Impairment of the cellulose degradation machinery enhances *F. oxysporum* virulence but limits its reproductive fitness

Francisco M. Gámez-Arjona¹, Stefania Vitale²*, Aline Voxeur³, Susanne Dora¹, Sascha Müller¹, Gloria Sancho-Andrés¹, Antonio Di Pietro², Clara Sánchez-Rodríguez*¹

¹Department of Biology, ETH Zurich, 8092 Zurich, Switzerland.  
²Departamento de Genética, Campus de Excelencia Internacional Agroalimentario ceiA³, Universidad de Córdoba, Córdoba, Spain.  
³Institut Jean-Pierre Bourgin, INRA, Centre National pour la Recherche Scientifique, AgroParisTech, Université Paris-Saclay, RD10, 78026, Versailles Cedex, France.  
*Actual address: Dipartimento Agricoltura, Ambiente e Alimenti, Università degli Studi del Molise, Campobasso, Italy.  
*Corresponding author  
corresponding_email@ethz.ch
Abstract

The plant vascular pathogen *F. oxysporum* (Fo) colonizes the different layers of the host root until it reaches the vasculature system, where it can proliferate quickly, causing plant wilting and decay. During this process, Fo grows mainly through the plant cell walls, that act as a barrier that hinders pathogen progression. Although numerous fungal cell wall degrading enzymes have been identified, many aspects of their role during infection remain elusive. Here, we show that the metabolic plasticity of Fo is capable of counterbalancing the inability of the fungus to degrade cellulose by enhancing the secretion of other virulence factors. As a consequence, Fo impaired in cellulase degradation become hypervirulent. On the other hand, the lack of cellulases has a severe negative effect on microconidial production, suggesting that cellulose degradation is a decisive process during the last stages of Fo-s life cycle in the plant. Our results indicate that, for Fo's evolutionary success, its pathogenicity needs to be coordinated with an optimal use of the resources derived from the host for fungal.

Introduction

The soil-borne fungus *Fusarium oxysporum* (Fo) Schlechtend. species complex contains plant and human pathogens, and nonpathogenic strains (Edel-Hermann and Lecomte, 2019). The plant pathogens affect a large number of plant species, causing losses in crops worldwide (Fisher et al., 2012; Michielse and Rep, 2009; Pennisi, 2010). Fo is genetically diverse, with individual strains, called *forme speciales* (f.sp.), infecting a narrow range of hosts (Baayen et al., 2000; Gordon and Martyn, 1997), such as Fo5176 and Fol4285, which infect *Arabidopsis thaliana* and *Solanum lycopersicum*, respectively (Nirmaladevi et al., 2016; Thatcher et al., 2012). We have recently contributed directly to the establishment of the model *Arabidopdid-Fo5176* (Fokkens et al., 2020; Huerta et al., 2020; Kesten et al., 2019; Menna et al., 2021; resubmitted to BMC Bio). Although Fo constitutes one of the world’s most economically important plant pathogens, many aspects of the first stages of its interaction with the host are vaguely understood.

After host perception and spore germination, Fo hyphae adhere to the root epidermal cells and grow intercellularly towards the cortex (Hall et al., 2013; Nahalkova et al., 2008). Pathogenic Fo advances through the inner roots cell layers and enters the xylem, where it proliferates blocking this vascular system, which translates in plant wilting and death. During the interaction, plants defenses are activated by perceiving the fungal directly through microbe-associated molecular patterns (MAMPs) and detecting endogenous signals released during microbe colonization, called damage-associated molecular patterns (DAMPs) (Hou et al., 2019; Husaini et al., 2018). Some DAMPs are molecules resulting from the plant CW degradation by fungal CW degrading enzymes (CWDEs) released to reduce the to cope with the mechanical barrier of the plant cell wall (CW) (Michielse and Rep, 2009), such as the pectin-derived oligogalacturonides (OGs; (Ferrari et al., 2013; Souza et al., 2017; Voxelur and Höfte, 2020). Cellulose degradation products, like cellobiose, have also been shown to induce plant defence are, therefore, considered potential DAMPs although their release upon microbe colonization has not been proved ((Ferrari et al., 2013; Souza et al., 2017; Voxelur and Höfte, 2020)

The composition of the CW is not homogenous among species or even plant tissues, but in general, the main components are the crystalline fibres of cellulose, embedded in a matrix of
hemicellulose, pectin, lignin and proteins (Cosgrove, 2005; Hématy et al., 2009; Kubicek et al., 2014; Underwood, 2012). This molecular complexity requires a broad set of CWDEs, each with different enzymatic functions (Glass et al., 2013). The majority of CWDEs are glycosyl hydrolases (GHs), divided based on their sequence similarity (Boraston et al., 2004; Henrissat and Bairoch, 1993). However, other essential CWDEs, such as lytic polysaccharide monoxygenases (AA9s), are not GHs but also contribute to crystalline cellulose digestion (Beeson et al., 2012; Quinlan et al., 2011).

