, permeability of a functional cuticular membrane can be investigated by this staining method, in contrast to Calcofluor staining. Two hours after the application of toluidine blue solution, Col-0 leaves showed a small stained spot covering less than 10% of the area on which the droplet was applied,



**Figure 3** Composition of aliphatic monomers of leaf polyesters. Large rosette leaves of 5- to 6-week-old plants of Col-0 and *lacs2-3* were analyzed. (A) Hydroxylated fatty acids and their derivatives and (B) fatty acids and fatty alcohols. DiOH, dihydroxy carboxylic acid; DiCA, dicarboxylic acid ( $n=3$ ;  $\pm$  s.e.; the experiment was repeated once with similar results).



**Figure 4** Cuticular transpiration. Loss of water from 5- to 6-week-old rosettes of Col-0 and *lacs2-3* plants was measured at the indicated time points ( $n=4-6$ ;  $\pm$  s.e.; experiment was repeated twice with similar results).

whereas *lacs2* leaves showed dark blue staining on 75–100% of the area. Interestingly, the *lcr* leaves showed staining on approximately 25–50% of the droplet area, whereas *hth* had a staining pattern similar to that of Col-0 (Figure 5A). A shorter incubation time, that is, 30 min, resulted in a similar pattern, but the staining was weaker (data not shown). Leaves of *lacs2-3* were already stained after only 5 min and the whole area underneath droplets was stained after 30 min (Figure 5B).

Taken together, our data demonstrate that the *lacs2-3* has a highly permeable cuticle that is even more permeable than that of *lcr*, in comparison with WT Col-0, whereas an

**Table I** Sensitivity to BASTA<sup>®</sup> of *hth*, *lcr*, and *lacs2* in comparison with WT Col-0

Basta (nl/ml)	Col	<i>hth</i>	<i>lcr</i>	<i>lacs2</i>
1	+++++	+++++	+++++	+++++
10	+++++	+++++	++++	++++
20	++++	++++	+++	+++
40	+++	+++	++	++
60	+++	+++	+/-	+/-

+++++: fully green; ++++: chlorotic tips or spots; +++: whole leaves chlorotic, some dying; ++: all expanded leaves died, but vigorous regrowth; +: poor or late regrowth; +/-: dead or regrowth after 10 days only.

increased cuticular permeability of *hth* leaves could not be confirmed in our experimental settings.

### Resistance to *B. cinerea* of mutants having altered cuticular permeability

Resistance of *lacs2* plants to *Botrytis* was compared with other mutants affected in cuticle formation. The visual phenotypes of the leaves 3 days after inoculation are depicted in Figure 6A. The number of lesions that were larger than the original infection site (outgrowing lesions) is given as a quantitative measure of the resistance level (Figure 6B). The *lacs2-3* mutant exhibited a strong resistance to infection with *B. cinerea* strain BMM, as described above, with less than 5% outgrowing lesions in comparison with WT Col-0 (75% outgrowing lesions). A significant increase in the resistance to *B. cinerea* was also observed in *lcr* (45% outgrowing lesions), whereas no increase in resistance was observed in *hth*. In addition, infection sites could be observed in *lcr* that did not develop any disease symptoms, similar to the symptom-free infection sites in the *lacs2-3* mutant (Figure 6A). The frequency of symptom-free lesions in *lcr* was significantly lower than that in the *lacs2-3* mutant (30 and 95%, respectively). Similar results were obtained with *B. cinerea* strain BO5.10.

### Analysis of leaf diffusates for antifungal compounds

The permeability of the cuticle correlated with the resistance to *B. cinerea* (Figures 5A and 6) but not with PDF1.2 and camalexin production that have often been correlated to *Botrytis* resistance (Penninckx *et al*, 1998; Berrocal-Lobo *et al*, 2002; Coego *et al*, 2005; Kliebenstein *et al*, 2005) (Supplementary Figure 4). In the search for antimicrobial compounds at the surface of the plant that might arrest the infection at an early stage, droplets of 1/4 strength potato dextrose broth (PDB) were incubated on the leaves so that antifungal substances could diffuse into them. The activity of these diffusates was analyzed in two assays: (1) spores of *B. cinerea* were germinated *in vitro* in the presence or absence of diffusates and the length of the hyphae was measured (*in vitro* assay). (2) *Botrytis* spores were also tested for their ability to infect WT plants in the presence or absence of diffusates (*in vivo* assay).

