Characterization of *Botrytis*–plant interactions using PathTrack[®]—an automated system for dynamic analysis of disease development

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SUMMARY

The measurement of disease development is integral in studies on plant–microbe interactions. To address the need for a dynamic and quantitative disease evaluation, we developed PathTrack[®], and used it to analyse the interaction of plants with *Botrytis cinerea*. PathTrack[®] is composed of an infection chamber, a photography unit and software that produces video files and numerical values of disease progression. We identified a previously unrecognized infection stage and determined numerical parameters of pathogenic development. Using these parameters, we identified differences in disease dynamics between seemingly similar *B. cinerea* pathogenicity mutants, and revealed new details on plant susceptibility to the fungus. We showed that the difference between the lesion expansion rate on leaves and colony spreading rate on artificial medium reflects the levels of the plant immune system, suggesting that this parameter can be used to quantify plant defence. Our results shed new light and reveal new details of the interaction between the model necrotrophic pathogen *B. cinerea* and plants. The concept that we present is universal and may be applied to facilitate the study of various types of plant–pathogen association.

Keywords: *Botrytis cinerea*, disease measurement, image analysis, necrotrophic development, plant resistance.

INTRODUCTION

The final step in the evaluation of the effect of specific elements, such as molecules, genes or environmental factors, on the interaction between plants and pathogens is the assessment of disease progression. Accurate measurement of disease levels is extremely important, e.g. for the evaluation of mutants, determination of the effects of growth conditions and treatments on disease progression, and comparison of field isolates. Unlike the impressive progress made in molecular and genomic analyses of plant–patho-

gen interactions, relatively little progress has been made in the development of methods for phenotypic analyses. A new approach, generally referred to as ‘phenomics’, aims to provide the means for high-throughput phenotype analysis (Furbank and Tester, 2011; Houle *et al.*, 2010; Junker *et al.*, 2014). The robust and very expensive systems used for the study of phenomics are designed mainly for high-throughput evaluation of plant development and are less suitable for disease analysis. In practice, disease levels are still estimated mainly manually by the scoring of arbitrary parameters, such as the number and size of lesions, plant weight, number of pustules, and so on. These methods suffer from several drawbacks: to name a few, they are prone to user bias, the results are qualitative or, at best, semi-quantitative, and the information is limited to specific time points. In addition, the procedures can be tedious and labour intensive, especially when trying to measure disease progression through time.

More recently, non-invasive methods that combine RGB (red, green, blue) and hyperspectral imaging with image analysis software have been applied to measure disease symptoms (Barbedo, 2013; Bardsley and Ngugi, 2012; Bock *et al.*, 2010; El-Hallem, 2012). Although helpful in scoring disease levels, these systems still come up short in several aspects. Primarily, the available methods are designed to collect data from single images, and hence the information remains static and values are relative. Because the user has to measure each leaf or sample separately, the number of scanned images scanned is restricted and the results are limited in scope. Most significantly, the information provides a snap shot, rather than a dynamic picture, of the disease. As such, the current disease measurement methods are not suitable, e.g. for the quantification of the contribution to plant defence of specific factors, such as a resistance gene, metabolic pathway, nutritional conditions or environmental parameters, and do not provide details of the dynamics of disease progression. The ability to measure such parameters is essential in order to translate the rich genomic and molecular information on plant defence and pathogen virulence factors into a clearer and more detailed picture of plant–pathogen interaction. Likewise, quantitative dynamic data of disease development are necessary to pinpoint the key genes/pathways, to construct the hierarchical order of

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defence genes, and to generate a uniform baseline for the measurement and comparison of diseases.

To address these needs, we have developed the PathTrack[®] package. The system is composed of a transparent chamber in which inoculated plants are placed, a computer-controlled time-lapse camera, and software for automated extraction and analysis of the data. The analysis is conducted simultaneously on large datasets (hundreds of images); the output includes video files and values of a set of parameters that describe disease progression in a numerical manner. Here, we introduce PathTrack[®] and present new details on the interaction between the grey mould fungus *Botrytis cinerea* and host plants obtained using this system.

RESULTS

The PathTrack[®] system

PathTrack[®] enables high-resolution, accurate tracking of necrosis progression on attached and detached leaves, as well as tracking of fungal growth on any other type of surface or growth medium. The system consists of a hardware component and a software package. The hardware includes a transparent infection chamber that can be sealed to maintain moisture, a set of leaf holders and a computer-operated camera (Fig. 1). The software performs continuous tracking and quantitative analysis of necrosis development. The necrotic area in each image is detected by custom-developed algorithms, and specific growth parameters are analysed as a function of time. For growth on medium, the software detects the hyphae and calculates the colony radius. A more complete description of the image acquisition and analysis systems is given in Experimental Procedures. Details of the software and tracking algorithms are provided as Supporting Information (Fig. S1).

