Complexity of Brassica Oleracea-Alternaria Brassicicola Susceptible Interaction Reveals Down-regulation of Photosynthesis at Ultrastructural, Transcriptional and Physiological Levels

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Research article

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Abstract

Background

Black spot disease, caused by *Alternaria brassicicola* in *Brassica* species, is one of the most devastating diseases all over the world, especially since there is no known fully resistant *Brassica* cultivar. In this study, the visualization of black spot disease development on *B. oleracea* var. capitata f. alba (white cabbage) leaves and subsequent ultrastructural, molecular and physiological investigations were conducted.

Results

Following germination on *B. oleracea* leaves, *A. brassicicola* invaded the host epidermal cells through appressoria, stomata or directly. The fungal attempts to penetrate epidermal cells usually evoked host cell death, and defense reactions manifested by the bright ‘halo’ around penetration site and host cell wall fortification were also observed. Inter- and intracellular hyphae growth within leaf tissues led to the loss of host cell integrity and various levels of organelle disintegration. Severe symptoms of chloroplast damage included the degeneration of chloroplast envelope and grana, and the loss of electron denseness by stroma at the advanced stage of infection. Transcriptional profiling of infected leaves revealed that photosynthesis was the most negatively regulated biological process. The down-regulation of 6 photosynthesis-related genes involved in light reactions and the Calvin cycle began as early as 12 hours post-inoculation (hpi) and extended to 44 genes at 48 hpi. Although in infected leaves, chlorophyll and carotenoid content did not decrease until 48 hpi, several chlorophyll *a* fluorescence parameters, such as photosystem II quantum yield (*F*<sub>v</sub>/*F*<sub>m</sub>), non-photochemical quenching (NPQ) or plant vitality parameter (Rdf) decreased significantly at 24 and 48 hpi compared to control leaves.

Conclusions

Our results indicate that the initial stages of interaction between *B. oleracea* and *A. brassicicola* are not uniform within an inoculation site and show a complexity of host responses and fungal attempts to overcome host cell defense mechanisms. The down-regulation of photosynthesis at the early stage of this susceptible interaction suggests that it may be a part of a host defense strategy, or alternatively, that chloroplasts are targets for the unknown virulence factor(s) of *A. brassicicola*. However, the observed decrease of photosynthetic efficiency at the later stages of infection is a result of the fungus-induced necrotic lesion expansion.

Background

*Brassica oleracea* var. capitata (head cabbage) from the *Brassicaceae* family is one of eight *Brassica* subspecies and an important vegetable in human diet due to its nutritional values [1]. Many white, red and Savoy cabbage cultivars are cultivated widely in Europe, Asia and North America, and used as a staple diet item, an ingredient of many national cuisine vegetable dishes or an addition to salads. In
Northern Europe and New Zealand, cabbage is often used as feed for sheep and cattle [1, 2]. As a widespread crop, cabbage is exposed to many bacterial and fungal diseases, which lower its yield all over the world. One of the most devastating fungus-induced diseases to all Brassicas is black spot disease, caused by *Alternaria brassicicola*. Cabbage cultivars show various levels of susceptibility to *A. brassicicola*, ranging from moderately to highly susceptible, but there is still not any known fully resistant cabbage cultivar [3].

*Alternaria brassicicola* belongs to the large fungal division ascomycota (*Ascomycetes*), order *Pleosporales*, and according to the novel taxonomic classification based on the molecular phylogenetic analysis, genus *Alternaria*, consists of 27 sections, with a distinct Brassicicola one [4]. As a necrotrophic fungus, *A. brassicicola* is seed-transmitted, and thus infects young seedlings causing their damping off [5, 6]. In the case of mature plants, the fungus is wind- and insect-spread, infecting preferentially older leaves. This results in black/brownish spreading lesions, which, at advanced stages of infection, can be responsible for the decay of the whole plant [7, 8, 9]. As a post-harvest pathogen, *A. brassicicola* has been found on cabbage debris and cabbage heads during storage [10]. The infection cycle of *A. brassicicola* is simple, from a single conidium to a mycelial network, often with overlapping stages typical for necrotrophic fungi [11]. After the initial attachment of the conidia to the leaf surface and germination, the fungus penetrates the host tissues through the appressoria and stomata, or invades them directly, using a preferential mode of penetration (depending on *Brassica* cultivar), or all of them simultaneously [3, 11, 12]. The fungus forms dome-shaped and usually aseptate appressoria at germ tube tips, similar to appressoria formed by *Botrytis cinerea*. Humidity, temperature and conidial concentration are factors that influence *A. brassicicola* germination and infection of a host under field and laboratory conditions [13, 14]. Prior to and during colonization, the fungus actively kills host cells primarily through the secretion of lipases, cell wall degrading enzymes, small secondary metabolites and recently identified as the most abundant phytotoxin in *A. brassicicola* cultures - brassicicolin A [15, 16, 17]. Many *A. brassicicola* mutants with depleted production of these secreted compounds, which also exhibit different levels of germination inhibition, were tested *in planta*, showing various degrees of virulence inhibition [16]. However, a primary virulence factor of *A. brassicicola* has not been discovered to date, thus it remains unknown how this fungus kills host plant cells. Moreover, it has been postulated that the possible resistance against *A. brassicicola* in Brassicas might be based on the cumulative effect of several genes rather than monogenicity [18, 19].

The knowledge concerning plant defense mechanisms against *A. brassicicola* infection is mostly based on the extensively investigated model pathosystem for *Brassicaceae* – non-host *Arabidopsis thaliana* and *A. brassicicola*. Arabidopsis resistance against the fungus is compromised in *pad3* and *coi1* mutants, indicating that it requires camalexin and jasmonic acid (JA)-dependent signaling, respectively [20]. The hypersensitive host cell death during this interaction is restricted to the inoculation site, and accompanied by generation of reactive oxygen species (ROS) and callose deposition in wild-type Arabidopsis Col-0 [21, 22]. On the other hand, camalexin is not metabolized by the fungus and inhibits its germination and development, but Brassicas phytoalexins induced during interaction with *A. brassicicola*, in most cases, are detoxified [23, 24, 25]. Although the transcriptome profiling of Arabidopsis Col-0 during...
A. brassicicola infection indicates that the photosynthesis-related genes remain unaffected in the resistant interaction [26; Zmienko and Macioszek, unpublished data], the microarray analysis of a susceptible pad3 mutant infected with A. brassicicola has demonstrated down-regulation of photosynthesis-related genes [27]. Moreover, recent research on susceptible Brassica juncea infected with A. brassicicola revealed severe changes in chloroplast ultrastructure, and a post-inoculation time-dependent decrease in chlorophyll a:b ratio and photosynthesis efficiency [9].

Here we report on the susceptible interaction between B. oleracea var. capitata f. alba (cultivar 'Glory of Einkhuizen') and A. brassicicola, both from the fungus and host plant perspective. In our work, we focused on the details of the fungal development and colony formation and the plant cell reactions during infection, at both light and transmission microscopy levels. Moreover, analyses of changes in the host transcriptional profiles with a special attention paid to photosynthesis-related genes and related physiological host responses to the fungus were conducted.

Results

Black spot disease development

The areas infected with A. brassicicola became macroscopically visible in the form of small brown necrotic spots on the second leaf of B. oleracea plants at 20-24 hours post-inoculation (hpi). The round-shaped necroses expanded beyond the inoculation sites at 48 and 72 hpi, and they were surrounded by a discrete chlorotic ring (Fig. 1a). Necrosis parameters such as area, perimeter and average radius increased in a time-dependent manner, indicating fast and massive fungal development on the susceptible host (Fig. 1b). Inoculation of all four fully developed leaves of B. oleracea plants revealed that the size of the necrotic spots was leaf position-dependent, the older the leaf the larger the necrosis, both on in planta and detached leaves assays (Additional file 1: Figure S1).

1. brassicicola development on leaf surface and host cell responses

The appearance of macroscopically visible necrotic spots at the inoculation sites on B. oleracea leaves at 24 hpi was strictly correlated with the overlapping stages of the A. brassicicola infection cycle, such as pre-penetration, penetration, colonization and conidiation, which started before this time point.

Following the inoculation of B. oleracea leaves, conidia began to germinate at 6-8 hpi, developing germ tubes at subsequent time points, which branched and elongated into hyphae at a later stage of infection (Additional file 1: Figure S2). Although the number of both germinating conidia and germ tubes increased gradually with the post-inoculation time flow (P < 0.001, r = 0.94), the number of germ tubes did not significantly increase over the number of germinating conidia at any investigated time points (Fig. 2). First dome-shaped appressoria were formed at 8 hpi and their number increased in a time-dependent manner (P < 0.001, r = 0.86), remaining at a similar level (not exceeding 20%) from 14 hpi (Fig. 2). The fungus penetrated host epidermal cells through appressoria, stomata or directly triggering various host
cell reactions at the same time (Fig. 3). At the beginning of penetration, a bright 'halo' could be observed under a scanning electron microscope (SEM) around the penetration sites, regardless of the mode of penetration (Fig. 3a, Additional file 1: Figure S3a and b). Direct penetration caused rapid disturbance and collapse of the host epidermal cells, visible as dark areas divided by light cell walls (Fig. 3b), but also dissolution of the leaf wax layer (Fig. 3c, Additional file 1: Figure S3c). Although penetration through stomata caused epidermal cell disturbance and death spreading to adjacent cells (Fig. 3d), host epidermal cells with fortified cell walls were also observed in this case (Fig. 3e). Regardless of the mode of fungal penetration, the first brownish host epidermal cells, indicating successful penetration sites, appeared as early as 14-16 hpi (Additional file 1: Figure S2). However, germinating conidia showing symptomless growth could also be observed on the leaf surface even at 24 hpi (Fig. 3f).