The results of *in vitro* assay are presented in Figure 7A. The 1/4 PDB diffusate from *lacs2-3* collected after 18 h of incubation inhibited the development of *Botrytis* hyphae, whereas the diffusates from Col-0, *lcr*, and *hth* did not. After 44 h, the 1/4 PDB diffusates collected from *lcr* and *lacs2-3* were active, whereas these from *hth* and Col-0 were not. The

growth-inhibiting effect of diffusate from *lacs2-3* was much stronger than that from *lcr*, whereas the 1/4 PDB diffusates from *hth* and Col-0 were not active. Results of the *in vivo* test are presented in Figure 7B. *Botrytis* spores that were mixed with 1/4 PDB diffusates, collected after 18 or 44 h from *lacs2-3* plants, were unable to infect Col-0 plants (0% outgrowing lesions). Spores incubated with 1/4 PDB diffusates from *lcr* collected after 44 h could not infect Col-0 plants (0% outgrowing lesions), but when collected after 18 h could still infect Col-0 normally (85% outgrowing lesions). Spores incubated with 1/4 PDB diffusates from *hth* or Col-0 had no significant reduction in infection capability (70–85% outgrowing lesions).

Both tests clearly indicate that the surface of plants with an increased cuticle permeability releases antifungal compounds against *Botrytis* and that the activity depends on the time of diffusion. Comparison of the *in vitro* and *in vivo* tests suggests that a reduction of 50% and more in the growth of the hyphae *in vitro* leads to an arrest in infection *in vivo*.

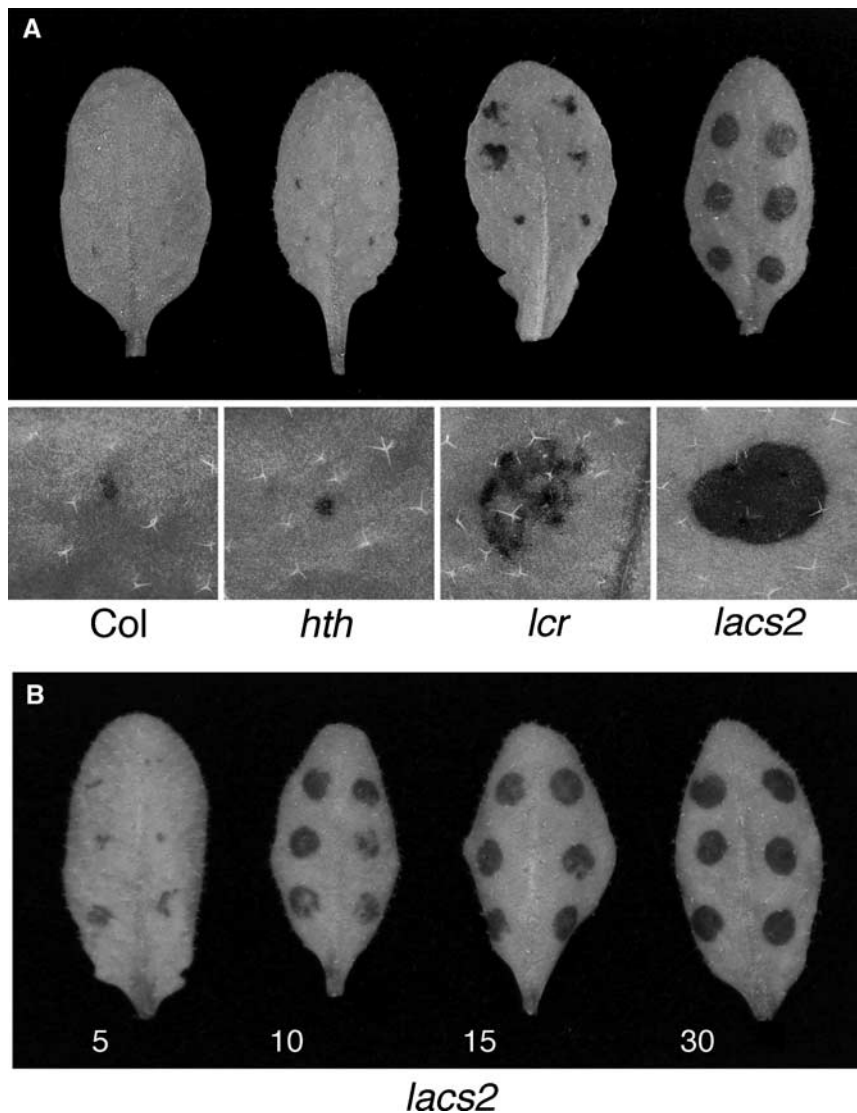
### Characterization of antifungal compounds and their induction

The antifungal compounds present in the 1/4 PDB diffusate obtained from *lacs2-3* plants after an incubation time of 42 h were characterized by testing their *in vitro* activity on *Botrytis* spores after different treatments: the active compound(s) were resistant to lyophilization and heat treatment and could not be extracted into ethyl acetate, hexane, or chloroform, indicating that they are of polar nature.