To quantify the growth dynamics, the main parameters analysed are the growth radius and growth rate. The growth radius is the necrosis average radius, calculated by $\sqrt{\frac{\text{Area}}{\pi}}$, and the growth rate is the time derivative of the growth radius. The rationale for choosing these parameters stems from the fact that fungal colonies spread radially from a single centre, as demonstrated by growth on potato dextrose agar (PDA) medium (Video 1). The detection accuracy of the system is limited by stability, primarily caused by leaf movement and light fluctuations. In addition, the level of colour change in the leaf, from green to different shades of brown, will set the detection sensitivity threshold. To increase the detection accuracy, we used leaf holders, softened light and kept the infection chamber in a darkened compartment. From the software end, a simple initial set-up procedure is included for calibration and error reduction. In addition, the images are aligned and their brightness is equalized before the detection procedure (for details, see Supporting Information, Fig. S2).

Definition and analysis of disease development parameters by PathTrack[®]

According to current paradigm, infection by *B. cinerea* includes an early stage, in which the fungus causes small local necrotic spots in the infected tissue, followed by a stage of spread (Shlezinger *et al.*, 2011a, b). We inoculated bean leaves with a spore suspension and used PathTrack[®] to obtain information on the kinetics of the infection process and on the transition from the first to the second infection stage. The time-lapse video reveals a clear 'break' stage, in which a necrotic lesion starts to spread around the initial inoculation spot (Video 2). Until this stage, sporadic micro-lesions develop within the initial inoculation site and eventually coalesce to a single or a few larger necrotic lesions that include most of the area under the inoculation droplet (Video 3). Following the break stage, infection seems to progress at a relatively constant average rate, with periodic halts, primarily when the fungus reaches a leaf vein.

In order to characterize disease dynamics in a numerical way, we analysed the growth rate data. Despite the seemingly constant average spreading rate that followed the break stage, data analysis revealed a previously unrecognized intermediate stage between the break stage and lesion expansion. According to these analyses, we defined four specific parameters of disease progression: break time (h), intermediate-stage duration (h), intermediate stage acceleration (mm/h^2), and late-stage lesion average expansion rate (mm/h). The break stage and the late-stage lesion expansion rate are rather uniform, whereas the intermediate-stage parameters are more variable (Fig. 2). The intermediate stage has not been reported previously. It is marked by the spread of necrosis out of the droplet boundaries (break time) and ends when the lesion spreads at a close to constant rate. During the intermediate stage, necrosis spread accelerates in a close to linear fashion until it reaches the final spreading rate. Therefore, the lesion expansion rate (at the late stage) is determined by the intermediate-stage acceleration rate and duration. The average lesion expansion rate remains until the end of the infection process with occasional fluctuations, which are caused, at least in part, by spatial changes in leaf architecture, mainly before (deceleration) and after (acceleration) the crossing of leaf veins (Video 4).

As a reference point to the internal maximum possible *B. cinerea* growth rate, we documented the growth of the fungus on PDA medium and calculated the mycelium area as a function of time (Video 1). The average fungal growth rate on PDA was $0.584 \pm 0.024 \text{ mm/h}$, three-fold higher than the average lesion expansion rate ($0.195 \pm 0.020 \text{ mm/h}$) on bean leaves (Fig. 2). In addition to a much higher growth rate, growth on PDA is highly stable compared with growth on a leaf, which varies considerably during each infection course and between different experiments. The variable growth rate on a leaf compared with stable growth on PDA shows that the fluctuation in growth rate during the

Fig. 1 Main components of the image acquisition system. The system includes a stand (top left), leaf holders of different sizes (middle left) and a transparent infection chamber (right). The inoculated leaf is placed on the bottom leaf holder and then a top leaf holder is placed on the leaf to keep it flat and motionless. Images are captured with a Nikon, New York USA D-90 camera.