New young conidia were produced by mature parental conidia (used as an inoculum) during direct conidiation, taking place between 14 and 24 hpi (Fig. 4a). The formation of the conidial chain developing on short conidiophores was observed later at 48 hpi.

The advanced stage of *A. brassicicola* development - colony formation - began as a tangle of elongating germ tubes developing into hyphae at 16-24 hpi (Fig. 4b, Additional file 1: Figure S2 and Figure S3d). Connections between different parts of a developing colony were provided by bridges formed directly between two mature conidia or germ tubes, in the form of conidial anastomosis tubes (CATs) (Fig. 4c and d) and fusions between hyphae - anastomoses (Fig. 4e and f). The expanded colony caused damage and overgrew host tissues from the surface to the bottom side of the infected leaf at 48 hpi (Additional file 1: Figure S3e-g) and sometimes chlamydospores could be observed on leaf surface at 72 hpi (Additional file 1: Figure S3h).

**Ultrastructure of infected host cells**

The ultrastructure of infected *B. oleracea* leaves revealed that *A. brassicicola* hyphae grew both inter- and intracellularly within the host leaf tissues (Additional file 1: Figure S4 and Figure S5a-d). Most of the hyphae had a regular ultrastructure, but a small number of hyphal cells with some level of degradation and osmiophilic granules were also observed (Additional file 1: Figure S5e). In the infected mesophyll cells, the changes such as damage to the cell wall, plasmolysis and various levels of organelle disintegration, were dependent on the distance from the necrotic area and presence of hyphae (Additional file 1: Figure S4). In the chlorotic area without hyphae, the host cell integrity was retained, although minor ultrastructural changes became evident.

In the control leaves, the mesophyll cells had a classical ultrastructure, with a large central electron-translucent vacuole and a narrow layer of homogeneously electron-dense cytoplasm, containing organelles, located parallel to the cell wall. Cell nuclei were surrounded by a continuous envelope perforated with nuclear pores only. Only few and small electron-dense heterochromatic grains were located next to the nuclear envelope. A well-distinguishable single nucleolus was present inside uniformly
electron-dense euchromatin (Fig. 5a). Lenticular chloroplasts with electron-dense stroma and a regularly arranged thylakoid and grana system contained up to 3 small starch grains (Fig. 6a).

The mesophyll cells at the border of the chlorotic and necrotic areas, especially in the close proximity of hyphae, showed various degrees of plasmolysis and organelle disintegration. They contained numerous lytic vacuoles, membranous multilamellar structures and vesicular bodies, as well as osmiophilic granules close to the cell wall and organelles (Figs. 5 and 6). Despite the appearance of symptoms of cell lysis, the ultrastructure of the nuclei was still well-preserved in some cells (Fig. 5b), showing only local nuclear envelope swelling. However, the nucleoplasm slowly lost its electron-density and acquired a fine granular and fibrillar appearance (Fig. 5c and d). In the more advanced stages of degradation, the nucleoplasm region turned strongly electron-translucent, and remnants of chromatin coalesced, forming electron-dense grains (Fig. 5e and f). With the exception of the terminal stages of nucleus degradation, the remnants of nucleoli remained recognizable (Fig. 5c and e) and the nuclear envelope was absent only in completely degraded cells (Fig. 5f).

The degradation of chloroplasts was more spectacular and distinct, and clear stages of their disintegration could be discriminated. First of all, numerous plastoglobuli appeared in still well-preserved chloroplasts (Fig. 6b). Subsequently, chloroplasts started to change their shape from lenticular to round (Fig. 6c and d). This phenomenon was accompanied by the swelling of the stroma that lost its electron-density, gradual disintegration of the chloroplast envelope, and formation of the cup-shaped or bowed arrangement of the thylakoid systems (Fig. 6c-e). Further degradation of the chloroplasts encompassed the final degeneration of the chloroplast envelope, disappearance of grana, distention and dilution of the thylakoid lumens and the disintegration of the stroma (Fig. 6f). Despite the level of degeneration of the lamellar system, the chloroplasts still contained starch grains or their hydrolyzed remains (Fig. 6).

Microarray analysis

The transcriptional profiling of *B. oleracea* leaves infected with *A. brassicicola* using 29k Arabidopsis Oligonucleotide Microarrays was performed. Out of 29,100 microarray oligonucleotide probes, 8,654 could be reliably mapped to *B. oleracea* nucleotide sequences, which represented 8,014 unique genes. The expression of these genes in infected plants at 12, 24 and 48 hpi was compared to control plants (0 hpi). Significant changes in gene expression (FDR<0.1) were observed as early as 12 hpi (27 genes) and they intensified while the infection progressed, with 189 genes regulated at 24 hpi and 410 genes at 48 hpi. Overall, 487 distinct genes were significantly affected in infected *B. oleracea* leaves (Additional file 2: Table S1).

Based on the results of the statistical analysis, the Gene Ontology (GO) and MapMan analyses were performed to annotate the significantly up- and down-regulated genes, and to determine the most affected biological processes and activation of individual genes during *B. oleracea* infection with *A. brassicicola*. Biotic stress response-related processes, such as the cell wall macromolecule catabolic process and defense response to fungus, were among the most extensively up-regulated GO terms (Fig. 7,
Additional file 2: Table S2). They were correlated to the microscopic results obtained in this study to uncover the differential responses of host cells to the infection within the inoculation site (Figs. 3 and 7, Additional file 2: Table S2 and S3). The up-regulation of host genes involved in aging, detection of ethylene stimulus and thiamine biosynthesis indicated general reprogramming of the host transcriptome in response to the infection. Analysis of stress-related genes at 48 hpi revealed differential expression of 167 genes, with 87 of them, mainly involved in cell response signaling, cell wall degradation, protein degradation, auxin-induced metabolism, being down-regulated. However, transcriptional activation of 80 stress-related genes among others \textit{LRR-RLK7} (AT1G09970) encoding leucine-rich repeat transmembrane protein receptor-like kinase 7, \textit{WRKY33} (AT2G38470) encoding transcription factor, \textit{PDF1.2} (AT5G44420) encoding jasmonate-responsive plant defensin or \textit{FAH1} (AT4G36220) encoding ferulate-5-hydroxylase 1 and \textit{CCoAMT} (AT1G67980) encoding caffeoyl-CoA 3-O-methyltransferase involved in lignin biosynthesis, even at 48 hpi, indicated that despite the successful colonization of the susceptible host by \textit{A. brassicicola}, some of the host cells battled with the fungus and basal defense signaling was still active (Additional file 2: Table S4). In turn, photosynthesis-related processes were strongly down-regulated (Fig. 7, Additional file 2: Table S2 and S3) in agreement with the observed severe changes in the ultrastructure of chloroplasts during infection (Fig. 6). Interestingly, gradual down-regulation of photosynthesis-related gene expression was observed during the entire course of \textit{B. oleracea-A. brassicicola} interaction investigated in this study (Additional file 2: Table S5). The majority of the 104 genes that were mapped to photosynthesis-related processes by the MapMan analysis were negatively regulated at one or more time points (Fig. 8a) and the number of significantly down-regulated genes (at FDR<0.1) increased from 6 at 12 hpi, through 32 at 24 hpi, up to 44 genes at 48 hpi (Fig. 8b, Additional file 2: Table S5). All crucial photosynthesis-related processes, such as light reactions, Calvin cycle, photorespiration, plastid ribosomal protein synthesis and tetrapyrrole synthesis were affected (Fig. 8c). The distinctive genes in the photosynthesis light reaction category were \textit{LHCb} and \textit{LHCa}, encoding chlorophyll \textit{a/b}-binding proteins, which are the part of photosystem II and photosystem I light harvesting complexes, respectively, and thus are important components of the chloroplast membrane system. These genes were down-regulated as early as 12 hpi, with an increasing effect at subsequent time points. In the Calvin cycle, constituting the dark reaction of photosynthesis, the genes encoding the RuBisCO small subunit and glyceraldehyde 3-phosphate dehydrogenase were also down-regulated at all investigated time points in infected \textit{B. oleracea} leaves. However, changes in the expression of genes involved in photorespiration, plastid ribosomal protein synthesis and tetrapyrrole synthesis started later, at 24 and 48 hpi (Additional file 2: Table S5). Additionally, consistent with the negative regulation of photorespiration-related genes, the down-regulation of genes involved in water homeostasis was also observed (Fig. 7). Remarkably, \textit{AtCLH2} (AT5g43860) encoding chlorophyll-chlorophyllido hydrolase 2 annotated by MapMan as a tetrapyrrole synthesis-related gene, was the only photosynthesis-related gene consistently up-regulated at all 3 examined time points of infection (however it did not pass the criteria of statistical significance) (Additional file 2: Table S5).

**Photosynthetic parameters analysis**
Based on the results of the host cell ultrastructure and microarray data, potential changes in photosynthetic pigment content and chlorophyll *a* fluorescence were investigated. As suspected, the content of chlorophyll *a*, *b*, *a+b* and carotenoids decreased in infected leaves compared to control at 48 and 72 hpi, but no significant differences between the content of any investigated photosynthetic pigments in the control and infected leaves were observed at 24 hpi (Fig. 9).

