Necrosis-Inducing Proteins of *Rhynchosporium commune*, Effectors in Quantitative Disease Resistance

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The barley pathogen *Rhynchosporium commune* secretes necrosis-inducing proteins NIP1, NIP2, and NIP3. Expression analysis revealed that *NIP1* transcripts appear to be present in fungal spores already, whereas NIP2 and NIP3 are synthesized after inoculation of host plants. To assess the contribution of the three effector proteins to disease development, deletion mutants were generated. The development of these fungal mutants on four barley cultivars was quantified in comparison with that of the parent wild-type strain and with two fungal strains failing to secrete an “active” NIP1 avirulence protein, using quantitative polymerase chain reaction as well as microscopic imaging after fungal green fluorescent protein tagging. The impact of the three deletions varied quantitatively depending on the host genotype, suggesting that the activities of the fungal effectors add up to produce stronger growth patterns and symptom development. Alternatively, recognition events of differing intensities may be converted into defense gene expression in a quantitative manner.

Qualitative disease resistance is usually defined as being complete and controlled by single resistance (*R*) genes, which mediate a very efficient defense frequently involving a hypersensitive response. In contrast, quantitative disease resistance is conditioned by multiple genes at quantitative resistance loci (QRL) exhibiting partial and inconsistent effects. The consequence is a reduction in disease instead of full resistance, with a wide range of disease phenotypes (Poland et al. 2009; St. Clair 2010). Qualitative disease resistance is generally associated with biotrophic pathogens, whereas quantitative disease resistance is found with biotrophic and necrotrophic pathogens. However, there is growing evidence that these definitions describe extreme positions of a continuum, with qualitative resistance and *R* genes at one end and quantitative resistance and QRL at the other end (Poland et al. 2009). Mechanistically, qualitative resistance usually requires the direct or indirect recognition of a single pathogen effector by a host resistance protein. In contrast, many different mechanisms are proposed to contribute to quantitative resistance (Poland et al. 2009; Schweizer and Stein 2011).

One of the host–pathogen interactions that appears to be positioned between the extremes of qualitative and quantitative resistance involves the barley pathogen *Rhynchosporium commune* (until recently, *R. secalis*) (Zaffarano et al. 2011). This fungus causes barley leaf scald, a destructive disease of great economic importance worldwide. The genetic basis of this interaction remains poorly understood. The fungus exhibits a high degree of pathogenic variability, easily rendering control measures based on resistance breeding and fungicides ineffective (Avrova and Knogge 2012; Shipton et al. 1974; Zhan et al. 2008). Over the years, a large number of fungal pathotypes has been detected in the different barley growing areas (Ali et al. 1976; Bouajila et al. 2006; Ceoloni 1980; Salamati and Tronsmo 1997; Takeuchi and Fukuyama 2009; Tekauz 1991). Phenotypic analysis of many interactions between fungal isolates and host cultivars revealed complex virulence patterns, with disease symptoms spanning from minute chlorotic and necrotic spots to complete leaf destruction. In addition, the interaction is characterized by a several-days- to weeks-long asymptomatic phase, frequently with pre-symptomatic spore formation, suggesting a reclassification of the fungus as a hemibiotroph rather than a true necrotroph (Zhan et al. 2008).

The quantitative and variable appearance of disease symptoms on different barley cultivars has rendered it difficult to unequivocally define *R* genes genetically. Nevertheless, 17 major gene loci have been identified that condition resistance to isolates of the fungus (Genger et al. 2005; Zhan et al. 2008); however, none of these *R* genes has been cloned to date. Several *R* alleles as well as QRL have been mapped at and around the complex *Rrs1* locus on chromosome 3H (Bjornstad et al. 2002; Zhan et al. 2008). Furthermore, numerous QRL were found at six of the seven barley chromosomes (Schweizer and Stein 2011), some of them in genomic regions that suggest them to be alleles of major *R* genes (Bjornstad et al. 2002; Cheong et al. 2006; Wagner et al. 2008). Best studied at the biochemical level is the effect of the *Rrs1* allele from ‘Turk’, *Rrs1*_{Turk}, which was introgressed into ‘Atlas’ to yield the near-isogenic ‘Atlas 46’ (Riddle and Suneson 1948; Wiebe and Reid 1960). The avirulence (*Avr*) gene corresponding to *Rrs1*_{Turk}, *AvrRrs1*, was among the first identified and characterized fungal *Avr* genes (Rohe et al. 1995). The gene encodes a small secreted necrosis-inducing protein, NIP1, which specifically induces defense reactions in *Rrs1* barley (Hahn et al. 1993). However, neither upon *Rrs1*-mediated recognition of NIP1 nor upon detection of the fungus through any other *R* gene does the plant appear to initiate a hypersensitive response to kill the invader.

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Nucleotide sequence data are available in the GenBank database under accession numbers JQ349028 (*NIP2*), JQ349030 (*GPD*), and JQ349029 (*NIP3*).

NIP1 and the structurally unrelated proteins NIP2 and NIP3 constitute a small family of secreted effector proteins that lack similarity to proteins from other fungi. The proteins were originally identified in fungal culture filtrates because they induce the formation of necrotic lesions on barley leaves resembling the disease symptoms (Wevelsiep et al. 1991). In addition, NIP1 and NIP3 stimulate the activity of the barley plasma membrane H^+ -ATPase (Wevelsiep et al. 1993), whereas no biochemical activity of NIP2 is known to date. Both necrotizing and H^+ -ATPase-stimulating activities of the fungal proteins occur on barley plants regardless of the *R* genotype. Therefore, both activities are presumed to be associated with fungal virulence. After expressing NIP1 in a heterologous system (Gierlich et al. 1999), the three-dimensional structure was solved (van't Slot et al. 2003) and an NIP1-binding site was identified on plasma membranes from barley regardless of the *R* genotype and from other cereals (van't Slot et al. 2007), again suggesting a relation to the virulence function of NIP1.

