The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*

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Background: Plants have evolved efficient mechanisms to combat pathogen attack. One of the earliest responses to attempted pathogen attack is the generation of oxidative burst that can trigger hypersensitive cell death. This is called the hypersensitive response (HR) and is considered to be a major element of plant disease resistance. The HR is thought to deprive the pathogens of a supply of food and confine them to initial infection site. Necrotrophic pathogens, such as the fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*, however, can utilize dead tissue.

Results: Inoculation of *B. cinerea* induced an oxidative burst and hypersensitive cell death in *Arabidopsis*. The degree of *B. cinerea* and *S. sclerotiorum* pathogenicity was directly dependent on the level of generation and accumulation of superoxide or hydrogen peroxide. Plant cells exhibited markers of HR death, such as nuclear condensation and induction of the HR-specific gene *HSR203J*. Growth of *B. cinerea* was suppressed in the HR-deficient mutant *dnd1*, and enhanced by HR caused by simultaneous infection with an avirulent strain of the bacterium *Pseudomonas syringae*. HR had an opposite (inhibitory) effect on a virulent (biotrophic) strain of *P. syringae*. Moreover, H_2O_2 levels during HR correlated positively with *B. cinerea* growth but negatively with growth of virulent *P. syringae*.

Conclusions: We show that, although hypersensitive cell death is efficient against biotrophic pathogens, it does not protect plants against infection by the necrotrophic pathogens *B. cinerea* and *S. sclerotiorum*. By contrast, *B. cinerea* triggers HR, which facilitates its colonization of plants. Hence, these fungi can exploit a host defense mechanism for their pathogenicity.

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Background

A large percentage of plant pathogens are biotrophs that require compounds from living host cells. Recognition of pathogen attack triggers a hypersensitive reaction (HR) in the plant, which includes generation of reactive oxygen intermediates (ROIs) and local cell death [1,2]. HR is considered to be one of the most important factors in impeding growth of biotrophic pathogens [3,4]. Pathogens that do not trigger HR can establish compatible interactions with the host, resulting in disease [5]. Recently, however, an *Arabidopsis* mutant (*dnd1)* was isolated that exhibits resistance to virulent pathogens in the absence of HR [6]. Although this does not rule out that HR plays an important role in resistance, it suggests that disease resistance can be achieved by a number of mechanisms, or that in some cases, a subset of defense mechanisms may be sufficient to stop pathogen growth.

One of the key HR components is the generation of ROIs that occurs within hours of attack by avirulent pathogens [4,7,8]. The following lines of evidence support the involvement of ROIs in HR: ROIs accumulate during pathogen attack; inhibition of ROI generation by diphenyleneiodonium in parsley, tobacco and in soybean reduces hypersensitive cell death; and ectopic expression of an H_2O_2-producing enzyme, glucose oxidase, in transgenic tobacco increases host cell death exclusively after pathogen challenge [9–11]. An increase in programmed cell death (PCD) caused by pathogen attack was also observed in engineered tobacco plants with reduced catalase activity, which accumulate higher levels of H_2O_2 [12]. It should be noted that ROIs alone are not always sufficient to cause PCD, and additional factors such as iron availability or production of nitric oxide may be required.

Botrytis cinerea is a necrotrophic fungal pathogen that attacks over 200 different plant species [13]. The disease is manifested by necrotic areas with extensive fungal growth, giving the characteristic appearance of grey mould. Contrary to biotrophs, aggressive strains of *B. cinerea* and the related necrotroph *Sclerotinia sclerotiorum* are not deterred by the plant cell death and can feed on dead tissue. We hypothesized that, whereas hypersensitive cell death can be an effective defence strategy against

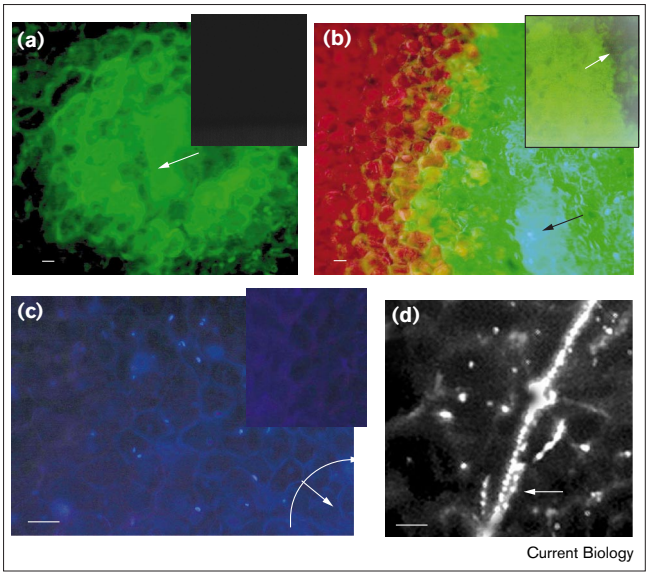
biotrophs [1,3], it may not be efficient against necrotrophic pathogens. Our results show that *B. cinerea* and *S. sclerotiorum* utilize plant hypersensitive response for rapid colonization of their hosts.