Fig. 2 Main stages and growth dynamic parameters of *Botrytis cinerea* wild-type on bean leaves. The graph shows the changes in lesion growth rate during the course of infection. The main stages of infection are marked with arrows. The inset graph shows the growth radius of the lesion over time. The table shows the mean values ± standard error of the disease parameters: 'break' is the time until the initiation of lesion spread; the intermediate-stage duration is the time from break until the stabilization of lesion expansion rate; the intermediate acceleration is lesion expansion acceleration rate; the late expansion rate is the average lesion expansion rate at the late stage.

course of infection is caused by physical barriers, such as veins, and possibly by spatial differences in the leaf chemical composition, such as sugars and anti-fungal metabolites. The difference in average growth rate on PDA and on a leaf reflects the suboptimal growth conditions that are present on leaves, and may be modulated by plant defence.

Measurement of plant defence

As pointed out above, at least three different sources of variance might contribute to the difference in average growth rate of the fungus on a leaf and on PDA: physical characteristics of the tissue, nutritional factors and plant defence. In order to estimate the net

Fig. 3 Comparison of disease symptoms on attached, detached and microwave-killed bean leaves. All leaves were inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL. Inoculation of detached leaves was performed on one of the first two leaves using the leaf holder. Detached leaves were placed in a Petri dish on a wet filter paper and incubated in the PathTrack[®] inoculation chamber. Microwave-treated leaves were placed in a Petri dish on a wet filter paper and then heated in a microwave for 5 s. Following microwave treatment, the leaves were inoculated and then incubated in the infection chamber. A water-soaked spot is visible in the dead leaf at 24 h post-inoculation (hpi) (arrows). The spot expands rapidly and symmetrically (36 h); fungal mycelium covers the entire spot (48 h) and keeps expanding at a constant rate (see also Video 5).

contribution of plant defence to the inhibition of fungal spread, we compared disease development in living and microwave-killed leaves. Microwave treatment eliminates active plant defence and destroys leaf architecture, and therefore the rate of spread on microwave-treated leaves is only restricted by nutrient availability and the possible presence of residual fungus-toxic plant molecules. Because the microwave treatment is performed on detached leaves, we used untreated detached leaves as a control. Unlike the brown necrosis on living tissue, the microwaved leaves did not develop clear necrosis and therefore it was not possible to determine a break time and intermediate-stage parameters. Instead, the leaf tissue around the inoculation site first changed to a water-soaked, darker green spot, but without any apparent external damage or necrosis (Fig. 3, Video 5). At a certain point, around 40 h post-inoculation (hpi), when the diameter of the water-soaked spot reached a size of several centimetres, the colour of the tissue changed to brown, together with the development of abundant aerial hyphae. The average expansion rate on the dead leaf was 0.369 ± 0.044 mm/h, which is 63% of the average colony expansion rate on PDA, and almost twice as fast as the late-stage average lesion expansion rate on attached bean leaves.

Unexpectedly, the entire process on control living detached leaves was slower than disease development on attached leaves, resulting in the formation of a delayed and smaller lesion on living detached leaves (Fig. 3, Table S1, see Supporting Information). Because there is no structural change in detached relative to attached leaves, the differences in kinetics of infection on living detached leaves might be attributed, at least in part, to the stimulation of the defence response caused by wounding of the leaf by the detachment process, and hence higher levels of resistance in the detached leaves (Degani, 2014; Degani *et al.*, 2004).

If plant resistance accounts for a significant proportion of fungal growth inhibition, the parameters of disease dynamics, as measured by PathTrack[®], might be used to estimate differences in defence levels between different plant species. To address this question, we used PathTrack[®] to analyse disease parameters on *Nicotiana benthamiana*, a solanaceous species that is commonly used as a host in studies of interactions with pathogens, including *B. cinerea*. As expected, disease development on *N. benthamiana* had the same general stages as those found on bean, including a break time, an intermediate lesion acceleration stage and a late stage with a constant lesion expansion rate (Video 6). However, parametric values of disease development differed from those on bean leaves (Fig. 4). The break time on *N. benthamiana* was slightly shorter, whereas the late-stage lesion expansion rate was slightly reduced compared with bean leaves. More significantly, however, the duration of the intermediate stage was almost doubled and, together with the reduced late-stage lesion expansion rate, resulted in a dramatically reduced intermediate-stage acceleration rate, from 0.017 mm/h² on bean to 0.006 mm/h² on *N. benthamiana*. These results demonstrate the utility of the PathTrack[®] system to identify specific parameters that differ significantly between two pathosystems, which might appear similar by lesion size.

