Review TRENDS in Plant Science Vol.11 No.5 May 2006

Licensed to kill: the lifestyle of a necrotrophic plant pathogen

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Necrotrophic plant pathogens have received an increasing amount of attention over the past decade. Initially considered to invade their hosts in a rather unsophisticated manner, necrotrophs are now known to use subtle mechanisms to subdue host plants. The gray mould pathogen *Botrytis cinerea* is one of the most comprehensively studied necrotrophic fungal plant pathogens. The genome sequences of two strains have been determined. Targeted mutagenesis studies are unraveling the roles played in the infection process by a variety of *B. cinerea* genes that are required for penetration, host cell killing, plant tissue decomposition or signaling. Our increasing understanding of the tools used by a necrotrophic fungal pathogen to invade plants will be instrumental to designing rational strategies for disease control.

Necrotrophic plant pathogens

Biotrophic plant pathogenic microbes such as downy or powdery mildews and rusts are generally accepted to have an intricate biological interaction with their host plant [1], presumably as a result of co-evolution [2]. Many plant pathologists have long considered that necrotrophic plant pathogenic fungi do not have much of a 'real' interaction with their host. Necrotrophs kill host cells by means of toxic molecules and lytic enzymes and they subsequently decompose the plant tissue and consume it for their own growth. If the toxic molecule shows differential activity to one or a few plant species, the pathogen has a limited host range and the metabolite is referred to as a host-selective toxin (HST) [3]. Several well-studied necrotrophs, in particular *Cochliobolus* and *Alternaria* spp., produce HSTs and have a limited host range and, thus, fulfill this criterion. There are also necrotrophic fungal pathogens with a broad host range, particularly those in the order of *Moniliales*, including *Monilinia fructicola*, *Sclerotinia sclerotiorum* (known as white mould) and *Botrytis cinerea* (known as gray mould, Figure 1). I will discuss our current knowledge of the mechanisms that enable *Botrytis cinerea* to infect >200 host plants to illustrate that the interaction between necrotrophic pathogens and their hosts is more subtle than previously anticipated.

Brief history of white and gray mould

The genus *Botrytis* was established as early as 1729. Fungi from the family of *Sclerotiniaceae* are among the earliest studied plant pathogens. In 1886, Anton De Bary [4] described the ability of *S. sclerotiorum*, a close relative of *B. cinerea*, to kill and macerate plant cells. He could microscopically distinguish both processes and could mimic these by administering cell-free extracts of fungal cultures to plant tissues. Similar studies were performed on *B. cinerea* in the first half of the 20th century (reviewed in [5]). Subsequent research on white and gray mould has often been descriptive and aimed at developing or evaluating methods for disease control, and progress in acquiring fundamental insight into the infection process has been slow. It has taken many decades to unravel, at the molecular level, the key factors involved in processes that De Bary [4] and William Brown [5] so elegantly described. Two reviews about the infection strategies of *S. sclerotiorum* have been published recently [6,7]. Compared with *B. cinerea*, *S. sclerotiorum* is less tractable to molecular-genetic studies because of its inability to produce conidia and the difficulty in achieving efficient transformation [8]. The development of molecular-genetic tools for *B. cinerea* [9] and the availability of an EST dataset [10] have been instrumental in elucidating the infection strategies of *B. cinerea*. Box 1 describes methods for targeted mutagenesis that can be applied for gene function analysis in *B. cinerea*. The rapid progress achieved over the past decade, as well as the recent determination of the genome sequences of strains B05.10 (assembled and annotated, available at http://www.broad.mit.edu/annotation/fungi/botrytis_cinerea/) and T4 (in progress; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=16118), has stimulated an increasing number of scientists to start working with *B. cinerea*, culminating in its emergence as a model for necrotrophic pathogens with a broad host range. The infection process of *B. cinerea* comprises several stages (Figure 2a), which I will sequentially discuss below in more detail.