Results and discussion

Production of oxygen radicals and induction of host cell death by *B. cinerea* infection

One of the first symptoms of *B. cinerea* infection is appearance of necrotic lesions, resembling HR. To examine whether plants inoculated with *B. cinerea* were producing ROIs, which are an important component of HR [8,14], we examined ROI production and localization with respect to hyphal growth. ROI production was detected [15] by 6 hours after inoculation of germinated spores (Figure 1a). The ROI-producing area spread with the progress of infection (Figure 1b). Double staining with trypan blue (which stains the hyphae) detected ROI production several cell layers away from the hyphae (Figure 1b, inset). Quantitative measurements of dye oxidation in a spectrofluorometer [15] corroborated the microscopy data (data not shown). Dye oxidation was reduced by addition of catalase, implicating H_2O_2 as the accumulating ROI (data not shown). A similar area, however, was also stained with a superoxide-specific dye, nitroblue tetrazolium [16] (data not shown). It is therefore possible that the H_2O_2 originated by dismutation of $^{\bullet}O_2^-$.

Figure 1

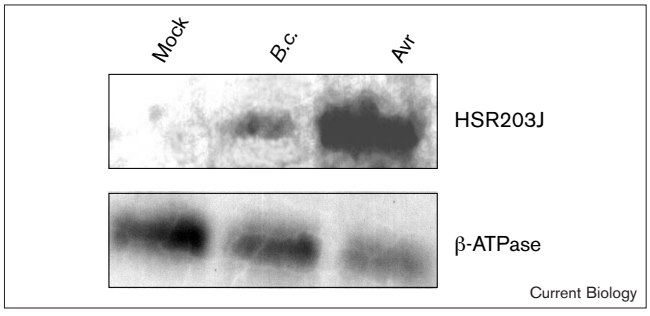


Production of ROI in plants infected with *B. cinerea*. **(a)** ROI production in wild-type *Arabidopsis* plants 6 h after *B. cinerea* inoculation, assayed by oxidation of 2′,7′-dichlorofluorescein diacetate [15]. Arrow points to the site of inoculation. The evenly stained area in the center is due to dye diffusion into the punctured area. Inset: puncture control showing no fluorescence in mock-infected leaves. **(b)** ROI production in leaves 36 h after infection. Red fluorescence is from chlorophyll. Inset shows double staining of ROI and fungal hyphae (arrow). **(c)** Nuclear condensation in cells of wild-type plants, as detected by DAPI staining 24 h after inoculation. Inset: DAPI fluorescence in mock-infected leaves. **(d)** Host cell death, as determined by Sytox staining, 24 h after inoculation in wild-type plants; arrow indicates fungal hyphae. The scale bar represents 10 μm in (a) and 20 μm in (b–d).

To determine whether ROI-accumulating cells exhibited signs of PCD, we examined nuclear condensation, which is strongly associated with PCD in animals and plants [5,17], using 4′,6-diamidino-2-phenyl-indole (DAPI), a membrane permeable DNA-binding dye [18]. Earlier reports detected condensed nuclei in hypersensitively reacting cells during pathogen attack [18–20]. Condensed DNA, apparent as bright DAPI stained spots as opposed to the diffuse, usually invisible, staining of normal nuclei [18,19], was observed around the infection site (Figure 1c). These nuclear changes preceded loss of plasma membrane integrity as determined by lack of nuclear staining with a membrane impermeable DNA-binding dye, Sytox, which stained only dispersed dead cells, usually close to hyphae (Figure 1d). This is in line with membrane rupture being a late event in the PCD process [14]. Interestingly, Sytox stained the fungal nuclei, probably due to different membrane composition.