Although both beneficial and phytopathogenic fungal organisms encode for CWDE, pathogens are known to express substantially more (Zhao et al., 2013). Additionally, there is increasing scientific support showing the indispensable role that CWDEs play upon fungal infection (ten Have et al., 1998; Isshiki et al., 2001; Martínez-Soto et al., 2013; Mathioni et al., 2011; Nguyen et al., 2011). Those findings, together with the fact that F. oxysporum lacks structures to penetrate the host (appressoria, penetration pegs), lead us to think that CWDEs are a crucial part of plant infection (Gibson et al., 2011). However, the functional redundancy found among CWDEs complicates a full assessment of their functions (Kurašin and Väljamäe, 2011), and in many cases, mutations of individual genes do not affect fungal virulence (Calero-Nieto et al., 2007; Pietro et al., 2003). Interestingly, fungi have evolved transcription factors to coordinate the expression of CWDEs based on the composition of their environment. In Neurospora crassa, a conserved zinc binuclear cluster transcription factor, named CLR1, was shown to be essential for fungal growth on cellulose (Coradetti et al., 2012; Craig et al., 2015a; Glass et al., 2013). Consequently, these transcription factors might be targeted as a way to circumvent the functional redundancy found between individual CWDEs, enabling the study of the function of groups of CWDEs (Tonukari et al., 2000).

One of the main targets of CWDEs is the cellulose because it provides mechanical strength to all plant cell types, and it is one of the most challenging materials within the CW to degrade (Glass et al., 2013). Therefore, cellulose is likely to be a significant barrier to Fo infection. To investigate how cellulose degradation impacts Fo infection, we have identified an orthologue of CLR1 from N. crassa in Fo. Furthermore, we have generated a Fo mutant line (clr1), which is unable to grow on cellulose. Here, we demonstrate that cellulose degradation is unnecessary during Fo infection, as shown by the increased virulence displayed by clr1. The mutant clr1 compensates cellulose degradation deficiency by secreting more virulence factors to compromise plant immune responses. Despite having increased virulence, the lack of cellulose degradation capacity severely compromises fungal metabolism during its saprophytic growth. Taken together, our findings expand the current understanding of root-pathogen interactions. Pathogenicity needs to be accompanied with an optimal use of the resources derived from the host to maximize the benefits obtained from the interaction.

Results

CLR1 is essential for cellulose degradation in F. oxysporum (Fo5176)

To understand the role of F. oxysporum (Fo) cellulases in root infection, we aimed to obtain a mutant impaired in cellulose degradation. We hypothesized that by inactivating a master regulator of cellulases, we could abolish the expression of a whole set of enzymes. In this way, we could avoid the high levels of functional redundancy present in CWDEs. CLR1 (NCU07705) has been previously described as a master regulator of cellulases in N. crassa (Coradetti et al., 2012). Using an in silico analysis, we found CLR1 orthologues in various Fusarium genomes, which shared a protein sequence identity above 70%, with the same DNA-binding domain sequence (Fig. 1a and 1b). Therefore, we generated a CLR1 null
mutant in the previously described Fo5176 pSIX1::GFP background, which allows for monitoring of Fo5176 colonization of the root vasculature (Huerta et al., 2020; Kesten et al., 2019). We obtained a deletion mutant that lacked the entire CLR1 coding region (clr1) and a complemented strain (clr1C) generated by reintroducing the wild-type pCLR1::CLR1 into the clr1 mutant background (Fig. S1). To determine whether mutants were affected in their response to stress, we grew the strains in control conditions (YPD), salt stress (NaCl), osmotic stress (Sorbitol), CW stress (Congo Red and CalcoFluor) and minimal media. There were no significant differences in the colony phenotype of clr1 or clr1C growing in different stress conditions compared to WT (Fig. S2).

To confirm that CLR1 can similarly regulate cellulose degradation pathways in Fo as in N. crassa, we tested the ability of clr1, clr1C and WT to utilize different carbon sources such as sucrose, cellulose and celllobiose. Fungal growth on sucrose was similar across all genotypes (Fig. 1c and S3). However, clr1 exhibited a significant reduction of growth on celllobiose and only residual growth was observed on cellulose compared to WT or clr1C (Fig. 1c and S3). These data indicate that CLR1 is essential for the catabolism of cellulose, in line with previous observations in N. crassa (Coradetti et al., 2012).

**clr1 reaches the root vascular system faster than WT**

To determine whether Fo needs cellulose degradation to infect its plant hosts, we performed plate infection assays as described before (Huerta et al., 2020). 8-day old Col-0 seedlings were transferred to plates containing the same number of WT, clr1, or clr1C microconidia. We monitored root vascular colonization by following GFP signal presence in the root vasculature for five days. clr1 showed an increase in pathogenicity compared to control lines as we counted a significantly higher number of vascular penetrations (Fig. 2a). The increased xylem colonization observed with clr1 correlated with higher expression of FoSIX1 relative to AtGAPDH and FoTUB, measured by qRT-PCR (Fig. 2b and 2c).

The expression of different cellulolytic genes is dependent on CLR1 in cultures growing on cellulose (Craig et al., 2015b). We chose nine of those genes and we found the orthologues in Fo, to study whether their expression would be also CLR1 dependent upon infection. Thus, we measured the gene expression in WT and clr1 of an endoglucanase, an intracellular β-glucanase, a secreted β-glucanase, a GH5, a plasma membrane transporter of hexoses (MFS), an expansin and two AA9s, 7-days after plants infection. The majority of them were downregulated in clr1 during infection (Fig. 2d). Additionally, we asked whether the decrease in cellulolytic enzyme expression would affect cellulose degradation in clr1 during root colonization. We quantified the residual root cellulose content 7-days after transferring the plants to plates with WT or clr1 microconidia. The cellulose content was lower in roots infected with WT than with clr1 (Fig. 2e), in line with the results of growth on cellulose (Fig. 1c). Taken together, these data suggest that cellulose degradation was affected in clr1 upon infection and it is not strictly necessary for Fo to reach the plant vasculature during infection.

