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Necrotroph Attacks on Plants: Wanton Destruction or Covert Extortion?

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Necrotrophic pathogens cause major pre- and post-harvest diseases in numerous agronomic and horticultural crops inflicting significant economic losses. In contrast to biotrophs, obligate plant parasites that infect and feed on living cells, necrotrophs promote the destruction of host cells to feed on their contents. This difference underpins the divergent pathogenesis strategies and plant immune responses to biotrophic and necrotrophic infections. This chapter focuses on Arabidopsis immunity to necrotrophic pathogens. The strategies of infection, virulence and suppression of host defenses recruited by necrotrophs and the variation in host resistance mechanisms are highlighted. The multiplicity of intraspecific virulence factors and species diversity in necrotrophic organisms corresponds to variations in host resistance strategies. Resistance to host-specific necrotophs is monogenic whereas defense against broad host necrotrophs is complex, requiring the involvement of many genes and pathways for full resistance. Mechanisms and components of immunity such as the role of plant hormones, secondary metabolites, and pathogenesis-related proteins are presented. We will discuss the current state of knowledge of Arabidopsis immune responses to necrotrophic pathogens, the interactions of these responses with other defense pathways, and contemplate on the directions of future research.

INTRODUCTION

Plant pathogens are categorized based on their modes of nutrition. Necrotrophic pathogens actively kill host tissue as they colonize and thrive on the contents of dead or dying cells (Stone, 2001). This lifestyle contrasts with that of biotrophic pathogens which derive nutrients from living cells and therefore must maintain host viability. A third group, hemibiotrophs, display both forms of nutrient acquisition shifting from an early biotrophic phase to necrotrophy at later stages of disease. The duration of the biotrophic or necrotrophic phase varies significantly among hemibiotrophic pathogens. Although this pathogen classification has been known for a long time, recent studies into Arabidopsis defense revealing differences in immune responses dependent on pathogen modes of nutrition have powered the widespread use of this terminology.

The relationship between a biotroph and its plant host is highly specialized as well as structurally and biochemically complex. Obligate biotrophs penetrate the host cell wall, colonizing the intercellular space using feeding structures such as haustoria to absorb nutrients and suppress host defenses without disrupting the plasma membrane (Schulze-Lefert and Panstruga, 2003; Mendgen and Hahn, 2004). The invading biotroph must strike a constant balance between virulence and evading host detection thus exhibiting a very sophisticated form of pathogenesis. By contrast, necrotrophs produce less specialized infection structures, instead overpowering the host by utilizing a variety of secreted pathogenicity and virulence factors throughout infection. Thus, fundamental differences exist between these two classes of pathogens in their infection related morphogenesis, the nature of causal agents, the disease symptoms they cause, their host range as well as the nature of plant resistance. We will expound on the implications of these differences on plant defense systems and the current state of knowledge derived from studying Arabidopsis interactions with necrotrophic pathogens.

General Description of Microbial Necrotrophy

Necrotrophic pathogens are bacterial, fungal and oomycete species that have very destructive pathogenesis strategies resulting in extensive necrosis, tissue maceration, and plant rots. To cause disease, necrotrophs secrete disease agents including phytotoxins, cell wall degrading enzymes (CWDEs), and other extracellular enzymes into host tissue both prior to and during colonization, with primary infection involving the formation of expanding necrotic lesions (Alfano and Collmer, 1996; Walton, 1996). When the host fails to constrain initial necrosis, diseases culminate in the death and decay of the entire plant. By contrast, biotrophic pathogens deploy complex and co-evolved biological strategies to exploit their hosts while keeping them alive in order to complete their life cycle.

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Infection by fungal necrotrophs generally involves stages of conidial attachment, germination, host penetration, primary lesion formation, lesion expansion, and tissue maceration followed by sporulation (Prins et al., 2000). Following germination, penetration may be achieved by active mechanisms such as appressoria formation and enzymatic degradation or passively through prior infection or wound sites as well as stomates (Prins et al., 2000). After entry, tissue is decomposed through further cellular dismantling using many of the lytic enzymes employed for initial penetration as well as toxic levels of reactive oxygen species (ROS). Many necrotrophs produce various low-molecular weight phytotoxic metabolites, ranging from host-specific to those having adverse effects on many diverse species (van Kan, 2006). Others secrete phytotoxic proteins known to induce necrosis, with the vast majority of broad host necrotrophs producing multiples of both (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006; Choquer et al., 2007). Throughout infection, these fungi also actively manipulate host cellular machinery in order to suppress defenses and/or aid in disease progression. Some necrotrophs influence host phytohormone levels or employ their own hormone biosynthesis thereby disrupting defense signaling (Prins et al., 2000; Sharon et al., 2004). Others have adapted mechanisms to detoxify host metabolites that interfere with virulence (Morrissey and Osbourn, 1999).

Overall, bacterial necrotrophs follow a similar mode of pathogenesis to their fungal counterparts, secreting virulence factors into host tissue to induce necrosis and progressive colonization of plant tissue (Alfano and Collmer, 1996). Some of these factors include pectic enzymes, toxins, and necrosis inducing proteins resulting in soft rot diseases (Alfano and Collmer, 1996; Pemberton and Salmond, 2004). However, in contrast to fungi, bacteria require a multi-protein secretion system for delivery of virulence factors into the host (Alfano and Collmer, 2004). Additionally, bacteria are not able to actively penetrate the leaf surface, relying instead on natural openings and wounds to gain entrance into the intracellular space. Some bacteria also utilize quorum sensing to enhance disease and evade host defenses (Alfano and Collmer, 1996). In quorum sensing, bacteria use cell to cell signaling to induce virulence gene expression only when the population density has reached a level high enough to overwhelm host responses.

Examples of Necrotrophic Infection Strategies

To highlight some specifics of the infection process, selected mechanisms employed in host colonization by stereotypical necrotrophs are presented here. *Botrytis cinerea* (telemorph *Botryotinia fuckeliana*), the causal agent of the grey mold disease, is a broad host fungal pathogen. The infection, colonization, and suppression of host defenses by *B. cinerea* is mediated by a number of lytic enzymes, toxins, high levels of ROS, necrosis-inducing factors and an array of secondary metabolites (van Kan, 2006; Choquer et al., 2007). In order to penetrate the host leaf cuticle, *B. cinerea* develops appressoria, infection structures that grow on the plant surface, from which oxidases, cutinases, and lipases are secreted to aid in actively dismantling the plant cutin and wax layers (van Kan et al., 1997; Tenberge, 2004). Once the cuticle has been weakened, the appressoria form penetration pegs which breach the epidermal cells via secretion of various CWDEs including laccases, proteases, pectinases, and specifically endopolygalacaturanses (endo-PGs) (Kars et al., 2005). *B. cinerea* also produces botrydial, a non-host selective toxin that functions as a strain specific virulence factor (Siewers et al., 2005). Exogenous application of botrydial produces chlorosis, cell collapse, and aids fungal penetration of plant tissue, most likely contributing to the host unspecificity of the fungus (Colmenares et al., 2002). *B. cinerea* also secretes oxalic acid (OA) which promotes infection by creating an optimal acidic environment for increased activity of secreted enzymes, disrupting cell wall integrity by chelating pectin calcium ions, and directly initiating plant cell death (Prins et al., 2000; Manteau et al., 2003; van Kan, 2006). Potentially, *B. cinerea* may activate specific virulence arsenals depending on host species and tissue type contributing to its success as a widespread pathogen of many plants. A detailed review of *B. cinerea* virulence factors has been described recently (Choquer et al., 2007).

Sclerotinia sclerotiorum, a close relative of *B. cinerea*, also secretes OA during infection. OA-deficient strains exhibit a total loss of pathogenicity (Godoy et al., 1990). The fungus utilizes oxalate to actively suppress host-induced oxidative bursts as well ABA-induced stomatal closure (Cessna et al., 2000; Guimaraes and Stotz, 2004). *S. sclerotiorum* also produces glucanases, glycosidases, cellulases, pectinases, xylanases, cutinases and proteases that aid in tissue degradation (Bolton et al., 2006). Polyketides such as scleroide, sclerone, isosclerone, and sclerin are also produced by *S. sclerotiorum,* however, only sclerin is shown to be a phytotoxic agent, inducing necrosis on select crucifer hosts (Pedras and Ahiahonu, 2004).

Different *Alternaria* species are known to produce numerous phytotoxic metabolites. Germinating spores of *A. brassicicola* secrete the atypical host selective toxin (HST) AB toxin, which is a protein rather than a characteristic low molecular weight secondary metabolite (Otani et al., 1998). *A. brassicicola* produces Brassicicolin A as a major HST and mannitol derivatives that exhibit some degree of host toxicity (Pedras et al., 2009). Other secondary metabolites generated by this fungus include brassicicenes A–F, phomapyrone A/F/G, and infectopyrone though their exact roles in pathogenicity are yet to be determined (MacKinnon et al., 1999; Pedras et al., 2009). *A. brassicicola* also produces the toxin depudecin, an inhibitor of histone deacetylases (Privalsky, 1998). Mutant strains impaired in depudecin biosynthesis display a 10% reduction in virulence on cabbage (*Brassica oleracea*) but not on Arabidopsis (Wight et al., 2009). *AbPro1* and *AbNIK1*, encoding a transcription factor and two-component histidine kinase, respectively, were recently identified as novel virulence factors of *A. brassicicola* (Cho et al., 2009). Deletions in either of these genes resulted in a significant loss of pathogenicity as well as altered vegetative growth. Interestingly, the fungus differentially regulates expression of specific cutinases depending on necrotrophic or saprophytic phase of infection (Yao and Koller, 1995).

Pectobacterium carotovorum (formerly *Erwinia carotovora)* is a bacterial necrotroph known to secrete various cellulases, proteases, phospholipases, and xylanases to actively degrade the plant cell wall (Barras et al., 1994). This pathogen also produces harpins, protein elicitors of the hypersensitive response (HR)

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(Cui et al., 1996; Mukherjee et al., 1997). Pathogen produced CWDEs and harpins are transported to the host via the type II/III secretion system (Sandkvist, 2001; Alfano and Collmer, 2004). Interestingly, the synthesis and subsequent release of these virulence factors amongst the infecting bacterial population is globally regulated by the diffusible signal molecules *N*-acyl homoserine lactones (*N*-AHLs) (Pirhonen et al., 1993; Barnard and Salmond, 2007). Disruptions in signal production, regulation, sensing, or type II secretion diminish *P. carotovorum* pathogenicity (Liu et al., 1999; Andersson et al., 2000; Smadja et al., 2004; Barnard and Salmond, 2007 2001).

Key Distinctions Between Biotrophic and Necrotrophic Pathogens

Fundamental differences exist between biotrophs and necrotrophs in many aspects of their pathogenesis. The infection processes, histology of disease, infection-related morphogenesis, nature of effector proteins, associated host defense responses, and most importantly their nutrient acquisition strategies vary significantly. These differences may account for why one defense strategy may effectively restrict biotrophs and enable necrotrophs. In contrast to necrotrophs, biotrophic pathogens secrete limited amounts of lytic enzymes, generally lack toxin production, and evade detection or suppress immune responses through manipulation of host defenses (Oliver and Ipcho, 2004). These pathogens create intimate relationships with their host cells using specialized structures such as haustoria and start slowly draining plant resources thereby gradually decreasing plant fitness (Schulze-Lefert and Panstruga, 2003). Necrotrophs, on the other hand, are facultative saprophytes that actively destroy host tissue using various toxins and CWDEs (Oliver and Ipcho, 2004). Generally, resistance to host specific necrotrophs is conditioned by single genes conferring complete immunity whereas resistance to broad-host necrotrophs is quantitative requiring many genes for full resistance.

HR cell death effectively restricts biotrophic infection and is the hallmark of race-specific resistance common to many plants (Jones and Dangl, 2006). HR confines biotrophs by abolishing nutrient supplies thereby limiting pathogen growth, however it may serve as a growth substrate for invading necrotrophs (Govrin and Levine, 2000). Thus, biotrophs actively suppress HR while necrotrophs promote HR-like cell death. During infection, *B. cinerea* and *S. sclerotiorum* induce HR-like symptoms with features of plant programmed cell death (PCD) (Govrin and Levine, 2000). Activation of the HR promotes susceptibility to *B. cinerea* yet whether this response extends to other necrotrophs or all forms of cell death lead to susceptibility to necrotrophic fungi is unknown (Govrin and Levine, 2000). Thus, biotrophs have evolved intricate infection strategies aimed at maintaining host viability whereas necrotrophs disrupt cellular integrity. The role of cell death control in determining the susceptibility of Arabidopsis to three different *Botrytis* species has been described (V. A. N. Baarlen et al., 2007). Similarly, defense responses that occur preceding or following the HR including the oxidative burst may also have contrasting defense functions depending on pathogen lifestyle. However, HR may override all other responses when they occur in unison.

Economic Impact of Diseases Caused by Necrotrophic Pathogens

Necrotrophic pathogens vary from host-specific species that infect only a single or small number of related species, to those capable of causing disease on hundreds of species belonging to different families. Consequently, crop losses resulting from these diseases are expected to be high though statistics on total losses attributed to these pathogens are difficult to obtain. The various necrotrophic fungi and their host plants are presented in Table 1. *Sclerotinia sclerotiorum,* the causal agent of white mold, infects more than 400 different species that collectively constitute the majority of global food production including major cereals, legumes, and many vegetable crops (Boland and Hall, 1994). Overall losses attributed to *Sclerotinia* across the U.S. are projected to be more than $200 million annually (Ramasubramaniam et al., 2009; Bolton et al., 2005). Crop losses resulting from *B. cinerea* are estimated to be on the order of 10-100 billion Euros per year (http://www.genoscope.cns.fr). Despite its sizeable amount, the revenue loss caused by these two fungi represents only a small fraction of the combined economic impact of necrotrophic pathogens worldwide indicative of the magnitude and prevalence of these diseases. In a single year, the cost of chemical control against *B. cinerea* can reach $780 million for just one crop with disease on treated plants still resulting in significant production loss (Genescope, 2002). Thus, as a result of the high cost of current controls as well as the frequency of fungicide resistance, there has been an increased effort to identify genetic resistance to necrotrophic pathogens.

Host-specific Necrotrophs

Many necrotrophic species have a very limited host range infecting only one or few related plant species. Important host-specific necrotrophs are listed in Table 1. Disease caused by such pathogens is linked to the production of host-selective toxins (HSTs) which are strain-specific effectors required for pathogenicity on natural hosts (Friesen et al., 2008). For example, *Cochliobolus carbonum* race 1 produces HC-toxin and its infection is restricted to maize plants where it causes the Northern corn leaf spot (Walton, 1996). A second *Cochliobolus* species, *C. victoriae* produces victorin and is responsible for Victoria blight of oat but does not infect any other host in nature (Wolpert et al., 2002a). Different *Alternaria* species and pathotypes also produce HSTs (Nishimura and Kohmoto, 1983; Wolpert et al., 2002a). Germinating spores of *A. brassicicola* secrete AB toxin on the surface of compatible hosts but not unrelated species, with the degree of toxin production correlating to plant susceptibility (Cooke et al., 1997; Kagan and Hammerschmidt, 2002).

Resistance to host-specific necrotrophs mirrors effector-triggered immunity (ETI) as it is conferred by single gene-encoded proteins that are insensitive to or able to detoxify HSTs (Wolpert et al., 2002b). In these interactions, toxins function as effectors because they suppress host defenses but are also determinants of host responses. The maize *HM1* gene encodes a carbonyl reductase that detoxifies HC-toxin thereby blocking its inhibition of histone deacetylases and conferring race-specific resistance (Johal and Briggs, 1992). Similarly, sensitivity to victorin and sus-

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Table 1. List of economically important fungal necrotrophs

Species	Host(s)	Major virulence factor(s)	Disease(s)
(a) Necrotrophic fungi causing foliar diseases.			
Cochliobolus heterostrophus	Maize	T-toxin	Southern leaf blight
Cochliobolus carbonum	Maize	HC-toxin	Northern leaf spot and ear rot
Cochliobolus victoriae	Oat	Victorin	Victoria blight
Alternaria alternata	Pear; strawberry; tangerine; apple; tomato; tobacco; citrus	AK-toxin; AF-toxin; ACT-toxin; AM-toxin; AAL-toxin; ACR (L)-toxin	Black/Dark leaf spot
Alternaria solani	Tomato and potato	Homozinniol; zinniol; a phthalide derivative; alternaric acid; tentoxin	Tomato early blight; collar and fruit rot
Alternaria brassicicola	*Brassica* species (broccoli, cabbage, canola, mustard; cauliflower; turnip)	Destruxin B; AB toxin	Black spot (leaf, stem, or pod spots)
Periconia circinata	Sorghum	Peritoxin (PC-toxin)	Milo
Pyrenophora tritici-repentis (Drechslera tritici-repentis)	Wheat	Ptr ToxA; Ptr ToxB	Tan spot
Bipolares sacchari	Sugarcane	HS-toxin	Eyespot
Phyllosticta maydis (Mycosphaerella zeae-maydis)	Maize	PM-toxin	Yellow corn leaf blight
Stagonospora nodorum (Phaeosphaeria nodorum)	Wheat	SnTox1	Stagonospora nodorum blotch
Stemphylium vesicarium	European pear	SV-Toxin	Brown spot
Botrytis fabae	Bellbean (*Vicia faba*)	Not known	Chocolate spot
Botrytis elliptica	Lilly	Not known	gray mold
Botrytis cinerea	Dicots; some monocots	CWDEs; oxalate	Gray mold “Botrytis blight”
Sclerotinia sclerotiorum	Cabbage; bean; citrus; celery; coriander; melon; squash; soybean; tomato; lettuce; cucumber	CWDEs; oxalate	White mold
Monilinia fructicola	*Prunus* species (apples; pears; and other pome fruits in Rosaceae	CWDEs; metabolites	Brown fruit rots
Fusarium graminearum/ Gibberella zeae	Cereals	Zearalenone; deoxynivalenol (DON)	Fusarium head blight
Septoria tritici (*Mycosphaerella graminis*)	Wheat	ABC transporters secreted toxins	Septoria tritici blotch
Cercospora zeae-maydis	Maize	Cercosporin	Gray leaf spot
Exserohilum turcicum	Maize	HT toxin	Northern leaf blight
Leptosphaeria maculans	Oilseed rape (or canola) (*Brassica napus*)	Sirodesmin PL-toxin	Blackleg or stem canker disease
Ascochyta rabiei	Chickpea	Solanapyrone	*Ascochyta* blight
Diaporthe toxica	Lupin	Phomopsin & glucoseamine	Phomopsis stem blight
Phoma medicaginis	Pea	Coumestrol	leaf spot and spring black stem
Colletotrichum gleosporoides	Lupin & Mango	N/A	Anthracnose
Leptosphaerulina trifolii	*Medicago* spp.	N/A	Lepto leaf spot
Pseudopeziza medicaginis	Lucerne	N/A	Common leaf spot
Stemphylium botryosum	Tomato; alfalfa; lettuce	Stemphyloxin I	Leaf spot and foliage blight
Stagonospora meliloti	Lucerne and medics	N/A	*Stagonospora* crown rot
Pleiochaeta setosa	Lupin	N/A	Brown leaf spot
(b) Soil-borne necrotrophic pathogens.			
Fusarium oxysporum	tomato, banana, cotton and many others	Fumonisins (FB1), fusaric acid, lycomarasmin, lycomarasmic-acid & Nep	*Fusarium* wilt
Rhizoctonia solani	lucerne, clovers, pasture grasses, grain legumes, cereals and oilseed crops	RS-toxin	Rhizoctonia canker/root rot
Pythium spp	Very broad	diverse	Seedling damping off

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ceptibility to *C. victoriae* is conditioned by the dominant Vb locus (Wolpert et al., 2002a). The host-specific necrotroph *Pyrenophora tritici-repentis* secretes proteins that serve as HSTs important for pathogenicity with host sensitivity conferred by a single gene for each toxin (Stock et al., 1996; Gamba et al., 1998). Conversely, the ToxA and ToxB insensitivity loci in wheat, *tsn1* and *tsc2* respectively, are race-specific as resistance is conferred only in the presence of two genetic determinants from the host and pathogen (Faris et al., 1996; Friesen and Faris, 2004). Interestingly, *Cochliobolus victoriae*, a host-specific necrotroph of oat (*Avena sativa*), can infect Arabidopsis. Many host interactions involving host-specific necrotrophs have been studied extensively (Wolpert et al., 2002a).