Penetrating the host tissue

Pathogens landing on a leaf must penetrate the host surface, which is composed of cutin covered with wax. There is cytological [11] and molecular-genetic [12] evidence for *B. cinerea* developing appressoria (infection structures that differentiate on the surface and form a penetration peg that breaches the cuticle) (Figure 2b) [11]. Appressoria of the rice blast fungus *Magnaporthe grisea* penetrate by exerting an extreme physical pressure on the host tissue, resulting from high osmotic turgor in the

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Available online 17 April 2006

 1360-1385/$ - see front matter doi:10.1016/j.tplants.2006.03.005

Figure 1. Symptoms of gray mould infection on a rose flower following inoculation with dry conidia.

appressorium [13]. Appressoria of *B. cinerea* are probably not capable of penetrating by physical pressure alone because they do not contain a septum that seals the appressorium to separate it from the germ tube. Even though *B. cinerea* germlings contain melanin [14], the appressorium does not produce the highly melanized wall needed to sustain physical pressure resulting from high osmotic pressure as generated in *M. grisea* [13]. Nevertheless, the structure that *B. cinerea* develops on a leaf surface is considered to be an appressorium [12] because penetration requires a membrane-associated protein BcPLS1 [12], which is homologous to a protein that is essential for appressorium function in *M. grisea* [15]. *B. cinerea Bcpls1*-deficient mutants form an appressorium of normal structural appearance but it cannot penetrate an intact plant surface [12]. The reasons for the failure to penetrate remain to be resolved.

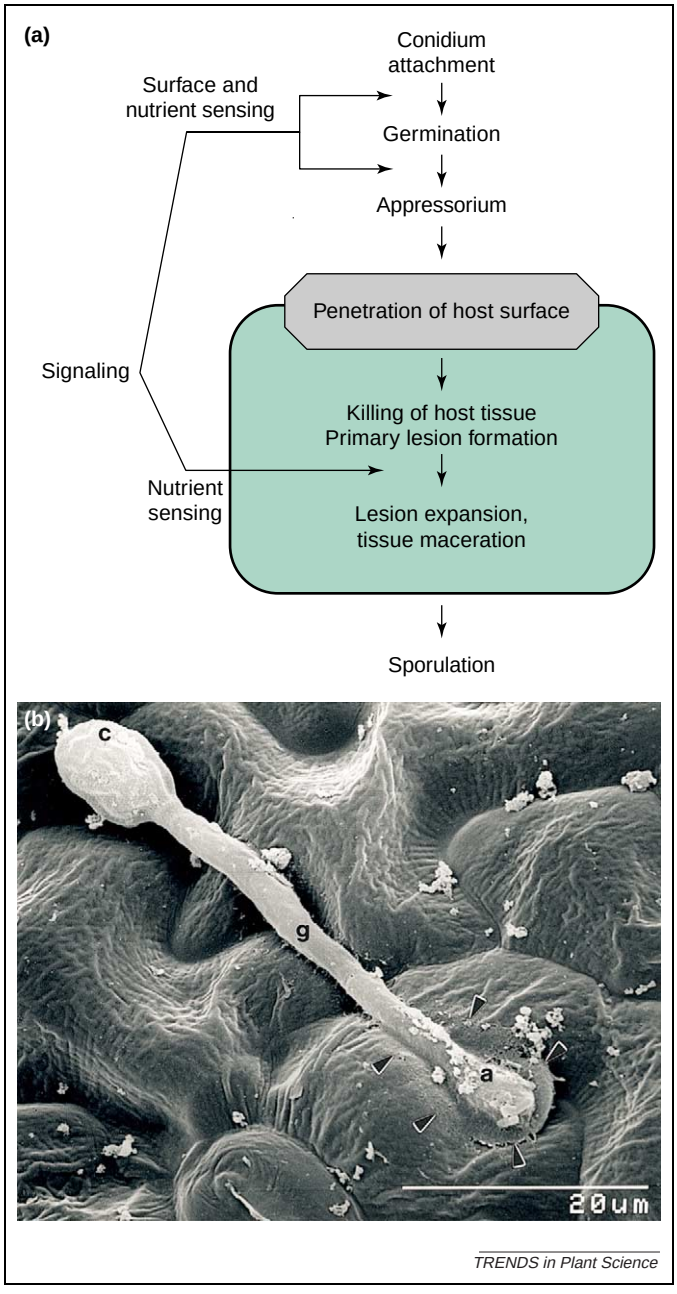

Figure 2. **(a)** Different developmental and physiological stages occurring during *Botrytis cinerea* infection of plants. These stages are discussed further in the text. **(b)** Germination and development of an appressorium on the surface of a tomato leaf. Abbreviations: a, appressorium; c, conidium; g, germ tube. Arrow heads indicate the demarcation of an area around the site of contact where the epidermal surface appears to be modified, possibly by polysaccharides covering the appressorium. Remnants of these polysaccharides remain visible as white flakes. Reproduced, with permission, from [76].