**Arabidopsis defense response is delayed upon clr1 infection compared to WT**

Plant defense-related gene expression is triggered upon fungal infection (Kesten et al., 2019; Masachis et al., 2016) and upon treatment with MAMPs or DAMPs (Souza et al., 2017). We reasoned that an inadequate activation of plant immune responses might be the cause of increased clr1 pathogenicity. To test this hypothesis, we measured the expression of three defense-related genes, At1g51890, WKRY45 and WKRY53, upon infection with clr1 and WT. We performed plate infection assays, as indicated above, and the gene expression was studied from infected roots 2,3,4,5 and 7-days after inoculation. After 2 and 3-days, defense gene expression was generally lower in plants infected with clr1 than WT (Fig. 3a). These findings suggest that the significantly higher number of vascular penetrations
observed upon clr1 infection (Fig. 2a) is a consequence of delayed activation of defense related genes. By contrast, 7-days after inoculation, plant defense gene expression was higher upon infection with clr1 than with WT (Fig. 3a). This increase coincides with higher plant vascular colonization by clr1 compared to WT, suggesting a possible correlation (Fig. 2a).

We next asked whether priming the plant with MAMPs (chitin) or DAMPs (cellobiose) could reduce plant susceptibility to clr1. To address this question, we performed plate infection assays with WT or clr1 in plants pretreated for 25 minutes with cellobiose or chitin to activate plant defense responses, or H2O as control. Later, pretreated plants were transferred to plates with two days pre-germinated clr1 or WT microconidia. The results showed that after priming plant immune responses, plant susceptibility to clr1 returned to WT levels (Fig. 3b and 3c). These findings suggest that clr1 recognition by the plant is compromised at the early stages of infection, probably because some DAMPs and MAMPs are less present upon clr1 infection.

Different amounts of cell wall degradation products are accumulated upon clr1 infection

Plant pathogens express a high number of CWDEs, and as a consequence, many different CW-derived molecules are expected to be generated upon infection. Some of these molecules are able to act as DAMPs. Consequently, we questioned whether the delay in clr1 perception was due to the absence or decreased production of DAMPs during clr1 infection compared to WT. To address this, we performed an analysis of infected root cell wall composition by using HP-SEC-MS/MS (Voxeur et al., 2019). We employed different commercial standards to identify the presence of OGs and hexoses (Fig. 4a). The results showed that between day 2 and 3-days after infection, the hexose profiles shifted from being basically composed of sucrose to cellobiose (Fig. 4b and S4). From 3-days after infection, the amount of hexoses (essentially cellobiose) were higher upon infection with clr1. These data could reflect a decreased mutant capacity to degrade cellobiose than WT (Fig. 1c and S3).

Interestingly, we found that, during infection, acetylated OGs are released in the CW of infected roots (Fig. 4b, 4c and S4). clr1 infection generally accumulated fewer OGs than WT, especially from 3- to 4-days post-inoculation. Concretely, the OG Gal4Ac was significantly more accumulated in roots infected with WT than with clr1 at 4-days after infection (Fig. 4c). The decreased accumulation of OGs during the early stages of plant infection are in line with the compromised perception of clr1 by the plant.

Pathogens are known to encode virulent factors in an attempt to undermine host defense responses (Rauwane et al., 2020). Therefore, we next questioned whether clr1 enhanced virulence is the consequence of decreased DAMP production upon infection, or conversely, clr1 has increased expression of virulence factors that act to counter any loss of virulence caused by clr1 decreased ability to express cellulases. To answer this question, we performed plate infection assays using the same number of clr1, WT or a mixed (50%clr1/50%WT) microconidia. We speculated that whether DAMPs accumulation was the only reason for increased plant susceptibility to clr1, by using a mixed WT/clr1 inoculum, we should observe a decrease in clr1 virulence proportional to the concentration of WT in the inoculum. The results showed that the mixed microconidia displayed the same pathogenicity as clr1 (Fig. 5a), observing a decrease only when 90% of the inoculum was composed of WT spores (Fig. 5b). Plant resistance did not increase proportionally to the amount of the WT strain. Our data indicate that clr1 infection generates different amounts of DAMPs than WT. However, they alone do not explain clr1 higher pathogenicity.

clr1 secretome has higher levels of virulence factors
A majority of the fungal virulence factors are secreted for suppression of host defense responses. We performed proteomic analysis on the secretomes of WT and clr1 during plant infection to determine whether their profile of secreted virulence factors differed significantly. 10-day old Col-0 seedlings, grown in hydroponics, were inoculated with either WT or clr1 microconidia. At 3-days post inoculation, we collected the liquid media, concentrated it, and carried out a proteomic MS/MS analysis using label-free quantification. 362 fungal proteins were identified, 75% with secretory signal peptides. Of these, more than 40% were already annotated as virulence factors. These include glycosyl hydrolases (25%), peptidases (13%), redox-related proteins (10%) and pectin and pectate lyases (3%) (Fig. 5c).

The results revealed a significant reduction in the abundance of cellulases in the clr1 secretome (Fig. 5d and table). For example, the levels of three GHs proteins (GH5, GH6 and GH7), which contain cellulose-binding domains and are predicted to have cellulolytic activity, were significantly reduced in clr1. Interestingly, the protein levels of two copper-dependent lytic polysaccharide monoxygenases (AA9s) were also found to be decreased in the clr1 secretome. These enzymes are not GHs, but they act on crystalline cellulose degradation, making it more accessible to cellulases (Zhang, 2020). The general decrease of cellulose degrading enzymes observed might explain why clr1 cannot degrade cellulose (Fig. 1c, 2d and S3).