To examine whether the nuclear changes in *B. cinerea*-infected plants coincided with induction of HR-specific markers, we assayed the expression of the *HSR203J* gene, which is activated rapidly and specifically during pathogen-triggered HR [21–23]. Since this gene has been identified only in tobacco and tomato plants and its cDNA does not cross-hybridize with *Arabidopsis* genes, we probed its expression in *B. cinerea*-infected tobacco (Figure 2), which exhibit disease symptoms similar to *Arabidopsis*. Induction of *HSR203J* was detected 16 hours after spore inoculation, and preceded lesion development by several hours, in line with induction of this gene early during

Figure 2



Northern analysis of *HSR203J* expression in plants infected with *B. cinerea*. Tobacco plants were infected with 500 spores of *B. cinerea (B.c.)* or 10^7 c.f.u. *P. syringae* pv *glycinea* (Avr). Leaf disks from infected and control areas were collected after 16 h. Total RNA was analyzed by northern blotting and probed with the HSR203J gene. Constitutively expressed β-ATPase served as loading control.

bacteria- or virus-caused HR [21]. As this gene is expressed only transiently during HR and is highly localized to the hypersensitively reacting area [21,23], it is hard to compare the expression levels between plants infected with *B. cinerea* and those infected with the bacterium *Pseudomonas syringae* pv *glycinea*. Moreover, in fungus-infected leaves necrosis develops with hyphal growth, thus at any given time point the HR is confined to a limited area, whereas bacteria cause a synchronized HR within the infiltrated area. Nonetheless, since *HSR203J* is not induced by H_2O_2 [22], this result indicates induction of HR-specific responses, independent of ROI.

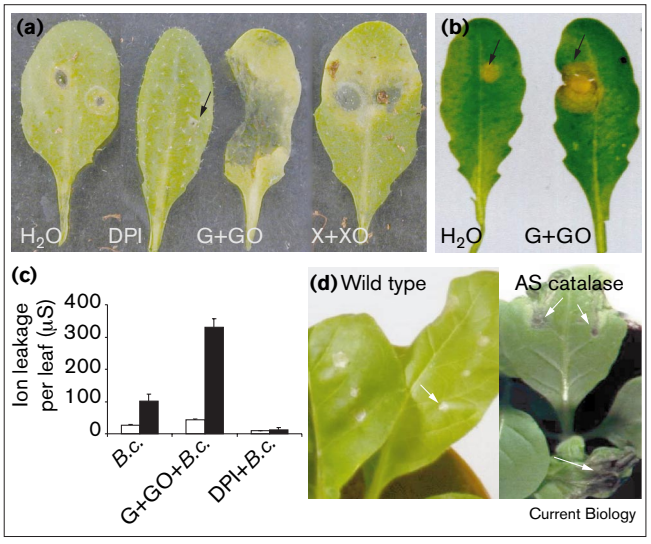
Manipulation of oxidative stress in inoculated leaves

To test whether plant-generated ROIs contributed to necrosis, as found in other plant pathogenesis systems [4,8], we experimentally raised or lowered the level of ROIs in the leaf. A number of inducible ROI-generating enzymes have been implicated in different plant–pathogen systems ([24–26], reviewed in [8,27]). Infiltration of 3 μM diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase and of other flavoprotein enzymes [28–30], into intercellular spaces prior to *B. cinerea* inoculation diminished ROI-dependent fluorescence by 65% and restricted necrosis for more than 3 days (Figure 3a). DPI was ineffective in stopping the disease when added into the germination (inoculation) medium, suggesting involvement of these plant enzymes in ROI production.

On the other hand, infiltration of mixtures of xanthine oxidase plus xanthine (X+XO) or of glucose oxidase plus glucose (G+GO), which produce $O_2^{\bullet-}$ or H_2O_2, respectively, greatly enhanced lesion formation (Figure 3a). Extensive necrosis occurred by 24–36 hours after inoculation, bypassing the quiescent phase that occurs in early stages of the *B. cinerea* infection [31]. No lesions were detected in leaves infiltrated with ROI-producing mixtures alone (data not shown), as previously reported [16]. Enhanced ROI generation also strongly stimulated necrosis induced by *S. sclerotiorum*, suggesting a common pathogenicity mechanism for both these necrotrophs (Figure 3b). The visual necrosis symptoms were also corroborated by quantitative measurements of ion leakage from the infected leaves, which is indicative of plant cell death [32] (Figure 3c). By contrast, similar treatment with G+GO-infiltrated into leaves reduced growth of a virulent (biotrophic) strain of *P. syringae* DC3000 (data not shown).