Box 1. Methods for gene function analysis in *Botrytis cinerea*

Targeted mutagenesis by gene disruption

An internal target gene fragment, devoid of translation start and stop codons, is inserted into a plasmid that contains an antibiotic selection marker cassette. The construct is transformed to the recipient fungus and integrates by homologous recombination (single crossover), resulting in a configuration that contains two truncated parts of the target gene interrupted by the plasmid with the selection marker cassette [69]. There is partial gene duplication.

Targeted mutagenesis by gene replacement

Flanking regions of the target gene are placed on both sides of an antibiotic selection marker cassette, either by cloning (e.g. [16]) or by overlap extension PCR [48]. The construct is transformed to the recipient fungus and integrates by homologous recombination (double crossover), resulting in a configuration in which the target gene is replaced by the selection marker cassette.

Random mutagenesis by T-DNA insertion

Agrobacterium tumefaciens can be used to transform fungi [70], including *Botrytis cinerea* [8]. Transformants frequently possess a single T-DNA copy that is inserted in the genome in a random manner (P. Tudzynski, personal communication). A collection of mutants can be generated, just as in *Arabidopsis* [71], and screened phenotypically. Mutants with a phenotype of interest must be analyzed to verify whether the mutation is tagged by the T-DNA insertion.

Gene silencing by RNA interference

Gene silencing is effective in plant pathogenic fungi [72,73], including *B. cinerea* (R. Patel, G.D. Foster and J. van Kan, unpublished) and might be particularly useful for genes that are important for viability. Simultaneous silencing of multiple genes is feasible using chimeric gene constructs [73]. Partial or inducible gene silencing strategies might enable the consequences of gene product depletion to be examined.

B. cinerea appressoria presumably secrete enzymes to breach the plant surface. Enzyme activities that were studied in this context include a cutinase and a lipase. Deletion of a cutinase gene and a lipase gene, either separately or together, did not detectably reduce virulence [16,17]. However, the genome sequence of *B. cinerea* appears to contain at least five additional cutinase genes and over a dozen lipase genes, as is also the case in *M. grisea* [18]. The role of cutinases and lipases in the pathogenesis of *B. cinerea* remains to be resolved. The tip of the penetration peg that breaches the cuticle generates H_2O_2 [11,19], which might assist in penetration by providing a substrate for oxidases that modify the cuticle, thereby rendering them easier substrates for decomposition. Upon breaching the cuticle, the penetration peg often grows into the anticlinal wall of the underlying epidermal cell [20]. The anticlinal cell wall is rich in pectin and the early invasion of the epidermal cell layer therefore involves the action of pectinases, specifically the endopolygalacturonase BcPG2. Mutants in which the *Bcpg2* gene was deleted showed a marked delay in primary lesion formation in bean and tomato [21], whereas mutants in other endopolygalacturonase genes did not show such a delay.

Host cell death requires the active participation of the pathogen and the host

B. cinerea possesses multiple tools that facilitate host cell death.

Phytotoxic metabolites

B. cinerea can produce a spectrum of phytotoxic metabolites of low molecular weight. The best-studied compound in this context is botrydial [22]. Botrydial was initially identified in liquid cultures and for a long time it was unclear whether it was produced during the infection at a level that was sufficient to exert toxic effects. Spectroscopic methods allowed the detection of botrydial in infected plant tissue [23] and the concentration was above the toxicity threshold. The biosynthetic pathway for botrydial has now been resolved [22] and a number of genes identified [24]. Deletion of one of these genes resulted in severe reduction of virulence in one strain, but not in two others [24], indicating that certain strains might strictly rely on botrydial to kill their host, whereas others can produce additional toxins, such as botcinolides [25].

Oxidative burst

B. cinerea actively triggers an oxidative burst during cuticle penetration [11,19] and primary lesion formation [26]. An oxidative burst occurs in many plant–pathogen interactions during a hypersensitive response that confers resistance to biotrophic pathogens [27]. In the case of a necrotroph such as *B. cinerea*, plant cell death is beneficial to the pathogen and leads to susceptibility [28]. Infection of bean and tomato leaves by *B. cinerea* results in massive accumulation of H_2O_2, both in the plant plasma membrane and in the extracellular sheath covering the surface of fungal hyphae [19,29]. *B. cinerea* infection can also lead to the accumulation of free radicals, in infected and uninfected tissue [30,31], culminating in lipid peroxidation [32–34] and depletion of antioxidants [34]. Altogether, these oxidative processes cause massive perturbation of the redox status in and around the infected tissue, thereby promoting disease progress [26]. One fungal enzyme that might play an important role in evoking the oxidative burst is a secreted superoxide dismutase (BcSOD1) that is expressed from the moment of penetration of the cuticle by the appressorium onwards. Deletion of the *Bcsod1* gene led to reduced virulence on multiple hosts [35]. The source of the superoxide that acts as substrate for BcSOD1 remains to be identified, but it might be produced by the plant.