In contrast, we found an increased presence of virulence factors in the secretome of clr1 compared to WT. Interestingly, it was significantly enriched in a cutinase, which is essential for the virulence of some necrotrophic fungal pathogens (Ma et al., 2019). Additionally, we found increases in other virulence factors like pectin and pectate lyases, which are released by the pathogen to degrade pectin in host CWs (Cuomo et al., 2007; Kars et al., 2005; Yang et al., 2018). The observed enrichment in these enzymes is line with the fewer OGs detected upon infection with clr1 (Fig. 4). Similarly, a set of peptidases (8), known as critical virulence factors (Monod et al., 2002), were more abundant in the clr1 secretome. These include subtilases (4), which are able to degrade plant proteins with antifungal activity, such as β1-3 glucanases (Cota et al., 2007). Also a Fungalysin, which compromises host defense by cleaving chitinases. The orthologues of this protein have been described as an extracellular metallopolypeptidase necessary for the virulence in U. maydis (Ökmen et al., 2018), F. oxysporum f sp. lycopersici (Jashni et al., 2015), F. verticillioides (Naumann et al., 2011) and C. graminicola (Sanz-Martín et al., 2016). Finally, we noticed an enrichment in a chitin deacetylase, whose orthologous impedes chitin-triggered immunity in cotton (Gao et al., 2019) by converting chitin into chitosan, preventing it from being degraded. In general, the enrichment of these virulence factors would explain why plants inoculated with clr1 show a delay in defense responses and increased susceptibility (Fig. 3a).

We next questioned whether the virulence factors were more abundant in clr1 independently of the media in which the fungus was growing. We chose four virulence factors (a pectate lyase, a cutinase, a subtilase and a peptidase) enriched in clr1 secretome, and we measured their gene expression from fungal cultures on sucrose media or plant infections. We observed an increase in the expression in 3 out of 4 (Fig. S5) in fungi derived from plant infections but not from fungi grown on sucrose. These results suggest that clr1 increases the number of virulence factors, expressed and secreted, in an attempt to compensate for its decreased ability to utilize cellulose as a primary carbon source.

**CLR1 is essential to complete F. oxysporum life cycle**

The saprophytic growth is an important part of Fo life cycle. We next asked whether lacking cellulose degradation would affect the clr1 saprophytic fitness. To address that question, we first studied the CLR1 expression in the wild type strain upon infection, for which infected roots were collected on different days post-inoculation. CLR1 expression increased significantly over the days, but especially after five days post-transfer, reinforcing the idea
that cellulases might have a critical role in the latest infection stages (Fig. 6a). Subsequently, we observed that CLR1 expression was significantly higher in fungi growing on dead material than on living plants after 4-days post-infection (Fig. 6b). Additionally, we asked whether cellulose degradation deficiency would impact clr1 cycle, by comparing the microconidia production of WT, clr1 and clr1C, once the plant was dead. The WT and clr1C produced significantly more microconidia than clr1 (Fig. 6c). These findings suggest that saprophytic growth is severely compromised in clr1.

Discussion

The CW has a crucial role during plant-microbes interaction since it constitutes a physical barrier between both organisms. Pathogenic and non-pathogenic fungi secrete a battery of CWDEs (Zhao et al., 2013), and the variety of the enzymes produced reflects the CW complexity and the fungal pathogenicity during plant-microbe interaction (Glass et al., 2013). For example, fungal pathogens are especially rich in the expression and diversity of CWDEs, which supports that CWDEs are essential for virulence (Kubicek et al., 2014; Lorrai and Ferrari, 2021; Rauwane et al., 2020). However, in many cases, mutations in those enzymes do not result in obvious phenotypes, probably due to functional redundancy (Apel-Birkhold and Walton, 1996; Di Pietro and Roncero, 1998; Espino et al., 2005; Gómez-Gómez et al., 2002; Huertas-González et al., 1999; Rogers et al., 2000; Ruiz-Roldán et al., 1999; Wu et al., 1997).

Cellulose is the most abundant CW polysaccharide. Despite its structural simplicity, it is recalcitrant for enzymatic degradation. Pathogenic strains are known to express high levels of cellulases during infection, increasing host penetration (Chen et al., 2005; Liu et al., 2005; Rispail and Di Pietro, 2009; Roberts et al., 1988; Van Vu et al., 2012). Nevertheless, in some cases the overexpression of cellulases caused defects in pathogenesis by affecting the carbon assimilatory balance during the early phase of infection (Fernandez et al., 2012). The complexity of this family of proteins and the importance of their well-orchestrated expression led us to probe if they may also participate in Fo-plant interactions.

Fungi possess transcriptional regulatory mechanisms to produce specific sets of CWDEs based on their environmental conditions and in anticipation of potential carbon sources (Wu et al., 2020). Some transcription factors control the expression of pectinases, xylanases or cellulases, but with a certain degree of overlap among them. For example, CLR1 is a master regulator of cellulases in N. crassa essential for cellulose degradation and whose expression increases significantly in culture with cellulose as the sole carbon source (Coradetti et al., 2012; Craig et al., 2015a, 2015b). Here, we found an orthologue of CLR1 in the pathogen Fo5176, which is highly virulent on Arabidopsis accessions (Thatcher et al., 2009). As it was shown in N. crassa, Fo mutants that lacked clr1 were not able to grow on cellulose, and the degradation of other cellooligomers such as cellobiose was severely but not completely compromised (Fig. 1c). In the case of clr1, we also observed a significant decrease in the secretion of cellulytic enzymes (Fig. 5d), especially those with known roles in the early stages of cellulolytic degradation, like the AA9s, explaining the lack of growth on cellulose. Our findings suggest that the role of CLR1 is well conserved among ascomycetes.