The relevance of NIP1 to barley attack in nature has been investigated by molecular population genetic analysis comprising several hundred fungal isolates. These studies (Schürch et al. 2004) revealed that the fungus is able to avoid *Rrs1*-mediated resistance by following either of two strategies: complete deletion of the *NIP1* gene (in 45% of all tested isolates) and alteration of the *NIP1* sequence to produce a modified ("inactive") protein that is no longer able to induce defense reactions. In contrast to *NIP1*, *NIP2* and *NIP3* were present in almost all (92 and 99.6%, respectively) of the analyzed fungal isolates, indicating their importance for the fungus. However, the role of the three proteins in fungal virulence remains to be resolved. In the present work, an attempt was made toward understanding the quantitative contribution of the three proteins to fungal pathogenesis. To this end, the three *NIP* genes were deleted and development of the deletion mutants was quantified and compared with that of the wild-type isolate, a natural *nip1*-isolate, and an isolate secreting an "inactive" version of the avirulence protein NIP1. The results suggest that the three proteins are part of the fungal repertoire of effectors, quantitatively supporting fungal growth and symptom expression on different barley cultivars to different extents.

RESULTS

NIP2 and *NIP3* gene structures.

After N-terminal sequencing of purified mature NIP2 and NIP3 (Wevelsiep et al. 1991), *NIP2* and *NIP3* clones were isolated from a fungal cDNA library (Rohe et al. 1995). In addition, the coding sequences (CDS) of both genes along with sufficient flanking sequence were obtained using a polymerase chain reaction (PCR) walking strategy (Devic et al. 1997), yielding sequences of 1,767 bp for *NIP2* and 2,563 bp for *NIP3*, that were deposited in the GenBank. A single 327-bp open reading frame translates into the 109-amino-acid (aa) NIP2. However, comparison of genomic and cDNA sequences revealed a 52-bp intron in the 3' untranslated region (UTR) starting immediately downstream of a tandem translation stop codon. The 115-aa NIP3 is encoded on two exons carrying 213 and 132 bp of the CDS, which are interrupted by a 61-bp intron. SignalP analysis (Emanuelsson et al. 2007) predicted signal peptides for NIP2 and NIP3 of 16 and 17 aa, respectively, which were confirmed by N-terminal sequencing. Mass spectrometry (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (not shown) verified a molecular mass of 10.03 kDa for the mature NIP2. In contrast, the mature NIP3 appears to be post-translationally processed proteolytically, because i) the deduced mass of the protein (10.97 kDa) exceeds the mass as determined by sodium dodecyl sul-

fate polyacrylamide gel electrophoresis (approximately 6.5 kDa) (Wevelsiep et al. 1991) and ii) the presence of a highly negatively charged C-terminal sequence leads to a calculated isoelectric point value of 4.52, which would have precluded

Fig. 1. Fungal growth and necrosis-inducing protein (*NIP*) gene expression. **A**, Growth of *Rhynchosporium commune* UK7 on susceptible barley 'Ingrid' and on moderately susceptible 'Atlas'. **B** and **C**, Relative abundance of *NIP* transcripts during growth of UK7 on 'Ingrid' and 'Atlas'.

protein purification by cation exchange chromatography. Near the N-terminus of the mature NIP3, a single glycosylation site was detected, which presumably carries the N-glycosidic carbohydrate side chain of the secreted protein (Wevelsiep et al. 1991).

Fungal development in planta.

Growth of fungal wild-type strain UK7 on susceptible barley 'Ingrid' and on moderately susceptible 'Atlas' was determined by quantitative (q)PCR-based relative quantification of fungal DNA. To this end, abundance of the fungal target gene, *GPD*, was measured relative to a barley reference gene encoding a putative trypsin-like serine protease. On 'Ingrid', a sigmoid growth curve was obtained, which describes an early phase

with slowly increasing fungal biomass as reflected by DNA amounts, followed by a phase with rapid fungal development and a late phase with strongly decelerated growth (Fig. 1A). During the first phase, fungal conidia germinated on the surface of host leaves and, after penetration of the cuticle, fungal colonies were established in the subcuticular region of the epidermis through the formation of thin, wide-septated hyphae (type I) (Kirsten et al. 2011), frequently along the anticlinal cell walls (Lehnackers and Knogge 1990; Linsell et al. 2011; Thirugnanasambandam et al. 2011). Approximately 5 days postinoculation (dpi), thicker, short-septated hyphae (type II) started to occur that later developed into a dense subcuticular stroma (Ayesu-Offei and Clare 1970; Hosemans and Branchard 1985; Lyngs Jorgensen et al. 1993) (Fig. 2). Under the growth

Fig. 2. Stages of fungal development in barley leaves. **A** and **B**, Early fungal hyphae (arrows) above the anticlinal walls of epidermis cells; **C** and **E**, few fungal hyphae (arrows) near leaf margins on resistant 'Atlas 46'; **D**, unsuccessful penetration of fungal hyphae on 'Atlas 46'; **F** through **J**, stages of stroma development. Bar = 50 μ m.

conditions, applied degradation of epidermis cells and underlying mesophyll cells became evident 7 to 10 dpi by the occurrence of macroscopically visible disease symptoms. New conidia, which were formed on the subcuticular stromata, could be observed in the lesion areas as well as protruding through the cuticle in symptomless leaf regions (Davis and Fitt 1990; Lehnackers and Knogge 1990). In contrast to 'Ingrid', a slowly increasing growth curve characterized fungal growth on 'Atlas' (Fig. 1A). Substantially fewer fungal hyphae and less stroma were found in the leaves and small, localized disease symptoms did not occur until approximately 14 dpi.

NIP gene expression during fungal development in planta.

In order to obtain a first clue to the functional relevance of the *NIP* genes to pathogenesis, their expression during fungal devel-

opment in planta was quantified by quantitative reverse-transcriptase (qRT)-PCR relative to a constitutively expressed fungal gene (Fig. 1B and C). In preliminary experiments, expression of *R. commune* *GPD*, *actin*, and *ubiquitin* was quantified during pathogenesis on barley by qRT-PCR using barley *actin* as the reference. Fungal *actin* and *ubiquitin* sequences came from an expressed sequence tag library (A. Navarro-Quezada and W. Knogge, *unpublished*). The obtained transcript profiles were then related to the increase in fungal biomass as measured by qPCR. Abundance of *ubiquitin* transcripts relative to fungal biomass increased after 10 dpi, whereas *GPD* and *actin* expression remained constant. *GPD* was selected as the constitutively expressed reference gene in all qRT-PCR experiments.