Oxalic acid

B. cinerea and *S. sclerotiorum* are notorious producers of oxalic acid [36]. Oxalic acid is a convenient and versatile molecule for these pathogens (Box 2). UV-generated oxalate-deficient mutants of *S. sclerotiorum* are entirely non-pathogenic but oxalate-producing revertants are restored in pathogenicity [37]. A similar phenotype can be expected for oxalate-deficient mutants of *B. cinerea*. Genes potentially involved in oxalate biosynthesis in *B. cinerea* are being investigated (J. van Kan, unpublished).

Host-selective toxin

The production of HSTs has been reported for two specialized *Botrytis* species: *Botrytis fabae* and *Botrytis elliptica* [38,39]. One class of microbial phytotoxic proteins that has recently gained interest is the class of NEP1-like proteins [40]. Species in the genus *Botrytis* each contain two paralogous NEP1-like protein-encoding genes that have undergone positive, diversifying selection during evolution (M. Staats and J. van Kan, unpublished). Diversification of NEP1-like proteins might have altered or expanded their toxicity spectrum, thereby possibly contributing to speciation in the genus. Whether NEP1-like proteins act as HSTs in the infection of

Box 2. Versatile role of oxalic acid in the infection process

Oxalic acid (dicarboxylic acid) is a simple, versatile and potentially convenient molecule for pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum*, for several reasons:

- Oxalate is a strong acid. Acidification stimulates the production and activity of a spectrum of secreted enzymes [74]. Enzymes secreted by *B. cinerea* that are active in an acidic environment include pectinases [21,74], proteinases [74,75] and laccases [74].
- Oxalate possesses strong metal-chelating activity, particularly for calcium and copper ions. The majority of calcium in plant cells is stored in the cell wall, embedded in pectin in a so-called 'egg-box' structure, where the Ca^{2+} ion takes the position of the egg. Oxalate is able to extract calcium ions from the pectin, particularly when the polymer is partially nicked by the action of pectinases. Chelation of oxalate by calcium leads to the formation of cubic crystals (see Figure 2 in [76]). Removal of oxalate opens up the egg-box structure and makes it even more accessible to pectinases for further degradation.
- Oxalate can reduce the oxidative burst and concomitant defense responses in plant tissues [77] and it can directly trigger programmed plant cell death (M.B. Dickman, personal communication), which is important for the infection process of *Botrytis* spp. [28,39].

different *Botrytis* species is currently under investigation (J. van Kan *et al*., unpublished).

The infection of *Arabidopsis* by *B. cinerea* is promoted by and requires an active cell death programme in the host [28]. The infection induces an oxidative burst and hypersensitive cell death concomitant with nuclear condensation and expression of the HR-specific gene *Hsr203*. Growth of *B. cinerea* was suppressed in the HR-deficient *Arabidopsis* mutant *dnd1* and stimulated by HR triggered by simultaneous inoculation with an avirulent bacterium [28]. Activation of metacaspase activity and expression of *Hsr203*, both indicative of the occurrence of programmed cell death, were observed in *B. cinerea*-infected tomato [41]. The specialized pathogen *B. elliptica* induces programmed cell death in its host plant lily by means of a secreted proteinaceous HST [39]. Application of this protein to lily facilitated successful infection by other *Botrytis* species that are otherwise non-pathogenic on lily. Chemicals that stimulated programmed cell death promoted disease whereas chemicals that prevented or delayed programmed cell death conferred some level of protection to lily against *B. elliptica* infection [39]. A general pattern is emerging that an infection by *Botrytis* species requires the active participation of the host (i.e. the induction of programmed cell death). It is likely, but remains to be established, that several of the toxic compounds mentioned above are inducers of programmed cell death (apoptosis), rather than 'plain toxins' causing disorganized plant cell death (necrosis).