The analysis of chlorophyll *a* fluorescence quenching revealed that the steady-state photosystem II (PSII) quantum yield (*F*v*/F*m_Lss*) and the empiric parameter used to assess plant vitality (Rdf) significantly decreased in infected *B. oleracea* leaves in a time-dependent manner (Fig. 10, Additional file 3: Table S6). The significant decrease of variable fluorescence photochemical quenching (qP), PSII open centers fraction (qL) and PSII efficiency factor (*F*q*/F_v*) in light adaptation periods and steady-state light during infection indicated partial blocking of electron transport to PSII reaction centers (Fig. 10). The temporary increase in the value of non-photochemical quenching (NPQ) in infected leaves at 48 hpi was only visible during one of the dark relaxation periods (NPQ_D3). In turn, NPQ in light adaptation periods (NPQ_L1-L3) showed a significant decrease in infected leaves compared to the control at 24 hpi and 48 hpi (Fig. 10, Additional file 3: Table S6). Despite this, the maximum quantum yield of PSII significantly decreased in infected *B. oleracea* leaves compared to the control at 48 hpi, as evidenced by the values of QY_max and QY_Lss (Fig. 10).

**Discussion**

Infections caused by necrotrophic fungi in host plants during susceptible interactions are related to unrestricted cell death visible as spreading lesions, the appearance of which is strictly correlated to a fungal infection cycle and the production of a wide set of virulence factors [28, 29]. The cabbage cultivar ‘Glory of Ehinkhuzen’ used in the presented study has been considered to be susceptible to *A. brassicicola* infection under laboratory conditions and moderately resistant in the field [3]. Macroscopically visible necrotic lesions on leaves during *A. brassicicola* infection spread gradually from small brown spots at 24 hpi to larger necroses surrounded by a chlorotic area in a leaf position-dependent manner similarly as described for other Brasicas [3, 9, 30].

**Timing of *A. brassicicola* infection cycle depends on host leaf surface**

In our study, the *A. brassicicola* infection cycle was delayed on the second leaf of ‘Glory of Ehinkhuzen’ compared to the susceptible white, red and Savoy cabbage cultivars infected with the same strain. The fungal germination began about 2 h later than on the white cabbage cultivar 'Stone Head', however the appearance of the first appressoria at 8 hpi was in accordance with the previous study [11]. The delayed germination of *A. brassicicola* conidia may be related to the thicker wax layer on the ‘Glory of Ehinkhuzen’ leaf surface that might have caused difficulties in conidial adherence to the leaf surface and/or a delayed recognition of the host surface signals by the conidia [31-33]. Following germination, the fungus
penetrated host epidermal cells through appressoria, stomata or directly without any preferential mode of penetration, but on other susceptible cabbage cultivars, *A. brassicicola* penetrated the leaf surface mainly through appressoria and rarely through stomata [11]. Nowakowska et al. [3] have claimed that the cabbage cultivars were penetrated mainly directly and through appressoria, and only rarely through stomata or without any preferential mode of penetration independently of a cultivar.

**Host cells respond differentially to penetration**

Using a SEM, we found that the fungus attempts to penetrate leaf surface, regardless of the mode of penetration, were accompanied by a bright 'halo' formation as early as 12 hpi (Fig. 3a, Additional file 1: Figure S3), indicating that at first, the host cell reaction to penetration was defensive. Such clear 'halos' around penetration sites have been observed during a resistant interaction of a biotrophic fungus *Blumeria graminis* f. sp. *tritici* on a wheat cultivar carrying effective resistance genes and also on a susceptible one, using cryoscanning electron microscopy [34]. Possibly these 'halos' were described as papillae formed around penetration sites of *A. brassicicola* on the leaves of both susceptible and moderately resistant cabbage cultivars after double staining with trypan blue and aniline blue using confocal laser scanning microscopy [3]. The biochemical analysis revealed that the 'halo' contains callose, phenolic compounds and an elevated level of calcium ions [34]. The 'halo' phenomenon indicates that the host cells are trying to combat fungal invasion at this point of the susceptible interaction and stay alive. It also shows that the *A. brassicicola* infection cycle probably contains a very short biotrophic phase, although there is a general agreement that a necrotrophic fungus first kills a host cell by secreting toxins, and then invades it. Interestingly, it has been postulated that another necrotrophic fungus, *B. cinerea*, should be considered as hemibiotroph having a short biotrophic phase. *Botrytis cinerea* suppresses early host defense reactions by the secretion of small RNAs (sRNAs), thus leading to the silencing of host genes, but to achieve this, the host cell must be alive [35]. It has been also shown that during invasion and the establishment of a necrotrophic interaction, *B. cinerea* 'sacrifices' many of its invading hyphae by subjecting them to cell death induced by plant-secreted cell death inducing factors, to get a chance to release fungal cell death inducing factors by the surviving hyphae into plant cells [36]. It could also be a mechanism used by *A. brassicicola* and an explanation for the presence of hyphae with deteriorating protoplasts (Additional file 1: Figure S5e).

At later stages of an *A. brassicicola* infection (from 12-16 hpi), the successful penetration sites appeared on SEM images as electron-dense collapsed epidermal cells (Fig. 3b) or brownish possibly dead cells under bright field light microscopy (Additional file 1: Figure S2), which have been previously described during *A. brassicicola* infection [11]. Brownish cells are also frequently found at penetration sites of other necrotrophic as well as biotrophic fungi [37]. Browning of successfully infected epidermal cells may have been caused by the activation of peroxidases and phenolics within the cell wall and/or a production of ROS by dying host cells, indicating the suppression of host cell defense reactions and a highly susceptible response at this stage of infection [11, 22, 38, 39].
1. *brassicicola* colony formation is triggered by successful penetration

Successful penetration of the susceptible host epidermal cells by *A. brassicicola* was a signal triggering colony formation at inoculation sites. Connections between different regions of an expanding mycelial network were established via CATs and anastomoses (Fig. 4). Their formation is a typical feature of many filamentous fungi [40, 41], but these phenomena are mostly described when fungi grow in media rather than *in planta* during infection. CATs are usually formed during the initiation of a colony, and facilitate the transport of water and nutrients as well as horizontal gene transfer [42, 43]. Similarly to CATs, the formation of fusions between hyphae (anastomoses) originating from the same or different conidia, allow them a proper distribution of nutrients, transduction of chemical signals and even exchange of genetic material [44]. Moreover, anastomosis formation is prerequisite for *A. brassicicola* virulence, as indicated by the development of an *A. brassicicola aso1* mutant that is unable to form anastomoses, and when tested *in planta* appears to be unable to spread beyond the inoculation site [45].

**Changes in host cell ultrastructure and transcriptome reprogramming**

The appearance of necrotic spots indicated successful invasion and colonization of the host tissues by *A. brassicicola*, although host cells were differentially affected within the inoculation site depending on the distance from the invading hyphae, as evidenced by the gradual degradation of organelles. Similar individual changes in the ultrastructure of infected plant host cells, such as cell lysis, disintegration of the nuclei and chloroplasts or the presence of osmiophilic granules have been observed in other pathosystems during infection with viruses, bacteria and fungi [46-49]. In the case of infected *B. oleracea* mesophyll cells, these changes could be a result of the action of toxins and secondary metabolites secreted by *A. brassicicola* [16] and general reprogramming of the host transcriptome and metabolome in response to the infection [50, 51]. *Alternaria* species secrete various non-host- (nHSTs) and host-selective toxins (HSTs) which are responsible for the degradation of different organelles within an infected cell in over 200 plant species. For example, *Alternaria alternata* pathotypes produce different HSTs depending on the plant species, such as the AM-toxin that degrades the plasma membrane and chloroplasts in apple; the ACR-toxin that causes damage of the mitochondria in lemon; the AK-toxin, AF-toxin and ACT-toxin that target the plasma membrane in susceptible cultivars of Japanese pear, strawberry and tangerine, respectively [52-54]. However, a possible effect of identified *A. brassicicola* secreted compounds during infection on plant cell structures or their influence on the host transcriptome has not been described yet. It has to be emphasized that visualized differential *B. oleracea* cell responses to *A. brassicicola* infection, from defensive to highly susceptible, were confirmed by our microarray results (Additional file 2: Table S4), and were also concordant with the previous studies on plant gene expression during infection of susceptible *B. oleracea* and *B. napus* with *A. brassicicola* [55, 56]. The most up-regulated categories revealed in the analysis of Gene Ontology of Biological Processes were aging, the
detection of ethylene stimulus, thiamine biosynthetic processes, cell wall macromolecule catabolic processes and defense response to fungus. Additionally, analysis of stress-related genes revealed up-regulation of the individual genes associated with hormone metabolism (brassinosteroids, ethylene and salicylic acid biosynthesis), peroxidases, glutathione S-transferases and secondary metabolism connected to flavonoid biosynthesis (Additional file 2: Table S4). Many of these genes have been previously described as important factors in general plant cell response to biotic stress and identified in transcriptome profile analyses of other susceptible hosts infected with *Alternaria* species [57, 58]. Some of these genes undoubtedly are characteristic for the *B. oleracea* response to infection by *A. brassicicola* [50, 55], albeit confirmation of their roles requires further investigations based on comparative transcriptomics and proteomic and metabolomic approaches.