On both cultivars, *NIP1* transcript levels exceeded those of *NIP2* and *NIP3*, with substantial mRNA amounts present at

Fig. 3. Disease phenotypes of the interaction between three strains and four deletion mutants of *Rhynchosporium commune* and four barley cultivars at 21 days postinoculation. 'Turk' and 'Atlas 46' but not 'Ingrid' and 'Atlas' carry resistance gene *Rrs1*.

the earliest harvesting time point (≤ 1 h postinoculation = 0 dpi) (i.e., presumably already in the spores). *NIP1* expression on 'Ingrid' showed a distinct maximum at 3 to 6 dpi and a subsequent rapid decline. By comparison, on 'Atlas', it followed a similar, albeit delayed curve with a maximum at 6 to 10 dpi. Similarly, in comparison with 'Ingrid', *NIP3* expression on 'Atlas' appears to be delayed, although more pronounced, with broad maxima approximately 6 and 10 dpi, respectively. In contrast, *NIP2* expression varied greatly on both cultivars, with maximum transcript abundance at 6 dpi on 'Atlas' but only weak expression on 'Ingrid'.

Generation and phenotypic analysis of *NIP* deletion mutants.

To generate deletion mutants for each of the *NIP* genes, replacement vectors were constructed, in which the CDS was interrupted or partially replaced by integration of a selectable marker gene cassette. Fungal deletion mutants UK7 Δ *NIP1* and UK7 Δ *NIP3* (hygromycin resistant) were obtained by homologous recombination following polyethylene glycol-mediated transformation of protoplasts of wild-type strain UK7 (Rohe et al. 1996). Among 60 transformants screened by PCR, two lacked the wild-type *NIP1* gene whereas, in two of 245 transformants, the disrupted *NIP3* gene had replaced the wild-type gene. Mutant UK7 Δ *NIP2* (phleomycin resistant) was generated by *Agrobacterium tumefaciens*-mediated transformation (ATMT) of fungal conidia (Kirsten et al. 2011). In addition to the *ble*-disrupted *NIP2* sequence, the T-DNA of the binary vector contained the enhanced green fluorescent protein (*EGFP*) gene for negative visual selection. Of the 56 mutants obtained after ATMT, 50 showed EGFP fluorescence, which is indicative of heterologous integration of the T-DNA into the fungal genome. PCR analysis revealed that five of the nonfluorescing mutants nevertheless contained the endogenous *NIP2* gene. However, in one mutant, the wild-type gene was replaced by the deletion construct. The molecular structure of mutant UK7 Δ *NIP2* was eventually confirmed by Southern hybridization (not shown). Finally, to study the effect of *NIP2* on *Rrs1* plants, an *NIP1NIP2* double-deletion mutant was generated by replacing the *NIP2* gene in UK7 Δ *NIP1* with an *NIP2*-UTR-flanked phleomycin cassette, an event that occurred in one of 31 analyzed fungal mutant colonies.

To assess the impact of *NIP* gene deletion on disease development and symptom expression, spores of wild-type strain UK7 expressing all three *NIP* genes; the three single-gene deletion mutants UK7 Δ *NIP1*, UK7 Δ *NIP2*, and UK7 Δ *NIP3*; and the double-deletion mutant UK7 Δ *NIP1* Δ *NIP2* were used to inoculate four different barley cultivars: susceptible 'Ingrid', moderately susceptible 'Atlas', near-isogenic 'Atlas 46' carrying the *Rrs1* resistance gene (Riddle and Suneson 1948), and the *Rrs1* donor 'Turk'. Two additional fungal strains were included in these studies: the natural *nip1*⁻ strain UK5 and strain AU2, which secretes an "inactive" isoform of the Avr protein NIP1 (Fiegen and Knogge 2002; Rohe et al. 1995; van't Slot et al. 2003). As expected, all interactions involving both the *R* gene *Rrs1* and the corresponding *Avr* gene *NIP1* remained symptomless, whereas loss of the *NIP1* gene rendered the fungal mutant UK7 Δ *NIP1* strongly virulent on the *Rrs1* cultivars Turk and Atlas 46 (Rohe et al. 1995) (Fig. 3). Fungal strains AU2, UK5, UK7 Δ *NIP1*, and UK7 Δ *NIP1* Δ *NIP2* produced disease symptoms on all four barley cultivars. Symptoms caused by UK5, UK7 Δ *NIP1*, and UK7 Δ *NIP1* Δ *NIP2* were similar on all plants, with small lesions on 'Atlas' and strong lesions on 'Turk' and 'Ingrid'. A difference was visible only on 'Atlas 46', where UK5-derived symptoms were stronger than those of UK7 Δ *NIP1* and UK7 Δ *NIP1* Δ *NIP2*. Strain AU2 induced the strongest symptoms on each of the four barley cultivars, whereas the deletion mutants UK7 Δ *NIP2* and

UK7 Δ *NIP3* were able to cause disease symptoms only on 'Ingrid' and 'Atlas' due to the continuing presence of a functional *NIP1*. Disease symptoms generated by mutant UK7 Δ *NIP2* on 'Ingrid' virtually did not deviate from wild-type symptoms. In contrast, the lesions visible on UK7 Δ *NIP2*-infected 'Atlas' remained smaller than wild-type lesions. Finally, UK7 Δ *NIP3*-generated necrotic lesions remained much smaller than the wild type on both cultivars.

Quantification of *NIP* deletion mutant growth in planta.

Biomass alterations of the different fungal strains and mutants on the four barley cultivars were measured by qPCR-based relative quantification of fungal DNA in all 28 interactions (four barley cultivars and seven fungal strains) at three time points: immediately after inoculation; at a mid-point of the development, 7 dpi ('Ingrid') or 10 dpi (other cultivars), and late in pathogenesis, at 14 and 21 dpi, respectively. The biomass of the avirulent strains (UK7, UK7 Δ *NIP2*, and UK7 Δ *NIP3* on 'Atlas 46' and 'Turk') remained low over this time, whereas that of the virulent strains increased without statistically significant alterations in their relation to each other (Fig. 4). Therefore, only the late time point was evaluated for comparing the different interactions.