Subsequently, we checked whether the impairment in cellulose degradation had consequences on Fo pathogenicity. CLR1 expression increased exponentially along with the infection (Fig. 5), which could imply a critical role during this process. However, clr1 reached the root vasculature system faster than WT (Fig. 2a and 2b), suggesting that Fo does not require cellulose degradation to colonize the root and reach the xylem. As we have demonstrated previously, root cellulose degradation occurs upon Fo infection (Kesten et al., 2019). Our data also revealed that WT degraded more cellulose upon infection than clr1 (Fig
Additionally, undermined defense responses, also host cell clr1 wall compromising (Fig. plant would explain OGs in vascular wilt diseases. Nevertheless, Pectin role expression media carbon rich virulence of in was S5). did in that by supported the fact in act also both in peptidases defense like it becomes 2015) (Jashni virulence (Gottwald et al., 2012; For example, in F. oxysporum f sp. lycopersici the protein Fungalysin is essential for virulence (Jashni et al., 2015). However, in a mutant like clr1, it becomes necessary to boost the acquisition of energy through other sources, and in this context, peptidases can not only undermine host defense response, but also provide both carbon and nitrogen. In addition, the higher levels of pectin and pectate lyases detected in the clr1 secretome might act also to obtain energy through other carbon sources, such as pectin. This might be supported by the fact that we did not observe and increase in the expression of virulence factors when the media was rich in carbon sources (Fig. S5). Nevertheless, Pectin and pectate lyases have also a fundamental role in the development of vascular wilt diseases (ten Have et al., 1998; Kars et al., 2005; Yang et al., 2018). Moreover, higher levels of pectin degradation would explain the decrease of OGs present in the root cell wall upon clr1 infection (Fig. 4), compromising also plant immune responses. Additionally, clr1 also undermined host defense responses, by secreting more chitin.
deacetylases, which decrease the amount of MAMPs available for plant perception (Gao et al., 2019). In general, the increase in the secretion of different virulence factors explain the delay in the perception of *clr1* at the early stages of infection (Fig. 3).

Pathogenicity is not the only factor to measure the success of a plant pathogen such as Fo. In fact, increased virulence shortens the time that Fo has to benefit from the nutrients derived from alive plants. Additionally, we show here that the exploitation of dead material promotes the completion of the Fo life cycle. Cellulose degradation is fundamental for Fo to produce microconidia because it allows the pathogen to assimilate cellulose, the most abundant biopolymer on earth (Fig. 6). In summary, we demonstrate that the impairment in cellulose degradation does not pose any obstacles for *F. oxysporum* plant infection, but it is essential during the saprophytic stage. This organism exhibits an extraordinary high degree of metabolic plasticity that serves to compensate for any lack or decrease in the production of cellulases upon infection. For example, by increasing the amount of virulence factors and decreasing DAMPs and MAMPs production. Nevertheless, the evolutionary pressure sets the use of virulence factors to maximize the benefits of interaction with the host.
Fig. 1. Cellulose degradation is regulated by CLR1 in *F. oxysporum* (Fo5176).

a) Pairwise comparison from 6 different fungi to identify potential CLR1 orthologous. Below the diagonal is indicated the percent of protein identity. b) Sequence alignment of the CLR1 DNA-binding domain. 6 protein sequences of CLR1 from different *Fusarium* species and *N. crassa* were used to study the conservation grade among them. The software CLC Genomic Workbench v.12 was used to perform the analysis. The conservation grade is shown. The domain Zn(2)-C6 is well conserved, ACEVCRSRKSRCDGTKPCKKLCTELGAECIY (Related with DNA-binding domain). c and d) Representative picture of the experiment to analyze growth phenotype of WT, clr1 and clr1C on sucrose or cellulose. Three replicates of each strain. Scale bar 1cm. d) Growth phenotype of WT, clr1 and clr1C growing on different carbon sources and measured as acid nucleic concentration (ng/µl). The strains were growing on sucrose 0.5% or cellobiose 0.5% for three days, or cellulose 0.5% for seven days. Shown are the Box plots: centerlines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum, n≥5. Anova was performed and Tukey’s multiple comparisons test; Asterisks show difference respect WT. ** p-value ≤ 0.01 or **** p-value ≤ 0.0001.
Fig. 2. *clr1* is more pathogenic than control strains.

a) Arabidopsis vascular penetration quantification at different days post-transfer to WT, *clr1* or *clr1C* microconidia containing plates. Values are mean +/- SEM, N≥ 28 plants from one representative experiment. We performed the experiment 3 times with the same result. RM two-way ANOVA on vascular penetration rate: p<0.0001 (fungal genotype), p<0.0001 (time), p<0.0001 (fungal genotype x time), day seven after exposition, Tukey’s multiple comparison test, p<0.0001. b) and c) *FoSix1* expression relative to *AtGAPDH* or *FoTub* respectively in infected Arabidopsis roots 7 days post-transfer to WT, *clr1* and *clr1C* microconidia containing plates. Shown are the box plots: centerlines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum. N=4 biological replicates; Welch’s unpaired t-test; p-value ≤0.05. c) Quantification of genes expression in WT and *clr1* by RT-qPCR. Infected Arabidopsis roots were collected seven days post-transfer to plates with WT or *clr1* microconidia. Expression was normalized to the fungal *FoTub* gene. Bar are means +/- SEM, N=3 biological replicates: Welch's unpaired t-test; * p-value<0.05. ** p-value<0.01. d) Infected Arabidopsis roots cellulose content compared to mock. Roots were collected 7 days after transfer to plates with WT or *clr1* microconidia. Shown are the Box plots as above. N=8 biological replicates. Welch’s unpaired t-test; p-value =0.087.
Fig. 3. The plant perceives later clr1 than WT.