Measurement of fungal pathogenicity

Botrytis cinerea pathogenicity mutants have been classified into four pathogenicity types according to lesion size and the stage at which infection is blocked (Giesbert *et al.*, 2012). We used PathTrack[®] to characterize *B. cinerea* strains that represent

Fig. 4 Main stages and growth dynamic parameters of *Botrytis cinerea* wild-type on *Nicotiana benthamiana* leaves. The graph shows the changes in lesion growth rate during the course of infection. The break and end of the intermediate stage are marked with arrows. The inset graph shows the growth radius of the lesion over time. The table shows the mean values $\pm$ standard error of the disease parameters.

different types of pathogenicity mutants (Table 1), and monitored disease progression with the system.

First, we reproduced the classical infection lesions by each strain and determined the growth rate on PDA, as a reference to the internal maximum possible growth rate of each strain. The mutants belonged to two main types: $\Delta bcbir1$ and $\Delta bcnoxa$ were categorized as type 3, which is characterized by reduced lesion size, and $\Delta bcnoxb$ and $ox-bcmcl1$ that were categorized as type 2, which is characterized by a highly restricted lesion (Fig. 5A). Despite these drastic changes in lesion size, except for a 20% reduced growth rate of $\Delta bcbir1$, all other pathogenicity mutant strains exhibited growth rates similar to growth rate of the wild type on PDA (Table 1). Next, we inoculated bean leaves with spores of the different strains and used PathTrack[®] to produce infection profiles (Video S1, see Supporting Information). Remarkably, PathTrack[®] analysis revealed distinct changes in disease aetiology, which highlighted the critical points during disease development at which each of the mutants was affected. For example, $\Delta bcnoxa$ and $\Delta bcbir1$ both belong to type 3 pathogenicity mutants. However, in $\Delta bcnoxa$, the intermediate-stage acceleration and final-stage lesion expansion rates were dramatically reduced, but the break time and intermediate-stage duration were almost unchanged, whereas, in $\Delta bcbir1$, the reduced lesion size resulted from the combination of a small delay in the break time

and slightly reduced intermediate-stage acceleration and late-stage lesion expansion rates (Fig. 5B). The type 2 mutants also differed significantly in the affected stages. The $ox-bcmcl1$ mutant showed a significantly delayed break time and shorter and slower, but highly variable, intermediate-stage parameters, whereas the most significant change in $\Delta bcnoxb$ was a drastically reduced acceleration rate at the intermediate stage. As expected, the late-stage (lesion expansion) was abolished in both type 2 strains. The combined results of growth on PDA and disease kinetics highlight the critical stages at which each mutant is affected. This information is useful, for example, for the evaluation of the importance of putative virulence factors for coping with the plant immune system.

We used $\Delta bcbir1$ to examine the ability of the PathTrack[®] system to discriminate between a general growth defect and specific role in pathogenicity. Infection of bean leaves by $\Delta bcbir1$ is characterized by a moderately reduced lesion size, possibly reflecting the reduced ability of this strain to cope with plant defence (Shlezinger *et al.*, 2011a, b). However, as shown in Table 1, the growth rate of $\Delta bcbir1$ is also moderately reduced on PDA, which might account for the reduced lesion size. As described earlier, inoculation of detached living leaves with the wild-type strain produced smaller lesions (Fig. 3) and all the disease parameters were reduced compared with those of attached leaves (Table S1),

Table 1 *Botrytis cinerea* strains used in this study.

Strain	Type*	Growth rate on PDA (mm/h)	Description	Reference	Source
B05.10	4	0.584 $\pm$ 0.024	Wild-type	Quidde <i>et al.</i> (1999)	P. Tudzynski, Muenster University, Muenster Germany
$\Delta bcbir1$	3	0.480 $\pm$ 0.023	Chromosomal passenger complex and anti-apoptotic gene	Shlezinger <i>et al.</i> (2011aa, b)	A. Sharon
$\Delta bcnoxa$	3	0.590 $\pm$ 0.027	NADPH oxidase catalytic unit A	Segmüller <i>et al.</i> (2008)	P. Tudzynski
$\Delta bcnoxb$	2	0.600 $\pm$ 0.015	NADPH oxidase catalytic unit B	Segmüller <i>et al.</i> (2008)	P. Tudzynski
$ox-bcmcl1$	2	0.613 $\pm$ 0.016	Methyl isocitrate lyase	A. Sharon, unpublished data	A. Sharon

*Pathogenicity type according to Giesbert *et al.* (2012).