The antifungal activity of the *lacs2* diffusate could be diminished by treatment with a lipase from *Mucor miehei*. The germlings of *Botrytis* were four to eight times longer than without lipase treatment of the diffusate (Figure 8 and Supplementary Figure 5). Depending on the PDB diffusate preparation, 12–60% of growth of the germlings in 1/4 PDB treated with lipase could be restored. Similar results were obtained with a lipase from *Candida antarctica* that had higher specific activity (data not shown). Proteinase K treatment has only a small effect on the activity of PDB diffusates (Supplementary Figures 5 and 8). Germlings were only 2–3 times longer than when incubated in untreated PDB diffusate. Between 5 and 18% of the germling length grown in 1/4 PDB treated with proteinase K could be restored (Figure 8).

These results indicate that the *lacs2* diffusate most likely contains several components. Differences in the efficiency of the treatments may reflect differences in the composition of diffusates that may result from slightly different induction kinetics of the compounds. One of the prominent active compounds of the PDB diffusate from *lacs2* plants may be a lipid or a compound that contains a linkage to an acyl group that is important for its activity. This lipase-sensitive compound is of relatively low molecular weight, as it could be filtrated through a membrane with a molecular weight cutoff of 3000 Da (data not shown).

Interestingly, only PDB itself—even when adjusted to different pH—induced the antifungal compounds in *lacs2-3* plants and not the major components of PDB (sucrose and starch), different pH, different ion concentrations, and osmotic potential (Table II). The inducer is, therefore, most likely a minor component of PDB that acts as an elicitor to induce antifungal compounds inhibiting the growth of the hyphae of *B. cinerea* and *S. sclerotiorum*, but not of *Alternaria*



**Figure 5** Permeability of leaf cuticles of mutants having an altered cuticular polyester. (A) Droplets of a toluidine blue solution were incubated on the leaves for 2 h and then washed with water. An overview of typically stained leaves from each genotype is given in the top panel. A microscopic view of the typical staining underneath a droplet that was incubated for 2 h on leaves from each genotype is given below its overview. (B) Toluidine blue droplets were incubated on *lacs2-3* leaves for the time points indicated below each leaf and then briefly rinsed with water. Typically stained leaves are presented. A colour version of this figure is available at the *EMBO Journal* online.

*brassicicola* and *Plectosphaerella cucumerina* (Supplementary Figure 6). In accordance with this observation, *lacs2-3* also showed increased resistance to infection by *Sclerotinia* (Figure 9). Interestingly, the resistance of *lacs2* to infection by *Sclerotinia* could be further increased by a 20-h preincubation period of the future inoculation sites with 1/4 strength PDB, again demonstrating the importance of PDB-mediated induction for the defence response observed in permeable cuticle mutants (Figure 9).

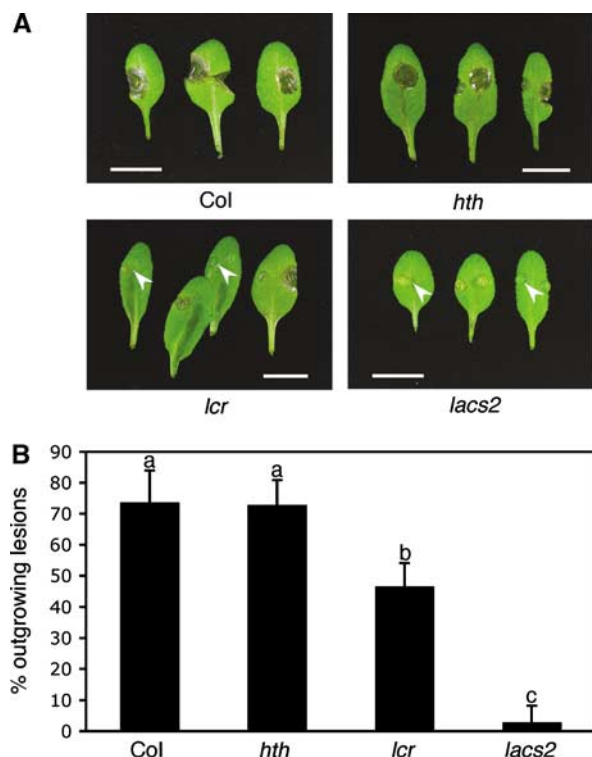
## Discussion

### *bre1/lacs2* is a cuticle mutant

Here, we describe the isolation of two new alleles of *lacs2* using a screening based on resistance to the fungal pathogen *B. cinerea*. The *LACS2* gene has previously been identified to be involved in cuticle formation (Schnurr *et al*, 2004).

Several methods were used to demonstrate an increased permeability of the native cuticle in the *lacs2-3* mutant; these methods include cuticular transpiration rate, herbicide sensitivity, and staining of leaves with toluidine blue (Figures 5 and 6; Table I). These studies corroborated and extended the findings that *lacs2-1* may have an increased permeability of the cuticle, as previously shown by increased chlorophyll leaching (Schnurr *et al*, 2004).