Both fungal strains lacking an "active" NIP1, AU2 ("inactive" NIP1) and UK5 (no *NIP1*), as well as the *NIP1* deletion mutants UK7 Δ *NIP1* and UK7 Δ *NIP1* Δ *NIP2*, were able to grow on all four barley cultivars, with AU2 always producing the highest biomass (Fig. 5). Unlike AU2, however, strain UK5 and the mutants UK7 Δ *NIP1* and UK7 Δ *NIP1* Δ *NIP2* grew substantially more poorly on 'Atlas' and 'Atlas 46' than on 'Ingrid' and 'Turk'. Fungal strains UK7, UK7 Δ *NIP2*, and UK7 Δ *NIP3* secreting an "active" NIP1 were hardly detectable on the *Rrs1* cultivars Turk and Atlas 46 (Fig. 6). However, as expected, removal of the *NIP1* gene from UK7 enabled strong growth of mutants UK7 Δ *NIP1* and UK7 Δ *NIP1* Δ *NIP2* on 'Turk'. Also, on 'Atlas 46', both mutants grew significantly better than UK7 ($P < 0.05$ and 0.01 , respectively) (Table 1). On the moderately susceptible 'Atlas', loss of *NIP1* caused a strong decrease in the biomass of both fungal mutants as compared with the wild-type situation (Table 1), whereas no statistically significant difference was observed on susceptible 'Ingrid'. Development of the *NIP2* deletion mutant on 'Atlas' was weak, resembling that of UK7 Δ *NIP1*, whereas it did not differ from the wild type on 'Ingrid'. Growth of mutants UK7 Δ *NIP1* and UK7 Δ *NIP1* Δ *NIP2* on 'Atlas 46' significantly exceeded that of UK7 ($P < 0.05$ and 0.01 , respectively) but, on 'Atlas', fell below it ($P < 0.01$ and 0.05 , respectively) (Table 1). The biomass of the double-deletion mutant did not significantly deviate from UK7 Δ *NIP1* and UK7 Δ *NIP2* on 'Atlas' or from UK7 Δ *NIP1* on 'Atlas 46' (Table 1). Finally, loss of *NIP3* drastically reduced fungal development on both susceptible cultivars.

During early stages of pathogenesis (3 dpi), when the fungus grows more or less two-dimensionally underneath the cuticle, a microscopic multiple-plane imaging analysis to quantify the fungus by determining colony areas was carried out (Seiffert and Schweizer 2005). For this purpose, UK7 and the single *NIP* gene deletion mutants were transformed with the *EGFP* gene (Kirsten et al. 2011) and fungal structures were monitored using a fluorescence-imaging acquisition protocol (Baum et al. 2011). On 'Ingrid', the areas covered by fungal wild-type colonies were significantly larger than those of all three deletion mutants (Fig. 7). In contrast, on 'Atlas', only the *NIP2* deletion mutant deviated from the wild type. On 'Atlas 46', the absence of the *Avr* gene became manifest in the *NIP1* deletion mutant growing faster than the wild type already at this early stage of the interaction. As in 'Ingrid' and 'Atlas', UK7 Δ *NIP2* colonies were smaller than the wild type in 'Atlas 46'.

Microscopic analysis of fungal growth.

All interactions of wild-type strains and single-gene deletion mutants resulting in a relative fungal biomass of >0.4 (Fig. 5) were characterized by a fungal development similar to that described for UK7 on 'Ingrid', except that the subcuticular fungal stroma grew more extensive and denser. In all other interac-

tions, fungal structures were reduced to various degrees. Both types of hyphae and little stroma were produced by UK5 on 'Atlas' and 'Atlas 46' and by UK7 on 'Atlas'. In contrast, only a few hyphae of strain UK7 and very little stroma were detectable at the leaf margins of 'Atlas 46' (Fig. 2). Along with sites of unsuccessful penetration, few subcuticular mycelia and

Fig. 4. Relative biomass of *Rhynchosporium commune* during growth of fungal strains and deletion mutants at three time points postinoculation of the indicated barley cultivars. Time point 0: 0 days postinoculation (dpi), time point 1: 7 dpi ('Ingrid') or 10 dpi (all other cultivars), time point 2: 14 dpi ('Ingrid') or 21 dpi (all other cultivars). Whiskers above bars mark the standard error.

small stromatal structures of mutant UK7 Δ NIP1 were found on 'Atlas'. On 'Atlas 46', single small stromatal structures were detected in the upper leaf part toward the tip, whereas more fungal hyphae but no stroma occurred toward the leaf base. Loss of the *NIP2* gene led to very little mycelia in small colonies and to increased wall appositions at sites of attempted penetration on 'Atlas', 'Atlas 46', and 'Turk', whereas dense fungal hyphae but little stroma occurred along the leaves of 'Ingrid'. Double-deletion mutant UK7 Δ NIP1 Δ NIP2 reached a biomass of >0.4 on 'Ingrid' and 'Turk', which mainly resulted from dense fungal hyphae, whereas only a few stromatal structures were detected. Similar to the interaction of UK7 with 'Atlas 46', only a few hyphae of UK7 Δ NIP1 Δ NIP2 were found near leaf margins of both 'Atlas' and 'Atlas 46' but no stroma was detected. Finally, the mutant UK7 Δ NIP3 generated very little subcuticular hyphae except in 'Ingrid', where local mycelia were visible, very few even beginning to form stromatal structures.

In many of the interactions, wall appositions were observed at sites of unsuccessful penetration (Fig. 2D). Although these appositions tended to appear more frequently in incompatible interactions, there was no clear correlation between number of appositions and symptom size or fungal biomass.

DISCUSSION

Effectors secreted by pathogens are molecules "that alter host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense responses (avirulence factors or elicitors)" (Kamoun 2009). Hence, dele-

tion of effector-encoding genes may affect pathogen development in planta and host interaction. When compared with the wild type, mitigated growth of a deletion mutant indicates removal of a virulence factor, whereas augmented growth follows from deletion of an avirulence factor. However, if detection of effectors contributes to quantitative resistance, their deletion may only cause marginal effects in the interaction with the host. Nevertheless, deletion mutants are crucial tools to an understanding of the function of genes encoding secreted fungal proteins.

When the *NIP* deletion mutants of *R. commune* were analyzed on different barley cultivars, both virulence and avirulence functions became apparent. On 'Atlas' (*rrs1*), all four deletion mutants exhibited weaker growth symptoms (Fig. 3) and a significantly attenuated growth compared with UK7 (Fig. 6). Near-isogenic 'Atlas 46', which carries the *R* gene *Rrs1*_{Turk}, and also the *Rrs1* donor 'Turk', both allow fungal growth in the absence of the corresponding *Avr* gene, *NIP1* (= *AvrRrs1*) beyond the very small colonies formed by wild-type strain UK7 near the leaf margins (Fig. 2). This was the case in the *NIP1* deletion mutants UK7 Δ NIP1 and UK7 Δ NIP1 Δ NIP2 but also in a natural *nip1*⁻ strain, UK5, and a strain expressing an "inactive" *NIP1*, AU2 (Fig. 5) (Rohe et al. 1995). However, also on 'Atlas 46', growth of mutants UK7 Δ NIP1 and UK7 Δ NIP1 Δ NIP2 failed to reach that of wild-type strain UK7 on 'Atlas'. Although the unavailability of a UK7 Δ NIP1 Δ NIP3 double-deletion mutant prevented a separate analysis of a possible *NIP3* effect on 'Atlas 46' (and 'Turk'), these results suggest that, against the 'Atlas' genetic background, the three *NIP* are potent virulence factors.