Fig. 5 Lesion formation and disease parameters on bean leaves caused by *Botrytis cinerea* pathogenicity mutants. (A) Leaves were inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL of the specified strain. Plants were incubated in a humid chamber and images were taken at 72 h post-inoculation (hpi). Bars show the average lesion radius $\pm$ standard error. Scale bar, 1 cm. (B) Parameters of disease progression: (a) break time; (b) intermediate-stage duration; (c) intermediate-stage acceleration rate; (d) late-stage lesion expansion rate. wt, wild-type B05.10; $\Delta bcbir1$, a knockdown strain of the anti-apoptotic gene *bcbir1*; $\Delta bcnoxA$, a deletion strain of the NADPH oxidase catalytic subunit A gene *bcnoxA*; $\Delta bcnoxB$, a deletion strain of the NADPH oxidase catalytic subunit B gene *bcnoxB*; *ox-bcmcl*, over-expression strain of the methyl isocitrate lyase gene *bcmcl1*. $\Delta bcbir1$ and $\Delta bcnoxA$ show a class 3 infection type, and $\Delta bcnoxB$ and *ox-bcmcl* show a class 2 infection type. Data are the average of four to six measurements; bars indicate the standard error.

probably reflecting elevated defence in the detached leaves. Therefore, if the development of the $\Delta bcbir1$ strain is delayed because of a reduced ability to cope with the plant immune system, then, compared with the wild-type strain, the disease parameters will be more significantly affected on detached leaves than on attached leaves. If, however, the reduced lesion size reflects a reduced growth rate *per se*, the difference between wild-type and $\Delta bcbir1$ strains would be expected to be similar on attached and detached leaves. In accordance with the expected reduced ability of the $\Delta bcbir1$ strain to cope with plant defence, the differences in disease parameters between the $\Delta bcbir1$ and wild-type strains were increased on detached leaves (Fig. 6, Video S2, see Support-

ing Information). Interestingly, the differences between wild-type and $\Delta bcbir1$ were observed only in the intermediate- and late-stage parameters, whereas the break time was extended on detached leaves in both strains, but the differences between the strains remained similar. In both strains, the intermediate- and late-stage parameters were delayed or reduced on detached leaves compared with attached leaves; however, the differences between the disease parameters of the wild-type and $\Delta bcbir1$ strains were increased considerably. The duration of the intermediate stage on detached leaves was almost twice as long in the $\Delta bcbir1$ strain than in the wild-type strain, compared with no or only a small difference on attached leaves, and the late-stage

Fig. 6 Growth dynamic parameters of *Botrytis cinerea* wild-type and $\Delta bcbir1$ on detached bean leaves. Detached leaves were placed in a Petri dish on a wet filter paper and then incubated in the PathTrack[®] inoculation chamber. (a) Growth radius. (b) Growth rate.

lesion expansion rate of the $\Delta bcbir1$ strain was reduced to half that in the wild-type strain, compared with a reduced expansion rate of only ~20% on attached leaves. These results not only support the role of *bcbir1* in coping with plant defence, but also highlight the stages at which the BcBir1 protein is necessary for infection.

DISCUSSION

The availability of complete genome sequences in many pathogens and in an increasing number of plants has led to the identification of numerous genes and proteins that are potentially involved in the regulation of plant–microbe interactions. However, in many cases, despite compelling biochemical and molecular evidence, disease evaluation fails to show a clear effect. In these cases, the lack of a phenotype is frequently attributed to reasons such as redundancy, epistatic relationships or unknown conditions at which a certain gene is involved. Although all these reasons are possible and should be considered, it is also possible that the failure to determine a clear phenotype results from the insufficient resolution of disease assessment procedures. In particular, most

of the currently available methods provide a snap shot of disease levels, but fail to detect valuable information related to the kinetics of disease development, which might vary in certain cases, even if the end result is similar. To address the need for continuous and quantitative disease measurement, we developed PathTrack[®], and used it to analyse the interaction between the model necrotrophic pathogen *B. cinerea* and plants. We show here that the *B. cinerea* infection process consists of several stages, including a previously unrecognized interphase between the initial and late stages. Furthermore, using PathTrack[®], we were able to identify specific differences in disease development between seemingly similar *B. cinerea* pathogenicity mutants. Finally, the system translates the visual input into a set of numerical values, which can be used as comparable parameters and as a quantitative measure of fungal virulence and plant resistance.