Broad-host Foliar and Soil-borne Necrotrophs

Fungal species belonging to the genera *Monilinia*, *Sclerotinia, Botrytis,* and *Alternaria* have a broad-host range as do species of the bacterial pathogen *Pectobacterium* (formerly *Erwinia*). The success of these pathogens on diverse crops is attributed to the production of an extensive array of compounds, enzymes, and toxins which singly or in combination likely interfere with common structural and functional features shared among different plant families. For instance, *B. cinerea* is able to infect more than 235 different plant species prevalent over geographically diverse regions (Jarvis, 1977). While *B. cinerea* is primarly a pathogen of dicotyledonous plants some monocot species including onions and lilies are also prone to infection (Prins et al., 2000; Staats et al., 2005). Different strains of *B. cinerea* may be armed with disparate arsenals of disease factors that enable it to colonize different plant species or even specific plant tissues. However, data supporting the concept of *forma specialis* indicative of host-specialized strains and genetically-defined races are lacking although variations in virulence of *B. cinerea* strains isolated from different host species have been documented (Denby et al., 2004). The broad-host pathogens *B. cinerea, Plectosphaerella cucumerina, A. brassicicola, S. sclerotiorum, Pythium irregulare*, *Pythium sylvaticum*, *Leptosphaeria maculans*, *Magnaporthe oryzae, Ralstonia solanacearum*, *Pectobacterium chrysanthemi* (formerly *Erwinia chrysanthemi*) and *P. carotovorum* have been used to study Arabidopsis immune responses to necrotrophic infection by different laboratories worldwide.

Although most of these pathogens infect the aerial parts of plants, there are also soil-borne necrotrophs that gain entry through root tissues causing root and vascular disease. Major soil-borne necrotrophs include species of the fungal genera *Rhizoctonia, Fusarium,* and *Colletotrichum* and bacterial species of *Streptomyces* and *Ralstonia* as well as the oomycete *Pythium*. These pathogens cause pre-/post-emergence damping-off in seedlings and are responsible for wilt and root-rot diseases that inflict an estimated loss of $4 billion annually to U.S. horticultural crops (Edgar et al., 2006). Principally, the lifestyle of root-infecting necrotrophs is akin to those causing aerial diseases, yet several distinctions in epidemiology and pathogenesis exist between these groups. Soil-inhabiting pathogens have to contend with an ecological system common to numerous potentially parasitic, degradative, or toxic microbes and are subject to environmental extremes (Okubara and Paulitz, 2005). Thus, these necrotrophs have evolved thick-walled often melanized structures allowing for increased persistence and defense. Inoculum of these pathogens persists in the soil until favorable conditions permit germination, which is followed by surface recognition, penetration, and intracellular colonization of the cortex. Initiation of germination is mediated by mechanisms of chemotaxis involved in sensing root exudates. Hyphae follow gradients of excreted host metabolites for directed growth toward plant tissue and, upon recognition, secrete various CWDEs that penetrate the root surface. Under wetter soil conditions, *Pythium* species form flagellated zoospores that actively swim towards the host and gain entrance using mechanical penetration via appressoria formation (Okubara and Paulitz, 2005). Subsequent colonization and tissue maceration is achieved by toxins, enzymes and various cell death elicitors. Soil-borne pathogens also cause vascular disease by producing oligosaccharides in the xylem that reduce water transport and host vitality (Okubara and Paulitz, 2005). These pathogens have limited ability for saprophytic competition and therefore generate survival structures as host-tissue collapses (Schroth and Hildebrand, 1964; Okubara and Paulitz, 2005). They also enlist methods of rapid colonization in order to overcome secondary invaders (Okubara and Paulitz, 2005). Thus, the speed of infection coupled with the limited movement of inoculum in the soil, only allows for disease on a minimal number of hosts per season hence soil-borne necrotrophs are monocyclic pathogens (Okubara and Paulitz, 2005). By contrast, foliar necrotrophs are polycylic resulting in the production of large amounts of spores that are dispersed over a wide area. To date, Arabidopsis has been used to study mechanisms of host resistance to the soil-borne *Pythium* and *Fusarium* species as well as *Ralstonia solanacearum* and *Rhizoctonia solani* (Deslandes et al., 2002; Perl-Treves et al., 2004; Okubara and Paulitz, 2005; Huffaker et al., 2006; van Loon et al., 2006b; Adie et al., 2007; Brooks, 2007; Berrocal-Lobo and Molina, 2008; Hu et al., 2008; Llorente et al., 2008; Diener, 2009).

SUPPRESSION AND MODIFICATION OF HOST DEFENSE RESPONSES BY PATHOGENS

Pathogens actively circumvent or suppress the plant defense weaponry and/or modify the host cellular and physiological environment in order to cause disease. Active but subtle suppression of host defenses is often attributed to obligate parasites as, generally, necrotrophs are considered less sophisticated organisms that deploy ‘brute force’ mechanisms to overcome the host. It is now understood that necrotrophic pathogens actively suppress, manipulate and/or evade host defenses during infection. Protection of fungal structures, inhibition of elicitor-induced defenses, de-toxification of plant defense compounds and manipulation of specific plant proteins or physiological processes are all common strategies employed to evade host immune responses.

Modification of Host Physiology

The *P. chrysanthemi* siderophore chrysobactin, required for systemic plant infection, modulates iron-acquisition in Arabidopsis (Dellagi et al., 2009). Chrysobactin is one of two ferric ion

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chelators that allow the pathogen to compete with plant cells in iron sequestration (Neema et al., 1993; Dellagi et al., 2005; Dellagi et al., 2009). Arabidopsis treatment with chrysobactin promotes bacterial growth while also activating host SA-regulated responses and expression of *FER1* encoding the iron storage protein ferritin (Dellagi et al., 2005; Dellagi et al., 2009). Plants infiltrated with a chrysobactin-deficient strain of *P. chrysanthemi* exhibited reduced *PR-1* but increased *PDF1.2* gene expression as compared to those infected with the wild-type strain. Thus, it appears chrysobactin promotes virulence by manipulating interactions in host hormone-mediated responses, eliciting SA dependent responses that oppose those mediated by JA/ET (Dellagi et al., 2009). Interestingly, SA responses and *FER1* are not induced by iron-bound chrysobactin (Dellagi et al., 2005; Dellagi et al., 2009). The degree of available iron also regulates host tissue degradation, with low levels activating transcription of pathogen pectinases (Franza et al., 2002). These data suggest a multiplicity of virulence function for *P. chrysanthemi* iron-chelators, contributing to pathogenesis through manipulation of host iron physiology and hormone-dependent defenses as well as the regulation of pectinase activity. Iron-chelating siderophores are also utilized by pathogens during infection (Haas et al., 2008). *NPS6*, encoding a non-ribosomal peptide synthetase involved in siderophore biosynthesis, is a functionally conserved virulence factor of different necrotrophic ascomycetes including *A. brassicicola* (Oide et al., 2006). Arabidopsis *rbohDrbohF* double mutants, disrupted in NADPH oxidase function, exhibit increased susceptibility to wild-type *A. brassicicola* infection but unaltered responses to a strain harboring a loss of function *NPS6* allele (Oide et al., 2006). This connection between iron-chelation, ROS, and virulence is consistent with the function of ferritin in host defense against free iron-induced oxidative stress (Ravet et al., 2009). Thus, *FER1* induction following necrotrophic infection appears to function as a host defense rather than as a source for pathogen iron assimilation (Ravet et al., 2009).

Owing to their defense functions, plant hormones are major targets of pathogen attack and manipulation (Berrocal-Lobo et al., 2002; Lorenzo et al., 2003; Thatcher et al., 2009). Many necrotrophic fungi produce plant hormones that may interfere with host physiology (Mobius and Hertweck, 2009). *B. cinerea* produces ET and ABA that may perturb host response pathways by promoting senescence and cell death (Sharon et al., 2004). Similarly, different bacterial and fungal pathogens manipulate JA-levels to enhance virulence (Feys et al., 1994; Zhao et al., 2003; Thatcher et al., 2009). For example, the *coi1* mutant exhibits increased resistance to *F. oxysporum* suggesting that it is target of fungal virulence (Thatcher et al., 2009).

Necrotrophs also co-opt or manipulate specific host proteins to suppress defenses during infection. The *B. cinerea* ABC transporter, atrB, exports camalexin *in vitro* and is required for pathogen tolerance to this phytoalexin (Stefanato et al., 2009). The mechanism of atrB camalexin detoxification, cellular export or enzymatic modification, is currently unknown however many necrotrophic fungi use different types of transporters to facilitate active removal of plant defense compounds (Stefanato et al., 2009); (Del Sorbo et al., 2000; Pedras and Ahiahonu, 2005; Stefanato et al., 2009). *S. sclerotiorum* glycosylates the indole ring of camalexin converting it to a less toxic form (Pedras and Ahiahonu, 2002, 2005).

The Role of Toxins and Necrosis-Inducing Proteins

As mentioned in the preceding sections, toxins and phytotoxic proteins are central to virulence strategies of necrotrophs. Many HSTs cause typical symptoms on Arabidopsis that are observed in host plants (Asai et al., 2000; Stone et al., 2000; Lorang et al., 2004; Desjardins et al., 2007). Thus, Arabidopsis responses to purified toxins and necrosis-inducing proteins have been used to study their virulence function, toxic effects, and modes of action. Toxins have cellular targets of virulence including specific host proteins and plant processes. A detailed analysis of the role of fungal toxins as suppressors of defense was recently presented (Mobius and Hertweck, 2009). The fungal toxins Fumonisin B1 (FB1) and AAL toxin are ceramide synthesis inhibitors that disrupt sphingolipid metabolism resulting in host accumulation of free sphingoid bases (Abbas et al., 1994). Both of these toxins, as well as free sphingoid bases, trigger cell death responses in Arabidopsis (Shi et al., 2007). Plants harboring loss of function alleles in *FB1-RESISTANT1* (*FBR1*), involved in sphingolipid biosynthesis, fail to generate ROS and are resistant to FB1-induced cell death due to attenuated accumulation of free bases (Shi et al., 2007). Interestingly, increases in free sphingoid base levels correlate with ROS and cell death, yet the phosphorylated forms of these same bases function to block these two responses (Shi et al., 2007). This suggests that maintenance of equilibrium between these two forms is critical in determining cell fate (Shi et al., 2007). Thus, some fungal pathogens alter the balance between phosphorylated sphingoid bases and their phosphorylated counterparts to induce necrosis. The Arabidopsis *AAL-toxin-resistant (atr1)* mutant exhibits enhanced tolerance to H_2O_2 and ROS-induced cell death suggesting the manipulation of ROS levels by AAL toxin is required for pathogen virulence (Gechev and Hille, 2005; Gechev et al., 2008). Fusicoccin, a toxin produced by *Fusicoccum amygdali*, impairs ROS scavenging in Arabidopsis cells by inducing accumulation of an unknown catalase inhibitor that significantly reduces the cellular capacity to degrade H_2O_2 (Beffagna and Lutzu, 2007).

Fusarium species produce several types of trichothecene phytotoxins that aid in host colonization (Desjardins et al., 1993). Their relative contributions to disease development are thought to be strain-dependent as individuals have distinct metabolic profiles (Ward et al., 2002). Several members of this family including deoxynivalenol (DON), T-2 toxin, and diacetoxyscirpenol (DAS) have been analyzed for their toxic effects on Arabidopsis (Nishiuchi et al., 2006; Masuda et al., 2007). Trichothecenes inhibit translation which was previously thought to be a mechanism of host defense suppression (Nishiuchi et al., 2006). However, it was found that T-2 toxin acts in an elicitor-like manner activating defense responses including SA biosynthesis/accumulation, ROS, MAP kinase signaling, callose deposition, and defense gene expression (Nishiuchi et al., 2006). Plants infiltrated with T-2 toxin form necrotic lesions independent of any elicited host-mediated cell death as lesions occur in the absence of SA and only exhibit hallmarks of the HR late in development (Nishiuchi et al., 2006). Plants treated with DAS also exhibit necrosis an activation of defense responses; however where T-2 toxin induces both *PDF1.2* and *PR-1* expression, DAS only induces *PR-1* (Nishiuchi et al., 2006). Similarly, at the same concentrations, DON fails to initiate the same responses and does not result in lesion formation

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though all three toxins were found to inhibit protein translation in Arabidopsis (Nishiuchi et al., 2006). All three also impede plant growth, showing individual variation in the degree of inhibition that is dependent on plant organ (Masuda et al., 2007). Expression profiling of T-2 toxin-treated Arabidopsis indicates the toxin induces defense responses but also inactivates brassinosteroid (BR) biosynthesis and increases generation of ROS (Masuda et al., 2007). BRs have been shown to induce broad-spectrum disease resistance as well as increased tolerance to abiotic stresses that elicit ROS and cell death (Krishna, 2003; Nakashita et al., 2003). Thus, it appears that some trichothecenes suppress BR-mediated defenses and regulation of ROS as a means of pathogenicity. Taken together, the disparities in host responses and symptom development elicited by DON, T-2 toxins, and DAS, despite their shared function in translational inhibition, suggest specificity in infection strategy by different phytotoxin-producing *Fusarium* species (Nishiuchi et al., 2006; Masuda et al., 2007). Interestingly, Arabidopsis BAK1 functions in innate immune responses that overlap with those induced by DAS and T-2 toxin, as well as necrotrophic defense and responses to BRs (Chinchilla et al., 2007; Kemmerling et al., 2007). Potentially, BAK1 may be a target of fungal virulence by species producing these toxins. Arabidopsis *NFXL1*, encoding a zinc finger type of transcription factor, was shown to be a signaling component of trichothecene-dependent responses (Asano et al., 2008).

In addition to toxins, necrotrophic pathogens produce phytotoxic proteins (Pemberton and Salmond, 2004). The *F. oxysporum* secreted necrosis and ethylene-inducing protein (NEP1) causes plant cell death and is the founding member of the NEP1-like family of fungal proteins (NLPs) characterized as being elicitors of necrosis (Bailey, 1995; Pemberton and Salmond, 2004). NLPs are small conserved molecules that induce HR-like cell death, ROS, and ET production (Bailey, 1995; Fellbrich et al., 2002; Pemberton and Salmond, 2004). Although NLPs elicit responses characteristic of plant defense, they contribute to the virulence of necrotrophic pathogens in a manner similar to toxins. Recent structural analyses indicate NLPs are similar to virulence promoting cytolytic toxins and function by interfering with integrity of the plasma membrane (Ottmann et al., 2009). This disruption of cellular integrity is then detected by host plants leading to the activation of defenses. In contrast to phytotoxins, NLPs are unique in their conserved toxicity to dicot plants and broad distribution across taxa (Ottmann et al., 2009). Functional NLPs have been demonstrated for *P. carotovorum* and *F. oxysporum* while two NLP-encoding genes have been identified in *Botrytis* species (Bailey, 1995; Mattinen et al., 2004; Staats et al., 2007).

In sum, plant responses to purified toxins/NLPs and the altered pathogenicity of fungal mutants impaired in their production generally establish these as agents of virulence. *In planta*, toxin production is likely induced following contact with the host tissue or its metabolites. Therefore, some differences between plant responses to purified toxins to that occurring during pathogen colonization may be expected. Finally, despite their importance as virulence factors, it is still unclear how toxins are internalized and the nature of the host cellular targets for many toxins, as well as the receptors and downstream components that mediate plant responses, are not known. Arabidopsis provides an ideal genetic host for identification of factors that mediate plant responses to toxins.

KEY DISTINCTIONS BETWEEN BIOTROPHIC AND NECROTROPHIC PATHOGENS

Fundamental differences exist between biotrophs and necrotrophs in many aspects of their pathogenesis. The infection processes, histology of disease, infection-related morphogenesis, nature of effector proteins, elicited host-defense responses and most importantly their nutrition acquisition strategies vary significantly. These differences may account for why one defense strategy may effectively restrict biotrophs and enable necrotrophs. HR cell death is a hallmark of race-specific resistances common to many plants and restricts bio(hemi)biotrophic pathogens (Jones and Dangl, 2006). The HR confines the pathogen by abolishing its nutrient supply thereby limiting pathogen growth but may serve as a substrate for necrotrophic pathogens (Govrin and Levine, 2000). Thus, biotrophs actively suppress the HR while necrotrophs promote HR-like cell death. *B. cinerea* and *S. scloretoruim* induce HR-like symptoms with features of plant programmed cell death (PCD) and activation of the HR promotes susceptibility to these pathogens (Govrin and Levine, 2000). If these responses extend to other necrotrophs and whether all forms of cell death enhance susceptibility to all necrotrophic fungi are not known. Thus, as biotrophs have evolved intricate infection strategies aimed at maintaining host viability, necrotrophs have developed tactics to disrupt cellular integrity. Similarly, defense responses that occur preceding or following the HR including the oxidative burst may also have contrasting defense functions depending on pathogen lifestyle. However, the HR may override all other responses when they occur in unison.