Fig. 5. Comparison of relative biomass of three *Rhynchosporium commune* strains and four deletion mutants on four barley cultivars at 14 days postinoculation (dpi) ('Ingrid') and 21 dpi (all other cultivars). Whiskers above bars mark the standard error.

Against the non-‘Atlas’ genetic backgrounds, the situation was found to be different. On susceptible ‘Ingrid’, *NIP3* also exerted a strong virulence effect, whereas *NIP1* and *NIP2* had only minute effects (Fig. 6). Hyphal growth and stroma density as well as disease symptoms of UK7Δ*NIP1*, UK7Δ*NIP2*, and UK7Δ*NIP1*Δ*NIP2* did not significantly differ from the compatible interaction of the parent strain UK7 with ‘Ingrid’ (Fig. 3). However, careful fluorescence-imaging-based evaluation of early fungal development on ‘Ingrid’ revealed an already reduced colony size not only of the *NIP3* deletion mutant but also of the mutants lacking *NIP1* or *NIP2* (Fig. 7). This suggests an early impact of these effectors on fungal virulence. Nevertheless, loss of *NIP1* did not have a persistent effect and no biomass deviation from the wild type was observable after a few days. Loss of *NIP2* took longer to be compensated because, at mid-point of the development, the biomass of UK7Δ*NIP2* still lagged behind the wild type. Finally, unlike on ‘Ingrid’, *NIP2* is a substantial virulence factor not only on ‘Atlas’ but also on ‘Turk’, as is suggested by the significantly lower biomass ($P < 0.05$) of mutant UK7Δ*NIP1*Δ*NIP2* as compared with UK7Δ*NIP1* on this cultivar (Fig. 6).

The function of genes in host–pathogen interactions may be foreshadowed by their expression profiles during pathogenesis. When relative expression of the *NIP* genes was compared with fungal growth, it became apparent that the rise in fungal biomass on susceptible ‘Ingrid’ coincides with the beginning of a decrease in *NIP* gene expression (Fig. 1). Furthermore, the generally decelerated and attenuated growth of UK7 on ‘Atlas’ as compared with ‘Ingrid’ coincides with a deferred and persisting *NIP* gene expression. This suggests that the *NIP* are functionally associated with the early period of *R. commune* development in barley leaves. In addition, transcript abundance of the genes *NIP2* and *NIP3* predicts their functional relevance.

NIP2 strongly affects virulence on ‘Atlas’ but not on ‘Ingrid’. This is reflected by the expression of the gene, which is high on ‘Atlas’ and low on ‘Ingrid’ and, hence, appears to be influenced by the host genotype. *NIP3* is a strong virulence factor and expressed to similar and medium levels on both cultivars. Only for the *NIP1* gene do transcript levels and functional importance not coincide. Unlike *NIP2* and *NIP3*, the gene is strongly expressed shortly after inoculation (i.e., presumably already in the spores), transiently reaching very high relative transcript abundance on both barley cultivars. Nevertheless, a strong virulence effect of *NIP1* was only observed on ‘Atlas’, whereas its impact on fungal growth on ‘Ingrid’ remained transient and very weak. This suggests that *NIP1*, possibly along with other genes, is preemptively expressed prior to host contact without being required or effective on all host genotypes. Not surprisingly, it is the recognition of the *NIP1* gene product by *Rrs1*-plants that leads to the early launching of defense reactions and to the efficient restriction of fungal growth (Hahn et al. 1993; Rohe et al. 1995). In contrast, expression of *NIP2* and *NIP3* occurs upon host contact and may be influenced by the host genotype.

To date, the biochemical function of *NIP2* remains unknown. In contrast, both *NIP1* and *NIP3* act at the interface with the host plant by stimulating the plant plasma membrane-localized H^+ -ATPase (Wevelsiep et al. 1993). As a consequence, an acidic environment would be generated, which has been described for several phytopathogenic fungi, including *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Prusky and Yakoby 2003). Adjusting the apoplastic pH may serve to optimize the conditions for enzymatic degradation of plant cell walls. Alternatively, the electrochemical gradient at the plant plasma membrane may be affected by activating H^+ -ATPase activity. As a consequence, the critical regulation of essential membrane transport processes

Fig. 6. Comparison of relative biomass of *Rhynchosporium commune* strain UK7 and its derivatives on four barley cultivars at 14 days postinoculation (dpi) (‘Ingrid’) and 21 dpi (all other cultivars). Asterisks indicate statistically significant (* indicates $P < 0.05$) and very significant (** indicates $P < 0.01$) deviation from wild-type UK7, in the ‘Turk’ diagram from UK7Δ*NIP1*. In contrast, there is no statistically significant difference between UK7Δ*NIP1* and the double-deletion mutant on ‘Atlas’ and ‘Atlas 46’. Whiskers above bars mark the standard error.

Table 1. Comparison of the relative biomasses of fungal wild-type strain UK7 and of the necrosis-inducing protein (*NIP*) deletion mutants in the interactions with barley ‘Atlas 46’ (lower left) and ‘Atlas’ (upper right)^a

	Atlas				
	UK7	Δ <i>NIP1</i>	Δ <i>NIP2</i>	Δ <i>NIP3</i>	Δ <i>NIP1/2</i>
‘Atlas 46’					
UK7	0.0466 *A	0.0068 **A	0.0058 **A	0.1617 ns	0.0407 *A
Δ <i>NIP1</i>	0.0113 *A	0.0656 ns	0.2041 ns	<0.0001 ***A46	0.1689 ns
Δ <i>NIP2</i>	0.4671 ns	0.0080 **A	0.1327 ns	0.0810 ns	0.1031 ns
Δ <i>NIP3</i>	0.1382 ns	0.0061 **A	0.1855 ns	0.5909 ns	0.0354 *A
Δ <i>NIP1/2</i>	0.0051 **A	0.1037 ns	0.0040 **A46	0.0034 **A46	0.7727 ns

^a Statistical significance of differences was assessed using the unpaired *t* test. *P* values and their statistical interpretation are shown. ‘A46’ or ‘A’ mark the cultivar with the significantly higher fungal biomass. Data in bold on the diagonal show the results of the comparisons between ‘Atlas 46’ and ‘Atlas’; ***, extremely significant, $P < 0.001$; **, very significant, $P = 0.001$ to 0.01 ; *, significant, $P = 0.01$ to 0.05 ; ns, not significant, $P > 0.05$.

would be altered (e.g., to facilitate nutrient export from host cells) (Elmore and Coaker 2011). With both functions, the extent of pH adaptation may quantitatively influence fungal virulence.