Our concept in developing PathTrack[®] was to produce a non-invasive, automated, low-cost system for live imaging and quantitative measurement of disease development. With these criteria in mind, we chose necrosis as the measurable parameter, as it is a good indicator of diseases caused by necrotrophic pathogens and can be easily captured by all types of imaging equipment, including home cameras. In this version of the system, we used a Nikon D90 camera (Nikon Inc, New York, USA) with two types of lens, which were used to obtain information from a relatively large area (18-105vr lens) or more detailed information from a smaller area (105/2.8vr lens). Depending on the needs and budget, one may consider using different types of imaging equipment, such as higher magnification lenses, hyper- and ultra-spectral cameras and sensors, etc. (Fiorani and Schurr, 2013). If hyper-spectral sensors are used, they may serve for the measurement of other parameters in addition to necrosis, which might reveal hidden aspects, such as plant responses that precede the appearance of necrosis. It is rather intuitive that the combination of multiple parameters over time would allow for a more detailed and precise analysis of the disease process.

Following the initial analyses of infected bean leaves, we defined three distinct stages of disease progression: an early stage in which there is no lesion spread, an intermediate stage in which lesion spread starts and the spreading rate increases, and a late stage in which the lesion expands at a close to constant rate. The intermediate stage has not been recognized previously and therefore changes the current paradigm of *B. cinerea* infection. One of the most important elements in the PathTrack[®] concept was to develop numerical values, which can be used as a stand-alone measure of disease intensity. Following the recognition of the three stages of disease development, we analysed the length of time and necrosis radial data of each stage, and extracted four different parameters that can be used to evaluate disease progression: break time, which characterizes the length of the first stage, intermediate-stage duration and acceleration rate, and late-stage

lesion expansion rate. Among these parameters, the break time and lesion expansion rate were relatively uniform between similar experiments, whereas the intermediate parameters were more variable, probably because of the relatively shorter time of this phase. Nevertheless, the intermediate phase always exists on leaves, whereas both the initial and intermediate stages do not exist on solid growth medium. Furthermore, the lesion expansion rate, which can be compared with colony spread on a solid growth medium, is significantly slower and less uniform than colony spread on artificial medium because of interruption by leaf architecture. Therefore, the four different parameters are specific to pathogenic development and can be used as a numerical estimate of disease development. With respect to the intermediate phase parameters, they are more prone to fluctuation because of the relatively short duration of this stage, and spatial differences on the leaf. It is likely that more accurate values might be obtained by increasing the quality of image acquisition, in particular by improving leaf stability and providing better control of humidity conditions inside the chamber.

The power of the numerical disease parameters is demonstrated by the improved characterization of previously characterized *B. cinerea* pathogenicity mutants. PathTrack[®] analysis of mutants that had been categorized previously in the same pathogenicity class (Giesbert *et al.*, 2012) revealed fundamental differences in the stages at which each of the specific mutants was affected. Furthermore, PathTrack[®] analysis indicated that the reduced virulence in the $\Delta bcbir1$ mutant is caused by a specific defect in the ability of the mutant to cope with plant defence, and not a general phenomenon of slow growth. In this case, combined analysis of attached and detached leaves highlighted the intermediate-stage duration and acceleration as the critical parameters in which the $\Delta bcbir1$ strain is affected. During interaction with plants, *B. cinerea* is exposed to plant-induced apoptotic-like programmed cell death (A-PCD), which halts fungal development and spread (Shlezinger *et al.*, 2011a, b). The ability of the fungus to recover and initiate the spreading phase depends on an anti A-PCD mechanism, in which BcBir1 plays a central role (Shlezinger *et al.*, 2011a, b, 2014). Accordingly, the smaller lesion size produced by $\Delta bcbir1$ was interpreted as reduced virulence caused by elevated A-PCD during the early infection stage (Shlezinger *et al.*, 2011a, b). However, we have shown here that the colony growth rate of $\Delta bcbir1$ on rich artificial medium (PDA) is reduced by 20% compared with the wild-type growth rate, which raises the possibility that the reduced lesion size might reflect the slow growth of the mutant rather than the impaired ability to cope with plant defence. PathTrack[®] analysis supports a specific role of BcBir1 in coping with plant defence, because it shows that the mutant is specifically affected in the intermediate-stage parameters, and that this defect is much more pronounced on detached leaves, which are less susceptible to infection.