### Defense-related genes are activated also in susceptible interaction

The up-regulation of several stress-related genes involved in the defense response to fungus in the examined susceptible cultivar of *B. oleracea* even at 48 hpi, when necrotic lesions were fully developed, indicates that some of the host cells still attempted to combat the invasion of the fungus. The fungal hyphae spreading beyond inoculation site, even at a later stage of infection, apparently induced the host defense response through PTI (pattern-triggered immunity) [50]. Probably, the perception of DAMP (damage-associated molecular pattern, e.g. products of the *A. brassicicola* cell wall degrading enzymes or plant secreted peptides) or PAMP (pathogen-associated molecular pattern, e.g. chitin) through a PRR (pattern recognition receptor) triggered the PTI-associated signaling cascade (albeit transcription of MEK1 was negatively regulated) and activated WRKY33 in infected *B. oleracea* cells, similarly to the situation observed in Arabidopsis during fungal infection [59]. Interestingly, the only up-regulated gene encoding PRR in our microarray analysis was *RLK7* (Additional file 2: Table S4). RLK7 belongs to category XI of RLKs and acts as a receptor for PIP1 secreted peptide. Elevated expression of *PIP1* has been described in Arabidopsis guard cells during *Pseudomonas*-induced PTI, and thus the peptide plays a role in stomatal immunity [60]. Moreover, RLK7 also contributes to Arabidopsis resistance to *B. cinerea* [61] and *Phytophthora irregulare* [62]. However, *LYK5*, the other gene encoding RLK, which is an important receptor engaged in chitin perception in Arabidopsis [63], was down-regulated. The up-regulated genes involved in defense signaling such as *WRKY33* [64] and *PDF1.2* [65] have been identified in *A. thaliana* signaling pathways and are required for signaling resistance to necrotrophic fungi *A. brassicicola* and *B. cinerea*. In Arabidopsis, WRKY33 transcription factor is responsible for the activation of camalexin biosynthesis [66], which efficiently inhibits development of necrotrophic fungi. Possibly, WRKY33 activates de novo biosynthesis of *B. oleracea* fungus-induced phytoalexins, such as brassinin, which can be metabolized by *A. brassicicola*, and thus the fungus suppresses the first line of host defense [67]. Subsequently, JA-dependent signaling, which is characteristic for host cells infected by fungal necrotrophs, activates transcription of JA-responsive defense genes encoding PR (pathogenesis-related) proteins such as PDF1.2 involved in resistance against *B. cinerea* [68] and *A. brassicicola* [23]. However,
the down-regulation of LOX2 chloroplast lipoxygenase required for JA accumulation may be also responsible for the impaired defense of the host and in turn, salicylic acid-dependent genes become activated, which promote host cell death [69]. Moreover, local fortification of the host cell walls microscopically observed within inoculation site in our study was reflected in the up-regulation of several stress-responsive cell wall and secondary metabolism-related genes, such as FAH1 involved in lignin biosynthesis and an important component of A. thaliana resistance mechanism to B. cinerea [70] and CCoAMT, which is involved in the biosynthesis of cell wall-bound phenolics and lignin [71]. The strengthening of host cell walls through lignin biosynthesis and its deposition at a pathogen entry site also constitutes the first line of the host defense reaction in order to slow down or restrict pathogen development, especially as lignin is not metabolized by most pathogens [72, 73]. Thus, even successful invasion of host tissues within inoculation sites does not signify that every single host cell immediately surrenders to a fungal invader. However, down-regulation of numerous stress-related genes found in our study indicates that A. brassicicola effectively overcomes the host arsenal of defenses (Additional file 2: Table S4). Although it has been reported that A. brassicicola secretes phytotoxin brassicicolin A, histone deacetylase inhibitor depudicin, siderophore N,N-dimethylcoproge [15, 17], a proteinaceous host-specific toxin - AB-toxin [74, 75] and many low molecular weight secondary metabolites e.g. brassicenes A to F [76], there is not yet any known putative A. brassicicola effector(s) targeting also still unknown Brassica receptor(s) and triggering ETS (effector-triggered susceptibility) or ETI (effector-triggered immunity), albeit many research groups all over the world work on A. brassicicola pathogenicity factors [16].

Down-regulation of photosynthesis is probably not only a part of susceptible interaction

Further, we have focused on changes in the ultrastructure of chloroplasts in infected mesophyll cells, mostly due to the observed clear stages of their degradation (Fig. 6) and the fact that analysis of our microarray data pointed out photosynthesis as the most negatively regulated process during infection of B. oleracea leaves with A. brassicicola (Additional file 2: Table S3). Chloroplasts are energy and carbon source organelles, and play an important role in plant immunity as a compartment for ROS generation and the production of phytohormones, secondary metabolites and their precursors [77]. As sensors of environmental changes, chloroplasts can shape nuclear gene expression and activate defense responses through redox flux [78]. Moreover, many pathogen-derived effectors target chloroplast-localized proteins, including components of the photosynthetic electron transport chain [79]. Therefore, changes in chloroplast ultrastructure are often used as good indicators of biotic/abiotic stress [80].

Gradual degradation of the chloroplast membrane system, such as widening of the thylakoid lumens and the disappearance of grana observed in infected B. oleracea leaves, indicated the suppression of photosynthesis light reactions and potential damage of light harvesting complexes and reaction centers. As a result, chlorophyll and carotenoid content and photosynthesis efficiency at a physiological level was decreased. Ultrastructural changes of the stroma, from its swelling to disintegration, suggested down-regulation of the photosynthesis dark reaction and a decrease of carotenoid content. In severely degraded
cells, the thylakoid system was still to some extent preserved, whereas the chloroplast envelope and stroma were totally disintegrated (Fig. 6f). The observed changes in the chloroplast ultrastructure were associated with changes in the expression of photosynthesis-related genes and the physiological response of the host cells. Early down-regulation of photosynthesis-related genes could be a result of the host cell defense strategy related to the shift from photosynthesis to non-assimilatory metabolism, as observed during plant infection by various pathogens, or an effect of action of an unknown A. brassicicola-secreted effector protein [81]. At later stages of infection (24 and 48 hpi), more genes involved in light reactions were down-regulated. Fluorescence decline ratio in steady-state (Rfd) and a significant decrease in steady-state PSII quantum yield ($F_v/F_m$-Lss) in infected B. oleracea leaves indicate inhibition of electron transport in light-dependent reactions. Confirmation of this was the decrease in the value of $F_q/F_v$, $q_L$, as well as the $q_P$ parameter, which describes the level of energy transferred to the reaction centers and informs about the proportion of open PSII reaction centers (Fig. 10) [82, 83]. The reduction of photosynthesis, determined by a decrease in the effective quantum yield of PSII, has been previously observed in the interaction of B. juncea-A. brassicicola [9] and in other plants infected with necrotrophic fungi [84, 85]. Also, in wheat plants infected with Bipolaris sorokiniana, a decrease in $F_v/F_m$ correlated with the loss of chlorophyll has been noted [86].

In severe stress caused by A. alternata in rice, a decrease in the electron transport rate was correlated with an increase in non-photochemical quenching (NPQ) [87]. An early increase in NPQ took place following a decline in photosynthetic electron transport activity. A similar PSII response was also observed in plants infected with viruses, e.g. Pepper mild mottle virus (PMMoV) infecting pepper leaves [88]. However, such an increase can be the result of both: protective processes of PSII and damage to the photosystem [82]. In infected B. oleracea leaves, we generally observed a decrease in NPQ (Fig. 10), which indicates a reduction in the dissipation efficiency of excess excitation energy as heat. However, it should be mentioned that not all processes associated with non-photochemical quenching lead to an increase in NPQ [89]. The decrease in NPQ in infected B. oleracea leaves may be due to reduced light absorption, e.g. as a result of the destruction of chloroplasts and a decrease in chlorophyll content (Fig. 9), rather than a lack of thermal dissipation. Also, in other plants (i.e. rice, tomato) exposed to necrotrophic fungi, a significant decrease in NPQ was observed both in the necrotic zone and in adjacent areas of the leaf blade, without significantly reducing the photosynthesis efficiency (measured as $F_v/F_m$) [85, 90].

In the case of progressive degradation of chloroplasts at the inoculation site, it is difficult to expect discrete changes in the photosynthesis light reactions. Therefore, the significant decrease in the values of chlorophyll $a$ fluorescence quenching parameters is not surprising. However, a slight decrease in QY_max and QY_Lss (Fig. 10) suggests that the destruction of the photosynthetic apparatus did not completely block the electron transfer in PSII in the analyzed leaf area. It is probable that the chloroplasts of cells that have not been invaded by the fungus allow photochemical reactions to occur, although it is known that even Alternaria spp. metabolites alone can cause a reduction or complete inhibition of the electron transport chain from QA to QB [91, 92]. This is due to competition between the toxin and QB for a binding site in the D1 protein on the thylakoid PSII membrane. In general, the photosynthetic yield of plants
infected with necrotrophic fungi presents complex spatial and temporal patterns, depending on the
degree of colonization of the individual regions of a leaf blade by the pathogen [93].

Conclusion

Our results show that initial stages of a susceptible interaction between *B. oleracea* and *A. brassicicola*
are complex, not uniform within an inoculation site and host cell defenses are still active even at later
stages of infection (48 hpi). Analysis of the microarray data suggested the possible involvement of
putative genes encoding LRR receptor-like kinases in PTI in this pathosystem. Ultrastructural, molecular
and physiological analyses of infected leaves revealed photosynthesis as the most down-regulated
process from the onset of the infection. This finding should be taken into consideration in further
research and work on a strategy for the management of black spot disease.