If the mRNA abundance reflects protein amounts and, thus, H^+ extrusion into the apoplast, expression of *NIP1* and *NIP3* during early stages of pathogenesis would be in agreement with such functions. During this period, fungal hyphae penetrate the host leaves and plant cell walls need to be at least partially degraded and nutrients taken up to establish subcuticular colonies. This may be more critical on the moderately susceptible ‘Atlas’, where the fungus is confronted with as-yet-unknown but apparently more challenging growth conditions

than on the susceptible ‘Ingrid’. On the latter, loss of *NIP1* does not affect fungal virulence, possibly because, under the more favorable growth conditions, enzymatic cell wall degradation does not need to be optimized, extracellular nutrient supply is satisfactory, or acidification induced by *NIP3* (and possibly other factors) suffices to optimize growth. Alternatively, H^+ -ATPase stimulation by the two proteins may affect different processes. For instance, the strong and very early *NIP1* expression may aim at efficiency of cell wall degradation, whereas the later-occurring *NIP3* may rather serve nutrient acquisition.

In addition to the *NIP*, many as-yet-unknown fungal and plant features can be suspected to affect the outcome of particular strain–cultural interactions. Evidence for the activity of additional plant and fungal factors comes from a quantitative comparison of *R. commune* growth on the different host cultivars. A conspicuous difference exists between the biomasses of virulent fungal strains on ‘Turk’ and ‘Ingrid’ on the one hand and on ‘Atlas’ and ‘Atlas 46’ on the other (Fig. 5). For instance, growth of strain UK5 on ‘Turk’ and ‘Ingrid’ clearly exceeded that on ‘Atlas’ and ‘Atlas 46’. A similar difference was found for the *NIP1* deletion mutants and also for UK7 and the *NIP2* deletion mutant on the *rrs1* ‘Ingrid’. This suggests that the ‘Atlas’ genetic background carries as-yet-unknown genes that slow down fungal development or, vice versa, lacks genes that enhance fungal development in ‘Ingrid’ or ‘Turk’. It remains to be shown whether the *R* gene *Rrs2*, which is present in ‘Atlas’ and ‘Atlas 46’ (Bjornstad et al. 2002; Dyck and Schaller 1961) is involved. This gene may recognize its corresponding *Avr* gene, *AvrRrs2*, if present in UK7—and its derivatives as well as in UK5.

The different genetic backgrounds of the host cultivars do not alter the interactions with fungal strain AU2, which grew equally well on all four barley cultivars (Fig. 5). The much higher biomass as compared with UK5 on ‘Atlas’ (60-fold) and ‘Atlas 46’ (16-fold) may be explained by the availability of additional, as-yet-unknown, very efficient virulence factors. Alternatively, this strain may be much better protected from or capable of eluding early recognition by the plant, possibly by not presenting avirulence factors. It is tempting to speculate that the significant growth difference ($P = 0.02$) between AU2 and UK5 on ‘Turk’ (*Rrs1*) is, at least in part, associated with the *NIP1* locus. UK5 lacks the virulence factor *NIP1*, whereas AU2 harbors an *NIP1* gene encoding an “inactive” *NIP1* isoform, which fails to initiate host defense reactions (Fiegen and Knogge 2002; Rohe et al. 1995; van’t Slot et al. 2007). However, its efficient binding to a membrane receptor (van’t Slot et al. 2007) suggests that it still fulfills its function for fungal virulence.

A comparison between the UK7-derived *NIP1* deletion mutants and the natural *nip1*[−] strain UK5 demonstrates that additional differences in the effector repertoire of the fungal strains are responsible for the observed differences in fungal growth (Fig. 5). For instance, UK5 grew significantly better ($P < 0.017$) than UK7Δ*NIP1* on ‘Turk’, which allows fungal growth only in the absence of an “active” *NIP1*. Similarly, the biomass of UK5 exceeded that of UK7Δ*NIP1* on ‘Ingrid’. The role of additional effectors is also evident, because the absence of the *NIP1* gene from strain UK5 appears to be compensated on ‘Atlas’. This enables a UK7-like growth in contrast to the UK7-derived *NIP1* deletion mutants.

The presented observations support the following interpretation of *R. commune* development on barley. After penetration of susceptible host tissue, the fungus grows macroscopically undetected in planta over long periods until disease symptoms eventually are formed. During this time, the fungus passes through two major morphological stages: a first hyphal stage

Fig. 7. Box plot comparison of area covered by colonies of green fluorescent protein (GFP)-tagged wild-type strain UK7 and GFP-tagged necrosis-inducing protein (*NIP*) gene deletion mutants at 3 days postinoculation. Quantification was based on fluorescence microscopic imaging. Boxes show upper and lower quartile and the median. Whiskers represent the upper and lower 1.5-fold interquartile ranges. Circles indicate outliers, squares the mean values. Figures beneath the boxes specify the number of fungal colonies analyzed in the given interaction. Gray lines separate independent experimental series, each containing the indicated deletion mutant and the associated reference wild-type strain UK7. Asterisks denote statistically significant deviation of the mutant from the wild type ($P < 0.01$).

to spread through the leaves and a second stage characterized by a dense subcuticular stroma that supports conidiation (Horbach et al. 2011; Kirsten et al. 2011). The NIP and, probably, additional effectors are expressed during the first stage, whereas no fungal genes have been identified yet that mark the second stage. The late occurrence of necrotic lesions through collapsing epidermal and mesophyll cells appears to correlate with a generally increased fungal biomass in the leaves without, however, coinciding with any conspicuous alteration in fungal morphology. Long asymptomatic fungal growth prior to a phase of severe plant tissue damage describes one of the current meanings of the term “hemibiotrophy” (Oliver and Ipcho 2004). Several phytopathogenic fungi, including a close relative of *R. commune*, *Pyrenopeziza brassicae* (Goodwin 2002), follow this growth pattern.