A main driver behind PathTrack[®] was the need for unbiased, absolute parameters that can be used to describe and evaluate plant–pathogen interactions. Having such parameters, it is now possible to compare different pathosystems and to extract the net effect of specific factors, such as plant resistance genes, fungal pathogenicity genes and environmental conditions. For example, comparison of *N. benthamiana*, and attached and detached bean leaves, shows that the break time is earlier on *N. benthamiana* and delayed on detached compared with attached bean leaves. However, the rest of the parameters are highly similar between *N. benthamiana* and detached leaves, and both differ considerably from the parameters of attached leaves (Table S1). These results hint at the increased susceptibility of *N. benthamiana* to *B. cinerea* at the early stage compared with bean, but reduced susceptibility in the following stages. Another example is the comparison of disease development on living and dead leaves. To evaluate the effect of the entire defence system on disease progression, we killed bean leaves by treatment for 5 s in a microwave. The video analysis showed that the entire course of disease was modified on killed leaves, including a lack of initial necrosis, which precluded the ability to determine a break time and intermediate-phase parameters. However, we could measure the expansion rate, which was twice as fast as the lesion expansion rate on a living plant. Furthermore, the expansion of the infected area on a dead leaf was smooth and continuous, similar to colony growth on PDA, and unlike the fragmentary and variable expansion of necrosis on a living leaf. Although the expansion rate on a dead leaf was still slower than the growth rate on PDA, the large difference in the lesion expansion rate on living and dead leaves can be attributed to the plant immune system, and hence this parameter can be used as an estimate of the level of plant defence. For example, the net contribution of a specific gene to immunity can be estimated by the difference in expansion rates on wild-type and mutant lines. Towards this goal, PathTrack[®] will be modified to allow the analysis of disease on *Arabidopsis thaliana*. The current system is unsuitable for this purpose because of technical difficulties, which will be addressed in the modified system.

EXPERIMENTAL PROCEDURES

Biological material

Fungi

Botrytis cinerea B05.10 wild-type and mutant strains were used (Table 1). Fungi were routinely cultured on PDA (Acumedia, Lansing, MI) and maintained at 21 °C under continuous fluorescent light.

Preparation of spores for plant inoculation

Spores were collected from 7-day-old PDA plates, washed with sterile water, counted and then diluted to 10⁵/mL in inoculation buffer [Gamborg's B5 medium (Duchefa, Biochemie, Haarlem, the Netherlands) with 10 mM glucose and 10 mM KH₂PO₄/K₂HPO₄ (pH 6.4)]. The spores were

incubated for 1.5 h at room temperature with gentle agitation and then used to inoculate the plants.

Plants

Bean (*Phaseolus vulgaris* cv. French bean, genotype N9059) and *N. benthamiana* were used. For bean, the first primary leaf of a 10-day-old plant was used. For *N. benthamiana*, the second leaf of a 10–14-day-old plant was used.

PathTrack[®] hardware

The hardware of the PathTrack[®] system includes a transparent Perspex chamber, a series of leaf holders and a digital camera that is placed on an adjustable arm above the chamber (Fig. 1). The chamber contains trays for water, a sealed lead and is lit by strips of light-emitting diodes (LEDs) from both sides. White filter paper is placed between the LED illumination source and the chamber to soften the light. The leaf holders are composed of four poles of 20 cm each that are placed on a Perspex base to form a square, and a series of flat Perspex holders of variable sizes that are used to fix the leaf. Images were captured using a Nikon D90 digital camera with a Nikkor 105 mm f/2.8 ED IF AF-S VS lens.

Set-up of inoculation experiments

Preparation of the inoculation chamber

The chamber was placed in a separate compartment at 22 °C. A tray with water was placed inside the chamber and the chamber was sealed for several hours to allow the accumulation of humidity.

Plant inoculation

A leaf was mounted in the leaf holder to keep the leaf surface flat and to prevent movement during the experiment (see Fig. 1). Care was taken to avoid pressure on the leaf. As a control for the experimental conditions, we inoculated the second leaf of the same plant, which was not mounted. Following the mounting and inoculation of the leaves, the plants were placed inside the inoculation chamber and the chamber was sealed.

Image acquisition

After set-up of the samples, the leaf was photographed at constant intervals for the entire duration of the experiment. As a standard, we used manual settings with a 0.07-s exposure time, 500 ISO, 6 F number and a resolution of 4288 × 2848 pixels. The time lapse was performed using Nikon Camera Control Pro 2 software, with 10-min intervals. Images were stored in Jpeg format. A typical experiment included around 500–600 images.