Decomposition and consumption of plant biomass

The ultimate goal of a necrotrophic plant pathogen is not to kill its host plant *per se* but to decompose plant biomass and convert it into fungal mass. A common feature shared by all plant species that are colonized by *B. cinerea* (i.e. dicots and corolliferous monocots) is their relatively high content of pectin in the cell wall. Plant species with low pectin contents are considered poor hosts for *B. cinerea*. It was therefore postulated that the host preference of *B. cinerea* reflects its possession of an effective pectinolytic machinery [42]. *B. cinerea* contains at least six endopolygalacturonase genes [43], which show differential regulation *in vitro* [44]. Expression of the endopolygalacturonase gene family during host infection depends on the plant species, tissue type and incubation conditions applied [45], suggesting some degree of versatility in the pectinolytic complex. Deletion of two endopolygalacturonase genes, separately, resulted in pronounced reduction of virulence on multiple host plants [21,46], whereas deletion of four other endopolygalacturonase genes had no notable effect on virulence (I. Kars *et al.*, personal communication).

The role of pectin methylesterases (PMEs) is controversial. It is generally assumed that endopolygalacturonases do not (efficiently) depolymerize highly methylated pectin, hence demethylation by PMEs facilitates the action of endopolygalacturonases. This predicts that PMEs are important for fungal growth when highly methylated pectin is the sole carbon source and for virulence on plant tissues with highly methylated pectin (such as leaves), but not on tissues with low pectin methylation (such as fruit). The phenotype of a *Bcpme1*-deficient mutant in one strain of *B. cinerea* supports this hypothesis [47]. However, results with single and double mutants in two *Bcpme* genes, including the same *Bcpme1* gene in a different strain, do not support this hypothesis [48]. Surprisingly, the wild-type strain and the *Bcpme*-deficient mutants grew better on 75% methylated pectin than on non-methylated polygalacturonic acid, suggesting that pectin demethylation by PMEs is not important for its depolymerization *in vivo* [48].

Other cell wall degrading enzymes produced by *B. cinerea*, such as cellulases and hemicellulases, have only recently been studied. Deletion of a cellulase gene did not affect virulence [49], whereas the deletion of a β-1,4-xylanase gene delayed lesion formation and reduced lesion outgrowth by more than 70% [50].

Infection requires intricate sensing and signaling

The infection of a host plant is a highly regulated process in which the pathogen must decide whether or not to germinate, when and where to develop an infection structure (appressorium) or produce enzymes and metabolites. There must be continuous sensing of the physical and chemical environment to make the correct decisions (Figure 2a). Sensing and signaling therefore play an important role in all stages of infection [51]. For example, *M. grisea* can germinate on hydrophilic as well as on hydrophobic surfaces but the formation of an appressorium is favored by a hydrophobic surface [52]. Germination of *B. cinerea* conidia on hydrophobic surfaces can occur in water, whereas germination on hydrophilic surfaces requires nutrients [53]. The process is also influenced by surface hardness [54], indicating that *B. cinerea* effectively senses the chemical and physical qualities of its environment during germination.

One problem of studying the role of signaling pathways by means of targeted gene deletion is the potential pleiotropy of mutations. Mutants in signaling pathways show defects in growth, conidiation, sclerotia formation or enzyme production *in vitro*. This was particularly evident in the first described *B. cinerea* signaling mutant, which was deficient in the MAP kinase gene *Bmp1* [55]. This mutant was severely affected in growth rate, spore production and production of several secreted enzymes. Consequently, it was difficult to conclude whether the non-pathogenic character [55] was due to inappropriate signaling or to pleiotropic physiological effects on fitness. More recent experiments have yielded a *Bmp1*-deficient mutant in a different strain that showed few pleiotropic defects *in vitro* and yet remained unable to infect host plants because of its inability to penetrate and invade the host, even when tissue was wounded [54].

Several studies have focused on G-protein-mediated signaling in *B. cinerea.* The genome contains three genes encoding Gα-proteins named BCG1, BCG2 [56] and BCG3 [54]. Deletion of the *bcg1* gene severely reduced virulence whereas deletion of the *bcg2* gene did not [56]. *bcg3*-deficient mutants were disturbed in nutrient-induced germination and sporulation and were defective in penetration, resulting in a delay in primary lesion