In contrast to necrotrophs, biotrophic pathogens secrete limited amounts of lytic enzymes, generally lack toxin production, and evade detection or suppress immune responses through manipulation of host defenses (Oliver and Ipcho, 2004). These pathogens create intimate relationships with host cells using specialized structures such as haustoria and start slowly draining plant resources thereby gradually decreasing plant fitness (Schulze-Lefert and Panstruga, 2003). Necrotrophs, on the other hand, are facultative saprophytes that actively destroy host-tissue using various toxins and CWDEs (Oliver and Ipcho, 2004). Generally, resistance to host-specific necrotrophs is conditioned by single genes conferring complete immunity whereas resistance to broad-host necrotrophs is quantitative requiring many genes for full resistance.

ARABIDOPSIS AS A MODEL SYSTEM FOR STUDYING RESPONSES TO NON-HOST, HOST-SPECIFIC AND BROAD-HOST NECROTROPHIC PATHOGENS

From its inception as a model organism nearly 25 years ago, Arabidopsis has become much like what the mouse is to humans with regard to pathological studies. The ability or inability of many pathogens to recognize, infect, and grow on Arabidopsis has provided an avenue for the molecular and genetic dissection of host immune response mechanisms to host and non-host pathogens (Ausubel et al., 1995; Glazebrook et al., 1997). Initial genetic analyses of different Arabidopsis pathosystems revealed the presence of conserved defense mechanisms analogous to those observed in other plants. These initial studies were largely

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focused on biotrophic and hemibiotrophic pathogens, leading to the establishment of molecular frameworks that now serve as the backbones for what we know of ETI (Jones and Dangl, 2006), systemic acquired resistance (SAR) (Vlot et al., 2009), and salicylate (SA)-dependent defenses (Kunkel, 1996; Glazebrook, 2005).

Compared to their biotrophic counterparts, knowledge on the biological processes underlying plant responses to necrotrophic infection are less understood. Despite their enormous economic impact and a lack of effective genetic resistance, research into plant immune responses to necrotrophic fungi has been fairly limited until recently. Research utilizing crop hosts has often proved challenging due in part to their genetic complexities, prolonged generation time, considerable size, and a lack of efficient procedures for hereditary manipulation. As a result, Arabidopsis has been adopted as a model system to study plant interactions with necrotrophic pathogens. Research into Arabidopsis immunity to necrotrophs goes as far back as 1965, but wasn't heavily pursued until after the early 1990s following the isolation of *R. solani* and *B. cinerea* from naturally occurring greenhouse infections (Berger, 1965; Koch and Slusarenko, 1990). Reports describing Arabidopsis responses to *S. sclerotiorum*, *A. brassicicola* and *F. oxysporum* began appearing soon thereafter (Dickman and Mitra, 1992; Penninckx et al., 1996). The finding that *A. brassicicola* infection induced expression of an Arabidopsis homolog of a radish defensin gene independent of SA responses led to the notion of pathogen-specific pathways functioning in resistance, enhancing molecular studies into mechanisms of plant defense against necrotrophs (Penninckx et al., 1996). The conserved radish defensin homolog, *PDF1.2,* is now routinely used as a molecular marker for the activation of defense against necrotrophic infection. Expounding upon this observation, it was later found that SA-dependent responses are typically associated with resistance to biotrophs whereas JA and ET synergistically regulate defense against necrotrophics (Penninckx et al., 1998; Thomma et al., 1998). It is now widely recognized that these two pathways generally function through mutual antagonism (Kunkel, 1996; Glazebrook, 2005). Currently, investigation into Arabidopsis immune responses to necrotrophs and their relationship with biotrophic infection is an active area of research that is gaining momentum.

Today, Arabidopsis serves as a model host to a wide spectrum of pathogens providing insight into plant responses to non-host, host-specific, and broad-host necrotrophs. *B. cinerea*, *A. brassicicola*, and *S. sclerotiorum*, represent some of the archetype broad-host necrotrophs utilized to probe into Arabidopsis immune responses. *S. sclerotiorum* readily infects Arabidopsis and requires oxalic acid for virulence mirroring its infection of natural hosts (Dickman and Mitra, 1992). The *F. oxysporum*-Arabidopsis pathosystem has provided an experimental platform for understanding host responses to soil-borne pathogens causing vascular and wilt disease (Diener and Ausubel, 2005; Berrocal-Lobo and Molina, 2008; Diener, 2009).

Overall, Arabidopsis has been successfully used to overcome the experimental limitations of crop hosts, greatly expanding our knowledge of the molecular genetics of resistance, cellular and biochemical basis of host responses to infection, variations in immune responses to different necrotrophs, and factors affecting pathogen virulence. Forward and reverse genetic screens have identified many regulatory components of host responses to necrotrophs as well as highlighted processes underlying defense. Arabidopsis genes implicated in innate immunity to necrotrophic pathogens and their biological functions are summarized in Supplemental Table 2. The availability of genomic and genetic resources, including mutant germplasm representing most cellular and physiological functions, has fueled a unique progress in Arabidopsis research unlike in any other plant system. Looking ahead, a closer examination of necrotroph-specific defense molecules using biochemical and cell biology approaches will shed light on how critical components of immune responses function to confer resistance. Identification of novel defense molecules, analysis of natural variation and complex genetic interactions, metabolic profiling, and the construction of regulatory networks using systems-level approaches are likely to put Arabidopsis at the forefront of research in plant immunity to necrotrophic infection.

MECHANISMS OF IMMUNITY TO NECROTROPHIC INFECTION

Defense against necrotrophic pathogens include constitutive and induced physical and chemical barriers. The plant cuticle and the cell wall inhibit the initiation and spread of infection while also serving as sources of elicitors that trigger induced defenses. Chemical defenses provide additional protection and include constitutively-present pre-formed compounds (phytoanticipins), as well as compounds that are produced only in response to infection (phytoalexins). Resistance involves an array of other responses including the accumulation of pathogenesis-related proteins, defensins, antimicrobial compounds and activation of hormone-regulated defenses. However, the identity of recognition factors, upstream components, and the signaling that links host recognition to resistance are not well understood in the context of necrotrophic infection. Despite overlapping pathogenesis strategies, defense against different necrotrophic species may vary depending on the primary determinant of virulence. Generally, the multiplicity of pathogen virulence and host defense molecules complicates the genetic dissection of resistance mechanisms and explains why our understanding of plant-interactions with necrotrophic pathogens lags behind.

The Intriguing Roles of the Plant Cell Wall and Cuticle in Arabidopsis Defense

The plant cell wall is an important component of defense and often a target of pathogen virulence particularly in the case of necrotrophs. The susceptibility of the host cell wall to degradation by CWDEs correlates with severity of necrotrophic infection. Necrotrophs secrete various CWDEs encoded by multigene families with the degree of diversity correlating with pathogen host range (Esquerre-Tugaye et al., 2000). Fungal polygalacturonases (PGs) hydrolyze the homogalacturonan of plant cell wall pectin and are important virulence factors for some pathogenic fungi including *B. cinerea* (Di Matteo et al., 2006). Plant polygalacturonase inhibiting proteins (PGIPs) counteract PGs contributing to basal resistance against necrotrophic infection in various species (Ferrari et al., 2003b; D'Ovidio et al., 2004). Intriguingly, mutations in Arabidopsis cellulose synthases (IRX/CESAs) required for secondary cell wall formation resulted in increased resistance to the necrotrophic fungi *P. cucumerina* and *B. cinerea*, attributed

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to a likely "hostile environment" created by the accumulation of secondary metabolites involved in defense at the site of primary infection (Hernandez-Blanco et al., 2007). Resistance attributed to altered cell wall composition by *IRX*/*CESA* mutations was also associated with increased endogenous ABA and a constitutive up-regulation of ABA-responsive genes but independent of SA- and JA/ET-regulated defenses. These observations indicate that various alterations in plant structural barriers, considered integral components of constitutive defense, can result in resistance to necrotrophic pathogens pointing to the possible co-evolution of pathogen virulence with host components. Conversely, mutation in *HISTONE MONOUBIQUITINATION1* (*HUB1),* encoding an E3 ligase required for histone H2B ubiquitination, reduces cell wall thickness resulting in specific but extreme susceptibility to *B. cinerea* and *A. brassicicola* (Dhawan et al., 2009). The altered cell wall thickness and hence pathogen susceptibility in *hub1* was partially attributed to impaired cell wall-related gene expression. Similarly, the Arabidopsis receptor-like kinase ERECTA contributes to defense against necrotrophs and cell wall biosynthesis (Llorente et al., 2005; Sanchez-Rodriguez et al., 2009). The *erecta* (*er*) mutant is susceptible to *P. cucumerina*, *R. solanacearum*, and *P. irregulare*, with altered resistance related to increased levels of uronic acid and cellulose in the plant cell wall (Llorente et al., 2005; Sanchez-Rodriguez et al., 2009). A second site suppressor of *er* restored susceptibility to *P. cucumerina* to wild type levels and partially restored cell wall modifications but had no effect on resistance to *R. solanacearum* or *P. irregulare.* These mutations also caused additional changes in cell wall composition suggesting a complex role for this physical defense in responses to necrotrophic infection that appears to be more dynamic than passive (Humphrey et al., 2007; Sanchez-Rodriguez et al., 2009).

Traditionally, the cuticle is known to protect plants against abiotic stresses and serves as barrier to fungal infection. Contrary to this notion, Arabidopsis mutants and transgenic plants altered in components of the cuticle, similar to cell wall mutants, were found to be completely resistant to *B. cinerea* (Kurdyukov et al., 2006; Bessire et al., 2007a; Chassot et al., 2007). Transgenic Arabidopsis plants constitutively expressing a fungal cutinase or lipase, each with cutin hydrolytic activity, exhibited enhanced resistance to *B. cinerea* infection (Chassot et al., 2007). Figure 1 shows the limited growth of *B. cinerea* in Arabidopsis plants expressing a fungal cutinase gene as revealed by trypan blue staining (Chassot et al., 2007). Interestingly, this resistance is specific to *B. cinerea* as plant responses to *P. cucumerina*, *A. brassicicola,* and *S. sclerotiorum* were unaltered (Chassot et al., 2007). Mutations in *LACERATA* (*LCR*), *FIDDLEHEAD* (*FDH*), *BODYGUARD* (*BDG*), *LACS2* (*BRE1*) and *RESURRECTION1* (*RST1)* also result in alter cuticle development and enhanced resistance to *B. cinerea* (Kurdyukov et al., 2006; Bessire et al., 2007b; Chassot et al., 2007; Mang et al., 2009; Voisin et al., 2009). In some of these cases, the altered cuticle composition was hypothesized to facilitate faster perception of fungal elicitors. This, coupled with an increased cuticle permeability that allows easier diffusion of defense signals to the infection site, is hypothesized to promote resistance (Bessire et al., 2007b; Chassot et al., 2007). However, mutant comparisons indicate that the degree of permeability does not directly correlate with level of resistance and is unaffected in *rst1* (Mang et al., 2009; Voisin et al., 2009). It was also suggested that mutant plants compensate the functional disorder of the cuticle by reinforcing their defenses thereby enhancing resistance independent of the changes in actual cuticle composition (Voisin et al., 2009).

Interestingly, the *lacs2* mutant is also resistant to *S. sclerotiorum* and compounds diffused at the leaf surface of *bdg1* mutant plants have inhibitory effects on *Monilia laxa* growth (Chassot et al., 2004; Bessire et al., 2007). Both of these fungi are closely related to *B. cinerea* suggesting a common element underlying cuticle-mediated defense against necrotrophs that may be

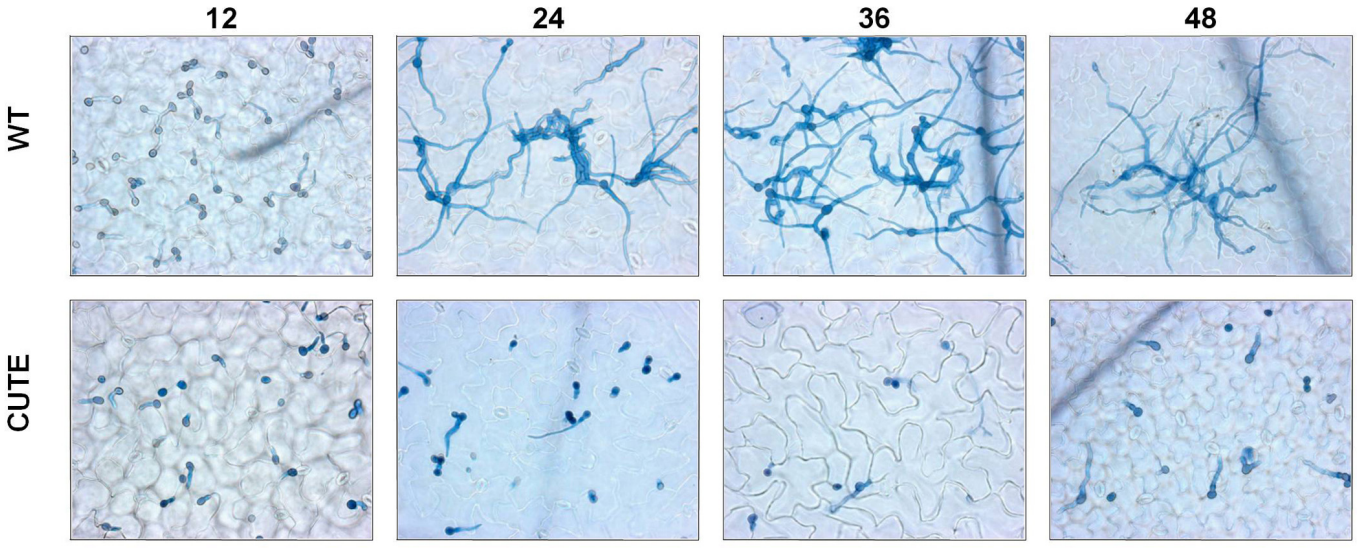


Figure 1. *B. cinerea* resistance in Arabidopsis transgenic *CUTE* plants expressing a *Fusarium pisi* cutinase gene.

Images show fungal growth as revealed by trypan blue staining at 12-48 h after inoculation (Pictures are courtesy of Jean-Pierre Metraux, University of Fribourg, Switzerland).

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specific to a certain group of these pathogens. The Arabidopsis *glycosylphosphatidylinositol-anchored lipid transfer protein (ltpg1)* mutant shows increased susceptibility to *A. brassicicola* attributed to changes in cuticle composition and structure further confirming the link between altered cuticles and resistance to necrotrophic fungi (Lee et al., 2009b). In the case of *rst1*, increased resistance to *B. cinerea* and *A. brassicicola* is accompanied with susceptibility to the biotrophic fungal pathogen *Erysiphe cichoracearum* (Mang et al., 2009). Mutation in *RST1*, encoding a transmembrane protein of unknown biochemical function, leads to increased cutin monomers on the leaf surface without altered permeability. Thus, the resistance of *rst1* may be due to its high level of cutin monomers which can serve as elicitors of defense, as discussed in the next section. Data from *rst1* mutants indicate a contrasting role for the plant cuticle in defense responses to different pathogens. In support of this observation, in addition to enhanced resistance to *B. cinerea*, *lacs2* plants exhibit increased susceptibility to avirulent strains of *P. syringae* indicating a loss of ETI (Tang et al., 2007).

Recognition-mediated Defense Against Necrotrophs

Pathogens elicit innate immune responses depending on the nature of the pathogen-derived elicitor. P/MAMP-triggered immunity (PTI) is a basal resistance response mediated by recognition of pathogen/microbe associated molecular patterns (P/MAMPs), regardless of pathogen lifestyle. P/MAMPs are evolutionarily conserved components of pathogens that resemble patterns of the innate immune system in mammals and insects that are mainly directed against epitopes characteristic for fungi or bacteria (Medzhitov and Janeway, 1997; Aderem and Ulevitch, 2000). P/MAMPs in general serve as non-self recognition mechanisms and vary in origin from those that are purely pathogen-derived to altered host molecules perceived as non-self. Effector-triggered immunity (ETI) is activated upon recognition of race-specific effectors by plant disease resistance proteins. ETI is a widespread form of resistance in the plant kingdom that is effective against (hemi)biotrophic pathogens but may have limited roles in resistance against typical necrotrophs (Jones and Dangl, 2006). Activation of ETI and PTI dispatches signals to uninfected tissues, leading to systemic resistance (Dempsey et al., 1999; Mishina and Zeier, 2007). These immune responses in the context of necrotrophic infection are highlighted in this section.

PAMP-triggered immunity (PTI)

Studies on plant responses to various P/MAMPs derived from different pathogens suggest that even though different elicitors are perceived by distinct plant receptors, the downstream responses converge into a common regulatory point to induce PTI (Pitzschke et al., 2009). The elongation factor Tu (EF-Tu) and flagellin are two bacterial proteins with completely separate functions in the bacterial cell yet perception of both activates the same downstream MAP kinase signaling cascade (Schuster and Khan, 1994; Rodnina and Wintermeyer, 2001). The flg22 (flagellin) and elf18 (EF-Tu) epitopes are recognized by Arabidopsis cells as MAMPs by the FLS2 and EFR receptors, respectively, and induce defense responses (Gomez-Gomez and Boller, 2000; Kunze et al., 2004; Zipfel et al., 2006). Interestingly, recognition of *B. cinerea* PAMPs and other fungal elicitors also activate components of this shared MAPK cascade including MPK3 and MPK6 (Ren et al., 2008; Pitzschke et al., 2009). PTI mediated by FLS2 confers resistance to *B. cinerea* in Arabidopsis (Asai et al., 2002). Similarly, pretreatment of plants with flg22 results in increased resistance to *P. syringae* and *B. cinerea* infection largely independent of SA, JA, and ET responses (Zipfel et al., 2004; Ferrari et al., 2007b). Flagellin-triggered resistance to *P. syringae* is only partially dependent on SA-signaling whereas induced defense against *B. cinerea* requires *PAD3* and *UPS1* function independent of SA, JA, and ET regulation (Ferrari et al., 2007a; Tsuda et al., 2008). PAD3 and UPS1 mediate accumulation of the Arabidopsis phytoalexin camalexin (Denby et al., 2005; Schuhegger et al., 2006). Interestingly, loss of BAK1, a receptor-like kinase that serves as part of the flg22-receptor complex together with FLS2 and BIK1 (Zhang et al., 2010), results in increased susceptibility to *B. cinerea* and *A. brassicicola* without altering resistance to *P. syringae* (Chinchilla et al., 2007; Kemmerling et al., 2007). BIK1 functions in resistance to necrotrophic fungi but suppresses resistance to *P. syringae* (Veronese et al., 2006). Intriguingly, *bik1* plants exhibit a loss of resistance to *P. syringae* hrcC-, a strain impaired in type three secretion but retains a repertoire of P/MAMPs, implicating BIK1 in the control of PTI (Lu et al., 2009a). Although recent data show interactions between BIK1 and FLS2/BAK1, the activation of MPK6, MPK3 and MPK4 by *B. cinerea* was independent of BIK1 function (Veronese et al 2006).