Ultrastructural analysis revealed that the cuticular membrane of *lacs2-3* was not visible at the outer extracellular matrix of the epidermis (Figure 3B). Previous findings showed a cuticular membrane of reduced thickness in the *lacs2-1* mutant (Schnurr *et al*, 2004). The differences in the appearance of the cuticular membrane in TEM may result from different growth conditions, developmental stage of the organ, and microscopic techniques. The surface of the epidermis collapsed in a very similar way when prepared for SEM in both *lacs2-1* and *lacs2-3* mutants (data not shown).



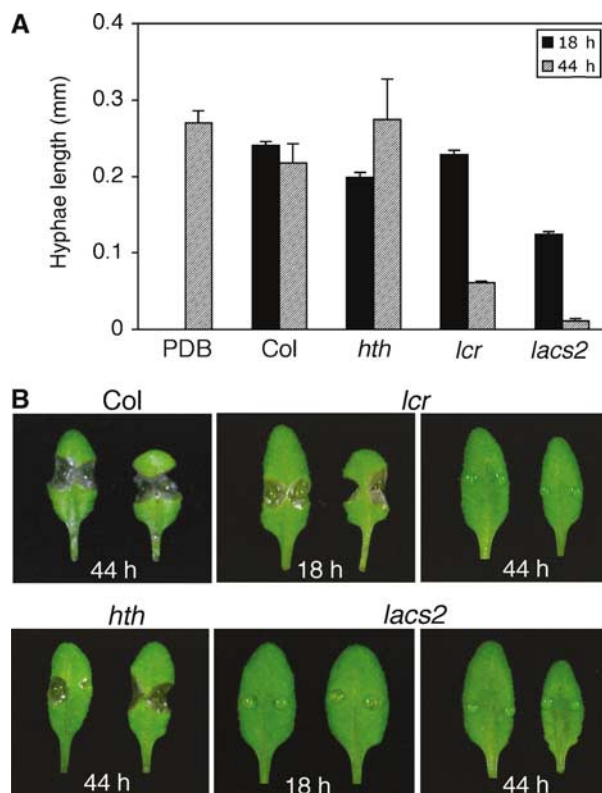
**Figure 6** Resistance to *Botrytis* of mutants having differences in their cuticular polyesters. (A) Macroscopic symptoms 3 days after inoculation with *B. cinerea* (BMM) on leaves of different genotypes. White arrowheads indicate examples of symptom-free inoculation sites. Scale bar: 1 cm. (B) Number of outgrowing lesions developed 3 days after inoculation with *B. cinerea* (BMM) in different genotypes ( $n=4$ ;  $\pm$ s.e.; different letters indicate significance at  $P<0.005$  using ANOVA). The experiment was repeated twice with similar results.

(Schnurr *et al*, 2004). Furthermore, the amount of cuticular waxes increased in *lacs2-3*, as reported for the *lacs2-1* mutant (data not shown) (Schnurr *et al*, 2004).

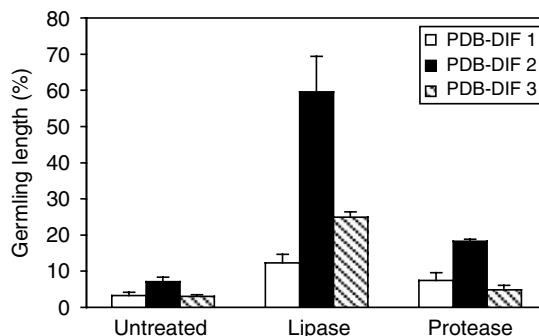
Developmental phenotypes described for *lacs2-1* were weaker under our growth conditions, that is, the rosettes were only slightly smaller and leaf deformations and/or organ fusions between leaves were only rarely observed (Schnurr *et al*, 2004). When organs were fused, the ultrastructure of the fusion was similar to that in other mutants defective in the cuticular membrane (Figure 2d) (Lolle *et al*, 1998; Wellesen *et al*, 2001; Yephremov and Schreiber, 2005; Nawrath, 2006; Kurdyukov *et al*, 2006a, b). Obvious macroscopic developmental phenotypes were thus different between the different *lacs2* alleles and most likely depended on the environmental conditions under which the plants were grown. In addition, our studies were performed in the Col-0 background, whereas *lacs2-1* is in the WS background (Schnurr *et al*, 2004).

### **LACS2 is necessary for the biosynthesis of the cutin polyester**

Analysis of the monomer composition of residue-bound polyesters of *LACS2* revealed that  $\omega$ -hydroxylated fatty acids and their derivatives, including  $\omega$ -hydroxy carboxylic acids, dihydroxy carboxylic acids, and dicarboxylic acids, were overall reduced to 20–25% of WT Col-0 amounts. This strong reduction in the typical cutin monomers of *Arabidopsis*



**Figure 7** Analysis of PDB diffusates for the presence of growth-inhibiting activities against *B. cinerea*. (A) Germination and growth of *B. cinerea* (BMM) *in vitro* in the presence of leaf diffusates collected at 18 h (grey bars) and 44 h (black bars) from plants of different genotypes. Three independent experiments were undertaken and six representative pictures were evaluated  $\pm$ s.d. (B) *B. cinerea* (BMM) spores were incubated on WT Col-0 leaves in the presence of 1/4 PDB diffusates collected from leaves of different genotypes at indicated time points. Typical symptoms 3 days after inoculation are presented.