Extensive lesions were also produced by *B. cinerea* when H_2O_2 accumulation was increased by suppression of catalase. Infiltration of *Arabidopsis* leaves with a specific catalase inhibitor, 3-aminotriazole [33], prior to inoculation was indistinguishable from the G+GO-treated leaves (data not shown). We also inoculated tobacco plants with reduced catalase activity due to constitutive expression of the catalase gene in an antisense orientation [34]. Infection of these plants resulted in both accelerated and enlarged lesion formation (Figure 3d) [34]. These data corroborate the role of ROIs, and specifically of H_2O_2, in fungus-induced lesion formation, and point to plant catalase as the enzyme that controls H_2O_2 levels during *B. cinerea* infection. It is noteworthy that our results are opposite to the increased resistance to other (biotrophic) pathogens observed in the catalase-suppressed plants [34,35]. They are also opposite to the resistance of transgenic potato plants expressing glucose oxidase against infection by *Phytophtora infestans* and *Erwinia carotovara* [36], and to the infiltration of G+GO on the *P. syringae* DC3000 strain described above.

Figure 3

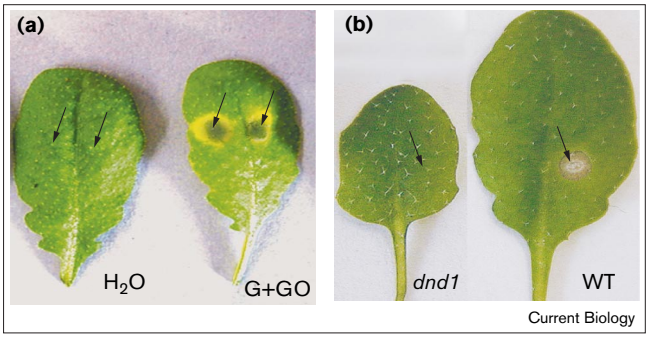


The effects of ROIs on *B. cinerea* and *S. sclerotiorum* infection. **(a)** The levels of ROI in *Arabidopsis* leaves were altered by infiltration of DPI (3 μM), or mixtures of xanthine oxidase (0.1 units) plus 1 mM xanthine (X+XO), or of glucose oxidase (100 units/ml) plus 2 mM glucose (G+GO); or treated with water (control). *B. cinerea* was inoculated in the center of the infiltrated area 1 h later. No lesions were seen in plants infiltrated with G+GO or X+XO mixtures alone (data not shown). The effects of the treatments on ROI were verified by spectrofluorometric measurements as described by Lu and Higgins [15] (data not shown). Photographs were taken 2 days after inoculation. **(b)** *Arabidopsis* leaves were infiltrated with water (control) or with G+GO and inoculated with *S. sclerotiorum*. Photographs were taken 2 days after inoculation. **(c)** Cell death as determined by measurements of ion leakage in *B. cinerea*-infected plants pre-treated with G+GO or DPI after 24 h (white) and 48 h (black). **(d)** 6-week-old wild-type and antisense (AS)-catalase-expressing tobacco plants [34] were inoculated with spores of *B. cinerea*, pictures were taken after 3 days. Note the delimited round lesions in wild-type plants, as opposed to spreading necrosis in AS catalase plants.

Infection of wild type and HR-deficient *Arabidopsis* plants by *B. cinerea* and *S. sclerotiorum*

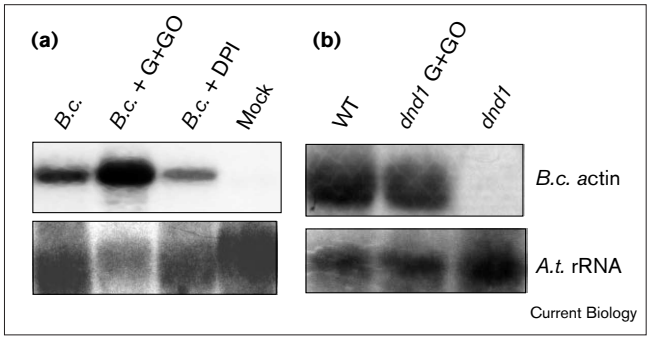
The above results demonstrate a strong correlation between the generation of ROIs and lesion formation in *B. cinerea* or *S. sclerotiorum* infections. To test the possible involvement