The MPK3 and MPK6 kinases are activated by flagellin in the presence of FLS2 with mutations in both genes resulting in altered responses to flg22 and enhanced susceptibility to *B. cinerea* (Ren et al., 2008). Further, activation of MPK6 by flagellin induces dissociation of the ERF104 transcription factor from an ERF104/MPK6 complex (Bethke et al., 2009). ERF104 also mediates plant responses to flagellin and *B. cinerea*. Additionally, the association of these two proteins is affected by ET-signaling, including that mediated by *EIN2,* which is required for resistance to *B. cinerea* (Bethke et al., 2009). The disease phenotypes of *MPK4* mutant alleles mirror those of *bik1* with plants displaying distinct responses to biotrophic and necrotrophic pathogens (Petersen et al., 2000; Brodersen et al., 2006; Veronese et al., 2006). MPK4 exists in a nuclear complex with MKS1 and WRKY33 that dissociates upon flagellin or pathogen detection (Qiu et al., 2008). *WRKY33* is also required for resistance to necrotrophs with the mutant exhibiting enhanced susceptibility to *A. brassicicola* and *B. cinerea* (Zheng et al., 2006). Evidence to date suggests that MPK4 functions in downstream signaling events regulated by MKK1, MKK2, and MEKK1 in response to bacterial and fungal P/MAMPs (Qiu et al., 2008; Pitzschke et al., 2009). Taken together, these studies on innate immune responses indicate a growing role for P/MAMP signaling in Arabidopsis responses to necrotrophic fungi. Additionally, BAK1, BIK1, MPK3, and MPK6 are involved in EF-Tu and chitin responses adding further complexity to PTI and the interplay between responses mediated by different PAMPs. The emerging picture is one of intertwined pathways and genetic elements in the control of basal resistance. Further studies are likely to address basal responses mediated by many orphan pattern-recognition receptors and their corresponding PAMPs in relation to defense against necrotrophic pathogens.

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Chitin-mediated immunity to necrotrophic fungi

Following infection by fungal pathogens, plant cells induce the expression of chitinases that accumulate at the site of penetration and hydrolyze chitin, a common constituent of fungal cell walls (Eckardt, 2008). The fragments resulting from chitin degradation are then perceived by host cells leading to the activation of PTI. The LysM Receptor Kinase1 (LysM RLK1), also known as CERK1, is required for chitin-elicited immune responses likely functioning in early recognition events (Miya et al., 2007; Wan et al., 2008). The lysine motif (LysM) for which LysM RLK1 is named binds a component of bacterial cell walls structurally similar to chitin, however, it is currently unknown whether LysM RLK1 directly binds chitin to activate basal defense (Bateman and Bycroft, 2000; Miya et al., 2007; Wan et al., 2008). Arabidopsis *LysM RLK1* mutants exhibit weak susceptibility to *A. brassicicola* and *Erysiphe cichoracearum* but are unaffected in their responses to *Colletotrichum higginsianum* and *P. syringae* suggesting a pathogen-dependent function of this gene in resistance. The expression of chitooligosaccharide-responsive genes was completely blocked in *LysM RLK1* mutant plants after chitin treatment yet showed induced expression following pathogen infection (Wan et al., 2008). This may account for the weak susceptibility of the mutant to necrotrophic fungi as other fungal elicitors induce immune responses overlapping with genes required for chitin responses. *LysM RLK1* mutant alleles are also altered in chitin-induced MAPK signaling, showing a lack of MPK3/MPK6 activation as well as altered expression of *WRKY* genes known to act in the PTI response pathway (Miya et al., 2007; Wan et al., 2008). Overall, these reports indicate LysM RLK1 is an upstream regulator of chitin-triggered immunity that shares overlapping components with other PTI responses.

Oligogalacturonides (OGs) as host-derived elicitors of defense

Polygalacturonases (PGs) are important pectolytic enzymes produced by necrotrophic fungi that hydrolyze the homogalacturonan of plant cell wall pectin and are important virulence factors for many necrotrophs including *B. cinerea*, *P. carotovorum, F. oxysporum*, and *S. sclerotiorum* (Niture, 2008). Plant polygalacturonase inhibiting proteins (PGIPs) counteract the actions of PGs contributing to basal resistance by not only directly blocking cell wall degradation but also by enhancing accumulation of long chain oligogalacturonides (OGs) (Cervone et al., 1989; D'Ovidio et al., 2004). OGs are fragments released following partial degradation of the primary cell wall capable of activating innate immune responses. As OGs are derived from host tissue rather than pathogen components, they cannot be described as *bona fide* PAMPs and are considered typical damage-associated molecular patterns (DAMPs) (Boller and Felix, 2009). Depending on the degree of chain polymerization, OGs induce oxidative bursts, cell wall lignification, phytoalexin accumulation, protease inhibitor expression, and changes in ion fluxes as well as SA, JA, and ET biosynthesis (De Lorenzo et al., 2001; Ridley et al., 2001; D'Ovidio et al., 2004). Due to their destructive pathogenesis strategies, DAMP-triggered immune responses may be commonly activated by necrotrophic pathogens as they actively release OGs during the course of infection.

Pre-treatment of Arabidopsis plants with OGs prior to *B. cinerea* inoculation increased resistance independent of SA- and JA/ET-mediated defenses (Ferrari et al., 2007b). Interestingly, the flagellin peptide flg22 induces similar immunity and transcriptional analysis revealed a high correlation between responses activated by these two elicitors (Ferrari et al., 2007b; Denoux et al., 2008). However, the overlap in transcript profiles is only comparable early after treatment, exhibiting differences in late gene expression, and, overall, flg22 elicits a stronger and sustained effect on gene expression relative to OGs. OGs induce *MPK3* and *MPK6* expression which represents a point of conserved convergence downstream of P/MAMP recognition (Denoux et al., 2008; Pitzschke et al., 2009). Both OG- and flg22-mediated *B. cinerea* resistance is dependent on *PAD3* and *UPS1* which are required for camalexin accumulation consistent with the role of camalexin in defense against necrotrophic fungi (Schuhegger et al., 2006; Ferrari et al., 2008). Overall, these observations suggest PTI is largely dependent on secondary metabolites indicating a growing role for hormone-independent defenses in resistance to necrotrophs (Ferrari et al., 2008; Galletti et al., 2009).

OG- and other P/MAMP-induced responses do interact with hormone-mediated defense pathways. Arabidopsis *PGIP1* and *PGIP2* are differentially regulated by JA and OGs, respectively, in response to *B. cinerea* (Ferrari et al., 2003b). Mutations in *ZFAR1*, encoding a putative zinc-finger protein containing ankyrin-repeat domains, cause sensitivity to ABA and susceptibility to *B. cinerea* at the site of infection (AbuQamar et al., 2006). *ZFAR1* is induced by OGs suggesting possible involvement in OG-induced resistance to *B. cinerea* that also requires ABA responses (AbuQamar et al., 2006; Ferrari et al., 2008). Similarly, OG-treatment affects expression of genes modulating ABA-levels (Denoux et al., 2008) and enhances ET-production that, in turn, suppresses JA-regulated defense gene expression in infected tissues (Rojo et al., 1999). The negative effect of ET on JA in damaged tissue is through mutual antagonism between *ERF1*- and *MYC2*-regulated responses (Lorenzo et al., 2004; Dombrecht et al., 2007). *MYC2* promotes ABA-signaling further linking OGs and ABA responses (Anderson et al., 2004).

Despite a clear role in the elicitation of immune responses, the receptors for OGs are not known. WAK1, a cell wall associated receptor kinase involved in pathogen defense, likely functions in receptor-mediated OG signaling (He et al., 1998; Decreux and Messiaen, 2005). WAK1 binds OGs in a calcium-dependent manner, with calcium fluxes altering the efficiency of interaction by changing the conformational state of OGs (Decreux and Messiaen, 2005; Cabrera et al., 2008). ABA has long been identified as a major regulator of calcium oscillations indicating a potential role for ABA in the regulation of OG responses (Evans et al., 2001; Federoff, 2002; Wasilewska et al., 2008).

In addition, OG-elicited immune responses trigger an oxidative burst generated by NADPH oxidase AtrbohD (Galletti et al., 2008). Interestingly, resistance responses mediated by OGs to *B. cinerea* are independent of AtrbohD-generated ROS. By contrast, AtrbohD generated oxidative burst is required for resistance to *P. chrysanthemi*, a bacterial necrotroph that also utilizes PGs during infection (Norman et al., 1999). Thus, OGs may trigger a resistance pathway that requires differential NADPH oxidase activity dependent on invading pathogen. Alternatively, ROS generated

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during OG-induced responses may have no direct role in limiting *B. cinerea* yet be sufficient to directly kill the bacterial pathogen.

It was previously stated that OG oligomer length lends specificity to activation of induced immune responses, therefore the degree of plant pectin methylation also influences defense by altering PG hydrolysis and the subsequent length of generated OGs (De Lorenzo et al., 2001; D'Ovidio et al., 2004; Lionetti et al., 2007). Over-expression of pectin methylesterase inhibitors in Arabidopsis effectively restricts *B. cinerea* infection with resistance related to the fungus's impaired ability to grow on methylesterified pectins (Lionetti et al., 2007). However, resistance could be also be ascribed to enhanced OG-mediated defenses resulting from an increased pool of effective elicitor fragments based on limited PG hydrolysis (Ferrari et al., 2007b).

Other endogenous elicitors of innate immunity

In addition to OGs, other cell wall components may serve as DAMPs. The *Phytophthora* cellulose binding elicitor lectin (CBEL) protein is a P/MAMP that is a potent inducer of innate immune responses (Khatib et al., 2004; Gaulin et al., 2006). CBEL is a cell wall glycoprotein from *Phytophthora parasitica var nicotianae* (Ppn), the causal agent of the black shank disease of tobacco. This glycoprotein is widely conserved in the genus *Phytophthora* and elicits HR-like lesions, defense responses, and protection against subsequent infection with this oomycete in host tobacco and non-host Arabidopsis plants (Khatib et al., 2004; Gaulin et al., 2006). Interestingly, the cellulose binding domain (CBD) of CBEL is essential and sufficient to induce immune responses. Since the CBD anchors CBEL to the cell wall, and CBEL binds cellulose, defense may result from responses of the plant to recognition of modified cellulose acting as a DAMP (Hematy et al., 2009).

The plant-derived peptides Pep1 and Pep2 were identified as elicitors of immune responses in Arabidopsis (Huffaker et al., 2006; Huffaker and Ryan, 2007). These peptides are required for defense signal amplification and communication between cells in a manner similar to tomato systemin signaling (Yamaguchi et al., 2006; Postel and Kemmerling, 2009). Constitutive expression of Arabidopsis *PROPEP1* or *PROPEP2*, which encode the precursors of these peptides, results in significantly increased basal *PR-1* and *PDF1.2* expression as well as enhanced resistance to *Pythium irregulare* (Huffaker and Ryan, 2007). The LRR-RLK PEPR1 was identified as the receptor for PEP1 (Yamaguchi et al., 2006). During necrotrophic infection, PEPR1 likely perceives PEP1 and amplifies defense signaling via a positive feedback loop as PROPEP1-generated PEP1 induces *PROPEP1* expression (Yamaguchi et al., 2006). PEPR1 and a close homolog, PEPR2, interact with BAK1 linking the function of endogenous peptides to innate immune responses triggered by pathogen-derived elicitors (Postel et al., 2010). BAK1 also interacts with BIK1, required for defense against *A. brassicicola* and *B. cinerea*, suggesting BAK1 may act as a universal adaptor for proper receptor kinase function during innate immune responses (Veronese et al., 2006; Lu et al., 2009a; Postel et al., 2010).

Finally, in addition to physical components, plant perception of altered cellular homeostasis resulting from pathogen infection can also induce innate immune responses (Nurnberger et al., 2004; Lotze et al., 2007; Tor et al., 2009). For instance, many necrotrophs generate ion fluxes and ROS as virulence factors during colonization (Shetty et al., 2008). Shifts in cellular status and/or intracellular signaling from pathogen-triggered oscillations have the potential to serve at DAMPs upon host-recognition (Nurnberger et al., 2004; Lotze et al., 2007; Tor et al., 2009). Cyclic nucleotide-gated ion channel (CNGCs) facilitate increases in cytosolic calcium ion concentrations sufficient for activation of signal transduction pathways required for innate defense responses (Lecourieux et al., 2006; Ma et al., 2009). Host perception of altered CNGC function may contribute to activation of defense during necrotrophic infection. For example, mutation in *DND1*, encoding a CNGC, eliminates the HR, conferring resistance to *B. cinerea* (Clough et al., 2000; Govrin and Levine, 2000). This suggests changes in cyclic nucleotide levels may alter DND1 function during infection, leading to a de-regulation of the HR and increased pathogen colonization. Host recognition of altered DND1 function or resultant shifts in cellular calcium may then be sufficient to induce defense. However, it is currently unclear if DND1 has a role in DAMP-mediated immune responses. Interestingly, PEPR1 is predicted to contain a guanylate cyclase (GC) catalytic motif which may contribute to the regulation of defense (Kwezi et al., 2007).

Overall, there is a very fine line between DAMP-induced immunity and facilitation of infection with regard to host responses upon perception of altered homeostasis (Tor et al., 2009). This line becomes further blurred by the intersection of endogenous elicitors and molecules synthesized by the pathogen that share the same identity when it comes to recognition of stressed and malfunctioning cells. This overlap likely accounts for the lack of identified receptors for this type of DAMP. However, heterotrimeric G-proteins, functioning in signal transduction pathways regulated by G-protein receptors, have been emerging as mediators of resistance to necrotrophic infection. G-protein-coupled receptors are able to bind diverse ligands ranging from metabolites to large peptides making them excellent candidates for potential receptors in DAMP-triggered immunity (Llorente et al., 2005; Trusov et al., 2006; Temple and Jones, 2007; Trusov et al., 2009). The RLK ERECTA, required for *P. cucumerina*, *R. solanacearum*, and *P. irregulare* resistance, may also be a good candidate for DAMP pereception in innate immune responses to necrotrophic infection (Llorente et al., 2005).

R-gene-mediated susceptibility or resistance?

In addition to PAMPs, many pathogens produce race-specific effectors that are directly or indirectly recognized by intracellular or extracellular resistance (R)-proteins to activate a particularly strong form of resistance. Effector recognition leads to activation of ETI responses including ion fluxes, production of ROS and antimicrobials, and the HR (Jones and Dangl, 2006). ETI effectively restricts biotrophs, however, it is not sufficient for defense against necrotrophic pathogens (Glazebrook, 2005). Until recently, neither R-protein recognition nor its downstream components have been implicated in resistance to necrotrophic pathogens. *RLM3*, a relative of the family of TIR-NB-LRR class of R-genes, confers protection to *B. cinerea*, *A. brassicicola* and *A. brassicae* by mediating callose and SA responses during pathogen infection (Staal and Dixelius, 2008; Staal et al., 2008). Interestingly, the *rlm3* mutant also shows a marginal increase in resistance to *P. syringae* without altered JA/ET-mediated responses suggesting it may function in novel crosstalk between biotrophic and necrotro-

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phic defense, distinct from the established antagonism between SA and JA/ET (Staal and Dixelius, 2008). *RLM3* encodes a truncated R-gene thought to act as an adaptor for specific receptors involved in defense (Staal and Dixelius, 2008).

Conversely, another truncated R-gene, *RPW8*, mediates susceptibility to necrotrophs (Wang et al., 2007b). The *RPW8.1* and *RPW8.2* homologs were originally identified as R-genes conferring broad-spectrum resistance to a wide range of powdery mildew pathogens (Xiao et al., 2001). It was later found that constitutive expression of the *RPW8* genes confers resistance to *Hyaloperonospora parasitica* as well as *Cauliflower mosaic virus* but increases susceptibility to *B. cinerea* and *A. brassicicola* (Wang et al., 2007b). Further, Arabidopsis susceptibility to the host-specific necrotrophic fungus *C. victoriae* is conditioned by *LOV1*, encoding a member of the CC-NBS-LRR class of R-proteins (Lorang et al., 2007). The susceptibility attributed to the *LOV1* gene is independent of major phytohormone response pathways, camalexin accumulation, and the HR suggesting other unspecified factors contribute to defense against necrotrophs (Lorang et al., 2007). R-gene-mediated susceptibility to necrotrophs has been also documented in sorghum where a NBS-LRR R-gene was found to mediate sensitivity to the *Periconia circinata* PC-toxin (Nagy et al., 2007). Thus, R-gene-mediated susceptibility is not unique to Arabidopsis and may be linked to activation of known immune responses such as the HR or currently uncharacterized immune responses against necrotrophs.

Toxin-mediated immune responses and susceptibility

Regardless of their specificity, toxins induce responses similar to those in PTI and ETI including but not limited to HR-like cell death, oxidative bursts, hormone and callose accumulation, and *PR*-gene expression. The significance of these responses has not been studied as toxins are generally considered virulence factors rather than elicitors of defense. By contrast, it is also possible that, similar to effectors from biotrophic pathogens, toxins suppress basal immune responses in the absence of toxin resistance genes.

As important virulence factors, toxins are major targets of host resistance. Members of the Arabidopsis *UDP-glycosyltransferase* superfamily of over 100 genes directly contribute to the inhibition of pathogen virulence through toxin modification (Poppenberger et al., 2003; Poppenberger et al., 2005). These proteins function in the transfer of sugar molecules to a wide range of acceptors thereby altering their physical and chemical properties (Li et al., 2001; Ross et al., 2001; Bowles et al., 2006). DOGT1 or UGT73C5, confers resistance to the *Fusarium* toxin deoxynivalenol (DON) by catalyzing the transfer of glucose to a hydroxyl group located on carbon 3 of the toxin (Poppenberger et al., 2003). DON contributes to virulence by inhibiting host protein synthesis, with inhibition abolished upon glycosylation by DOGT1. DOGT1 can also detoxify an acetylated derivative of DON but not nivalenol despite a difference of only one hydroxyl group between these two toxins. Expression of *DOGT1* is induced with DON, as well as JA, ET, and SA suggesting it may also play a role in hormone-mediated defense responses during necrotrophic infection (Poppenberger et al., 2003). Consistent with this function, DOGT1 catalyzes glucosylation of the brassinosteroids brassinolide and castasterone (Poppenberger et al., 2005). Constitutive expression of *DOGT1* significantly reduces plant stature and leads to decreased accumulation of 6-deoxocastasterone and castasterone. Interestingly, the Micro-Tom dwarf cultivar of tomato, which is resistant to *Fusarium*, harbors a mutation in a P450 protein catalyzing the oxidation of 6-deoxocastasterone to castasterone (Scott and Harbaugh, 1995; Marti et al., 2006). Modification or detoxification of toxins is a major means of plant resistance to host-specific necrotrophs. However, the role of toxin-detoxification and modification in resistance to broad-host pathogens is not well known.