Substantial lesions are usually accompanied by extensive fungal growth and modest lesions by moderate growth. Even in resistant plants lacking any visible symptoms, extremely decelerated fungal development can be observed that is restricted to the marginal leaf tissues. This suggests a quantitative model for the interaction of *R. commune* and barley, in which fungal effectors may play distinct, important roles. Effectors may be active toward multiple plant susceptibility targets (encoded at quantitative susceptibility loci) (Denby et al. 2004; Kamphuis et al. 2008), their impact adding up to produce stronger growth patterns and symptom development. This situation would be reminiscent of pathosystems involving multiple necrotrophic effectors (proteinaceous host-selective toxins) such as *Stagonospora nodorum* blotch of wheat (Friesen et al. 2008). Alternatively, recognition of multiple fungal effectors by the plant may lead to a gradually increasing plant defense response to decelerate fungal growth without, however, reaching a complete arrest or killing of the fungus as in hypersensitively responding plants. The underlying recognition factors may be encoded at QRL (Poland et al. 2009).

In both models, a series of input signals (effector recognition by susceptibility or resistance target), each of low intensity, would be converted into response intensity (plant susceptibility and defense) in a quantitative manner (Tao et al. 2003). However, depending on the genetic background of the particular host, in both models, the contribution of a single factor may dominate the outcome, such that resistance appears to be controlled by a single gene. The drastically differing outcome of the interactions of ‘Turk’ with UK7 and UK7 Δ NIP1 may serve as an example. Nevertheless, because UK5 and AU2 exhibit an even stronger growth on this cultivar, additional resistance factors appear to be involved in the interaction with UK7.

This quantitative model for the interaction of *R. commune* and barley is underpinned by many reports over the years showing a widely varying degree of symptoms caused by highly variable fungal isolates as well as by population genetic evidence of high genotypic diversity worldwide (Zaffarano et al. 2006). Future transcript-profiling-based experiments will need to verify the quantitative nature of the host response. Furthermore, it would be interesting to find out whether the response of the NIP is encoded by known barley quantitative trait loci.

MATERIALS AND METHODS

Plant and fungal culture conditions.

Conditions for plant growth and fungal cultivation *ex planta* as well as for plant inoculation were described previously (Hahn et al. 1993; Lehnackers and Knogge 1990).

Isolation of NIP2 and NIP3 cDNA clones.

Sequencing of the purified proteins by automated Edman degradation yielded N-terminal sequences of 56 and 49 aa for

NIP2 and NIP3, respectively. Back translation of these sequences allowed designing degenerate PCR primers (Rohe et al. 1995). Using DNA from fungal strain UK7 as a template, genomic fragments were amplified and used as probes to screen a λ ZAP (Stratagene, La Jolla, CA, U.S.A.) cDNA library from fungal strain US238.1 (Rohe et al. 1995). For this purpose, the amplification products were 32 P-dCTP labeled during the PCR reaction (NIP2 cDNA) or digoxigenin (DIG) labeled (NIP3 cDNA) using the DIG DNA labeling kit and the DIG luminescence detection kit (Boehringer, Mannheim, Germany). In both cases, four independent cDNA clones were isolated and sequenced.

Isolation of genomic NIP2 and NIP3 clones.

Genomic sequences of the NIP2 (1,767 bp) and NIP3 (2,563 bp) gene were obtained by PCR walking on uncloned fungal DNA (Devic et al. 1997). To this end, DNA from isolate UK7 was digested with restriction enzymes generating blunt ends. DNA templates for walking were generated by ligating the resulting fragments with an adaptor (adaptor oligonucleotide 1: 5'-CTAATACGACTCACTATAGGGCTCGAGCG GCCGCCCC GGGGAGGT-3' and adaptor oligonucleotide 2: 5'-P-ACCTCC CC-NH₂-3'). Subsequently, the NIP2 gene was cloned in four steps. PCR using the primers adaptor 1 and NIP2 antisense1 on DNA walking templates derived from *EcoRV* digestion and adaptor ligation, followed by a second PCR using the nested primers adaptor 2 and NIP2 antisense2 on the amplification product of the first PCR, yielded an NIP2 5' fragment of 1,079 bp. Similarly, an NIP2 3' fragment of 991 bp was obtained using the primers adaptor 1 and NIP2 sense1 for the first PCR and primers adaptor 2 and NIP2 antisense2 for the second PCR. Both fragments were cloned separately into pGEM-T Easy (Promega Corp., Madison, WI, U.S.A.). The 1,020-bp *AatII* fragment from the clone containing the 3' region of the NIP2 gene and 3' untranslated sequence was then inserted into the *AatII* site of the 5' clone to yield the complete NIP2 CDS flanked by a 655-bp 5' sequence and 819-bp 3' sequence. Subsequently, the entire NIP2 fragment was inserted into the *EcoRI* site of pBluescript KS⁻ (Stratagene).

The NIP3 gene was cloned in a similar way using *PvuII*-digested DNA. The 5' and 3' fragments of 1,866 and 1,486 bp, respectively, were obtained using the primer combinations adaptor 1/NIP3 antisense1 and adaptor 2/NIP3 antisense2 and adaptor1/NIP3 sense1 and adaptor2/NIP3 sense2. After cloning both fragments into pGEM-T Easy, a 1,911-bp *EcoRI* fragment from the clone containing the NIP3 CDS along with 1,144 bp of 5'-untranslated sequence was ligated into the *EcoRI* site of pBluescript KS⁻. Subsequently, a 1,568-bp *SacI* fragment from the 3' clone was used to replace the 847-bp *SacI* fragment in the pBluescript KS⁻ 5' clone to yield the entire NIP3 CDS flanked by approximately 1.1 kb each of 5' and 3' untranslated sequence. Finally, both NIP2 and NIP3 genomic clones were sequenced.

Generation of gene replacement vectors.