Figure 4



Pathogenesis of *B. cinerea* and *S. sclerotiorum* on wild-type and HR-deficient *dnd1* mutants. **(a)** 5-week-old *dnd1* plants were pre-treated with water or G+GO, as in Figure 3, and infected with germinated spores of *B. cinerea.* Note the near absence of lesions in water pre-treated *dnd1* plants, as compared to wild-type plants in Figure 3a. The photograph was taken 2 days after inoculation. Replacement of G+GO with X+XO had a similar effect (data not shown). **(b)** Comparison of disease symptoms in wild-type (WT) and *dnd1 Arabidopsis* plants inoculated with *S. sclerotiorum* mycelium disks (arrows). Picture was taken 3 days after infection.

Figure 5



Northern analysis of *B. cinerea* actin accumulation. **(a)** *B. cinerea* growth in plants with altered ROI levels was assessed by northern analysis of a constitutively expressed actin gene. Total RNA was extracted 3 days after inoculation and hybridized with a specific fragment of the *B. cinerea* actin gene that is not homologous to the plant actin [31]. Where indicated, plants were pre-treated with DPI or G+GO as in Figure 3. rRNA, *A. thaliana* 18S rRNA. Note the decrease of plant rRNA in the highly infected sample. **(b)** Growth of *B. cinerea* in wild-type (WT) and HR-deficient *dnd1* plants. Leaves were taken 3 days after inoculation. No signal was seen in the *dnd1* plants even after overexposure of the film.

of HR in necrotroph pathogenicity, we undertook two approaches: infection of HR-deficient *dnd1* mutants [6] and infection of *B. cinerea* following inoculation of an avirulent (HR-inducing) strain of *P. syringae*. Wild-type and HR-deficient *dnd1* mutants were inoculated with *B. cinerea* or *S. sclerotiorum*. Differences in *B. cinerea* penetration were excluded by puncturing leaves in the inoculum center. Wild-type plants infected with *B. cinerea* began to develop necrotic lesions 18–21 hours after inoculation, whereas in *dnd1* mutants almost no symptoms were seen, even after 5 days (Figure 4a). By this time, the majority of wild-type leaves had extensive lesions and were almost completely deteriorated. The striking difference between the two plants was also seen in *S. sclerotiorum* infections. Placement of mycelium disks of *S. sclerotiorum* produced visible lesions in the wild-type plants after 2 days, but *dnd1* mutants remained symptomless even after 7 days (Figure 4b). Thus, the absence of lesions in *dnd1* suggests that the lesions in wild-type plants were caused by a host-activated HR, and not by fungus toxicity.

As the mechanism of HR suppression in *dnd1* is not known, it is possible that these plants are generally more resistant. Indeed, *dnd1* plants exhibit high levels of salicylic acid (SA), a molecule that induces broad-spectrum resistance [6]. However, assessment of *B. cinerea* pathogenicity in SA-treated wild-type plants did not show inhibition of lesion formation or growth of *B. cinerea* (E.G. and A.L., unpublished observations). To explore the possibility that *dnd1* mutants were resistant to *B. cinerea* by means of a preformed SA-independent resistance mechanism, we infiltrated X+XO or G+GO into the *dnd1* plants prior to *B. cinerea* inoculation, as in Figure 3a. Both pre-treatments resulted in development of necrotic lesions (Figure 4a). In fact, some *dnd1* plants became infected even without ROI pretreatment when kept under constant light for extended time periods (>10 days). This result further supports the link between ROI, HR and necrotroph pathogenicity.

The effects of ROIs and HR on fungal growth

The above results demonstrate that ROI production influenced disease symptoms, as manifested by visible HR. But did lesion formation facilitate *B. cinerea* growth? To examine this link, we probed accumulation of *B. cinerea* actin (Figure 5a), which is expressed constitutively during infection [31], and counted conidiospores (Table 1). The latter assay also analysed the development of fungus inside the plant. Both assays clearly showed that *B. cinerea* growth was enhanced by elevated generation of ROIs and the consequent increase in hypersensitive cell death [7,8,14] but was diminished by reduction in ROIs. Moreover, ROI supplementation supported *B. cinerea* growth in the *dnd1* mutants (Figure 5b). These results are also in agreement with higher ROI levels that were found in plants infected with more aggressive strains of *B. cinerea*, when compared to less aggressive strains [37].