**Figure 8** Analysis of the activity of different PDB diffusates from *lacs2* after enzymatic digestions. Germination and growth of *B. cinerea* (BMM) in the presence of different 1/4 PDB diffusates (PDB DIF) from *lacs2* that had undergone no treatment (control), treatment with lipase from *M. miehei* at 32 U/ $\mu$ l (lipase), or proteinase K (protease) are shown. Lengths of the germlings grown in treated 1/4 PDB diffusates from *lacs2* were normalized to those grown in treated 1/4 PDB (100%). Three to four representative pictures were evaluated  $\pm$ s.d.

coincided with a complete loss of insoluble lipids that can be normally visualized as a distinct osmium-dense layer present on the outer epidermal extracellular matrix (Figures 2B and 3A) (Bonaventure *et al*, 2004; Franke *et al*, 2005). The

**Table II** *In vitro* hyphal growth of *Botrytis* in the presence of various diffusates

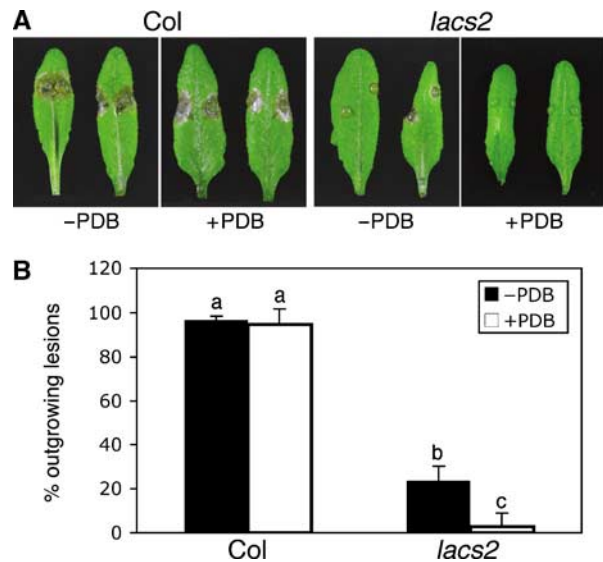
	Col-0	<i>lacs2</i>
1/4 PDB	+++	–
1/4 PDB pH 4.0	+++	–
1/4 PDB pH 8.0	+++	–
Glucose (5 g/l)	+++	+++
Starch (1 g/l)	+++	+++
1 M NaCl	+	+
150 mM NaCl	+++	+++
300 mM mannitol	+++	+++
0.1 M Na-acetate buffer pH 4.0	–	+/-
0.1 M K-phosphate buffer pH 8.0	–	+/-
H <sub>2</sub> O	+++	+++
H <sub>2</sub> O/ <i>Botrytis</i> (5 × 10 <sup>4</sup> spores/ml)	+++	+++

+ : more than 90% hyphal growth in comparison with 1/4 PDB; +/- : reduced growth of hyphae with abnormal morphology; – : less than 10% growth of hyphae.

reduction in hydroxylated fatty acids corroborates studies on LACS2 expressed in *E. coli* showing a preference for ω-hydroxy palmitic acid over palmitic acid (Schnurr *et al*, 2004). Interestingly, the reduction in dicarboxylic acids in the polyester (to 85%) was higher than that of 2-hydroxy acids, dihydroxy acids, and ω-hydroxy acid, suggesting that dicarboxylic acids may be the preferred substrate of LACS2. Reduction in 2-hydroxy acids was not always significant. Comparisons of the polyester composition between total extracted plant material and cuticles of *Arabidopsis* had previously shown that only 20–25% of 2-hydroxy acids detected in total extracted leaves are present in the cuticle, whereas 75–100% of ω-hydroxylated fatty acid derivatives are in the cuticle (Franke *et al*, 2005). Thus, a small reduction in total 2-hydroxy acids may still represent significant changes in cuticular 2-hydroxy acids. Further studies on the LACS2 enzyme using a broad range of hydroxylated fatty acids and their derivatives will be necessary to determine the substrate specificity more accurately.

The broad impact of LACS2 on polyester composition is interesting, indicating that LACS2 may be important for a general step in cutin biosynthesis. Nothing is known about the transport mechanism of cutin monomers and the assembly of the cutin polyester. Other isoenzymes of long-chain fatty acid-CoA synthetases are however involved in the CoA activation of fatty acids before their transfer to glycerol during lipid biosynthesis (Shockey *et al*, 2002). Therefore, an attractive idea is that LACS2 is essential for activation of cutin monomers for their assembly into cutin domains, potentially containing glycerol or other molecules rich in hydroxy groups still inside the cell, that are then transported across the membrane to the outermost layer of the cell wall for their final polymerization.