Methods

Plant growth, fungal strain and inoculation

The seeds of *Brassica oleracea* var. *capitata* f. *alba* (white cabbage) early cultivar 'Glory of Einkhuizen'
were obtained from a Polish seed company and grown in soil:perlite mixture (15:1) in a plant growth
room under fluorescent light (Super TLD Philips 865) at 100 µmol m⁻² s⁻¹, 16 h day/8 h night photoperiod,
at temperature 22±1°C and relative humidity of approximately 65%. The wild type strain of *A.
brassicicola* (ATTC 96836) was grown on potato dextrose agar plates (PDA; Difco, the Netherlands) for 7-
10 days under the same conditions as plants, but in the dark. The second leaves of four-leaf mature *B.
oleracea* plants were inoculated with the *A. brassicicola* conidial suspension at a concentration of 5 x 10⁵
conidia per ml of distilled water, or distilled water in case of control plants, by putting one or two 10 µl
drops per leaf or spraying the leaves using a Nalgene aerosol spray bottle (Sigma-Aldrich, USA) [9, 11]. In
all experiments, plants were inoculated 4-6 h after switching on the light (about 10.00-12.00 a.m. local
time). The inoculated plants were incubated in transparent plastic boxes to maintain high humidity.

Disease progression analysis

*Brassica oleracea* plants were drop-inoculated, and necrotic spot parameters such as area, perimeter and
average radius were measured using the WinDIAS Leaf Image Analysis System (Delta-T Devices, UK). For
each time point the second leaf of at least 6 plants was detached and images were taken immediately at
24, 48 and 72 hours post-inoculation (hpi) in 3 independent experiments (n=3).

Light microscopy

To investigate the stages of *A. brassicicola* development during leaf infection, aniline blue-lactophenol
(Sigma-Aldrich, USA) stain was applied on drop-inoculated leaves at 4, 8, 12, 14, 16, 20 and 24 hpi [11].
The numbers of germinated conidia, germ tubes and appressoria were counted per 100 conidia at random sites of the inoculation area on the second leaf of 3 plants per each time point.

**Scanning and transmission electron microscopy**

To investigate the changes of leaf surface and fungal growth during infection, 1.5 cm diameter leaf discs were cut out from the *B. oleracea* second leaves sprayed or drop-inoculated with the conidial suspension. The samples were examined under a tabletop scanning electron microscope TM-1000 (SEM; Hitachi, Japan) operating at 15 kV, without any pre-processing [11]. Samples from 6 plants per experiment were harvested at 12, 24, 48 and 72 hpi. The experiment was repeated independently twice.

For the ultrastructural investigations, samples from the second leaf of the control and drop-inoculated plants were collected at 48 hpi and embedded in Epon-Spurr resin mixture [9]. The semi-thin (1 µm thick) and ultra-thin sections (80 nm thick) were obtained using a Reichert Jung ultramicrotome (Leica, Germany). The ultra-thin sections, after staining with uranyl acetate and lead citrate [94], were examined under a transmission electron microscope JEOL 1010 (TEM; JEOL, Japan) operating at 80 kV. The ultrastructure of the plant cells was observed on at least 50 micrographs per each treatment.

**Microarray experiment**

The time-course pattern of gene expression was examined in the second leaves of *B. oleracea* sprayed with *A. brassicicola* conidial suspension by cDNA hybridization to 29k Arabidopsis Oligonucleotide Microarrays (University of Arizona) (GEO platform GPL7725). Each sample treated as a single biological replicate was collected from 3 control or 3 infected plants. For each time-point (0, 12, 24 and 48 hpi), 2 biological replicates were analyzed and a common reference design was applied, where each sample was labeled with Cy5 and hybridized against the Cy3-labeled pool of the control plants.

Each microarray probe sequence (~70-mer) was used as a query in a BLASTN search against *Brassica* nucleotide sequences available in the GenBank database. The best hit was recorded for each probe. Probes with high homology to *Brassica* targets were selected by applying a Bit score cutoff value of 40. For 66% of them, probe:target alignments were >60 nt in length and displayed >75% sequence identity.

The total RNA was extracted with RNeasy Plant Mini Kit (Qiagen, Germany) and samples were DNase-digested with a TURBO DNA-free kit (Ambion, USA). The sample quality was analyzed with a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA) and 2100 Bioanalyzer (Agilent Technologies, USA). For each labeling reaction, 15 µg of high-quality RNA ($A_{260}/A_{280}$ and $A_{260}/A_{230} \geq 2$, RIN $\geq 9$) was reverse-transcribed using the SuperScript Indirect cDNA Labeling System (Invitrogen, USA) according to the manufacturer’s protocol. Aminoallyl-modified cDNA was coupled with 5 µl of Cy3 or Cy5 ester dye (Amersham Pharmacia, UK) and unbound dye was removed with a MinElute Reaction Cleanup Kit (Qiagen, Germany). The efficiency of the labeling was monitored with NanoDrop measurements.
Corresponding Cy3 and Cy5-cDNA were mixed, the volume was adjusted to 10 µl and the samples were heat-denatured at 95°C for 10 min and added to 115 µl pre-warmed (68°C) hybridization buffer SlideHyb #3 (Ambion, USA). Hybridization was carried out in a HybArray 12 workstation (PerkinElmer, USA) at 42°C for 18 h. The microarrays were washed with 2x SSC (Ambion) supplemented with 0.1% SDS (Sigma-Aldrich, USA) at 42°C, 0.5x SSC at 30°C and 0.005x SSC at 25°C, each for 5 cycles (20 s flow, 40 s hold) and dried by centrifuging. Images were collected with a ScanArray Express scanner (PerkinElmer, USA) at 100% laser intensity and variable PMT settings.

The images were analyzed with GenePix 6.1 software (Molecular Devices, USA) using the morphological opening background subtraction method with default parameters. The data was further analyzed in an R/Bioconductor environment using the limma package [95, 96, 97]. Foreground and background intensities were read with “genepix.custom” function. After background subtraction and printtip-loss normalization within arrays, followed by scaling the log-ratios to have the same median-absolute-deviation (MAD) across arrays, a linear model was constructed for the contrasts of interest. Differential expression of genes for which the probes passed homology-based filtering criteria (see above) was assessed by moderated t-statistics and correcting for multiple testing using the false discovery rate (FDR) [98]. Adjusted p-value 0.1 was chosen as a significance threshold.

For functional analysis and gene annotation, A. thaliana gene IDs originally assigned to the microarray probes were used. Only 8,014 genes with high homology to Brassica were taken into consideration and used as a background in the Gene Ontology (GO) and MapMan analyses.

A GO enrichment analysis of differentially regulated genes was performed separately for each time point against the list of all genes represented on the microarray using the ThaleMine v.4.1.1 online analysis tool [99]. The p-value was calculated using hypergeometric distribution test and adjusted with the Holm-Bonferroni multiple test correction, and the significance threshold was set at p-value <0.05. The visualization of significantly regulated GO terms was performed using REVIGO [100]. The MapMan (v.3.5.0) analysis was performed based on the probe mapping files for A. thaliana and pathways downloaded from the MapMan repository [101].

**Photosynthetic pigment content**

Chlorophyll was extracted in 100% methanol and measured using a spectrophotometer PowerWave XP (BioTek, USA). The content of chlorophyll a, chlorophyll b and carotenoids was calculated according to Wellburn [102]. The samples were harvested from 3 control and 3 infected plants per time point, and each experiment was repeated independently 3 times (n=3).

**Chlorophyll a fluorescence**
The control and drop-inoculated infected plants were dark-adapted for 30 min, then each second leaf was detached and immediately subjected to analysis of the kinetics of chlorophyll a fluorescence quenching using a Handy FluorCam 1000-H System (Photon Systems Instruments, Czech Republic) according to the manufacturer's built-in protocol. The measurements were performed on the selected area (diameter about 1.5 cm) of control untreated leaves, as well as infected ones containing necroses. The duration of the reading procedure per one leaf was 4 min, and parameters such as $F_0$ (minimum fluorescence), $F_m$ (maximum fluorescence), $F_v$ (variable fluorescence) as well as $F_p$ (peak fluorescence during the initial phase of Kautsky effect) were measured accordingly during 4 light adaptation, steady-state in light and/or 3 dark relaxation periods. Based on the measured chlorophyll a fluorescence parameters, automatic calculation of over 45 parameters such as QYmax (maximum photosystem II quantum yield, $F_V/F_M$), $F_V/F_m$ (photosystem II quantum yield), $F_q/F_v$ (photosystem II efficiency factor), Rfd (fluorescence decline ratio) and $q_L$ (fraction of photosystem II open centers) in light adaptation periods and steady-state in light was performed and NPQ (non-photochemical quenching) and $q_P$ (photochemical quenching of variable fluorescence) were calculated also in the dark relaxation periods. All the measured and calculated chlorophyll a fluorescence parameters are shown in Additional file 3: Table S6. In each experiment, 3-4 control and 3-4 infected plants were used per time point. The experiment was repeated independently 3 times (n=3).

**Statistical analysis**

The statistical analysis of all the data obtained in this work, except the microarray data, was performed using analysis of variance (one-way or two-way ANOVA) and post-hoc Duncan’s test ($P<0.05$) using STATISTICA v.13.3.