Replacement vectors were constructed by inserting selectable marker gene sequences into the CDS of the three NIP genes. For NIP1 disruption, a 2,410-bp *SphI* fragment from vector pAN7-1 containing the 3' region of the *gpdA* promoter, the *hph* gene conferring hygromycin resistance, and the 5' region of the *trpC* termination sequence was ligated into the *SphI* site of pAG-NIP1 (Rohe et al. 1995) to yield the vector Δ NIP1. For NIP2 disruption, the *AatII* sites at positions 2,349 and 3,928 in the vector pAN8-1 were first mutated using the unique site elimination system (Amersham Biosciences, Freiburg, Germany). Using the primers BleA and BleB, both containing an *AatII* restriction site, and the mutated vector pAN8-1 as a tem-

plate, a fragment was generated that contained the 3' region of the *gpdA* promoter, the *ble* gene conferring resistance to phleomycin, and the 5' region of the *trpC* termination sequence. Cloning of this 1,895-bp fragment into the *AatII* site of *NIP2* yielded the vector $\Delta NIP2$. For *NIP3* disruption, the same *SphI* fragment from vector pAN7-1 as was used to construct vector $\Delta NIP1$ replaced a 93-bp *SphI* fragment within the *NIP3* CDS to yield the vector $\Delta NIP3$. The vectors $\Delta NIP2$ and $\Delta NIP3$ were linearized with *BamHI* and *HindIII*, respectively, prior to transfer into protoplasts of fungal strain UK7 (Rohe et al. 1996). Primary screening to identify fungal replacement mutants was performed by PCR using fungal DNA as a template and primers 5 and 6 (Rohe et al. 1996), which directly flank the *NIP1* CDS; primers *NIP2IV* (39 bp upstream of the *NIP2* translation start codon) and *NIP2* antisense2 (105 bp downstream of the *NIP2* stop codon); and primers *NIP3* sense2 (32 bp upstream of the *NIP3* translation start codon) and *NIP3* antisense2 (303 bp downstream of the *NIP3* stop codon), respectively. Binary vector pNIP2KO.bin was constructed from vector pPZP200 (Hajdukiewicz et al. 1994) and contains the *ble*-disrupted *NIP2* sequence from vector $\Delta NIP2$ and the *EGFP* gene adjacent to the right T-DNA border, both under the control of *gpdA* promoter and *trpC* termination sequence from *Aspergillus nidulans*. To generate an *NIP1/2* double mutant, a 4,215-bp fragment was amplified from pNIP2KO.bin using the primers NIP2KOs and NIP2KOas. The fragment containing the *ble* gene cassette flanked by 969 bp of 5' UTR and 1,015 bp of 3' UTR of the *NIP2* gene was used to transform protoplasts of mutant UK7 $\Delta NIP1$.

Primers.

Adaptor 1: 5'-CTAATACGACTCACTATAGGGCTCGAGC GGCCGCCCGGGGAGGT-3', *NIP2* sense2: 5'-CGCAAACG CGGTCTGTCGACGTCGTGGCC-3', *NIP2* anti-sense2: 5'-CA TCCGCTTTGTCGGTTGAAGCTAG-3', *NIP2 IV*: 5'-TCCCA CCACAAC CACGCCGC-3', *NIP3* sense2: 5'-CAAGAGCTC TCAAATCACATCTATTT CAACC-3', *NIP3* antisense2: 5'-C GAATTCTATCT GTCACTACCTGTGATA-3', MutAatII₂₃₄₉: 5'-ACCGCGCGCGACGTTGCCGGA GCG-3', MutAatII₃₉₂₈: 5'-TGGTTTCTTAGACTTCAGGTGGCAC-3', *BleA*: 5'-GAC TCAAATCGACGTCAGCA ACATC-3', *BleB*: 5'-AGG TCTC TTGACGTCGTT GATCTG-3', *NIP2KOs*: 5'-GAATGTATC CAATTCAATCT-3', *NIP2KOas*: 5'-AACAGGTCAATAGTTA GTTC-3'. Underlined sequences indicate *AatII* and mutated *AatII* restriction sites.

Fungal transformation.

Transformation of fungal protoplasts was carried out as previously described (Rohe et al. 1996). For ATMT of fungal conidia, the protocol of Kirsten and associates (2011) was used. Candidate mutants were verified by PCR analysis. Before further use, all fungal mutants were grown on susceptible barley 'Ingrid' and reisolated. To this end, leaf sections were harvested 14 dpi, briefly washed in 70% EtOH, surface sterilized with 2% hypochloride, washed with water, and incubated at 17°C in the dark on Lima bean agar containing ampicillin (100 µg/ml) and tetracycline (10 µg/ml). After fungal mycelia had grown out of the tissue onto the agar, mycelial pieces were transferred to Lima bean agar plates without antibiotics. After 10 to 14 days at 17°C in the dark, spores were harvested to inoculate new Lima bean agar plates for inoculum multiplication.

qPCR and qRT-PCR.

To monitor fungal growth, DNA was extracted from inoculated barley leaves at 0, 7, and 14 dpi ('Ingrid') or 0, 10, and 21 dpi (all other cultivars). Relative quantification of fungal biomass was achieved by qPCR applying the efficiency cali-

brated model (Pfaffl 2001). Fungal *GPD* served as the target gene and a barley gene (accession code CD058235) encoding a predicted trypsin-like serine protease as the reference. Biomass data resulted from three independent biological experiments, except for fungal strain UK7 and mutant UK7 $\Delta NIP1$ on 'Ingrid' at 14 dpi and for UK7 on 'Atlas 46' and 'Turk' at 21 dpi, where four biological replicates were carried. In each case, three technical replicates were measured. To analyze the expression of the *NIP* genes, total RNA was extracted from inoculated leaves at six time points. Four independent inoculation experiments were carried out and all samples were measured in duplicate. Transcript amounts of the target genes were determined relative to the transcript amounts of the constitutively expressed *GPD* gene by qRT-PCR.

Microscopic analysis.

At 3, 7, and 10 dpi, inoculated leaves were harvested; cleared overnight in a mixture of 80% (vol/vol) ethanol, and 20% (vol/vol) chloroform (0.15% [wt/vol] trichloroacetic acid); and stained overnight with 1.25% (wt/vol) trypan blue in 50% (vol/vol) ethanol, 16.7% (vol/vol) lactic acid, and 16.7% (vol/vol) glycerol (vol/vol). Subsequently, the leaves were washed twice in 33% (vol/vol) lactic acid and 33% (vol/vol) glycerol, transferred into in 50% (vol/vol) glycerol, and examined under the microscope (Axioplan 2; Carl Zeiss, Jena, Germany).

To compare and quantify fungal colony areas early during pathogenesis, the fungal wild-type strain UK7 and the three single-gene deletion mutants were transformed with the *EGFP* gene (Kirsten et al. 2011). The experimental set-up along with the protocol for multiplane fluorescence image acquisition was described previously (Baum et al. 2011). To minimize the biological error, inoculation experiments with mutant and wild-type spores were carried out in parallel (i.e., each deletion mutant was compared with its own wild-type reference).

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