In a second approach to explore the relationship between HR and *B. cinerea* pathogenicity, we asked whether (classical) HR, caused by avirulent pathogens, influences subsequent colonization by *B. cinerea*. Leaves were infected with 10^5 or 10^7 c.f.u./ml *P. syringae* pv *tomato (P.s.t.)* carrying the *avrRpm1* avirulence gene, or with nearly isogenic *hrp*$^-$ mutants (which do not cause HR or disease), or the virulent DC3000 strains [38]. Spores of *B. cinerea* were

Table 1

Production of *B. cinerea* conidiospores in *A. thaliana*.

Treatment	Conidia per leaf
X+XO	604 ± 86
G+GO	1383 ± 340
dnd1	0 ± 0
Wild type	30 ± 8

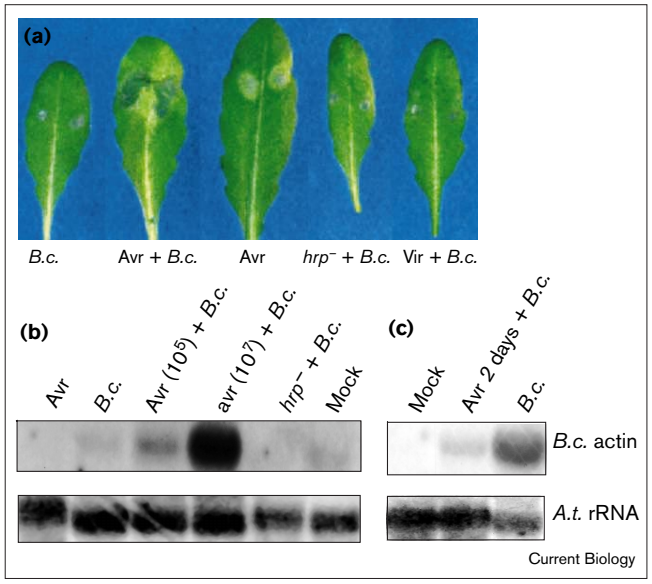
Arabidopsis plants were pre-treated with mixtures producing $^{\bullet}O_2^-$ (X+XO) or H_2O_2 (G+GO) and inoculated 1 h later with *B. cinerea*, with a single inoculation in each leaf. Conidiospores were isolated after 7 days. The experiment was repeated three times with similar results.

placed in the center of the bacterial infiltration zone 6 hours later. Massive spreading necrosis was observed in plants preinfected with HR-causing bacteria but not in plants pretreated with *hrp*⁻ bacteria or with disease-causing virulent bacteria (Figure 6a). In some experiments, bigger lesions were seen in leaves pre-infected with a high dose of the virulent DC3000 strain, which may reflect the limited ROI production and cell death often observed in response to high concentration of virulent pathogens. These results indicate that HR triggered by a different pathogen promoted *B. cinerea* infection. The latter conclusion was also verified by probing accumulation of the fungal actin (Figure 6b). To rule out the possibility that *B. cinerea* simply grew better on dead tissue, we inoculated germinated spores in the center of typical HR lesions after the HR caused by *P.s.t.(avrRpm1)* was complete. The reduced accumulation of fungal actin RNA observed in the dead tissue indicated slower growth of the fungus (Figure 6c).

The effects of ROIs and HR on bacterial growth

The enhanced growth of *B. cinerea* in hypersensitively reacting tissues was contrasted by inoculating the hypersensitively reacting leaves with a biotrophic pathogen. HR was triggered with bacterium *P. syringae* pv *glycinea (P.s.g.)* which induces a non-host type of HR, and after 3 hours the reacting area was challenged with the virulent strain of *P.s.t.* DC3000, which grows in the intercellular space. Bacteria were extracted from leaves 3 days after inoculation and plated on selective medium that allowed growth of *P.s.t.* only. No growth of *P.s.t.* occurred in HR-reacting leaves (Figure 7). Moreover, compared with immediate extraction the number of virulent bacteria was reduced from 10^5 to 3×10^2 c.f.u.. To exclude the possibility that inhibition of *P.s.t.* growth was due to competition with *P.s.g.*, we grew 10^5 c.f.u. *P.s.t.* DC3000 (without antibiotics) with or without the same amount of *P.s.g.* outside the plant, and then plated them on the selective medium. In the test tube we did not detect a negative effect of *P.s.g.* on growth of *P.s.t.*, which implies that inhibition of *P.s.t.* growth *in planta* was caused by the plant-produced