Wound-induced resistance to B. cinerea

Wounding Arabidopsis leaves prior to inoculation induces strong immunity to *B. cinerea* (Figure 2) (Chassot et al., 2008). This form of wound-induced immunity (WII) is contrary to data implicating tissue damage in promoting colonization by necrotrophic pathogens during infection by providing leaked nutrients and sites of necrosis (Baudoin, 1986; Prins et al., 2000). In fact, many infection protocols include steps of leaf perforation prior to inoculation with *B. cinerea* spores to support disease development in laboratory settings. Punctures have also been used as a means to negate differences in fungal penetration when assaying for altered virulence on genetically modified plants (Govrin and Levine, 2000). Thus, not only are the results published by Chassot et al. unexpected but also surprising in that they are fairly recent observations despite the inclusion of wounding in many older inoculation procedures (Govrin and Levine, 2000; Thomma et al., 2000). The WII to necrotrophs was likely unnoticed in previous reports because leaves were not wounded to a level sufficient for inducing resistance (Chassot et al., 2008). Adequate puncturing induces transient protection that inhibits *B. cinerea* growth at the primary site of infection (Chassot et al., 2008). While protection is transient with regard to the timing of inoculation subsequent to wounding, the resistance to fungal infection that is induced is relatively lasting (Chassot et al., 2008).

Interestingly, WII functions independently of SA- and JA/ET-mediated responses, relying instead on camalexin and glutathione accumulation (Chassot et al., 2008). The *pad3*, *pad2*, and *ups1* Arabidopsis mutants, impaired in camalexin accumulation, are all compromised in WII. Mutation in *PAD2*, encoding an enzyme involved in glutathione biosynthesis (GSH1), largely eliminates wound-induced resistance whereas *pad3* and *ups1* plants exhibit only partial losses (Parisy et al., 2007; Chassot et al., 2008). Partial losses were also observed for the glutathione biosynthesis mutants *rax1* and *cad2* despite their wild-type levels of camalexin accumulation (Cobbett et al., 1998; Ball et al., 2004; Chassot et al., 2008). Thus, camalexin and glutathione appear to be important factors in WII to *B. cinerea*.

As camalexin is variably toxic to other pathogens and glutathione has a direct function in regulating its biosynthesis as well as ROS-elicited cell death, it seems likely that the protection conferred by wounding may not be specific to *B. cinerea*. In fact, in light of recent publications, these primed defenses may correspond to components of innate immunity (Chassot et al., 2008; Bednarek et al., 2009; Clay et al., 2009). Camalexin represents only one of several derivatives stemming from tryptophan me-

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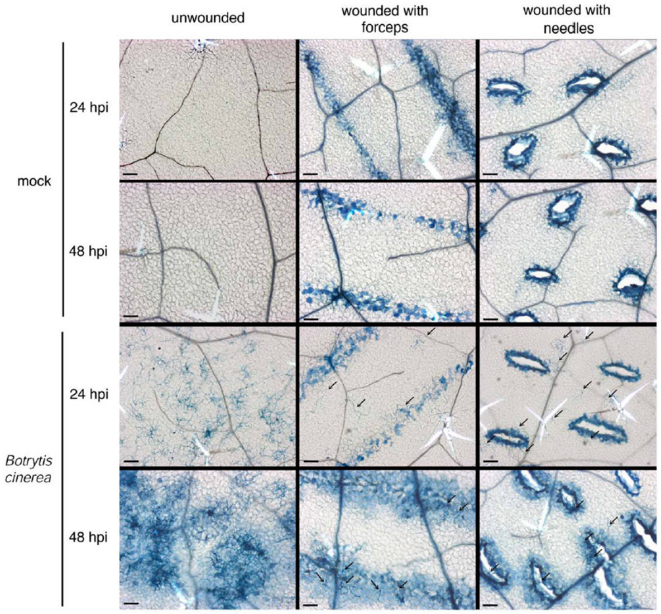


Figure 2. Arabidopsis wound-induced immunity to *B. cinerea*.

Fungal growth in plants inoculated with *B. cinerea* with and without wounding as revealed with trypan blue staining. hpi, hours after inoculation. (Pictures are courtesy of Jean-Pierre Metraux, University of Fribourg, Switzerland)

tabolism (Glawischnig, 2007). Initial conversion of tryptophan generates indole-3-acetaldoxime which serves an intermediate in camalexin as well as indole glucosinolate biosynthesis (Glawischnig, 2007; Rauhut and Glawischnig, 2009). Recent studies indicate an active glucosinolate hydrolysis pathway is involved in PTI (Bednarek et al., 2009; Clay et al., 2009). Callose deposition during PTI was found to be dependent on *PEN2*-regulated glucosinolate biosynthesis and degradation (Clay et al., 2009). Thus, it is plausible that WII may be based on camalexin and glutathione as well as glucosinolate metabolism and export. Consistent with this notion, PAD2-generated glutathione is thought to function as a cysteine donor for tryptophan indole derivatives involved in pre-invasive defense against fungal pathogens (Bednarek et al., 2009). Additionally, Arabidopsis plants harboring loss of function *PAD2* alleles exhibit decreased glucosinolate accumulation (Schlaeppi et al., 2008). The added dependence of wound-induced protection on glucosinolate could account for the partial losses in resistance exhibited by *pad4* and *ups1* relative to complete suppression in *pad2* (Chassot et al., 2008). Indole glucosinolate derivatives have previously been shown to contribute to resistance against *B. cinerea* as well as *F. oxysporum* (Tierens et al., 2001; Kliebenstein et al., 2005). Recent evidence also suggests a role for the *PEN2*-dependent defense pathway in mediating plant responses to *B. cinerea* infection (Bednarek et al., 2009; Consonni et al., 2009). Based on these data, together with the emerging role of PTI in governing plant response to necrotrophs, it seems unlikely that WII is specific to *B. cinerea.* Future, studies will define the role of WII to other pathogens, interactions with glucosinolate-dependent defenses, and upstream regulatory elements of the pathway leading to wound-induced resistance.

Hormone-regulated Immune Responses: Complexities and Relevance

Plant responses to pathogens have been studied extensively in connection with plant hormone synthesis and signaling. Changes in hormonal homeostasis promote resistance or susceptibility by creating a range of pathological conditions exploited by pathogens (Grant and Jones, 2009). It is now known that all the major plant hormones have some role in plant immune responses although SA, JA, and ET were the predominant players for a long time. Abscisic acid (ABA), auxin, and gibberellins (GAs) have recently emerged as critical factors of host resistance. Although a general consensus of hormone-related defenses has emerged through studies of single hormone-mediated responses, data on interactions of different hormones during pathogen-infection have revealed the limitations of some of these conclusions. The published literature is replete with data that makes it hard to draw generalized conclusions due to the complex interactions between hormone responses and biosynthesis as well as the external and endogenous signals that regulate them. However, over the last two decades it has been, perhaps overwhelmingly, stated that JA and ET synergistically function in defense against necrotrophs, generally, in direct antagonism to SA-mediated defenses (Glazebrook, 2005). These relationships have been substantiated by extensive genetic evidence, such as the distinct pathogen responses of numerous Arabidopsis mutants including *bik1*, *wrky33*, *bos3*, *ssi2*, and *rst1* to biotrophic and necrotrophic pathogens (Thomma et al., 1998; Petersen et al., 2000; Kachroo et al., 2003a; Kachroo et al.; Veronese et al., 2004; Veronese et al., 2006; Zheng et al., 2006; Mang et al., 2009). Plants harboring loss of function alleles of genes involved in JA/ET signaling/biosynthesis including the *aos*, *jar1*, *Atpla1*, *coi, fad3/fad7/fad8, ein2, ein3, eil1,* and *ora59* mutants display increased susceptibility to necrotrophic infection (Bonaventure et al. 2007; Ferrari et al., 2003; Yang et al., 2007; Thomma et al., 1998; Stintzi et al., 2001; Alonso et al., 2003; Pre et al., 2008). However, SA is also implicated in local resistance to necrotrophs (Ferrari et al., 2003a). Currently, many reviews exist highlighting the interrelations between these three hormones in plant defense (Thomma et al., 2001; Pieterse et al., 2001; Rojo et al., 2003; Glazebrook, 2005; Bari and Jones, 2008; Pieterse et al., 2009; Van der Ent et al., 2009). Thus, rather than "beat a dead horse/infect dead plants" so to speak, we will focus on recent findings as well as the growing roles of ABA, auxin, and GA in resistance to necrotrophic infection.

Ethylene and its interactions with jasmonate

In Arabidopsis, predominant genetic data implicates ethylene (ET) in resistance to necrotrophic fungi. Early observations on the role of ET were made in Arabidopsis *ein2* which displays enhanced susceptibility to some necrotrophic fungi (Thomma et al., 1999a). Now, many components of the ET-response pathway including *EIN3*, *EIL1* and the *"WEAK" ETHYLENE-INSENSITIVE* loci (*WEI1–WEI5*) are known to have a defense function against necrotrophic pathogens based on the disease responses resulting from loss of function mutations (Thomma et al., 1999a; Alonso et al., 2003; van Wees et al., 2003a). The role of ET is complex and may also vary depending on the specific necrotroph. For ex-

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ample, mutation in *EIN2* has no effect on resistance to *A. brassicicola* but contributes to resistance against *B. cinerea*. Yet, double mutant analysis involving *ein2* and the *B. cinerea* and *A. brassicicola* susceptible mutant *hub1*, revealed ET-responses promote susceptibility to *A. brassicicola* at the site of infection as demonstrated by reduced disease symptoms and pathogen growth in *hub1ein2* relative to infected *hub1* (Dhawan et al., 2009a). In contrast, *hub1ein2* plants show increased susceptibility to *B. cinerea* as compared to both parental mutants.

Consistent with the overall role of ET in defense, the molecular components of ET response pathways and their biochemical regulation are important components of immune responses to necrotrophic infection. EIN3 levels are modulated by phosphorylation resulting from two branches of MAPK signaling that have opposite effects on accumulation (Yoo et al., 2008). MPK6 phosphorylation stabilizes EIN3 and both of these proteins are required for resistance to *B. cinerea* (Ren et al., 2008; Yoo et al., 2008). MPK6 also phosphorylates the ET-response factor ERF104 (Bethke et al., 2009). MPK6 and ERF104 exist in a nuclear complex that dissociates upon flagellin perception in an ET-dependent manner (Bethke et al., 2009). Loss of function and over-expression of *ERF104* results in enhanced susceptibility to *B. cinerea* implying a strict regulation of this gene is essential to plant resistance (Bethke et al., 2009). ERF104 is thought to regulate *PDF1.2* expression downstream of EIN3 during ET-dependent defenses.

Interestingly, treatment with the flagellin peptide flg22 prior to infection confers protection against *B. cinerea* (Ferrari et al., 2007b). The flg22 epitope induces PTI, including the deposition of callose which is dependent on ET-regulated MYB51 accumulation (Clay et al., 2009). Flagellin treatment also positively affects expression of the ET-response factor *ERF1* (Clay et al., 2009). *ERF1* is a downstream target of *COI1*-mediated signaling and a point of convergence between the JA and ET signaling pathways (Lorenzo et al., 2003). Recent studies that revealed the susceptibility of *etr1*, *ers1*, *etr2*, *ers2*, and *ein4* mutants to Fumonisin B1 (FB1) demonstrated that ET-receptors have specific roles in mediating responses to toxin-elicited cell death (Plett et al., 2009). Loss of *ETR1* and *EIN4* resulted in delayed and accelerated necrosis, respectively, attributed to altered *ERF1* transcript levels. In addition, in protoplast-based assays, FB1-induced cell death is dependent on ET, SA, and JA (Asai et al., 2000). Thus, ET may modulate defense through its effects on cell death or interaction with other hormone responses. *ETR1* is also involved in the regulation of resistance mediated by Arabidopsis *GDSL LIPASE-LIKE 1* (*GLIP1*) (Kwon et al., 2009). *GLIP1* encodes a secreted antimicrobial protein proposed to function in the generation and amplification of signals required for ET-mediated systemic resistance (Oh et al., 2005; Kwon et al., 2009). Plant treatment with recombinant GLIP1 or its constitutive expression *in planta* confers local and systemic protection against *A. brassicicola* and *E. carotovora* (Kwon et al., 2009). GLIP1-elicited resistance correlates with increased systemic expression of *PDF1.2* and is dependent on ET-signaling mediated by *ETR1*. These results support the presence of a novel ET-regulated but JA-independent branch of systemic resistance against necrotrophs.

In Arabidopsis, ET generally functions synergistically with JA to promote resistance to necrotrophs. Both the JA and ET response pathways are required for induction of the plant defensin *PDF1.2* during infection. Global gene expression in *B. cinerea* inoculated *ein2* and *coi1* plants suggest a significant overlap between the two pathways consistent with their synergistic function in resistance (AbuQamar et al., 2006). ERF1 is a downstream component of COI1-mediated signaling and a point of convergence between the JA- and ET-signaling pathways (Lorenzo et al., 2003). Over-expression of *ERF1* results in resistance to necrotrophs and rescues the disease susceptibility of *ein2* and *coi1* (Berrocal-Lobo et al., 2002). By contrast, Arabidopsis MYC2, a basic helix-loop-helix-leucine zipper transcription factor, is induced by JA, in a COI1-dependent manner, but represses resistance to necrotrophic infection. Consequently, the *jin1/myc2* mutant shows increased resistance to *B. cinerea* (Lorenzo et al., 2004). The *MYC2*-regulated branch of JA signaling, downstream of *COI1*, directly antagonizes JA/ET defense responses to necrotrophs (Lorenzo et al., 2004). ERF1 activates the same genes MYC2 represses in response to pathogen infection and represses those MYC2 up-regulates following wounding. Thus, the synergy and antagonism between JA and ET can be delineated downstream of *COI1*, with elicitor-dependent mutual repression occurring between both branches. Consistent with this, the transcription factor ORA59, acting downstream of COI1, integrates these two pathways in the regulation of defense gene expression following infection (Pre et al., 2008). Plants harboring loss of function mutation in *ora59* are susceptible to *B. cinerea* indicating the requirement of both JA and ET signals for defense. Recently, the simultaneous activation of these pathways was proposed to negate the suppressive effects of SA on JA responses (Leon-Reyes et al., 2010). When activated prior to or at the same time, SA-responses can specifically inhibit JA-mediated *ORA59* and *PDF1.2* induction (Leon-Reyes et al., 2010). However, concurrent ET- and JA-signaling overcomes this antagonistic effect. *EIN2* bypasses *NPR1*-dependent crosstalk between SA and JA responses allowing for direct ET-mediated antagonism during pathogen infection. Consistently, EIN3 and EIL1 negatively regulate SA-accumulation and subsequent responses by blocking *SID2* transcription (Chen et al., 2009).

Jasmonate

In parallel with ET, the role of JA in defense is well established in many pathosystems. Exogenous application of JA confers resistance to necrotrophic infection with extensive genetic data reinforcing its role in plant defense against these pathogens. Primary observations of JA's function in defense were made in the *fad3fad7fad8* triple mutant which lacks JA and displays enhanced susceptibility to *Pythium mastophorum* (Vijayan et al., 1998). Subsequent studies indicated that many other genes in JA-response or synthesis pathways including *AOS1* and *JAR1* contribute to defense as loss of function alleles show enhanced susceptibility to necrotrophs (Lorenzo and Solano, 2005). The JA-receptor *COI1* is required for most JA-dependent responses and thus has a widespread impact on *B. cinerea* and *A. brassicicola*-induced gene expression and resistance to necrotrophic pathogens (van Wees et al., 2003a; AbuQamar et al., 2006). The Arabidopsis *rst1* mutant exhibits extreme resistance to *B. cinerea* and *A. brassicicola* dependent on *COI1* (Mang et al., 2009). The phenotypes of *rst1* correlate with increased levels of JA-biosynthesis and responsive gene expression as well as elevated cutic-

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ular lipids. Increased resistance of the o*verexpressor of cationic peroxidase 3 (ocp3)* mutant to *B. cinerea* and *P. cucumerina* is also *COI1*-dependent (Coego et al., 2005). *PFT1,* which encodes the MED25 subunit of Mediator, functions downstream of *COI1* in *A. brassicicola* and *B. cinerea* defense (Kidd et al., 2009). Gene expression studies in the *pft1* mutant revealed impaired induction of JA-responsive genes associated with increased levels of *WRKY70*, a negative regulator of JA-signaling (Kidd et al., 2009). *COI1* is also required for induced trichome formation mediated by JA in response to wounding and appears to regulate a *GL1*-mediated branch of JA-dependent pathogen defense (Calo et al., 2006; Yoshida et al., 2009).

COI1-regulated *ERF1* expression positively correlates with *F. oxysporum* resistance, yet *COI1* was found to enhance susceptibility to this pathogen (Berrocal-Loco et al., 2002; Lorenzo et al., 2003; Thatcher et al., 2009). After *F. oxysporum* inoculation, *coi1* mutants display minimal to no visible symptoms and have a 100% survival rate whereas wild-type plants exhibit severe chlorosis coinciding with higher mortality (Thatcher et al., 2009). Interestingly, the resistance of *coi1* to *F. oxysporum* is independent of JA-mediated pathogen responses and biosynthesis (Thatcher et al., 2009). The roots of infected *coi1* have decreased expression of an ET-independent branch of JA-signaling known to antagonize defense against necrotrophic pathogens (Lorenzo et al., 2004; Dombrecht et al., 2007; Thatcher et al., 2009). This suggests *F. oxysporum* exploits the function of *COI1* in signal integration to promote disease and underscores the importance of *COI1* in defense as it is a target of pathogen virulence (Thatcher et al., 2009). *F. oxysporum* was previously shown to secrete at least 22 JA- and octadecanoid-derived compounds which may serve as pathogen effectors targeting COI1 (Miersch et al., 1999). The α-subunit of heterotrimeric G-protein GPA1 may also be an effector target as it was recently shown to regulate a subset of genes functioning in non-pathogenic JA-responses downstream from COI1 (Okamoto et al., 2009). Consistent with this idea, the *gpa1* mutant has increased resistance to *F. oxysporum* and *A. brassicicola* (Trusov et al., 2006).