formation. However, the growth rate of expanding secondary lesions of *bcg3*-deficient mutants was similar to that of the wild type [54]. In mammals and yeast, G-protein signaling usually requires the upstream perception of an environmental stimulus by a G-protein coupled receptor. A gene encoding such a putative receptor, named BTP1, was functionally analyzed. Deletion of the *btp1* gene led to the altered regulation of several genes, but the *btp1*-deficient mutants were not reduced in virulence [57]. Target genes that are under downstream control of the Gα-protein BCG1 include proteinases, glycohydrolases, cytochrome P450 monooxygenases, sugar transporters and a polyketide synthase [58]. Gα-mediated signaling acts via the cAMP pathway. The adenylate cyclase is essential for full virulence of *B. cinerea* [59] but there are also processes in *B. cinerea* that are regulated by BCG1 via cAMP-independent pathways [58], such as the calcium signaling pathway acting via calcineurin [60]. Target genes regulated by calcineurin comprise the endopolygalacturonase gene *Bcpg1* and a group of cytochrome P450 monooxygenase genes [60], including the *Bcbot1* gene, which is required for biosynthesis of the phytotoxin botrydial [24]. Both Gα-protein BCG1 and calcineurin regulate the *Bcbot1* gene [24] – this is the first evidence of converging signaling pathways in *B. cinerea*.

Perspectives

Botrytis cinerea uses multiple strategies to subdue its host plant(s). So far, the 'silver bullet', which is crucial for an infection to succeed on all host tissues without affecting fungal growth *in vitro*, has not been identified and might not exist. Certain virulence factors can be important for one isolate on one particular host species, but they might be dispensable on another host species, or they might be dispensable for a different isolate [24,47,48]. I prefer not to use the term 'redundancy' in such cases because these factors might still be relevant for virulence on certain host species or even only on certain tissue types. This situation provides the fungus with multiple tools to cause 'overkill'.

Increasing our knowledge of the infection strategies of necrotrophic pathogens in general, and more specifically *B. cinerea*, should be helpful in designing novel rational disease control strategies. In the 1990s, the emphasis of control strategies was on transgenic plants containing antifungal compounds of heterologous origin (e.g. [61]). However, necrotrophic pathogens such as *B. cinerea* are well adapted to surviving in a hostile environment full of antifungal compounds. The wide host range of *B. cinerea* relies on the ability of the fungus to counteract the toxic action of a broad range of plant metabolites, either by its ability to detoxify (e.g. [62]) or secrete [63] antifungal compounds. Such strategies are therefore less likely to be successful. More recent attempts to delay disease by expressing polygalacturonase inhibitor proteins (plant proteins that inhibit fungal endopolygalacturonases) have been partially successful [64–66]. Furthermore, transgenic plants expressing enzymes that degrade oxalate produced by the pathogen have been successful against the white mould *S. sclerotiorum* (e.g. [67]). In the future it might be more rewarding to focus on strategies that prevent cell death. Infection by necrotrophic pathogens can largely be prevented by abolishing plant cell death via the expression of anti-apoptotic genes from animals [68]. However, such a strategy might impede HR mechanisms that confer resistance to biotrophic pathogens. It is therefore crucial to determine the precise order of events during these two types of plant cell death and identify the steps that distinguish HR from necrotroph-inflicted (programmed?) cell death. Tweaking the cell death process, either by chemicals, or by the application of transgenes or by classical breeding might enable plants with an enhanced resistance level to *B. cinerea* to be developed without compromising resistance to biotrophs. I am optimistic about the prospects of designing such a strategy in the coming decade.

Acknowledgements

I am grateful to Bettina and Paul Tudzynski, Matthias Hahn, Günther Doehlemann, Peter van Baarlen and Jos Raaijmakers for critical reading of the manuscript.

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Plant Science meetings in August 2006

ASPB Plant Biology 2006
5–9 August 2006
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http://www.aspb.org/meetings/pb-2006/

Agricultural Biotechnology International Conference (ABIC 2006)
6–9 August 2006
Melbourne, Australia
http://www.abic2006.org/index2.html

11th IAPTC&B Congress: International Congress of Plant Tissue Culture and Biotechnology
13–18 August 2006
Beijing, China
http://www.genetics.ac.cn/IAPTCS1/Intvitation.htm

Tropical Crop Biotechnology Conference
16–19 August 2006
Cairns, Australia
http://www.tcbc2006.com.au/

8th International Congress of Plant Molecular Biology (ISPMB)
20–25 August 2006
Adelaide, Australia
http://www.sallyjayconferences.com.au/ispmb2006/program.htm

Photosynthesis in the Post-Genomic Era
20–26 August 2006
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