The high permeability of the cuticle of *lacs2-3* can thus be attributed to the large reduction in the cutin polyester. The slight increase in cuticular waxes of *lacs2* that has also been observed in transgenic *Arabidopsis* plants expressing a fungal cutinase and in the *bdg* mutant might represent a compensation reaction to improve inadequate cuticular properties (Sieber *et al*, 2000; Schnurr *et al*, 2004; Kurdyukov *et al*, 2006b). The results obtained with *lacs2* support the idea that the cutin polyester could serve as a scaffold in which



**Figure 9** Influence of PDB preincubation on the resistance of *lacs2* to *Sclerotinia*. (A) Macroscopic symptoms 3 days after inoculation with *S. sclerotiorum* on leaves of Col-0 and *lacs2* without (–PDB) and with preincubation (+PDB) with 1/4 PDB for 20 h. (B) A number of outgoing lesions developed 3 days after inoculation with *S. sclerotiorum* in Col-0 and *lacs2* plants without (–PDB) and with preincubation (+PDB) with 1/4 PDB for 20 h ( $n=6$ ;  $\pm$ s.e.; different letters indicate a significance at  $P<0.005$  using ANOVA). The experiment was repeated with equivalent results.

intracuticular waxes are packed to seal the cuticle (Goodwin and Jenks, 2005).

### Role of cuticle in plant pathogen interactions

The characterization of *lacs2* as a cutin biosynthetic mutant that is strongly resistant to *Botrytis* and *Sclerotinia* is *per se* a counter-intuitive result. In the past, the cuticle has been seen as a barrier that protects plants from invading pathogens. However, *lacs2* did not show an altered resistance or susceptibility to a number of pathogens, such as the adapted and non-adapted powdery mildew isolates *Golovinomyces orontii*, *Erysiphe pisi*, and *Blumeria graminis* f. sp. *hordei* (Supplementary Figure 7) as well as certain necrotrophic fungi such as *A. brassicicola* (data not shown) and *P. cucumerina* (Supplementary Figure 8) (Thomma *et al*, 1999; Berrocal-Lobo *et al*, 2002; Consonni *et al*, 2006). Similarly, cutinase-expressing *Arabidopsis* plants did not show an altered susceptibility to *E. cichoracearum*, *B.g. hordei*, *Hyaloperonospora parasitica* (NOCO), and *Phytophthora brassicae* (Sieber *et al*, 2000; Chassot and Métraux, 2005). The cuticle of *Arabidopsis* is therefore not a major barrier to prevent pathogen invasions.

As cutinase-expressing plants as well as the *Arabidopsis* mutant *bdg* were both immune to infection by *Botrytis*, it had been speculated that cutin monomers or the action of cutinase/hydrolase that are released into the surface of these plants may be relevant for the resistance to *Botrytis* (Chassot and Métraux, 2005; Chassot *et al*, 2007). However, studies presented here show that the phenotype common for all mutants exhibiting an increased resistance to *Botrytis* is an increased permeability of the cuticle (Lolle *et al*, 1998; Sieber *et al*, 2000; Wellesen *et al*, 2001; Schnurr *et al*, 2004; Kurdyukov *et al*, 2006a). Most likely a direct effect of the permeable cuticle, such as an altered transpiration rate or



stomata closure, does not confer resistance to *Botrytis*; resistance is probably due to an indirect mechanism.

### **A permeable cuticle allows the induction of antifungal compounds**

Detailed studies on the permeability of the cuticle by using toluidine blue evidenced a direct correlation between increased permeation through the cuticle into the apoplast (Figure 6A) to the appearance of antifungal compounds active against *Botrytis* in leaf diffusates (Figure 8) and the degree of resistance to *B. cinerea* (Figure 7).

*lacs2-3* showed a very high permeability of the cuticle (Figure 6), a rapidly appearing strong antifungal activity in leaf diffusates (Figure 8), and very strong resistance to *B. cinerea* (Figure 7). *lcr* has a cuticle of intermediate permeability that is not as high as that of *lacs2-3* plants (Figure 6A). Antifungal activities of *lcr* could only be identified at late time points of the incubation period (Figure 8). In addition, *lcr* had an intermediate level of resistance to *Botrytis* that was clearly lower than that of *lacs2-3* plants (Figure 7). The *hth* mutant did not show any increased cuticle permeability in our experimental settings, no antifungal activities (Figure 8), and no resistance to *Botrytis* (Figure 7), in accordance with the hypothesis that an increased permeability of the cuticle is necessary for the presence of antifungal compounds and *Botrytis* resistance.