Abscisic acid

ABA has long been known for its role in plant responses to abiotic stresses, yet recent data have brought ABA to the forefront as a major regulator of plant immunity (Ton et al., 2009). Exogenous application or high endogenous ABA generally correlates with susceptibility to necrotrophic pathogens (Mauch-Mani and Mauch, 2005; Fujita et al., 2006). The Arabidopsis *aba2* mutant, deficient in ABA biosynthesis, displays enhanced resistance to *F. oxysporum* as well as increased basal and induced JA/ET-dependent defense gene expression (Anderson et al., 2004). Loss of *ABA2* function, in addition to mutations in *AAO2* and *ABI4,* involved in ABA synthesis and signaling, respectively, also increase resistance to *B. cinerea* infection (Adie et al., 2007). Arabidopsis *AGB1* encodes the β-subunit of heterotrimeric G-protein which functions as a negative regulator of ABA responses (Pandey et al., 2006). The *agb1* mutant is susceptible to *F. oxysporum, B. cinerea,* and *P. cucumerina* (Llorente et al., 2005; Pandey et al., 2006; Trusov et al., 2006). However, ABA positively contributes to defense against *A. brassicicola, S. sclerotiorum, L. maculans,* and *P. irregulare* (Guimaraes and Stotz, 2004; Adie et al., 2007; Kaliff et al., 2007; Asselbergh et al., 2008).

Based on literature, the role of ABA in immune responses to necrotrophic pathogens appears to be very intricate, with the mechanisms of resistance or susceptibility mediated by ABA largely unclear. ABA modulates processes that impact resistance including stomatal closure, defense gene expression, and ROS production/scavenging, indicative of an extensive role for ABA in defense (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008; Bari and Jones, 2009; Grant and Jones, 2009; Maksimov, 2009; Ton et al., 2009). Recent models suggest ABA may have differing roles that vary dependent on the type of tissue infected, stage of disease, the pathogen infection strategy, and/or the pathogen (Ton et al., 2009). *S. sclerotiorum*-generated oxalate facilitates pathogen entry by inducing stomatal opening while simultaneously repressing host-mediated closure by ABA (Guimaraes and Stotz, 2004). As *B. cinerea* also utilizes oxalate to promote host-colonization, it is interesting that mutation in *ABA2* confers resistance to this pathogen while enhancing susceptibility to *S. sclerotiorum* (van Kan, 2005; Adie et al., 2007; Guimarães and Stotz, 2004). Loss of Arabidopsis *BBD1*, encoding a bifunctional nuclease, results in susceptibility to *B. cinerea* attributed to decreased ABA-dependent callose deposition (You et al., 2009). In addition, susceptibility of *bbd1* correlates with reduced *PDF1.2* and *RD29a* expression supporting a concerted function of JA and ABA in defense (You et al., 2009). The non-protein amino acid β-amino butyric acid (BABA) primes ABA-regulated callose-accumulation sufficient for resistance to *B. cinerea* infection in Arabidopsis (Ton and Mauch-Mani, 2004). Consistent with this, the callose synthase mutant *pmr4* is susceptible to *A. brassicicola* but not to *B. cinerea*. ABA-mediated callose deposition also contributes to defense against *L. maculans, P. irregulare, A. brassicicola,* and *P. cucumerina* (Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). Resistance to *L. maculans* requires *RLM1*-dependent activation of ABA-biosynthesis for induction of unknown defense mechanisms regulated by *ABI4* (Kaliff et al., 2007). In response to *A. brassicicola* and *P. irregulare*, ABA induces defense gene expression by activating JA-biosynthesis (Adie et al., 2007). The Arabidopsis *constitutive disease susceptibility 2-1D* (*cds2-1D)* mutant is a gain of function allele of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE5* (*NCED5*), encoding an ABA biosynthetic enzyme, that results in constitutive activation of ABA-biosynthesis and resistance to *A. brassicicola* (Fan et al., 2009). The increased levels of endogenous ABA in *cds2* promoted JA-accumulation suggesting resistance may be a result of a synergistic defense regulated by ABA and JA. ABA may also modulate defense against necrotrophs associated with PTI. Flagellin perception by FLS2 elicits ABA-dependent stomatal closure, a process that is blocked by the bacterial toxin coronatine (Melotto et al., 2006). FLS2-mediated responses regulate callose accumulation and confer resistance to necrotrophs (Asai et al., 2002).

Currently, the role of ABA in defense cannot be generalized as it appears to have a pathogen- and context-dependent role (Asselbergh et al., 2008; Maksimov, 2009; Ton et al., 2009). Divergent ABA functions have been reported amongst the different necrotrophic species, including between *B. cinerea, S. sclerotiorum,* and *A. brassicicola* despite their common virulence and pathogenicity strategies (Guimaraes and Stotz, 2004; Adie et al., 2007). Elucidating the nature of ABA function is further confounded by its interactions with other resistance pathways and

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the potential trade-offs resulting from the occurrence of abiotic stresses during infection (Asselbergh et al., 2008; Maksimov, 2009; Ton et al., 2009). Overall, ABA is a key regulator of defense against necrotrophs with both negative and positive contributions. Extensive genetic data also suggest ABA mediates crosstalk between and interacts with JA- and SA-responses. Additional levels of complexity are likely to emerge as new ABA functions in defense are described.

Auxin

Auxin is one of the most well characterized phytohormones with regards to plant growth and development. Recent experiments have revealed a previously unknown function for auxin in plant immunity (Wang et al., 2007a; Llorente et al., 2008; Navarro et al., 2008; Bari and Jones, 2009; Kazan and Manners, 2009). The Arabidopsis mutants *axr1*, *axr2*, and *axr6*, impaired in auxin signaling, exhibit increased susceptibility to *P. cucumerina* and *B. cinerea* (Llorente et al., 2008). These genes function in SCF-mediated degradation of auxin/IAA transcriptional repressors consistent with an observed global down-regulation of auxin-responsive gene expression (Llorente et al., 2008). This suggests necrotrophic pathogens may actively suppress auxin-responses to promote disease. Transcriptional analysis of the *agb1* mutant which exhibits enhanced susceptibility to several fungal necrotrophs indicated a significant repression of auxin-inducible genes (Ullah et al., 2003; Llorente et al., 2005; Pandey et al., 2006; Trusov et al., 2006). AGB1 is required for G-protein-mediated defense against necrotrophs and physically interacts with NDL1, a positive regulator of auxin transport (Llorente et al., 2005; Pandey et al., 2006; Trusov et al., 2006; Mudgil et al., 2009). Loss of *axr6* also disrupts JA-responses by preventing COI1 association with SCF which is required for ubiquitin-mediated degradation of repressors of this pathway (Ren et al., 2005). The *aux1* mutant is susceptible to *P. irregulare* and exhibits compromised *Trichoderma*-mediated induced systemic resistance (ISR) against *B. cinerea* (Tiryaki and Staswick, 2002; Korolev et al., 2008). Metabolic profiling of systemic leaves from *aux1* indicates the mutant has distorted ratios of the hormones and indolic compounds required for the establishment of ISR (Truman et al., 2010). Similar to *AXR6*, JA-signaling is dependent on *AUX1* function in ubiquitin-mediated proteolysis pathways (Tiryaki and Staswick, 2002; Ren et al., 2005). Flagellin-perception leads to accumulation of the microRNA miR393, which represses auxin receptors thereby decreasing auxin signaling, and enhanced resistance to *P. syringae* indicating a negative regulatory role of auxin in bacterial defense (Navarro et al., 2006). Consistent with this, *GLIP2* positively contributes to resistance against *P. carotovorum* through the negative-regulation of auxin (Lee et al., 2009a). Thus, these observations implicate auxin-signaling/accumulation in plant responses to pathogen infection adding a further level of complexity to hormone-mediated plant innate immunity.

Gibberellins

Gibberellins (GAs) were discovered because of their association with the rice foolish seedling ("Bakanae") disease caused by the necrotrophic fungus *Gibberella fujikuroi (Fusarium moniliforme).* The pathogen produces GAs which cause disease symptoms such as pale yellow, elongated seedlings with slender leaves and stunted roots in rice. Mechanistic explanations for how GA affects plant immune responses have been provided only recently using Arabidopsis responses to pathogens. The DELLA proteins are a family of transcriptional repressors of GA responses, the accumulation of which was recently implicated in resistance to necrotrophic infection (Navarro et al., 2008; Smirnoff and Grant, 2008). GA-activated degradation of DELLA proteins leads to GA-responses indicating the likely function of this hormone as a negative regulator of defense against necrotrophs (Achard et al., 2008; Navarro et al., 2008). Constitutive expression of *GAI* encoding a DELLA protein results in increased resistance to *A. brassicicola* and DELLA stabilization contributes to flagellin-mediated responses that are sufficient for protection against *B. cinerea* infection (Navarro et al., 2008; Ferrari et al., 2007).

The Control of Chromatin-Modifications and Transcription in Defense Against Necrotrophic Pathogens

Regardless of the immune response pathway, plant defense against pathogens requires transcription of a wide range of genes encoding different regulatory and antimicrobial proteins, secondary metabolites, histological barriers and many other components. Accordingly, genome-wide analysis of expression reveals that necrotrophic fungi induce an array of genes belonging to diverse functional groups. *B. cinerea* induces genes encoding pathogenesis-related (PR) proteins and hormones, proteins involved in the metabolism of ROS, cell wall formation, and abiotic-stress responses (AbuQamar et al., 2006). The global expression profiles from *B. cinerea*- and *A. brassicicola*-infected Arabidopsis overlap significantly based on an analysis of gene expression consistent with an overall overlap of plant defense mechanisms against these two pathogens (van Wees et al., 2003b; AbuQamar et al., 2006). The functional significance of most of the changes in gene expression is unclear. However, loss of function mutations in some induced genes reveals a direct role in resistance (Veronese et al., 2006; Zheng et al., 2006). Mutations in others have no observable affects on disease resistance possibly owing to functional redundancy, or lack of sensitive assays that discriminate subtle changes.

Transcriptional control of gene expression plays a major role in determining the outcome of plant-pathogen interactions. Often, the strength and speed of expression determines the difference between resistance and susceptibility. DNA-binding proteins including ERFs, zinc-finger, MYB, WRKY, and HD-ZIP transcription factors (TFs) are all induced in response to *B. cinerea* infection suggesting a potential role in plant resistance (AbuQamar et al., 2006). Among these, *WRKY33*, *WRKY70*, *ZFAR1*, *ERF1* and *ERF104* are required for responses to *B. cinerea* (Mengiste et al., 2003; Lorenzo et al., 2004; AbuQamar et al., 2006; Zheng et al., 2006; Bethke et al., 2009). Genetic screens identified other important TFs encoding MYB, AS1, and HD-Zip homeodomain proteins (Mengiste et al., 2003; Coego et al., 2005; Nurmberg et al., 2007). The transmission of signals from upstream pathogen recognition factors to the activation of plant defense responses via these TFs is poorly understood especially for responses to important necrotrophic fungi. However, the plant-specific WRKY

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transcription factors are intermediate signaling components of various PTI responses (Asai et al., 2002).

In addition to sequence-specific TFs, a range of co-activators and chromatin-modifications modulate gene expression during defense. Modifications of histones and chromatin-remodeling have a fundamental role in gene regulation thus affecting many physiological processes including responses to environmental stresses (Bastow et al., 2004; Tsuji et al., 2006). The SWI/SNF chromatin-remodeling ATPase SPLAYED is required for defense against *B. cinerea* by regulating expression of several genes in the JA/ET-regulated defense pathway (Walley et al., 2008). Interestingly, some necrotrophs produce toxins that interfere with plant chromatin-modification machinery to suppress defense. The HC-toxin produced by strains of *C. carbonum* and depudecin, a structurally unrelated toxin produced by *A. brassicicola,* inhibit host histone deacetylases (HDs) and thereby may suppress defense (Brosch et al., 1995; Ransom and Walton, 1997; Privalsky, 1998). Consistent with this, the Arabidopsis *histone deacetylase19* (*hda19*) mutant is compromised in resistance to *A. brassicicola* (Zhou et al., 2005). It is thought that HDA19 functions in the regulation of JA- and ET-responsive genes during necrotrophic infection (Zhou et al., 2005). Interestingly, COI1 has been shown to interact with HISTONE DEACETYLASE6 (HDA6) (Xie et al., 1998; Devoto et al., 2002). Loss of function mutations or decreased expression of *HDA6* through RNAi results in down-regulation of JA-responsive genes including *PDF1.2* and *ERF1* (Wu et al., 2008).

More recently, histone H2B ubiquitination mediated by the RING E3 ligase, HISTONE MONOUBIQUITINATION1, has been shown to have a specific defense function against necrotrophic fungi (Dhawan et al., 2009). The *hub1* mutant displays extreme susceptibility to *B. cinerea* and *A. brassicicola*. Interestingly, HUB1 interacts with MED21, a subunit of an evolutionarily conserved multi-subunit Mediator complex that regulates the function of RNA polymerase II. *MED21 RNAi* lines are susceptible to the same two necrotrophic fungi providing a strong link between HUB1 and MED21 functions in defense. HUB1 and MED21 are both induced by chitin suggesting that they may be involved in PTI. Two additional subunits of Mediator complex, MED25 (PHYTOCHROME AND FLOWERING TIME1, PFT1) and MED8, are also required for resistance to necrotrophic pathogens (Kidd et al., 2009). Mediator complex is required for diverse transcriptional activation processes including the expression of antimicrobial peptides in Drosophila (Kim et al., 2004). Thus, the defense function of Mediator is broadly conserved suggesting that the subunits may relay signals from upstream regulators and chromatin-modifications to RNA polymerase II which, in turn, regulates transcription during infection. Mediator complex interacts with chromatin modification complexes, such as SWI/SNF and histone-modifying enzymes including histone de-/acetylases and HUB1 (Malik and Roeder, 2008; Dhawan et al., 2009). Both HUB1 and Mediator functions are likely linked to the expression of critical genes contributing to defense (Dhawan et al., 2009). Thus, chromatin-modifications have regulatory functions in host responses as well as pathogen virulence likely linked to the modulation of target gene expression. Future studies on the state of chromatin during resistant or susceptible responses as well as how modification is accomplished at chromatin of defense genes will shed light on the mechanisms underlying plant responses to pathogens.

Downstream Components of Defense Against Necrotrophic Fungi

There has been an overall paradigm that basic incompatibility between host and potential pathogens is provided by passive defenses such as the cuticle, cell wall, and various pre-formed molecules including phenolics and alkaloids. When this incompatibility is overcome, it leads to an activation of appropriate defenses that may vary depending on the nature of the pathogen and are regulated through complex networks of interacting pathways. Yet recent observations suggest that active defense responses involving the HR, accumulation of ROS, callose-deposition and synthesis of secondary metabolites occur in some incompatible interactions (Mysore and Ryu, 2004). In general, pathogen-recognition activates host immune responses composed of molecules that make the plant environment inhospitable thereby limiting pathogen ingress or the extent of damage. Among these molecules, phytoalexins, various secondary metabolites, antimicrobial peptides and PR-proteins have been studied in connection with plant immunity to necrotrophs. Generally, the relative importance of many of these compounds in resistance is not clear, with some implicated in defense based purely on correlative data whereas others have direct antimicrobial activity against necrotrophic pathogens. It is also possible that accumulation of some of these metabolites simply mark cellular perturbation rather than a direct role in pathogen-inhibition during infection.

Phytoalexins and other secondary metabolites

Secondary metabolites are dispensable chemical agents with a predominant function in aiding plant fitness to broad environmental stimuli (Bennett and Wallsgrove, 1994; D'Auria and Gershenzon, 2005). Plant infection with necrotrophs as well as treatment with toxins induces the biosynthesis of secondary metabolites (Thomma et al., 1999b; Stone et al., 2000; Rauhut and Glawischnig, 2009). These metabolites are constitutively present, generated from pre-existing constituents (phytoanticipins), or synthesized *de novo* in response to pathogen ingress (phytoalexins) (VanEtten et al., 1994). Derivatives of indole compounds, glucosinolates, phenylpropanoids, fatty acids, and flavanoids have all been implicated in defense against necrotrophs.

Camalexin, an indole derivative of tryptophan, is considered a characteristic phytoalexin and the most well-described secondary metabolite involved in Arabidopsis defense (Glawischnig, 2007; Rauhut and Glawischnig, 2009). Infection by different microbes induces camalexin synthesis at the site of infection but its antibiotic activity is limited to some pathogens. Although the exact mechanism is currently unknown, its microbial toxicity is attributed to pathogen membrane disruption as high concentrations induce ion leakage and inhibit proline uptake in bacterial cells (Rauhut and Glawischnig, 2009). Camalexin has long been associated with defense against necrotrophic fungi (Thomma et al., 1999b; Ferrari et al., 2007b; Chassot et al., 2008; Lu et al., 2009b; Stefanato et al., 2009). Plant infection with necrotrophs as well as treatment with fungal elicitors, including the *Fusarium* toxin Fumonsin B1 and a *Pythium* Nep1-like protein, induce biosynthesis of this metabolite (Thomma et al., 1999b; Stone et al., 2000; Rauhut et al., 2009). The Arabidopsis mutants *pad3*, *pad2*, *bos2*, *bos4* and

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esa1, impaired in camalexin synthesis or accumulation, exhibit enhanced susceptibility to *B. cinerea, A. brassicicola* and *P. cucumerina* (Thomma et al., 1999b; Tierens et al., 2002; Ferrari et al., 2003a; Veronese et al., 2004). Among these, *PAD3* encodes the P450 monooxygenase responsible for the synthesis of camalexin (Schuhegger et al., 2006). Plants harboring loss of function alleles of *UPS1* (*UNINDUCER AFTER PATHOGEN AND STRESS1*), involved in tryptophan biosynthesis, have reduced camalexin but show wild type levels of resistance to *B. cinerea* (Denby et al., 2005). Despite increased camalexin levels, the Arabidopsis *bos3* mutant exhibits extreme susceptibility to *B. cinerea* and *A. brassicicola* (Veronese et al., 2004). The *bos3* mutant is unable to control the extent of pathogen- or stress-induced cell death which may override the contributions of camalexin to resistance. The variation in camalexin-based resistance to necrotrophs is likely a result of interplay between multiple defense factors and disparities in isolate sensitivity to camalexin (Kliebenstein et al., 2005; Rowe and Kliebenstein, 2008). The virulence of many fungal species has been linked to their ability to metabolize or detoxify phytoalexins (Pedras and Ahiahonu, 2005). This was recently confirmed with identification of the *B. cinerea* ABC transporter BcatrB, involved in the active export of camalexin from the fungal cell (Stefanato et al., 2009). Interestingly, camalexin is also involved in wounding-, flagellin-, and OG-induced resistance to *B. cinerea*, with the *pad3* mutant displaying compromised protection in response to all three treatments (Ferrari et al., 2007b; Chassot et al., 2008).