Image analysis

The PathTrack[®] software package for data acquisition and analysis is scripted in MATLAB. The software reads the images in chronological order, detects differences in leaf coloration (necrosis) caused by fungal activity and analyses the determinants of disease development. The output file includes the true values of the following parameters: duration of the initial stage, time of transition between stages, acceleration rate during transition between stages (mm/h²), average radius and radii for prede-

defined angles of necrosis perimeter, necrotic area and average rate of disease expansion (mm/h). In addition, the software generates two types of visual output: video clips and graphs showing the kinetics of disease progression. The detection by the software is performed by a simple initial set-up procedure, followed by automatic object recognition algorithms. The object recognition algorithms are based on image processing methods, such as colour transformation, clustering and morphological operations. A detailed description of the software and tracking algorithms is provided in Supporting Information (Fig. S3).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 PathTrack[®] initial set-up procedure. The user selects (by mouse clicks) the initial drop regime, necrosis ranges, reference range and the length scale for the conversion of pixels to centimetres.

Fig. S2 Detection of necrosis area. Example of segmentation of necrosis from an image. Left, original image. Middle, final binary image. Right, original image with the detected necrosis marked as orange lines.

Fig. S3 Detection of hyphal colony. Example of segmentation of the hyphal colony from an image. Left, original image. Middle, final binary image. Right, original image with the detected hyphal colony marked as orange lines.

Video S1 Development of *Botrytis cinerea* mutant strains on bean leaves. The leaf was inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL of each of the strains. Following inoculation, the plant was incubated in the PathTrack[®] infection chamber at 22 °C with continuous light. The video shows the development of wild-type (pathogenicity group type 4, top left), $\Delta bcnoxa$ (pathogenicity group type 3, top right) and two strains of pathogenicity group type 2 ($\Delta bcnoxb$ and *ox-bcmcl1*, bottom left and bottom right, respectively). The full orange line marks the necrotic lesion boundaries, as detected by PathTrack[®] software.

Video S2 Comparison of development of *Botrytis cinerea* wild-type and $\Delta bcbir1$ strain on bean leaves. The leaf was inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL of each of the strains. Following inoculation, the plant was incubated in the PathTrack[®] infection chamber at 22 °C with continuous light. The full orange line marks the necrotic lesion boundaries, as detected by PathTrack[®] software.

Table S1 Data on parameters of growth dynamics.

Video 1 Growth of *Botrytis cinerea* wild-type strain on potato dextrose agar (PDA). A 5- μ L droplet of spore suspension containing 10^5 spores/mL was placed in the centre of a Petri dish

with PDA medium. The plates were incubated in the PathTrack[®] infection chamber at 22 °C with continuous light. The time lapse shows colony spread starting 30 h after the addition of the spores. The full orange line marks the fungal colony boundaries, as detected by PathTrack[®] software.

Video 2 Spreading stages of *Botrytis cinerea* wild-type strain on bean leaves. The leaf was inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL. Following inoculation, the plant was incubated in the PathTrack[®] infection chamber at 22 °C with continuous light. The video shows the intermediate and late stages of necrosis development. The full orange line marks the necrotic lesion boundaries, as detected by PathTrack[®] software.

Video 3 Early stage of *Botrytis cinerea* wild-type strain on bean leaves. The video shows a close up of necrosis development within the infection droplet during the early stage of infection.

Video 4 Leaf architecture causes spatial fluctuations in the lesion expansion rate. Each of the four arrows marks the necrotic lesion growth in a specific radial direction. Note the halt of progression of necrosis expansion in the direction marked by the blue arrow after 85 h as a result of encounter with a central vein.

Video 5 Fungal spread on a dead bean leaf. A leaf was detached from a 10-day-old bean plant, placed on a wet filter paper in a Petri dish and subjected to 5 s in a microwave operating at the maximum level. Following microwave treatment, the leaf was inoculated with a 5- μ L droplet of a spore suspension of wild-type (left) and $\Delta bcbir1$ strains containing 10^5 spores/mL. Note the appearance of a fast-spreading water-soaked lesion between 20 and 40 h post-inoculation (hpi), and the abrupt appearance of fungal mycelia at 42 hpi.

Video 6 Spreading stages of *Botrytis cinerea* wild-type strain on a *Nicotiana benthamiana* leaf. The leaf was inoculated with a 5- μ L droplet of a spore suspension containing 10^5 spores/mL. Following inoculation, the plant was incubated in the PathTrack[®] infection chamber at 22 °C with continuous light. The video shows the intermediate and late stages of necrosis development. The full orange line marks the necrotic lesion boundaries, as detected by PathTrack[®] software.