Plant stress related genes expression (At1g51890, WRKY45 and WRKY53) 2, 3, 4, 5 or 7-days post-transfer to plates with WT or clr1 microconidia. Expression relative to WT (Col-0). We use AtGAPDH as a reference gene. Shown are box plots: centerlines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum, n ≥ 4 biological replicates; Welch’s unpaired t-test; p-value of each comparison is indicated. b) and c) Ten days old roots were treated with cellobiose b) or of chitin d) during 25 minutes. They were subsequently transferred to a plate with already germinated WT or clr1 microconidia. The number of vascular penetrations was monitored afterwards. Values are mean +/- SEM, N=14, we repeat three times the experiment with the same result. RM two-way ANOVA on vascular penetration rate: p<0.0001 (fungal genotype and treatment), p<0.0001 (fungal genotype x time), day seven after exposition. Tukey’s multiple comparisons test, * p<0.05. Asterisk indicated a statistically difference with respect to WT.
Fig. 4. *clr1* infections accumulate different amounts of cell wall-derived molecules in the CW than WT.

a) Base peak chromatogram obtained by HP-SEC-MS analysis to study the hexoses present in the root cell wall in mock conditions. An example of a commercial standard (cellobiose) is shown to identify the elution time of the different sugars. The material was collected from 12-days old seedlings. This is a representative profile from one experiment. 

b) Kinects of OGs (blue) and Hexoses (yellow) produced along five days post-transfer to plates with WT or *clr1* microconidia. DP indicates the degree of polymerization of OGs. This is the average from three biological replicates.

c) Kinetic of GalA4Ac produced in Arabidopsis roots by WT or *clr1* four days post-infection. Bars are means +/- SEM, n=3 biological replicates. Asterisk indicates statistical significance, One-way ANOVA with Tukey post hoc comparison. p<0.05.
**Fig. 5.** *clr1* secretes more virulence factors upon infection than WT.

a) Quantification of vascular penetration of Arabidopsis plants at different days post-transfer to plates with WT, *clr1* or a mix with 50% *clr1* + 50% WT microconidia. Values are mean +/- SEM, n=30 plants from one representative experiment, we performed the experiment 3 times with the same result. RM two-way ANOVA on vascular penetration rate: p<0.0001 (fungal genotype), p<0.0001 (time), pp ≤ 0.0001 (fungal genotype x time), day seven after exposition, Tukey’s multiple comparisons test, ***p<0.0001 and ****p<0.0001 both relative to WT.

b) Quantification of vascular penetration of Arabidopsis plants seven days after transfer the plants to plates with WT, *clr1* or a mix with 25%, 10% or 2,5% of *clr1* + WT microconidia. Bars represent means +/- SEM, N≥30, from one independent experiment. We performed 2 times the experiment with similar results. Letters indicate statistically difference between samples, Unpaired t-test with Welch’s correction; p<0.05.

c) Number of total proteins identified in WT Fusarium secretome studies. Percentages of the most representative family proteins found are indicated.

d) Volcano plot of differences in the abundance of proteins present in the secretome of WT relative to *clr1* secretome. Values are from four biological replicates. Green dots represent significantly less abundance in *clr1* secretome than WT. Red dots represent significantly more abundance in *clr1* than WT. p-values < 0.05 (moderated t-test). The name of several identified proteins is indicated next to the dot.
Fig. 6. CLR1 is crucial during saprophytic growth.

a) CLR1 expression analysis relative to FoTub. Arabidopsis infected roots were collected at different days post-transfer to WT microconidia containing plates. Values represent means +/- SE from four biological replicates. b) CLR1 expression relative to FoTub. WT and clr1 were growing with alive plants or dead material for four days. Shown are the Box plots: center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum, N ≥ 4 biological replicates; Welch’s unpaired t-test; * p-value<0.05. c) Microconidia production in WT, clr1 and clr1C from dead material. Box plots are as above. N ≥ 11 biological replicates. Asterisks indicate statistical difference relative to WT, Welch’s unpaired t-test. p<0.001 and p<0.0001.
Fig. 7. model of *Fusarium oxysporum* life cycle.

(A) *clr1* secretes more virulences factors and produces less DAMPs and MAMPs. Consequently, *clr1* colonizes faster the xylem (B). (C) Finally, during the saprophytic growth WT is able to use cellulose as carbon sources producing more microconidia.
Fig. S1. Identification *clr1* mutant.

a) Scheme of *CLR1* gene and the neomycin cassette use for homologous recombination. b) PCR screening to identify WT, *clr1* and *clr1C*. The mutant and wild type insertion were tested with the primers ForCLR1genotyping and RevCLR1genotyping (indicated with arrows in panel a)). The wild type fragment length is 2589 pb and the neomycin fragment approx. 2872 pb. c) Rt-PCR to check *CLR1* expression with the primers CLR1rtpcrfor and CLR1rtpcrrev. Rt-PCR of *FoTubulin* was used as expression control.
Fig. S2. No difference in the colony growth phenotype of WT, clr1 and clr1C in different stress media.