Figure 6

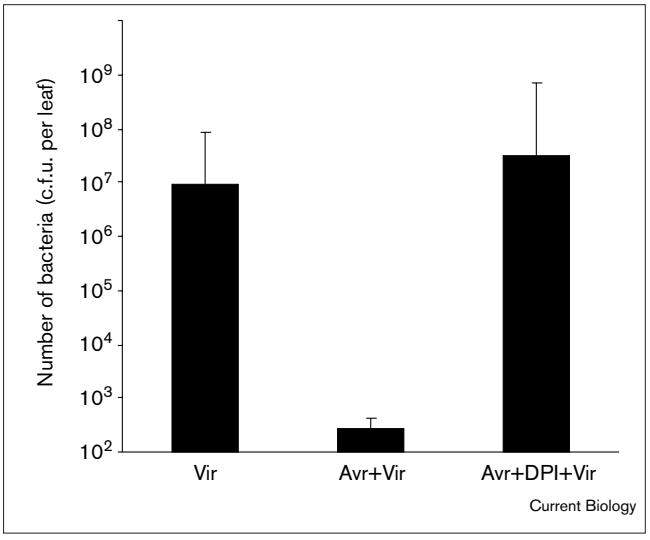


HR-dependent lesion formation and *B. cinerea* growth in plants inoculated with *B. cinerea* and/or *P. syringae* pv. *tomato (avrRpm1)*. **(a)** Wild-type *Arabidopsis* plants were infiltrated with 10^7 c.f.u. avirulent *P.s.t.(avrRpm1)* or *hrp*⁻ mutants, or 5×10^6 c.f.u. virulent *P.s.t.* DC3000. After 4 h, a drop of *B. cinerea* spores was placed in the center of the infiltration site. Photographs show lesions after 24 h. Leaves from left to right: *B.c.*, *B. cinerea* alone; *avr+B.c., P.s.t.(avrRpm1)* followed *by B. cinerea*; *avr, P.s.t.(avrRpm1)* alone; *hrp⁻+B.c.*, *P.s.t.(hrp⁻)* followed by *B. cinerea; vir+B.c.*, *P.s.t.* DC3000 followed by *B. cinerea.* **(b)** Growth of *B. cinerea* in hypersensitively reacting tissue analyzed by northern blotting of leaves infected with 10^5 or 10^7 c.f.u. *P.s.t.(avrRpm1)* or 10^7 of *hrp*⁻ mutants and inoculated with *B. cinerea* 2 h later. At 3 days after the *B. cinerea* inoculation, leaves were collected and total RNA from each treatment probed for the *B. cinerea* actin gene. The blot was autoradiographed for 16 h. **(c)** Northern analysis of *B. cinerea* growth within dead tissue. Plants were challenged with 10^7 c.f.u. *P.s.t.(avrRpm1)* and 2 days later (after HR was clearly visible) inoculated with *B. cinerea* (avr 2 days+*B.c.*). Leaves were collected 4 days after *B. cinerea* inoculation, and total RNA probed for the fungal actin gene. The blot was autoradiographed for 4 days.

HR. This conclusion is further supported by inhibition of *P.s.g.*-caused HR with DPI (Figure 7). DPI has been shown to inhibit hypersensitive cell death from avirulent strains of *P. syringae* in soybean cell cultures and in tobacco plants [7,12]. Infiltration of DPI into leaves rescued growth of *P.s.t.* in the presence of *P.s.g.*, whereas G+GO decreased growth of *P.s.t.* below 10 c.f.u., in line with the antimicrobial nature of H_2O_2. These results are opposite to the growth of *B. cinerea* seen in DPI and G+GO treated plants (see Figure 5), indicating that the two types of pathogens fundamentally differ in their mode of pathogenesis, with respect to oxidative burst and to HR.

In summary, we show that the HR response of plants to pathogens facilitated rapid growth and spread of *B. cinerea* and very probably also of *S. sclerotiorum*. *B. cinerea* triggered oxidative burst and hypersensitive cell death. Fungal

Figure 7



Growth of *P. syringae* pv. *tomato* in hypersensitively reacting tissue. *Arabidopsis* plants were inoculated with 10^7 c.f.u. virulent *P.s.t.* DC3000 either alone (vir) or 3 h after infiltration of same amount of avirulent (HR-causing) *P. syringae* pv glycinea (avr+vir). DPI (3 μM) was infiltrated 1 h prior to *P.s.g.* inoculation (avr+DPI+vir). Bacteria were extracted 3 days later and plated on selective medium that supported growth of *P.s.t.* but not *P.s.g.*. Extraction of *P.s.t.* DC3000 immediately after infiltration resulted in 10^5 c.f.u.. Similar results were obtained after 6 days.