Glucosinolates are indole compounds that share the same primary biosynthesis steps as camalexin (Rauhut and Glawischnig, 2009). Glucosinolate derivatives are increasingly being associated with innate immune responses to necrotrophic pathogens (Bednarek et al., 2009; Clay et al., 2009). Glucosinolates are constitutively present in plant tissue, located in sulfur rich cells adjacent to cells containing myrosinases (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006). Upon tissue damage, glucosinolates come into contact with myrosinases and are hydrolyzed resulting in the generation of antimicrobial derivatives. In addition to serving as a passive defense, glucosinolates also participate in induced responses (Bednarek et al., 2009; Clay et al., 2009; Consonni et al., 2009). Recent data show that a *PEN2*-dependent glucosinolate metabolism pathway functions in broad-spectrum antifungal defense (Bednarek et al., 2009; Clay et al., 2009). PEN2 acts as an atypical myrosinase that actively hydrolyzes glucosinolates allowing the directed movement of derivatives to sites of fungal penetration (Bednarek et al., 2009). This pathway is required for resistance to *P. cucumerina* and overlaps with PTI (Bednarek et al., 2009; Clay et al., 2009). Upon recognition of flagellin, FLS2-mediated responses to adapted and non-adapted pathogens induce several defenses including callose-deposition at the site of infection (Clay et al., 2009). Callose-deposition was dependent on *de novo* glucosinolate biosynthesis activated by ET-signaling and subsequent cleavage by PEN2. Hydrolytic products then likely form cadmium-bound complexes with the phytochelatin synthase PCS1 facilitating their export via PEN3 to the apoplast for initiation of callose-biosynthesis. Tryptophan-metabolism is also required for flagellin-mediated resistance to *B. cinerea* (Ferrari et al., 2007b). Additionally, mutation in the Arabidopsis *MLO2*, involved in powdery mildew defense, confers resistance to *B. cinerea* in a glucosinolate- and camalexin-dependent manner (Consonni et al., 2009). These data indicate *MLO2*-mediated resistance shares components with *PEN2*-regulated defense responses, supporting molecular interaction between these pathways during fungal infection (Bednarek et al., 2009; Clay et al., 2009; Consonni et al., 2009).

Arabidopsis produces a wide array of other secondary metabolites that also contribute to immunity to necrotrophic pathogens, (D'Auria and Gershenzon, 2005) here we will just briefly highlight a few examples. (*E*)-2-hexenal and (*Z*)-3-hexenal, two volatile C6-aldehydes produced from phenylpropanoid, enhance resistance to *B. cinerea* (Kishimoto et al., 2005; Kishimoto et al., 2006). Plant treatment with these C6-aldehydes induces significant tissue lignification that acts as a physical barrier against fungal penetration (Kishimoto et al., 2005; Kishimoto et al.). C6-aldehydes also induce camalexin-accumulation and increased expression of defense-related genes as well as the *COMT* transcript (Kishimoto et al., 2005; Kishimoto et al.). Arabidopsis *COMT*, encoding a 5-hydroxyguaiacyl *O*-methyltransferase involved in lignin biosynthesis, is required for resistance to *A. brassicicola* and *B. cinerea* (Quentin et al., 2009). The susceptibility of *comt* mutants is independent of hormone-mediated defenses and correlates with a significant decrease in sinapoyl malate, a hydroxycinnamate ester also derived from the phenylpropanoid pathway (D'Auria and Gershenzon, 2005; Quentin et al., 2009). Plant treatment with flg22, harpins, and necrosis-inducing *Phytophthora* protein 1 (NPP1) induces *COMT* expression suggesting it may function in PTI (Quentin et al., 2009). Several other hydroxycinnamate derivatives functioning in resistance to *A. brassicicola* were also recently described (Muroi et al., 2009). *ACT* encodes an agmatine coumaroyltransferase that catalyzes the last step of hydroxycinnamic acid amides (HCAAs) biosynthesis in Arabidopsis (Muroi et al., 2009). The *act* mutant exhibits enhanced susceptibility to necrotrophs attributed to impaired pathogen-induced accumulation of the HCAAs *p*-coumaroylagmatine, feruloylagmatine, *p*-coumaroylputrescine and feruloylputrescine (Muroi et al., 2009). *p*-Coumaroylagmatine directly inhibits *A. brassicicola* growth *in vitro* further supporting the role of HCAAs in defense against nectrophs (Muroi et al., 2009).

Finally, JA-mediated immunity to necrotrophic pathogens is associated with the regulation of secondary metabolite biosynthesis (Xie et al., 1998; Lorenzo et al., 2004; Dombrecht et al., 2007; Kidd et al., 2009; Shan et al., 2009). The susceptibility of *pft1*, impaired in the MED25 subunit of Mediator complex, was linked to the attenuated JA-responses including a lack of anthocyanin, an antimicrobial flavonoid secondary metabolite (Hatier and Gould, 2008; Kidd et al., 2009; Shan et al., 2009). *PFT1* integrates JA-signals downstream of COI1 which is required for JA-induced anthocyanin biosynthesis (Kidd et al., 2009); (Xie et al., 1998; Kidd et al., 2009; Shan et al., 2009). Additionally, MYC2 suppresses tryptophan and its derivatives such as indole glucosinolate biosynthesis during JA-signaling consistent with the resistance of the *myc2* mutant to necrotrophic fungi.

Pathogenesis-related proteins in the context of defense against necrotrophic fungi

Arabidopsis accumulates many classes of pathogenesis-related proteins (PR) in response to infection. Currently, 17 PR-protein families that mark active defense are recognized and include

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antifungal proteins, protease inhibitors, defensins, and other small peptides (Van Loon and Van Strien, 1999; van Loon et al., 2006a). Despite variations in pathogen-recognition and signaling pathways, it is now understood that plant infection results in the expression of overlapping sets of *PR*-genes independent of the infecting pathogen. A detailed analysis of the function of PR-proteins in plant immunity was recently reviewed (Narasimhan et al., 2009). This section highlights PR-proteins that have been linked to immune responses to necrotrophic fungi.

Plant defensins (PR-12) are small cysteine rich peptides stabilized by disulfide bonds that exhibit "cationic charges at physiological pH" (Selitrennikoff, 2001). In Arabidopsis, defensins are encoded by a multigenic family of at least 13 putative genes encoding 11 different defensins including three closely related *PDF1.2* genes (*PDF1.2a*, *PDF1.2b*, and *PDF1.2c*). The exact mechanism of fungal inhibition by these plant proteins is largely unknown mainly due to species-dependent variability in mode of action. Suggested mechanisms include disrupting calcium ion concentrations required for hyphal tip growth, inducing ion-permeable pore formation via direct insertion into the fungal membrane or adverse electrostatic interactions, and/or effecting cytosolic targets (Selitrennikoff, 2001). Currently, the role of *PDF1.2* in Arabidopsis defense is unclear though genetic and biochemical data support a function in resistance to necrotrophic fungi. Mutations disrupting JA- and ET- defense responses abrogate *PDF1.2* expression and resistance to many necrotrophic fungi. Numerous mutants including *bik1*, *esa1*, *wrky33*, and *ssi2* exhibit enhanced susceptibility to necrotrophic infection coincident with decreased and/or delayed *PDF1.2* induction associated with antagonism from increased SA levels (Tierens et al., 2002; Veronese et al., 2004; Veronese et al., 2006; Zheng et al., 2006). By contrast, plants harboring loss of function *HUB1* or *BOS1* alleles are not altered in *PDF1.2* expression yet display extreme susceptibility to *B. cinerea* and *A. brassicicola* (Mengiste et al., 2003; Dhawan et al., 2009). *HUB1* and *BOS1* function in hormone-independent resistance to necrotrophs. Interestingly, the *ERECTA* mutant is susceptible to *P. cucumerina* although it shows increased *PDF1.2* expression relative to wild type plants in response to infection (Llorente et al., 2005). Thus, while a factor, *PDF1.2* expression is not the sole determinant of plant resistance to necrotrophs. Functional ET- and JA-response pathways are required for *PDF1.2* expression during pathogen infection (Penninckx et al., 1996; Penninckx et al., 1998). However, this requirement can be bypassed through the constitutive expression of *ERF1*, a downstream component of the JA/ET-signaling pathway (Lorenzo et al., 2003).

Similar to *PDF1.2*, JA and ET also synergistically regulate expression of *CHI-B*, *HEL*, and *Thi2.1* in response to pathogen infection. *CHI-B* and *HEL* encode a PR-3 class Ib basic chitinase and an acidic hevein-like PR-4 protein, respectively (Van Loon and Van Strien, 1999; Selitrennikoff, 2001). Class I chitinases are synthesized as prepropeptides that undergo intracellular trafficking to the vacuole where they are processed to mature proteins (Sticher et al., 1993). Disruption of the vacuole during pathogen infection releases these enzymes allowing for active degradation of chitin, a major constituent of fungal cell walls (Collinge et al., 1993; Kasprzewska, 2003). Chitinases catalyze cleavage of β-1,4 bonds in chitin polymers, rendering hyphae more sensitive to osmotic stress which ultimately inhibits pathogen growth (Selitrennikoff, 2001). Chitin-oligosaccharide byproducts resulting from this hydrolysis are also sufficient for the induction of innate immune responses following host perception (Miya et al., 2007; Eckardt, 2008; Wan et al., 2008). Thus, chitinases contribute twofold to defense, through direct pathogen inhibition as well as indirect activation of PTI (Kasprzewska, 2003; Huckelhoven, 2007; Miya et al., 2007; Eckardt, 2008; Wan et al., 2008).

Interestingly, *in vitro* assays using purified CHI-B indicate it is not an effective growth inhibitor of *Alternaria, Fusarium*, or *Sclerotinia* species despite contributing to defense against these pathogens in Arabidopsis (Verburg and Huynh, 1991; Dai et al., 2006). Thus, the primary function of this protein in resistance to necrotrophs may be in the initiation of basal immune responses. Alternatively, the lack of antifungal activity *in vitro* could signify the requirement of synergistic interaction for effective CHI-B-mediated cell wall hydrolysis. Many studies have shown that the combined activity of chitinases and β-1,3-glucanases results in enhanced resistance compared to that imparted by the individual proteins (Mauch et al., 1988; Sela-Buurlage et al., 1993; Jach et al., 1995). Similar to CHI-B, purified tobacco PR-3b is unable to directly affect *F. solani* growth *in vitro* (Verburg and Huynh, 1991; Sela-Buurlage et al., 1993). Yet, in combination with the β-1,3-glucanase Glu-I, PR-3b effectively restricts fungal development (Sela-Buurlage et al., 1993). β-1,3-glucanases are PR-2 family proteins that hydrolyze β-1,3-linked glucan polymers which, when cross-linked to chitin, represent the structural core of fungal cell walls (Selitrennikoff, 2001; van Loon et al., 2006a). As cell wall chitin is interior to glucan with respect to the plasma membrane, β-1,3-glucanases likely increase chitin accessibility during infection accounting for the synergy between these and PR-3 proteins (Selitrennikoff, 2001). In Arabidopsis, *PR-2/BGL2* expression is induced with activation of SA-dependent defense responses which generally enhance susceptibility to necrotrophs (Thomma et al., 2001; Glazebrook, 2005; Veronese et al., 2006; Seo et al., 2008). However, local resistance to *B. cinerea* does require SA, suggesting CHI-B and BGL2 may function together in pathogen inhibition at the site of infection (Ferrari et al., 2003a). Consistent with this idea, both genes are induced in response to *A. brassicicola* and *S. sclerotiorum* (Oh et al., 2005; Dai et al., 2006).

In addition to CHI-B, HEL also contributes to defense against necrotrophs in a chitin-dependent manner. HEL is a hevein-like protein that binds to chitin in developing hyphae (Bormann et al., 1999; Selitrennikoff, 2001; van Loon et al., 2006b). Through an unknown mechanism, HEL attachment to areas of fungal cell wall synthesis disrupts cellular polarity resulting in inhibited growth (Bormann et al., 1999; Selitrennikoff, 2001). Over-expression of the ET-response factor *ERF2* elevates basal transcript accumulation of *HEL* and increases plant resistance to *F. oxysporum* (Brown et al., 2003; McGrath et al., 2005). ORA59, also an ERF family member, integrates JA- and ET-signaling responses downstream of COI1 following necrotrophic infection (Pre et al., 2008). *ORA59* gene silencing enhances susceptibility to *B. cinerea* and significantly reduces expression of several defense genes including *HEL* (Pre et al., 2008). Interestingly, constitutive expression of *ORA59* in *coi1* is sufficient for restoring *HEL* induction in an elicitor-dependent manner (Pre et al., 2008). Mutation in *MYC2*, also acting downstream of *COI1*, results in increased levels of pathogen-induced *HEL* correlated with enhanced resistance to *B. cinerea* and *P. cucumerina* (Lorenzo et al., 2004).

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The PR-13 family is composed of small cysteine rich antifungal thionins that increase hyphal membrane permeability and cellular lysis through a currently undefined mechanism though several competing models have been proposed (Thevissen et al., 1999; Stec, 2006). Thionins may form rigid peptide carpets across the bilayer, induce ion-selective channel formation, and/or initiate phospholipid removal from the membrane causing it to become unstable and collapse (Stec, 2006). However, thionins inhibit protein and DNA synthesis as well as regulate cellular redox suggesting loss of fungal membrane integrity could actually be a secondary effect of thionin action (Stec, 2006). In support of a role in direct membrane disruption, thionins function synergistically with lipid transfer proteins (LTPs) to inhibit fungal growth (Molina et al., 1993). LTPs are PR-14 proteins that bind and transfer membrane phospholipids, an action that corresponds to a proposed mechanism for thionin activity (Selitrennikoff, 2001). LTPs act as antimicrobials as well as receptors and signaling molecules for the activation of plant defense responses (Segura et al., 1993b; Arondel et al., 2000; Buhot et al., 2001; Maldonado et al., 2002; Chassot et al., 2007). Purified Arabidopsis LTP-a1 and LTP-a2 inhibit *F. solani* growth *in vitro* (Segura et al., 1993a). Plants harboring mutation in *LTPG1,* encoding a membrane-anchored LTP, form diffuse cuticles with altered lipid composition and are more susceptible to *A. brassicicola* infection (Lee et al., 2009a). Conversely, transgenic expression of a fungal cutinase (CUTE plants) or loss of *BDG1* function also result in diffuse cuticle formation but enhanced resistance to *B. cinerea* (Sieber et al., 2000; Kurdyukov et al., 2006; Chassot et al., 2007). Resistance in both lines correlated to increased expression of several *LTP* family members and was attributed to the combined action of these members with other defense response proteins (Chassot et al., 2007). However, over-expression of three of the induced *LTPs* (*At4g12470*, *At4g12480*, and *At4g12490*) individually also resulted in increased resistance suggesting they may be sufficient for *B. cinerea* defense (Chassot et al., 2007). Constitutive expression of *Thi2.1* also confers resistance to necrotrophic infection, significantly inhibiting *F. oxysporum* growth and symptom development (Epple et al., 1997).

PR-1 is the most widely studied PR-protein in relation to plant immune responses. *PR-1* expression is the standard molecular marker for SA-dependent responses and correlates with resistance to biotrophs. Although *PR-1* expression is induced in response to *B. cinerea*, *A. brassicicola* and *P. cucumerina* in Arabidopsis early in infection, it is not sufficient to confer resistance to these pathogens (Veronese et al., 2004). Indeed, higher basal and induced expression of *PR-1* correlates with enhanced susceptibility to necrotrophs in many Arabidopsis mutants (Veronese et al., 2004; Veronese et al., 2006). This is consistent with the suppression of resistance to necrotrophs by increased endogeneous and exogenous SA-levels. However, exogenous SA-treatment has also been shown to promote resistance to *B. cinerea* but increased susceptibility to *A. brassicicola* at the site of infection (Ferrari et al., 2003a; Spoel et al., 2007). Consistent with this observation, *PR-1* is highly expressed in cells immediately surrounding areas of necrosis, yet it does not appear to play a significant role in limiting disease lesions (Ferrari et al., 2003). The exact function of PR-1 in plant immunity in general and to necrotrophic pathogens in particular has not been defined and its biochemical functions are unknown. Thus, PR-1 may just be a marker of induced cell death or necrosis during infection.

Proteins belonging to the PR-6 family of protease inhibitors (PIs) are well-described plant defense molecules functioning as anti-feedants and antimicrobial agents (Ryan, 1990; Koiwa et al., 1997; Joshi et al., 1999; Kim et al., 2009). Generally, characterization of PIs in Arabidopsis has been limited. However, several have recently emerged as mediators of necrotrophic defense (Chassot et al., 2007; Li et al., 2008; Sels et al., 2008). The Arabidopsis serine protease, *KUNITZ TRYPSIN INHIBITOR 1 (KTI1)* modulates pathogen-elicited cell death and can specifically antagonize necrosis caused by fumonisin B1 (Li et al., 2008). Over-expression of *ATTI1*, encoding a defensin-like trypsin inhibitor, or *At2g38870,* encoding a putative PR-6 PI enhances resistance to *B. cinerea* (Chassot et al., 2007; Sels et al., 2008). Arabidopsis contains six predicted PR-6 proteins that exhibit differential regulation in response to pathogens (Sels et al., 2008). Other than the over-expression of *At2g38870*, no other studies have been reported on the function of these proteins in defense.

In sum, several PR-proteins are clearly linked to defense against necrotrophs whereas others, despite accumulation after infection, have no genetic or biochemical data supporting a role in resistance. Genetic studies on the functions of *PR*-genes is hampered due to the multigenic nature of these families and their likely redundant functions. Mechanistically, many enhance pathogen cell wall permeability or degrade major cell wall components of filamentous fungi. Other PR-proteins, including PR-1, may simply mark a perturbation in cellular homeostasis.

PERSPECTIVE

Arabidopsis basal resistance and non-host responses to necrotrophs have provided avenues for the molecular, genetic and biochemical dissection of plant immunity. Immense progress has been made in understanding different processes and genetic regulators underlying host responses to necrotrophs. Plant hormone signaling and synthesis have become integral to plant responses to necrotrophic infections with JA and ET being critically important for systemic and local resistance. ABA has also come to the forefront as a major regulator of plant responses to pathogens, with auxin and GA slowly becoming recognized factors in defense against necrotrophic infection. Additionally, SA appears to have a context-dependent role in resistance to necrotroph. The host cell wall and cuticle are now recognized as dynamic players in resistance rather than functioning solely as static barriers to necrotrophic infection. Genetic data from cell wall and cuticle mutants have also revealed an evolutionary specialization of necrotrophic fungi in adapting to host components. Host-resistance mechanisms that counter fungal toxins or their effects have been described. Chromatin-modifications have recently been established as important regulators of basal resistance to necrotrophic infection. Analysis of Arabidopsis secondary metabolites implicate glucosinolate metabolism as regulator of broad-spectrum resistance to different fungal pathogens. The prevalence of contrasting plant immune responses to pathogens of different lifestyles has been established, with pathway crosstalk recognized as a fine-tuning mechanism between these active defenses. Interestingly, despite their disparate pathogenesis and nutrient acquisition strategies, and often antagonistic resistance mechanisms, downstream components of basal resistance to biotrophic and necrotrophic pathogens also share

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significant overlap. However, there still exists immense variation in innate immune responses to necrotrophic infection in general.