To test a potential susceptibility of clr1 and clr1C to different stresses we use YPD as a control media, Minimal Media (nutrient limitation), YPD with 1 M NaCl (salt stress), YPD with 50 μg/mL Congo Red or YPD with 40 μg/mL Calcofluor White (cell wall perturbations) or 1 M of Sorbitol (osmotic stress). Plates were spot-inoculated with 20 μl of decimated dilution from $10^7$ microconidia/ml.
Sucrose, cellobiose or cellulose, 0.5% wt/vol for all the assays. After two (sucrose and cellobiose) or seven (cellulose) days growing the mycelia dry weight was quantified. Shown are the Box plots: center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum, n=6. One-way Anova was performed and Tukey's multiple comparisons test; **** p-value ≤ 0.0001. Asterisks indicate difference respect to WT.
Fig. S4. Kinetics of Hexoses and OGs produced in Arabidopsis roots by WT and clr1.

a) Examples of detected OGs modification. Acetylation or oxidation. b) Kinetics of OGs and hexoses identified at the root cell wall by HP-SEC-MS at different days post-transfer to plates with WT or clr1. Bars represent means +/- SD from 3 biological replicates.
Fig. S5. Fungal virulence factor expression in different growth conditions.

The gene expression of 4 virulence factors enriched in clr1 secretome was measured. (Pectate lyase (g16048), cutinase (g2038), peptidase (g7764) and subtilisin (g1489)) in cultures growing on sucrose (2 days) or from infected Arabidopsis roots (seven days post-transfer). FoTub gene was used as a reference gene. Shown are the Box plots: center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to the minimum and maximum, n ≥ 4. Asterisks indicate differences respect WT; p-value<0.05. ** p-value<0.01.
| Primer               | Sequence 5’→ 3’                                                                 | USE                               |
|---------------------|-------------------------------------------------------------------------------|-----------------------------------|
| gpdA15B             | GGATCCCGAGACCTAATACAGGCCCT                                                   |                                   |
| trpter8B            | GGATCCAAAACAAGTGATCCTGTGCTT                                                  | HygBr cassette/Phleor             |
| Neo G               | TGCCCTGAATGAACCTGCAAGA                                                       | cassette/Neor cassette            |
| Neo Y               | CCAAGTCTTCTCAGAATATCAC                                                      |                                   |
| CLR1PromFor         | TCCAGTAAGCATCAAGTCG                                                         |                                   |
| CLR1PromForNest     | GGCTTCTTCTCCTCTCGG                                                          |                                   |
| CLR1PromRev         | TTTACCCCAGAATGCAAGTTAGCTTTTGTATTAGGTCTC TAGAAACGGTTATCGAGGAGCGG*             |                                   |
| CLR1TerFor          | TGGTCTTTGATTAGGCTGTATTAGGTCTC TTCTGGGTGTATATTCTCTGGG*                       | CLR1                              |
| CLR1TerRevNest      | TTTTGAATGGCCAGGGGGGGT                                                    | knockout/complementation          |
| CLR1TerRev          | AGAGTAGAGAGATGAACGGAA                                                      |                                   |
| CLR1 rev gene       | ATGATGAGACGCGTTGAGG                                                        |                                   |
| cldr1 rev nest 2    | TTCATGTTGGTCTCAGAAGCT                                                       |                                   |
| for CLR1 genotyping | GCCGCTACACCAACTCACT                                                       |                                   |
| rev CLR1 genotyping | TGCTGGGCTCAACATCCAACCAA                                                   |                                   |
| CLR1 RTPCR FOR      | GCTTGCCCTGTGGATTG                                                         |                                   |
| CLR1 RTPCR REV      | TCCAGGCTTTGTGGTGAT                                                        |                                   |
| At1g51890_qPCR-Fwd  | CTAGCCGACTTTGGGCTAT                                                        |                                   |
| At1g51890_qPCR-Rev  | CGAGTGGATTGGTGTTACTCAGG                                                    |                                   |
| WRKY45_qPCR-Fwd     | GAACAATCCATTCCCAGG                                                        |                                   |
| WRKY45_qPCR-Rev     | GGAGGAGGATGTGAGTTT                                                        |                                   |
| WRKY53_qPCR-Fwd     | GCCACAAGACACAGAGTCA                                                       |                                   |
| WRKY53_qPCR-Rev     | ACCGTTGGATTGGAGAGG                                                         |                                   |
| FoSIX1_qPCR-Fwd     | TCAAGAGGCTGGGTTG                                                        |                                   |
| FoSIX1_qPCR-Rev     | GAGCGCTACAGTGCGG                                                         |                                   |
| FoβTUB_qPCR-Fwd     | AACTCAGATGAGACCCTTGG                                                       |                                   |
| FoβTUB_qPCR-Rev     | GACATGACAGAAGAAGAG                                                         |                                   |
| GAPDH_qPCR-Fwd      | AGGTGGAAGAGCTGCTTCCCT                                                       |                                   |
| GAPDH_qPCR-Rev      | GCAACACTTTCCCCAACAGCCT                                                  | Real time PCR                     |
forrtpcrg16048 GCAGTACCGTGTGATCATGG
revrtpcrg16048 CATCGAGAACACTGCTTCCA
forrtpcrg2038 TGGCAATAAGATTGCTGCTG
revrtpcrg2038 GGTATGCCGTTCTTGAA
forrtpcrg7764 TATCGAGGCCGATACCAAAG
revrtpcrg7764 AGGTGTCTGTGGTACCTTG
forrtpcrg1489 GCACCCTGCCTGACATCTTT
revrtpcrg1489 ACCAGCAATTGCGGGAGTAC