growth was accelerated by concurrent infection with avirulent bacteria, or by enhanced generation of ROI, as opposed to pathogenicity of virulent biotrophic pathogens which was reduced by enhanced ROI production and by HR. Thus, HR induced by other pathogens or elicited by the fungi themselves restricts spread of biotrophic pathogens, but has an opposite effect against necrotrophs. Moreover, it may provide the necessary window for establishment of disease during the critical early stages of necrotroph attack, which usually lack specific mechanisms for penetration [39].

Materials and methods

Chemicals

Dichlorodihydrofluorescein diacetate (DCFH-DA) and Sytox were from Molecular Probes and glucose oxidase from Worthington. All other chemicals were from Sigma.

Biological materials

dnd1 mutants were a gift from A. Bent (University of Wisconsin, Madison). *Arabidopsis* plants (strain Col-O) were grown for 4–5 weeks at 22°C. *B. cinerea* strain INRA (kept in A. Mayer collection, Hebrew University, Israel) was grown on 1.5% malt agar at 20°C and continuous light. *S. sclerotiorum* (from Y. Elad, Volcani Center, Israel) were grown on potato dextrose agar (DIFCO). *P. syringae* (from N. Keen, University of California, Riverside) were grown in Kings B medium at 29°C.

Pathogen manipulation

B. cinerea: spores were germinated in 0.05% glucose, 0.03 M KH_2PO_4 pH 5, for 2–3 hours, filtered through 25 μm nylon mesh and resuspended at 10^5 spores/ml. Leaves were inoculated with 5 μl of an 80–100 spores/μl suspension, and the droplets punctured with a 25G needle. Conidiospores were isolated by shaking one leaf in 1 ml water; five replicates of 10 μl aliquots were counted under the microscope. *S. sclerotiorum* were inoculated by placing 3 mm disks excised from agar on the upper leaf side as described in [40]. Inoculated plants were kept at 20°C in a box covered with nylon. *P.s.t.* strains were from J. Dangl (University of N. Carolina), *P.s.g.* were from N. Keen (University of California) inoculations were as described in [6]. The fungus was stained with 5.5% trypan blue for 60 min. Growth of *P.s.t.* was assayed by counting c.f.u. as described [6] on selective medium supplemented with 100 μg/ml rifampicin. *P.s.g.* were grown as described [7].

ROI and death measurements

Leaves were incubated in 0.2 ml 10 μM DCFH-DA for 15 min in phosphate buffer pH 7.5 in the dark. Leaves were taken out for microscopic examination, and the remaining medium measured in a BioTek FL600 fluorometer set at 485 nm/525nm excitation/emission wavelengths, respectively. Similar results were obtained by infiltration of the dye directly into leaves as described [15]. Cell death was assayed by staining leaves in 2 μM Sytox for 5 min. Ion leakage was determined by measuring conductivity in a Consort K511 instrument according to [32].

Fluorescence microscopy

ROI dependent fluorescence in leaves was analyzed in an Olympus IF70 epifluorescent microscope with narrow-band excitation emission filters: DAPI, Ex: 365HT25nm; Em: 450DF65nm [16]; Sytox and DCFH-DA: 485DF22/535DF35. A wide-band emission filter was used to simultaneously visualize chlorophyll and DCFH-DA in Figure 2b. Photographs were taken with an Olympus SLR camera and Fuji 200 ASA film, or with a Nikon Coolpix950 digital camera.

Northern analysis

For RNA preparation 100 mg leaves were homogenized in liquid nitrogen and resuspended in 4 M guanidinium thiocyanate, 0.2 M sodium acetate pH 4, 0.1 M β-mercaptoethanol, 0.5% sarkosyl. RNA was extracted with 1 volume water saturated phenol and 1/4 volume chloroform and precipitated twice in isopropanol. Hybridization was done in Amersham, USA QuickHyb buffer according to manufacturer's instructions. *B. cinerea* actin (*actA*), kindly provided by E. Benito [31], was labeled with [^{32}P]deoxycytidine. Exposure time varied according to signal strength as indicated in figure legends. 18S chloroplast rRNA from *Arabidopsis* was used as RNA control.

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