**Abbreviations**

**CAT**: conidial anastomosis tube

**DAMP**: damage-associated molecular pattern

**ETI**: effector-triggered immunity

**ETS**: effector-triggered susceptibility

**$F_0$**: minimum fluorescence

**FDR**: false discovery rate

**$F_m$**: maximum fluorescence

**$F_p$**: peak fluorescence during the initial phase of Kautsky effect
$F_v$: variable fluorescence

$F_q/F_v$: PSII efficiency factor

$F_v/F_m$: photosystem II (PSII) quantum yield

**GO:** Gene Ontology

**HST:** host-selective toxin

**JA:** jasmonic acid

**LRR:** leucine-rich repeat

**nHST:** non-host-selective toxin

**NPOQ:** non-photochemical quenching

**PAMP:** pathogen-associated molecular pattern

**PDA:** potato dextrose agar

**PR:** pathogenesis-related

**PRR:** pattern recognition receptor

**PSII:** photosystem II

**PTI:** pattern-triggered immunity

**QA:** plastoquinone A

**QB:** plastoquinone B

**qL:** PSII open centers fraction

**qP:** photochemical quenching of variable fluorescence

**QY:** photosystem II quantum yield

**QYmax:** maximum photosystem II quantum yield

**Rdf:** plant vitality parameter

**RLK:** receptor-like kinase

**SEM:** scanning electron microscopy
sRNA: small RNA

TEM: transmission electron microscopy

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

All the data analyzed during this study is included in this article or its supplementary information files. The microarray gene expression data generated during the current study is available in the Gene Expression Omnibus (GEO) repository under experiment number GSE155051 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE155051].

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

VKM conceived, designed and performed experiments. MG carried out the TEM experiments. AZ performed the microarray experiments and analysis. MS discussed the microscopy and ultrastructure results. AS and JO discussed the chlorophyll a fluorescence results. AKK helped with the manuscript preparation. V KM wrote the manuscript with input from all the authors. All authors read and approved the final manuscript.
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References

1. Babula D, Kaczmarek M, Ziółkowski PA, Sadowski J. Brassica oleracea. In: Kole C, editor. Genome Mapping and Molecular Breeding in Plants, Volume 5 Vegetables. Berlin, Heidelberg: Springer-Verlag; 2007. p. 227-285.
2. Ryder EJ. Cabbage. In: Ryder EJ, Leafy salad vegetables. Netherlands: The AVI Publishing Company, Springer; 1979. p. 127-169.
3. Nowakowska M, Wrzesińska M, Kamiński P, Szczechura W, Lichocka M, Tartanus M, Kozik EU, Nowicki M. Alternaria brassicicola—Brassicaceae pathosystem: insights into the infection process and resistance mechanisms under optimized artificial bio-assay. Eur J Plant Pathol. 2019;153:131-151.
4. Lawrence DP, Rotondo F, Gannibal PB. Biodiversity and taxonomy of the pleomorphic genus Alternaria. Mycol Progress. 2016;15:3.
5. Maude RB, Humpherson-Jones FM. Studies on the seed-borne phases of dark leaf spot Alternaria brassicicola and grey leaf spot Alternaria brassicae of brassicas. Ann App Biol. 1980;95:311-319.
6. Humpherson-Jones FM, Maude RB. Studies on the epidemiology of Alternaria brassicicola in Brassica oleracea seed production crops. Ann App Biol. 1982;100:61-71.
7. Dillard HR, Cobb AC, Lamboy JS. Transmission of Alternaria brassicicola to cabbage by flea beetles (Phyllotreta cruciferae). Plant Dis. 1998;82:153-157.
8. Nowicki M, Nowakowska M, Niezgoda A, Kozik EU. Alternaria black spot of Crucifers: symptoms, importance of disease, and perspectives of resistance breeding. Vegetab Crops Res Bulletin. 2012;76:5-19.
9. Macioszek VK, Wielanek M, Morkunas I, Ciereszko I, Kononowicz AK. Leaf position-dependent effect of Alternaria brassicicola development on host cell death, photosynthesis and secondary metabolites in Brassica juncea. Physiol Plant. 2020;168:601-616.
10. Humpherson-Jones FM. Survival of Alternaria brassicae and Alternaria brassicicola on crop debris of oilseed rape and cabbage. Ann App Biol. 1989;115:45-50.
11. Macioszek VK, Lawrence CB, Kononowicz AK. Infection cycle of Alternaria brassicicola on Brassica oleracea leaves under growth room conditions. Plant Pathol. 2018;67:1088-1096.
12. Sharma P, Deep S, Bhati DS, Sharma M, Chowdappa P. Penetration and infection processes of Alternaria brassicicola on cauliflower leaf and Alternaria brassicae on mustard leaf: a histopathological study. Plant Pathol J. 2014;13:100-111.
13. Humpherson-Jones FM, Phelps K. Climatic factors influencing spore production in *Alternaria brassicae* and *Alternaria brassicicola*. Ann App Biol. 1989;114:449-458.

14. Chen LY, Price TV, Park-Ng Z. Conidial dispersal by *Alternaria brassicicola* on Chinese cabbage (*Brassica pekinensis*) in the field and under simulated conditions. Plant Pathol. 2003;52:536-545.

15. Pedras MSC, Chumala PB, Jin W, Islam MS, Hauck DW. The phytopathogenic fungus *Alternaria brassicicola*: Phytotoxin production and phytoalexin elicitation. Phytochemistry. 2009;70:394-402.

16. Cho Y. How the necrotrophic fungus *Alternaria brassicicola* kills plant cells remains an enigma. Eukaryot Cell. 2015;14:335-344.

17. Pedras MSC, Park MR. The biosynthesis of brassicicolin A in the phytopathogen *Alternaria brassicicola*. Phytochemistry. 2016;132:26-32.

18. Aneja JK, Agnihotri A. Alternaria blight of oilseed Brassicas: epidemiology and disease control strategies with special reference to use of biotechnological approaches for attaining host resistance. J Oilseed Brassica. 2013;4:1-10.

19. Saharan GS, Mehta N, Meena PD. In: Saharan GS, Mehta N, Meena PD, editors. *Alternaria* Diseases of *Crucifers*: Biology, Ecology and Disease Management. Singapore: Springer Science+Business Media; 2016. p. 175-210.

20. van Wees SCM, Chang H-S, Zhu T, Glazebrook J. Characterization of the early response of *Arabidopsis* to *Alternaria brassicicola* infection using expression profiling. Plant 2003;132:606-617.

21. Su’udi M, Kim MG, Park SR, Hwang DJ, Bae SC, Ahn IP. *Arabidopsis* cell death in compatible and incompatible interactions with *Alternaria brassicicola*. Mol Cells. 2011;31:593-601.

22. Kámán-Tóth E, Dankó T, Gullner G, Bozsó Z, Palkovics L, Pogány M. Contribution of cell wall peroxidase- and NADPH oxidase-derived reactive oxygen species to *Alternaria brassicicola*-induced oxidative burst in *Arabidopsis*. Mol Plant Pathol. 2019;20:485-499.

23. Thomma BP, Nelissen I, Eggermont K, Broekaert WF. Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. 1999;19:163-171.

24. Pedras MS, Minic Z, Sarma-Mamillapalle VK. Substrate specificity and inhibition of brassinin hydrolases, detoxifying enzymes from the plant pathogens *Leptosphaeria maculans* and *Alternaria brassicicola*. FEBS J. 2009;276:7412-7428.

25. Pedras MSC, Abdoli A. Pathogen inactivation of cruciferous phytoalexins: detoxification reactions, enzymes and inhibitors. RSC Adv. 2017;7:23633.

26. Bilgin DD, Zavala JA, Zhu J, Clough SJ, Ort DR, DeLucia EH. Biotic stress globally downregulates photosynthesis genes. Plant Cell Environ. 2010;33:1597-1613.

27. Narusaka Y, Narusaka M, Seki M, Ishida J, Nakashima M, Kamiya A, Enju A, Sakurai T, Satoh M, Kobayashi M, Tosa Y, Park P, Shinozaki K. The cDNA microarray analysis using an *Arabidopsis*pad3 mutant reveals the expression profiles and classification of genes induced by *Alternariabrassicicola*. Plant Cell Physiol. 2003;44:377-387.
28. van Kan JAL. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends Plant Sci. 2006;11:247-253.

29. Mengiste T. Plant immunity to necrotrophs. Ann Rev Phytopathol. 2012;50:267-294.

30. Doullah MAU, Meah MB, Okazaki K. Development of an effective screening method for partial resistance to Alternaria brassicicola (dark leaf spot) in Brassica rapa. Eur J Plant Pathol. 2006;116:33-43.

31. Łaźniewska J, Macioszek VK, Kononowicz AK. Plant-fungus interface: The role of surface structures in plant resistance and susceptibility to pathogenic fungi. Physiol Mol Plant Pathol. 2012;78:24-30.

32. Kou Y, Naqvi NI. Surface sensing and signaling networks in plant pathogenic fungi. Seminars Cell Develop Biol. 2016;57:84-92.

33. Ziv C, Zhao Z, Gao YG, Xia Y. Multifunctional roles of plant cuticle during plant-pathogen interactions. Front Plant Sci. 2018;9:1088.

34. Babosha AV, Ryabchenko AS, Avetisyan GA, Avetisyan TV. Visualization of the halo region in plant-powdery mildew interactions by cryoscanning electron microscopy. J Plant Pathol. 2020;102:103-111.

35. Veloso J, van Kan JAL. Many shades of grey in Botrytis-host plant interactions. Trends Plant Sci. 2018;23:613-622.

36. Shlezinger N, Minz A, Gur J, Hatam I, Dagdas YF, Talbot NJ, Sharon A. Anti-apoptotic machinery protects the necrotrophic fungus Botrytis cinerea from host-induced apoptotic-like cell death during plant infection. PLoS Pathog. 2011;7:e1002185.

37. Fung RWM, Gonzalo M, Fekete C, Kovacs LG, He Y, Marsh E, McIntyre LM, Schachtman DP, Qiu W. Powdery mildew induces defense-oriented reprogramming of the transcriptome in a susceptible but not in a resistant grapevine. Plant Physiol. 2008;146:236-249.