Toluidine blue diffused through the cuticular membrane of *lacs2* and *lcr* within less than 1 h, whereas antifungal compounds appear only much later on the surface of *lacs2* and *lcr* (18–44 h), indicating that the production of these antifungal compounds has to be induced. Induction studies with different solutions evidenced that the inducer of *Botrytis* and *Sclerotinia* resistance in *lacs2* plants is most likely a minor component present in PDB. Molecules acting as elicitors could be, for example, cell-wall degradation products of potato, such as oligosaccharides that have been shown to elicit defence responses (Côté and Hahn, 1994; Ridley *et al*, 2001). The fact that pretreatment with PDB increases resistance of *lacs2* to *Sclerotinia* indicates that the induction of defence responses is a critical step for this type of resistance.

PDB-induced defence responses include production of antifungal compounds that are active against certain necrotrophic fungi. Antifungal compounds have also been found in cutinase-expressing plants and in the *bdg* mutant (Chassot *et al*, 2007). PDB diffusates collected from *lacs2* plants could be partially inactivated by treatment with lipases, indicating that an acyl group is necessary for the activity of some of its components. Oxylipins are potent antimicrobial compounds and *Botrytis* is sensitive to some of them (Prost *et al*, 2005). Oxylipins of plant origin have also been shown to activate biological responses in fungi, showing that oxylipins can be transferred from a plant to a fungus (Tsitsigiannis and Keller, 2006). However, the inactivation of the active compound by lipases is somewhat surprising as *Botrytis* secretes lipases (Reis *et al*, 2005). Inactivation may thus also result from an additional activity, that is, an esterase activity, at high enzyme concentrations. Only the determination of the chemical structure of this antimicrobial compound will solve this enigma. In addition, the PDB diffusate from *lacs2* lost some activity on treatment with proteinase K. A large number of peptides and antimicrobial proteins are encoded in the *Arabidopsis* genome, among which are 300 defensin-like

genes (Garçia-Olemedo *et al*, 1998; Silverstein *et al*, 2005). Antimicrobial peptides may thus contribute to the described resistance response. A specific sensitivity of a pathogen to different antimicrobial compounds has been found in a number of cases and could explain why *lacs2* is resistant only to *Botrytis* and *Sclerotinia* and not to other necrotrophs (Thomma *et al*, 1999; Prost *et al*, 2005).

A number of different genes conferring *Botrytis* resistance when overexpressed in WT plants were induced in cutinase-expressing plants and in *bdg* (Chassot *et al*, 2007). Although the genes characterized by Chassot *et al* were not induced in *lacs2* plants under our experimental settings (data not shown), it is well possible that additional defence mechanisms, that is, strengthening of the cell wall, contribute to the resistance to *Botrytis* upon induction with PDB, explaining why the effect of the PDB diffusate on *Botrytis* resistance *in vivo* seems to be stronger than *in vitro* (50% reduction in hyphal length in *in vitro* results in 100% resistance).

The basis of the resistance described here in plants having a permeable cuticle thus lies in a better perception of elicitors from PDB leading to a number of defence responses, including the release of antifungal compounds, that arrest the invasion of certain necrotrophic pathogens. Most likely both the elicitor and the antifungal compounds are restricted in their diffusion across the cuticle, resulting in the observation that resistance to *Botrytis* is directly correlated to permeability of the cuticle of different *Arabidopsis* mutants. *Botrytis* resistance in permeable cuticle mutants is therefore an example of the importance of the timing of defence responses for the outcome of a plant–pathogen interaction (Tao *et al*, 2003). The cuticle is usually seen as a protective layer because it slows down interactions between the plant and its environment. We present here an example where this reduced diffusion rate is to the disadvantage of the plant, an aspect of cuticle biology that has not been reported before.

## **Materials and methods**

### **Plant material**

Plants were grown on a pasteurized soil mix under a 12 h light and 12 h dark cycle, with an average night temperature of 16°C (up to 90% humidity) and a day temperature of 20°C (60% humidity). WT plants are *Arabidopsis* accession Col-0, obtained from the *Arabidopsis* Biological Research Center (Columbus, OH). The mutants *lcr* (*lcr1*) and *hth* (*hth-12*), both in the Col-0 background, were obtained from Alexander Yephremov (MPI für Züchtungsforschung, Cologne) (Wellesen *et al*, 2001; Kurdyukov *et al*, 2006b). EMS-mutagenized M2 seeds accession Col-0 were provided by the laboratories of Chris Somerville, Carnegie Institution of Washington, Stanford, USA and Fred Ausubel, Massachusetts General Hospital, Boston, USA. The T-DNA insertion line in the *LACS2* gene (At1g49430) is the GABI-Kat line 368C02 (Rosso *et al*, 2003).