It is no doubt that Arabidopsis has revolutionized the concepts of plant innate immunity and has provided a conceptual framework for progress in crop plants. First, the wealth of information generated in Arabidopsis serves as a springboard for the initiation of research in other plant systems. Second, direct transfer of important regulatory components of plant immunity from Arabidopsis to other hosts suggests a significant functional conservation exists in crop plants. For instance, Arabidopsis *NPR1,* a central regulator of SAR, confers resistance to *F. graminearum* in wheat (Makandar et al., 2006). Many other examples also exist in the literature demonstrating the positive impact of Arabidopsis in advancing research in basic plant immunity and transfer into crop plants.

Despite this significant progress, a critical knowledge gap still remains in understanding factors involved in plant defense. The components of pathways linking recognition to downstream immune responses limiting pathogen ingress need to be mapped. The complex nature of resistance and diversity of disease factors makes these tasks challenging. Research focused on complex mechanisms of defense, interactions between pathways, metabolic regulation, the quantitative genetics of resistance, and utilizing systems level approaches will undeniably catapult our understanding of plant immunity not only to necrotrophs but many other pathogens as well.

DISEASE ASSAY PROCEDURES FOR *B. cinerea*

Described below are the procedures our lab routinely uses for the maintenance of *B. cinerea* cultures and our protocol for disease assays on Arabidopsis. We also show specific examples that illustrate the progress of infection, typical symptoms, and different methods of disease assessment for *B. cinerea*.

Maintenance of *B. cinerea* and Plant-inoculation

1. Initiate fungal cultures by transferring pieces of agar containing mycelium to fresh 2xV8 agar
2. [2x V8 agar (36% V8 juice, 0.2% CaCO3, 2% Bacto-agar, Becton, Dickinson and Company, Sparks, MD USA)]
3. Incubate for 10 days in the dark at 20-25° C, (in lab drawers or cabinets is okay). Make sure you keep the culture in the dark without sealing the plates.
4. At around 10 days, there is enough spores to spray-inoculate roughly 5 trays containg around 40 plants each. *B. cinerea* sporulates profusely on 2xV8 media.
5. To collect conidia, take pieces of agar containing actively growing *B. cinerea* and suspend them in 1% Sabouraud maltose broth (SMB).
6. Shake vigorously and filter suspension through cheese cloth to separate the agar pieces and mycelium from detached conidia.
7. Take an aliquot and determine the conidial density. Adjust the conidial suspension to desired density in SMB buffer and proceed with disease assays.
8. To infect plants, spray the *B. cinerea* spore suspension onto Arabidopsis plants using a Preval sprayer (Valve Corp., Yonkers, NY, USA). Spray a very fine mist, ensuring equal volumes for different treatments. Spraying too much or larger droplets may macerate tissue too fast.
9. Alternatively, drop-inoculate (3-4 µl from a $2.5x10^5$ spore suspension) leaves and measure disease lesions (we don't really like drop-inoculation on detached leaves).
10. To establish infection, plants should be kept under a sealed transparent cover to maintain high humidity in a growth chamber with (21° C) day and (18° C) night temperature with a 12-h light/12-h dark cycle. Keeping the assays at room temperature has also worked for us but the room temperature should not exceed 25° C.

Notes on Maintenance and Storage of Fungus

a. Subculture by transferring pieces of agar with fungus to new 2xV8 plates.
b. Prepare glycerol stocks from virulent cultures.
c. After several subcultures virulence of the pathogen goes down dramatically. At this time, go back to your glycerol stock and start a new culture. Alternatively you can always start from a glycerol stock.
d. No more than 10 subcultures are recommended.
e. High temperatures >25°C are not good for infection. *B. cinerea* likes cool and humid temperatures.
f. Adding antibiotics such as ampicillin or kanamycin in the fungal growth V8 media will help avoid bacterial contamination.

Problems and Critical Factors in *B. cinerea* Disease Assays

The most common problem with *B. cinerea* fungal cultures is a loss of virulence. This can be unpredictable but corrected by initiating fungal cultures from a glycerol stock or re-isolating the fungus from infected plants. In order to observe the expected plant responses reflecting the functions of genes defined by loss or gain of function mutations, it is important to keep as much of the disease variables constant. Uniform plant growth conditions and using healthy unstressed plants is important as stressed plants show exaggerated disease symptoms.

Inoculation and Scoring Disease Symptoms and Pathogen Growth

Our preferred method of *B. cinerea* inoculation on Arabidopsis is spraying a conidial suspension on 4-5 week old plants. However, visual assessment of disease symptoms from spray-inoculated plants may be tricky unless the disease response phenotypes of the mutants are clear. Visual assessment of subtle variations in disease symptoms are often difficult to determine in spray-inoculated plants.

In wild type Arabidopsis plants sprayed with a conidial suspension, early disease symptoms include small necrotic lesions which are the sites of primary infection, chlorosis surrounding these areas of necrosis, tissue maceration and occasional leaf collapse on the lower leaves. Small necrotic sites are observed between 24-48 h after inoculation and indicate the primary sites of infection (Figure 3). The onset of diseases symptoms and progress vary depending on the environmental conditions and the amount of inoculum used.

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The extent of chlorosis, necrotic spots and tissue maceration and, in later stages, plant decay relative to the wild type plants is indicative of altered disease responses. In whole plant disease assays, plants could be completely decayed if the pathogen is left to overgrow particularly after spray-inoculation of plants. In those cases, the relative susceptibility could be assessed by counting the percent of decayed plants after extended incubation under conditions that promote disease. The nature and strength of disease symptoms vary significantly between mutants depending on the contribution of the genes involved. Loss of chlorophyll, quantitative PCR (qPCR) for constitutively expressed fungal transcripts or for fungal DNA relative to those in the plant in infected tissue, and measuring fungal ergosterol via HPLC are all possible methods to determine fungal growth in inoculated plants.

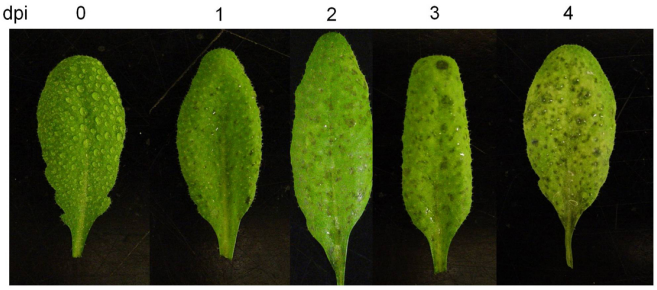


Figure 3. *B. cinerea* disease symptoms in wild type (Col-0) Arabidopsis

B. cinerea causes necrotic lesions on a wild type Arabidopsis. Images show progress of typical disease symptom from 1 to 4 dpi. Plants were sprayed with 2.5 x 10^5 spores/ml *B. cinerea* suspension. dpi, days post infection.

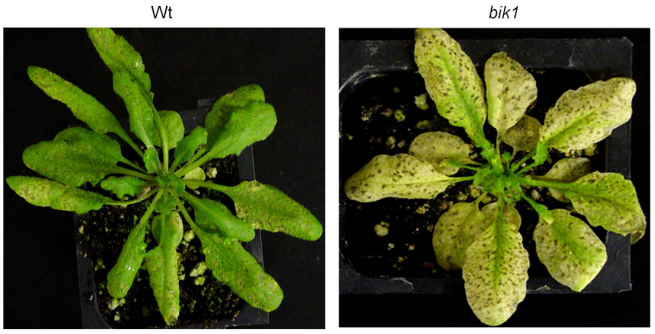


Figure 4. Disease symptoms in wild type (Col-0) and *bik1* mutant plants after spray-inoculation with a *B. cinerea* conidial suspension (2.5x10^5 spores/ml).

Sites of infection are clearly visible as restricted necrotic sites in the wild type plants. The *bik1* plants show enhanced chlorosis as an early disease symptom.

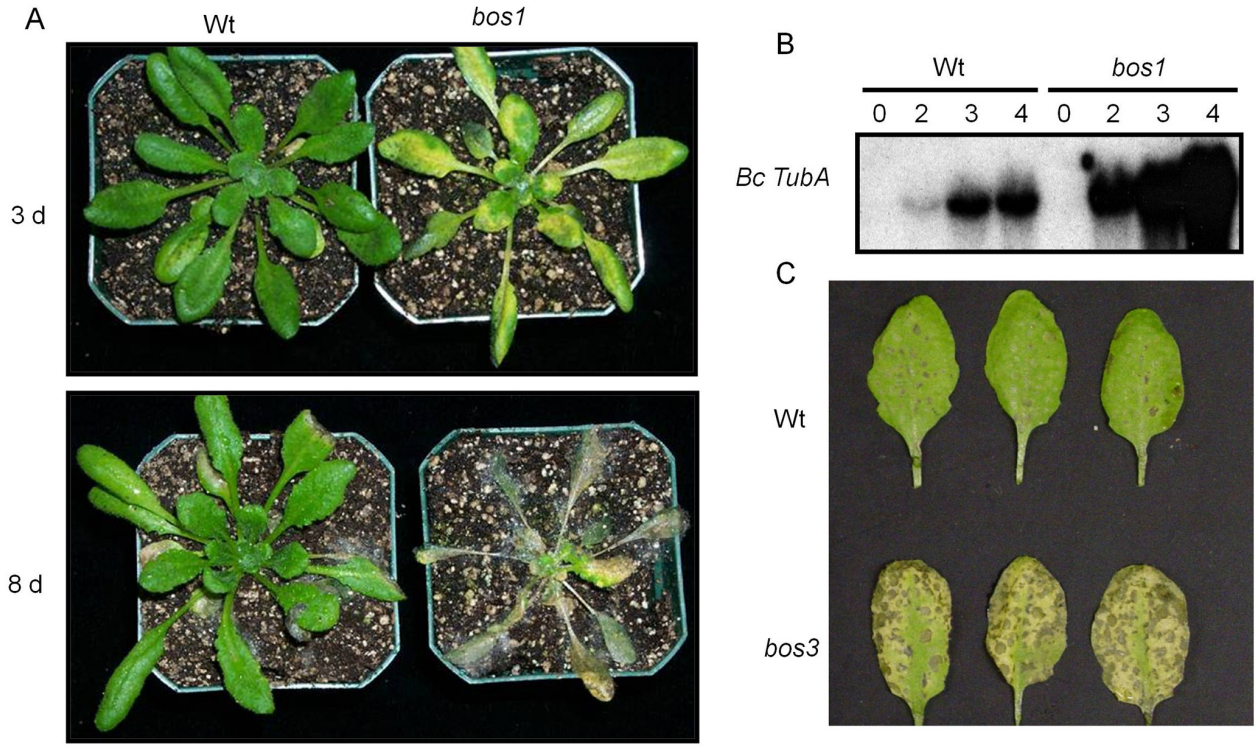


Figure 5. Disease symptoms in wild type (Col-0), and **(A)** *bos1* and, **(C)** *bos3* mutant plants after spray-inoculation with a *B. cinerea* conidial suspension (2.5x10^5 spores/ml).

The RNA-blot in **(B)** shows accumulation of the *B. cinerea Tublin A* gene transcript as a measure of fungal growth. The *bos3* plants show increased necrosis and chlorosis after inoculation with *B. cinerea*. d, days after inoculation.

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Genetic screens in our laboratory identified many mutants that show strong disease susceptibility phenotypes suggesting a clear function in immunity to necrotrophic pathogens for the genes defined by these mutations. Among these are the *botrytis-induced kinase1* (Veronese et al., 2006), *botrytis-susceptible1* (Mengiste et al., 2003), *botrytis susceptible 3* (Veronese et al., 2004) and *histone monoubiquitination1* (Dhawan et al., 2009a) mutants described in the previous sections of this chapter, used here to illustrate the various disease symptoms caused by necrotrophic infection. Figure 4 shows typical early disease symptoms in wild type (Col-0) and the *bik1* plants consisting of areas of leaf chlorosis and necrosis. The enhanced susceptibility of *bik1* is apparent at early stages of infection, primarily consisting of increased leaf chlorosis.

Following *B. cinerea* infection, the *botrytis susceptible1* mutant shows aggressive chlorosis, without apparent necrotic sites, which spreads and consumes the entire plant (Figure 5A). RNA-blot analysis clearly marks increased accumulation of the constitutive *B. cinerea Tubulin A* gene (*Bc TubA*) transcript indicative of increased fungal growth in *bos1* plants relative to infected wild type (Figure 5B). *bos3* plants display a run-away cell death and increased necrosis in response to infection (Figure 5C).

The progress of disease symptoms in spray-inoculated wild type, *wrky33* and 35S:*WRKY33* plants is shown in Figure 6. The *wrky33* mutant is very susceptible to *B. cinerea* as observed from the early tissue maceration and complete decay of plants at later stages of disease.

B. cinerea disease assays using detached leaves, arranged on water-saturated Whatman filter papers, produces inconsistent results but works fine for assessing plant responses to *A. brassicicola*. Drop-inoculation of *B. cinerea* on leaves still attached to plants produces reproducible results and quantifiable disease symptoms as the size of disease lesions can be measured. In this case, disease at the site of inoculation is severe due to the application of thousands of spores at one spot.

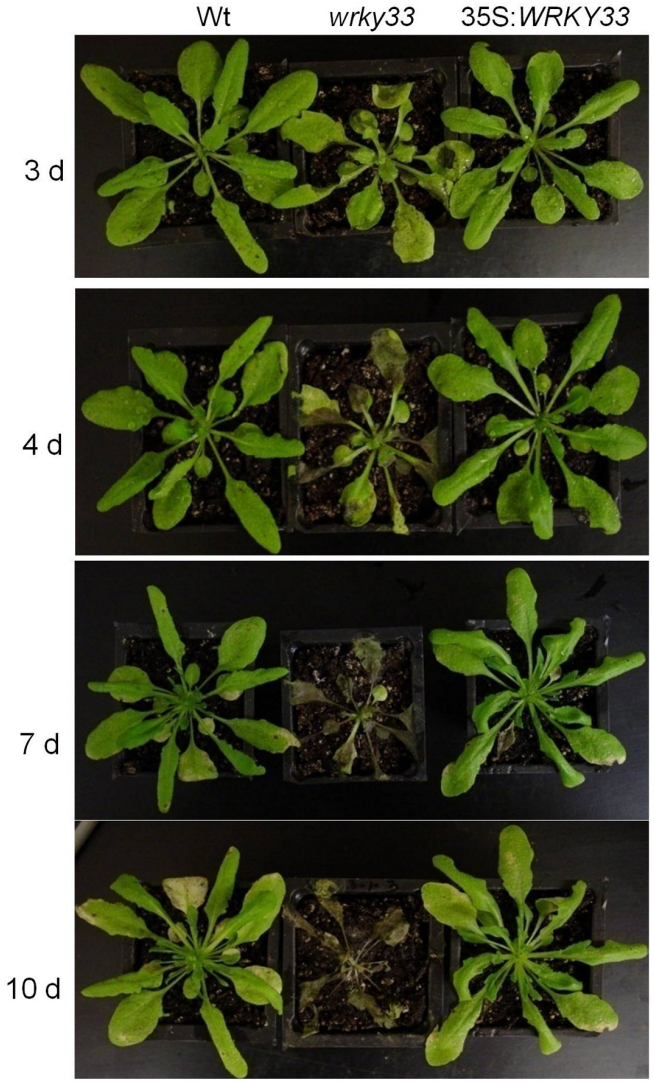


Figure 6. Progress of disease symptom in wild type, *wrky33* and 35S:*WRKY33* plants sprayed with 2.5 x 10^5 spores/ml *B. cinerea* suspension.

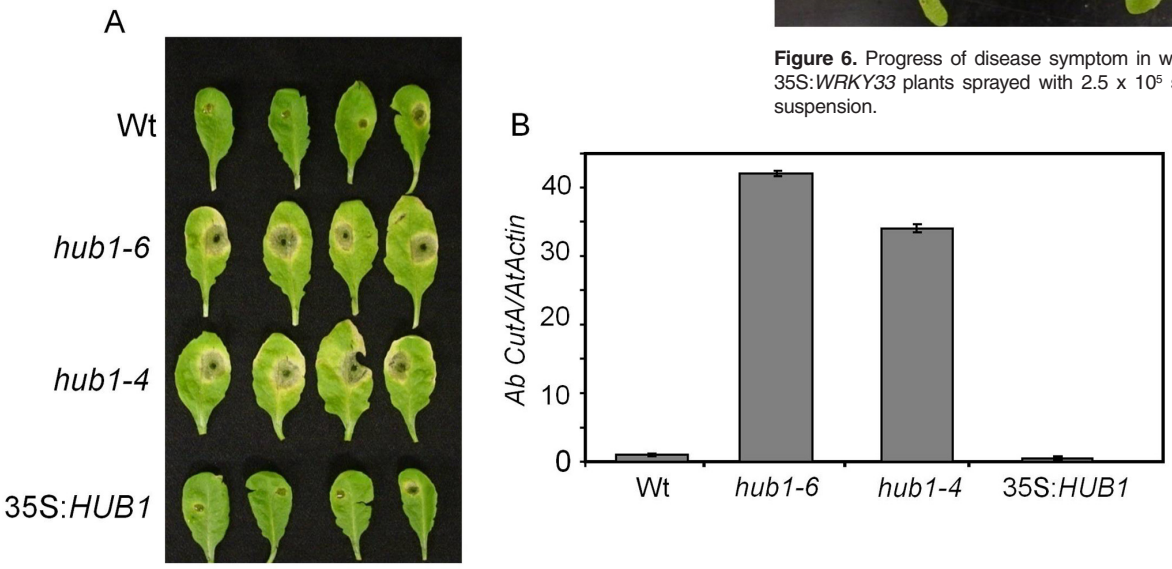


Figure 7. Disease symptoms caused by *Alternaria brassicicola* in Arabidopsis wild type, *hub1* and 35S:*HUB1* plants.

(A) Detached leaves were drop-inoculated (5 μl, 5x10^5 spores/ml). **(B)** Disease symptoms and measurements of fungal growth are from 5 dpi. dpi, days post infection.

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For comparison, the disease symptoms and fungal growth in *A. brassicicola*-inoculated wild type and *hub1* plants are presented in Figure 7. At 5 dpi, wild type Arabidopsis plants show resistance with a very limited disease lesion. The *hub1* mutant produces larger disease lesions and supports increased fungal growth. Fungal growth was determined as the proportion of plant to fungal DNA in inoculated plants using quantitative PCR (Figure 7B).

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