38. Nokthai P, Lee VS, Shank L. Molecular modeling of peroxidase and polyphenol oxidase: substrate specificity and active site comparison. Int J Mol Sci. 2010;11:3266-3276.

39. Mandal S, Kar I, Mukherjee AK, Acharya P. Elicitor-induced defense responses in Solanum lycopersicum againstRalstonia solanacearum. Sci World J. 2013;

40. Roca MG, Read ND, Wheals AE. Conidial anastomosis tubes in filamentous fungi. FEMS Microbiol Lett. 2005;249:191-198.

41. Read ND, Lichius A, Shoji Y-J, Goryachev AB. Self-signalling and self-fusion in filamentous fungi. Curr Opin Microbiol. 2009;12:608-615.

42. Kurian SM, Di Pietro A, Read ND. Live-cell imaging of conidial anastomosis tube fusion during colony initiation in Fusarium oxysporum. PLoS ONE. 2011;13:e0195634.

43. Read ND, Goryachev AB, Lichius A. The mechanistic basis of self-fusion between conidial anastomosis tubes during fungal colony initiation. Fungal Biol Rev. 2012;26:1-11.

44. Shahi S, Fokkens L, Houterman PM, Rep M. Suppressor of fusion, a Fusarium oxysporum homolog of Ndt80, is required for nutrient-dependent regulation of anastomosis. Fungal Gen Biol. 2016;95:49-
57.

45. Craven KD, Vélèz H, Cho Y, Lawrence CB, Mitchell TK. Anastomosis is required for virulence of the fungal necrotroph *Alternaria brassicicola*. Eukaryot Cell. 2008;7:675-683.

46. Koziel E, Otulak-Koziel K, Bujarski JJ. Modifications in tissue and cell ultrastructure as elements of immunity-like reaction in *Chenopodium quinoa* against prune dwarf virus (PDV). Cells. 2020;9:148.

47. Otulak K, Chouda M, Bujarski J, Garbaczewska G. The evidence of tobacco rattle virus impact on host plant organelles ultrastructure. Micron. 2015;70:7-20.

48. Krzymowska M, Konopka-Postupolska D, Sobczak M, Macioszek VK, Ellis BE, Hennig J. Infection of tobacco with different *Pseudomonas syringae* pathovars leads to distinct morphotypes of programmed cell death. Plant J. 2007;50:253-264.

49. Gabara B, Kuźniak E, Skłodowska M, Surówka E, Miszalski Z. Ultrastructural and metabolic modifications at the plant-pathogen interface in *Mesembryanthemum crystallinum* leaves infected by *Botrytis cinerea*. Environ Exp Bot. 2012;77:33-43.

50. Lai Z, Mengiste T. Genetic and cellular mechanisms regulating plant responses to necrotrophic pathogens. Curr Opin Plant Biol. 2013;16:505-512.

51. Chen F, Ma R, Chen X-L. Advances of metabolomics in fungal pathogen-plant interactions. Metabolites. 2019;9:169.

52. Tsuge T, Harimoto Y, Akimitsu K, Ohtani K, Kodama M, Akagi Y, Egusa M, Yamamoto M, Otani H. Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. FEMS Microbiol Rev. 2013;37:44-66.

53. Akimitsu K, Tsuge T, Kodama M, Yamamoto M, Otani H. *Alternaria* host-selective toxins: determinant factors of plant disease. J Gen Plant Pathol. 2014;80:109-122.

54. Meena M, Samal S. *Alternaria* host-specific (HSTs) toxins: An overview of chemical characterization, target sites, regulation and their toxic effects. Toxicol Rep. 2019;6:745-758.

55. Cramer RA, La Rota CM, Cho Y, Thon M, Craven KD, Knudson DL, Mitchell TK, Lawrence CB. Bioinformatic analysis of expressed sequence tags derived from a compatible *Alternaria brassicicola-Brassica oleracea* Mol Plant Pathol. 2006;7:113-124.

56. Schenk PM, Thomas-Hall SR, Nguyen AV, Manners JM, Kazan K, Spangenberg G. Identification of plant defence genes in canola using *Arabidopsis* cDNA microarrays. Plant Biol. 2008;10:539-547.

57. Upadhyay P, Rai A, Kumar R, Singh M, Sinha B. Differential expression of pathogenesis related protein genes in tomato during inoculation with *solani*. J Plant Pathol Microb. 2014;5:1.

58. Zhu L, Ni W, Liu S, Cai B, Xing H, Wang S. Transcriptomics analysis of apple leaves in response to *Alternaria alternata* apple pathotype infection. Front Plant Sci. 2017;8:22.

59. Gong B-Q, Wang F-Z, Li J-F. Hide-and-seek: chitin-triggered plant immunity and fungal counterstrategies. Trends Plant Sci. 2020;25:805-816.

60. Hou S, Shen H, Shao H. PAMP-induced peptide 1 cooperates with salicylic acid to regulate stomatal immunity in *Arabidopsis thaliana*. Plant Signal Behav. 2019;14:1666657.
61. Liu Z, Wu Y, Yang F, Zhang Y, Chen S, Xie Q, Tian X, Zhou J-M. BIK1 interacts with PEPRs to mediate ethylene-induced immunity. Proc Natl Acad Sci USA. 2013;110:6205-6210.

62. Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA. PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. Plant Cell. 2010; 22:508-522.

63. Erwig J, Ghareeb H, Kopischke M, Hacke R, Matei A, Petutschnig E, Lipka V. Chitin-induced and CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) phosphorylation-dependent endocytosis of Arabidopsis thaliana LYSIN MOTIF-CONTAINING RECEPTOR-LIKE KINASE5 (LYK5). New Phytol. 2017;215:382-396.

64. Zheng Z, Qamar SA, Chen Z, Mengiste T. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 2006;48:592-605.

65. Manners JM, Penninckx IA, Vermaere K, Kazan K, Brown RL, Morgan A, Maclean DJ, Curtis MD, Cammue BP, Broekaert WF. The promoter of the plant defensin gene 2 from Arabidopsis is systemically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. Plant Mol Biol. 1998;38:1071-1080.

66. Zhou J, Wang X, He Y, Sang T, Wang P, Dai S, Zhang S, Meng X. Differential phosphorylation of the transcription factor WRKY33 by the protein kinases CPK5/CPK6 and MPK3/MPK6 cooperatively regulates camalexin biosynthesis in Arabidopsis. Plant Cell. 2020; org/10.1105/tpc.19.00971.

67. Srivastava A, Cho IK, Cho Y. The Bdtf1 gene in Alternaria brassicicola is important in detoxifying brassinin and maintaining virulence on Brassica Mol Plant Microbe Interact. 2013;26:1429-1440.

68. Meng X, Xu J, He Y, Yang K-Y, Mordorski B, Liu Y, Zhang S. Phosphorylation of an ERF transcription factor by Arabidopsis MPK3/MPK6 regulates plant defense gene induction and fungal resistance. Plant Cell. 2013;25:1126-1142.

69. Vidhyasekaran P. Plant Hormone Signalling Systems in Plant Innate Immunity. Dordrecht: Springer Science+Business Media; 2015.

70. Lloyd AJ, Allwood JW, Winder CL, Dunn WB, Heald JK, Cristescu SM, Sivakumaran A, Harren FJM, Mulema J, Denby K, Goodacre R, Smith AR, Mur LAJ. Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against Botrytis cinerea. Plant J. 2011;67:852-868.

71. Vidhyasekaran P. Cell wall degradation and fortification. In: Vidhyasekaran P, editor. Fungal Pathogenesis in Plants and Crop. Molecular Biology of Host Defence Mechanism. New York: 2nd edition, CRC Press, Taylor and Francis Group; 2008. p. 275-320.

72. Bhuiyan NH, Selvaraj G, Wei Y, King J. Role of lignification in plant defense. Plant Signal Behav. 2009;4:158-159.

73. Laluk K, Mengiste T. Necrotroph attacks on plants: wanton destruction or covert extortion? Arabidopsis Book. 2010;8:e0136.

74. Otani H, Kohnobe A, Kodama M, Kohmoto K. Production of a host-specific toxin by germinating spores of Alternaria brassicicola. Physiol Mol Plant Pathol. 1998;52:285-295.
75. Oka K, Akamatsu H, Kodama M, Nakajima H, Kawada T, Otani H. Host-specific AB-toxin production by germinating spores of *Alternaria brassicicola* is induced by a host-derived oligosaccharide. Physiol Mol Plant Pathol. 2005;66:12-19.

76. MacKinnon SL, Keifer P, Ayer WA. Components from the phytotoxic extract of *Alternaria brassicicola*, a black spot pathogen of canola. Phytochemistry. 1999;51:215-221.

77. Kretschmer M, Damoo D, Djamei A, Kronstad J. Chloroplasts and plant immunity: Where are the fungal effectors? Pathogens. 2020;9:19.

78. Rossi FR, Krapp AR, Bisaro F, Maiale SJ, Pieckenstain FL, Carrillo N. Reactive oxygen species generated in chloroplasts contribute to tobacco leaf infection by the necrotrophic fungus *Botrytis cinerea*. Plant J. 2017;92:761-773.

79. Xu Q, Tang C, Wang X, Sun S, Zhao J, Kang Z, Wang X. An effector protein of the wheat stripe rust fungus targets chloroplasts and suppresses chloroplast function. Nat Commun. 2019;10:5571.

80. Zechmann B. Ultrastructure of plastids serves as reliable abiotic and biotic stress marker. PLoS ONE. 2019;14:e0214811.