### **Inoculation with *B. cinerea* and *S. sclerotiorum***

*B. cinerea* strains BMM and BO5.10 were provided by Brigitte Mauch-Mani, University of Neuchâtel, Neuchâtel, Switzerland and Jan van Kan, Wageningen University, Wageningen, The Netherlands, respectively. *S. sclerotiorum* was provided by Henk-Jan Schoonbeek, University of Fribourg, Fribourg, Switzerland. Both pathogens were grown on 1 × PDA (potato dextrose agar; Difco). Conidia of *Botrytis* were harvested in water and filtered through glass wool. A liquid culture of *S. sclerotiorum* in 1/2 PDB was prepared from 2 cm<sup>2</sup> quickly growing hyphae and incubated for 2 days at 20°C under constant agitation. A *Sclerotinia* suspension containing 1 ml of mycelium/10 ml 1/2 PDB was prepared by disruption of the mycelium by a short treatment in a Polytron. Plants were inoculated with 5- $\mu$ l droplets of a suspension of

$5 \times 10^4$  spores/ml in 1/4 PDB of *Botrytis* (Difco) or of the hyphal preparation of *Sclerotinia* and kept under 100% humidity. Control plants were inoculated with 1/4 PDB (mock). The growth of both pathogens was quantified by monitoring the presence of lesions and their diameter 2–4 days after inoculation. The level of resistance was estimated by the potential of the pathogen to cause soft rot symptoms extending beyond the inoculation site (outgrowing lesions).

#### Assessment of cuticle permeability

For staining with Calcofluor white, plant material was decolorized in 95% ethanol, equilibrated in 0.2 M NaPO<sub>4</sub> (pH 9.0) for 1 h, and incubated for 1–3 min in 0.5% Calcofluor in 0.2 M NaPO<sub>4</sub> (pH 9.0). Tissues were viewed under a Leica MZ16 fluorescence microscope equipped with a UV filter. Toluidine blue staining was adapted from a previously described protocol (Tanaka *et al*, 2004). Tissues were incubated with 5- $\mu$ l droplets of a 0.025% solution of toluidine blue in 1/4 PDB for different time periods (5 min to 2 h).

Herbicide sensitivity was tested by spraying BASTA® on the adaxial side of the leaves of 4- to 5-week-old rosettes at concentrations of 5, 10, 20, 30, 40, 50, and 60  $\mu$ l/ml. Plants were kept under a transparent lid for 1 day and evaluated 1 and 2 weeks after the treatment. Water loss from 6-week-old rosettes excised at the hypocotyl was measured by weighing plants at different time points. 100% equals the total water content of a plant.

#### Analysis of residue-bound lipids

Monomers were liberated by hydrolysis with sodium methoxide of total extracted leaf material of 6-week-old *Arabidopsis* plants according to an established protocol (Bonaventure *et al*, 2004). Trimethylsilyl derivatives were analyzed by gas chromatography using an HP-5MS column (J&W Scientific) either in a unit from Hewlett Packard (model 5890) coupled to a flame ionization detector or in a unit from Agilent (Agilent 6890N Network GC system) linked to a mass spectrometer (Agilent 5973 Network Mass Selective Detector) using the following temperature profile: after 2 min at 90°C, the temperature was increased at a rate of 8°C/min to 290°C and held for 10 min.

#### Characterization of leaf diffusates

Droplets (5  $\mu$ l) of 1/4 PDB were incubated on 5-week-old plants under 100% humidity. For the *in vitro* test, 9  $\mu$ l of PDB diffusate was mixed with 3  $\mu$ l of *B. cinerea* spores to a final concentration of

$5 \times 10^4$  spores/ml and placed on a microscope glass slide. Fungal growth was observed under the microscope after incubation under high humidity conditions for  $\pm 14$  h (*in vitro* test). Diffusates without PDB were adjusted to 1/4 PDB before carrying out the *in vitro* test. Microscopic pictures were taken with the help of WinRhizo software (www.regent.qc.ca/products/rhizo/Rhizo.html). For the *in vivo* test, 15–20 5- $\mu$ l droplets of the same mixture were directly applied on WT plants and formation of lesions was evaluated after 3 days.

For characterization of 1/4 PDB diffusate, 20  $\mu$ l of diffusate was incubated in 32 U/ $\mu$ l of the lipase from *M. miehei* (Sigma), 3.2 U/ $\mu$ l of the lipase from *C. antarctica* (Sigma), or 0.035 U/ $\mu$ l of proteinase K at 37°C for 15 min. Molecular weight was estimated by using a Microcon® 3 (Millipore Corp.) column followed by the *in vitro* growth test.

#### Transmission electron microscopy

Samples were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 0.3 mM calcium chloride in 0.1 M cacodylate buffer, precontrasted with 2% uranyl acetate in 10% ethanol, and then dehydrated in a graded series of ethanol and propylene oxide. Specimens were embedded in a mixture of epon/araldite. Ultrafine sections of 50–70 nm were cut on a Reichert ultracut microtome with a Diatome diamond knife and mounted on either copper or nickel parallel bar grids. The grids were then contrasted with 2% aqueous uranyl acetate for 10 min and saturated lead citrate for 10 min. The samples were observed with a Phillips CM12 transmission electron microscope.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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