81. Kangasjärvi S, Neukermans J, Li S, Aro E-M, Noctor G. Photosynthesis, photorespiration, and light signalling in defence responses. J Exp Bot. 2012;63:1619-1636.

82. Maxwell K, Johnson GN. Chlorophyll fluorescence - a practical guide. J Exp Bot. 2000;51:659-668.

83. Nosek M, Kornaś A, Kuźniak E, Miszalski Z. Plastoquinone redox state modifies plant response to pathogen. Plant Physiol Biochem. 2015;96:163-170.

84. Chaerle L, Hagenbeek D, De Bruyne E, Valcke R, Van Der Straeten D. Thermal and chlorophyll-fluorescence imaging distinguish plant-pathogen interactions at an early stage. Plant Cell Physiol. 2004;45:887-896.

85. Pérez-Bueno ML, Pineda M, Barón M. Phenotyping plant responses to biotic stress by chlorophyll fluorescence imaging. Front Plant Sci. 2019;10:1135.

86. Rios JA, Aucique-Pérez CE, Debona D, Cruz Neto LBM, Rios VS, Rodrigues FA. Changes in leaf gas exchange chlorophyll *a* fluorescence and antioxidant metabolism within wheat leaves infected by *Bipolaris sorokiniana*. Ann Appl Biol. 2017;170:189-203.

87. Yang Z-X, Yang Y-F, Yu S-Z, Wang R-G, Wang Y, Chen H-L. Photosynthetic photochemical and osmotic regulation changes in tobacco resistant and susceptible to *Alternaria alternata*. Trop Plant Pathol. 2018;43:413-421.

88. Rys M, Juhász C, Surówka E, Janeczko A, Saja D, Tóbiás I, Skoczowski A, Barna B, Gullner G. Comparison of a compatible and an incompatible pepper-tobamovirus interaction by biochemical and non-invasive techniques: Chlorophyll *a* fluorescence, isothermal calorimetry and FT-Raman spectroscopy. Plant Physiol Biochem. 2014;83:267-278.

89. Malnoë A. Photoinhibition or photoprotection of photosynthesis? Update on new sustained quenching component, qH. Environ Exp Bot. 2018;154:123-233.
90. Ghosh S, Kanwar P, Jha G. Alterations in rice chloroplast integrity photosynthesis and metabolome associated with pathogenesis of *Rhizoctonia solani*. Sci Rep. 2017;7:41610.

91. Chen S, Dai X, Qiang S, Tang Y. Effect of a nonhost-selective toxin from *Alternaria alternata* on chloroplast-electron transfer activity in *Eupatorium adenophorum*. Plant Pathol. 2005;54:671-677.

92. Chen S, Kim C, Lee JM, Lee H-A, Fei Z, Wang L, Apel K. Blocking the Q$_B$-binding site of photosystem II by tenuazonic acid, a non-host-specific toxin of *Alternaria alternata*, activates singlet oxygen-mediated and EXECUTER-dependent signalling in *Arabidopsis*. Plant Cell Environ. 2015;38:1069-1080.

93. Barón M, Pineda M, Pérez-Bueno ML. Picturing pathogen infection in plants. Z Naturforsch C. 2016;71:355-368.

94. Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol. 1963;17:208-212.

95. Smyth GK, Speed T. Normalization of cDNA microarray data. Methods. 2003;31:265-273.

96. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:3.

97. Ritchie ME, Silver J, Oshlack A, Holmes M, Diyagama D, Holloway A, Smyth GK. A comparison of background correction methods for two-colour microarrays. Bioinformatics. 2007;23:2700-2707.

98. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B. 1995;57:289-300.

99. *Krishnakumar V, Hanlon MR, Contrino S, Ferlanti ES, Karamycheva S, Kim M, Rosen BD, Cheng C-Y, Moreira W, Mock SA, Stubbs J, Sullivan JM, Krampis K, Miller JR, Micklem G, Vaughn M, Town* Araport: the Arabidopsis Information Portal. Nucleic Acids Res. 2015;43:D1003-D1009.

100. Supek F, Bošnjak M, Škunca N, Šmuc T. REVIGO summarizes and visualizes long lists of Gene Ontology terms. PLoS ONE. 2011;6:e21800.

101. Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M. MAPMAN: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. 2004;37:914-939.

102. Wellburn AR. Spectral determination of chlorophylls $a$ and $b$, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol. 1994;144:307-313.

Figures
Figure 1

Analyses of necrotic lesion development on the second fully developed leaf of B. oleracea infected with A. brassicicola a time-course images of necrosis development on the drop-inoculated leaves; b quantitative analysis of necrotic spot size changes. Data was gained using the WinDIAS system. The means ± SE were calculated from at least 18 measurements per time point obtained in 3 independent experiments (n=3).
Figure 2

Quantitative data of A. brassicicola development on B. oleracea leaves The values are means ± SE of 3 replicates.
Figure 3

A. brassicicola penetration modes and different host cell reactions. The images were captured using SEM at 24 hpi. 

a. A bright 'halo' (asterisk) around the appressorium (arrow) penetrating the host cell; 

b. Direct penetration (arrows) caused a collapse of epidermal cells (asterisks) underneath germinating conidia; 

c. Dissolved wax layer (asterisk) around germinating conidium (arrowhead points to stoma); 

d. Germination tube (arrow) entering the leaf via stoma (arrowhead) surrounded by collapsed epidermal cells (asterisks);
e germination tube (arrow) forming an appressorium (double tail arrow) over stoma (arrowhead). Neighboring epidermal cells (asterisks) have fortified cell walls; f symptomless growth of hypha (arrow) on leaf surface (arrowhead points to stoma). Abbreviation: C – conidium

Figure 4

A. brassicicola mycelium formation on B. oleracea leaves The images were captured using SEM. a direct conidiation - new conidia (arrows) bud off from a mature conidium (24 hpi); b massive elongation and
branching of hyphae (arrows) on the leaf surface (24 hpi); c-d conidial anastomosis tubes (CAT, arrows) form connections between conidia (24 hpi); e anastomoses (arrows) between hyphae (48 hpi); f fusion of hyphae (arrow) (48 hpi). Abbreviations: C – conidium, St – stoma

Figure 5

Degradation of mesophyll cells in B. oleracea leaf infected with A. brassicicola at 48 hpi. The images were captured using TEM. a a typical nucleus with a nucleolus and small heterochromatin grains
(arrowheads), and chloroplasts in control mesophyll cell; b fragment of cell with degraded protoplast and nucleus with well-preserved nucleolus and fine granular chromatin; c nucleus with a nucleolus, condensed clumped chromatin and locally dilating nuclear envelope (arrowheads); d fragment of cell with degraded protoplast detached from the cell walls (asterisks), nucleus with granular chromatin, locally dilated envelope (arrowheads) and electron-dense deteriorating nucleolus; e fragment of strongly degraded cell with protoplast detached from cell walls (asterisk), pycnotic stage nucleus with osmiophilic remnants of chromatin (arrows) and dilated nuclear envelope (arrowheads); f terminal stage of nucleus degradation with clumps of chromatin (arrows), broken and dilated envelope (arrowheads). Abbreviations: Ch – chloroplast, CW – cell wall, ER – endoplasmic reticulum, G – granum, GA – Golgi apparatus, IS – intercellular space, LV – lytic vacuole, M – mitochondrion, MS – multilamellar structure, N – nucleus, Nu – nucleolus, VB – vesicular body
Figure 6

Disintegration of chloroplasts in B. oleracea leaf mesophyll cells infected with A. brassicicola at 48 hpi. The images were captured using TEM. a lenticular-shaped chloroplast with typical thylakoid and grana structure, and small starch grains in uninfected leaf; b fragment of mesophyll cell with degraded cytoplasm (asterisks), osmiophilic granules in plasmalemma (arrows) and a typical well-preserved chloroplast with plastoglobuli; c round-shaped chloroplast with a still preserved thylakoid and grana...
system, disintegrated chloroplast envelope (arrowheads) and swollen electron-translucent stroma; d round-shaped chloroplast with cup-shaped thylakoid system, reduced number of grana, locally swollen envelope (arrowheads) and electron-translucent stroma; e strongly disintegrated chloroplast with flocculent remnants of stroma, locally disintegrated envelope (arrowheads) and collapsed thylakoid system (asterisks) devoid of grana; f remnants of chloroplast with destroyed envelope (arrowheads) and thylakoids with widened lumens (arrows). Grana and stroma are missing. Abbreviations: Ch – chloroplast, CW – cell wall, G – granum, P – plastoglobule, S – starch grain, St – stroma

Figure 7

Gene Ontology of Biological Processes in B. oleracea leaves during A. brassicicola infection
Figure 8

Microarray-based analysis of expression changes of photosynthesis-related B. oleracea genes during the course of A. brassicicola infection a MapMan analysis of all mapped photosynthesis-related genes (104 genes); b MapMan analysis of photosynthesis-related genes significantly regulated at FDR<0.1; c MapMan diagram of significant expression changes of photosynthesis-related genes at 48 hpi.
Figure 9

Time-course analysis of photosynthetic pigment content. The means ± SE were obtained in 3 independent experiments (n=3). Asterisks indicate significant differences between control and infection at each time point according to Duncan test (P<0.05).
Figure 10

Analysis of chlorophyll a fluorescence parameters in B. oleracea leaves infected with A. brassicicola at 48 hpi. Values are the means obtained in 3 independent experiments (n=3; detailed analysis is available as Additional file 3: Table S6).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
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