g10314 for qPCR endo TTGCGCCAAGAATGTATCC

g10314 rev qPCR TGAAGAGAAAAACCTTGCC

g07437 forw qPCR TGACCAGAAATCCCGATG

g07437 rev qPCR TGGGCTTGAATTTCCTCAG

g13011 for qPCR expansin TCCTGCAGAAACCGAAGGC

g13011 rev qPCR TTTCCGCTTTGGTGCATAC

g15609 forw qPCR gh5 TCGCAACGATGTCACATC

g15609 rev qPCR AGCCTTTATCGTCGCGATGC

g14977 for qPCR aa9 GCTGGTTCAAGATCAAGGACTG

g14977 rev qPCR ATATTGCAGCGTTGGTACATGC

qpcr 09696 forw aa9 ACGGACCTTTGAGGATGTC

qpcr 09696 rev GACGGGTCAACACTCGATG

qpcr 02860 for mfs CTCTGCCAAAGTTCCTCCAG

qpcr 02860 rev CTGCCGTCCTTGACATGAC

qpcr 04972 forw periplasmi TGATGCTGAGTGAGGATCTG

qpcr 04972 rev TGAAGAATCACAGGCACTCG

qpcr 07642 for bglucosidase TTACATCGCAAACGAGCAAG

qpcr 07642 rev GAGCTTGAGCTTGAGGACAC
Methods

Plants material and growth conditions.

Arabidopsis thaliana (Col-0) were used in the analysis of fungal pathogenicity. Arabidopsis seeds were sterilized with chlorine gas (50 ml 14% sodium hypochlorite solution and 2 ml 37% HCl) in a sealed bell jar for 4h and the seeds were stratified for 2 days at 4°C in darkness before using them. Growth conditions were 16-h light (24°C)/8-h dark cycle at 21°C for all Arabidopsis experiments. In the case of plate experiments, seedlings were grown upright with ½ MS media (ph adjusted to 5.7 but without buffering). When the experiment was performed in a hydroponic system, the seeds were germinated on 2 mm foam plugs floating in 330 ml pots on ½ MS + 1% sucrose media at pH 5.7 adjusted by KOH. The media was exchanged 6 days after germination to ½ MS and seedlings were further grown.

Fungal strains and culture conditions.

Fusarium oxysporum strain 5176 (Fo5176) was originally isolated in Australia from infected Arabidopsis plants (Thatcher et al. 2012). The strains were routinely cultured in potato dextrose broth (PDB) at 28°C with orbital shaking at 170 rpm. Where necessary the following antibiotics were added to the culture medium: hygromycin B (55 µg/ml), neomycin (100 µg/ml) and phleomycin (5.5µg/ml). For microconidia collection, 3 to 5 day-old cultures were collected by filtration through a nylon filter (Monodur; mesh size 10 µm). Filtrates were centrifuged at 12000 g for 10 min, the pellet containing the microconidia was washed using deionized water, resuspended in water to reach the desired concentration. For long-term storage of Fol strains freshly obtained microconidia were resuspended in 30% glycerol and stored at -80°C.

Fungal Growth in different carbon sources.

F. oxysporum 5176 cultures were grown on ½ MS media (Murashige and Skoog media, Difco). Carbon sources were added to 0.5% wt/vol. Conidia were inoculated into 3 ml liquid media at 10⁶ conidia/mL and grown at 28° in dark and shaking (180 rpm), three days for sucrose and cellobiose (Fluka) and seven days for cellulose (Sigma, cellulose type 50). The material was dried at 60° for one day to further weight it or extract nucleic acids. For the nucleic acids extraction, we follow previous protocol but without degrading RNA (Raeder and Broda, 1985). The nucleic acids were quantified by using a nanodrop.

Colony growth phenotype.

To analyse the colony growth phenotype, drops containing serial dilution (1x10⁵, 1x10⁶ and 1x10⁷) of freshly obtained microconidia were spotted on different agar plates with different media as indicated in (Segorbe et al., 2017). Normal media with (YPD), for cell wall stress assay Congo red (5 µg/mL) or 5 µg/mL CalcoFluor White prepared in 0.5% KOH, for analysis in a deficient media nutrient-poor minimal media (MM) (Di Pietro and Roncero, 1998), for hyperosmotic stress assay 1M sorbitol. Plates were incubated at 28°C and imaged.

Fungal transformation.

Using an in silico approach, we identified an orthologous of CLR1 (N.crassa, NCU07705) (Coradetti et al. 2012) in F. oxysporum, Fo5176 (FOXB_08021) and Fol4287 (FOXG_08626). Targeted gene deletion of the entire CLR1 gene in the Fol4287-3XmClover3 genetic background, constitutively expressing three copies of the mClover3 fluorescent protein, and in the Fo5176-pSIX1::GFP background, expressing GFP under the control of SIX1 promoter. Gene complementation in the Δclr1 mutants backgrounds were performed as
reported previously (López-Berges et al., 2009). Gene replacement was performed using the split-marker method (Catlett et al., 2003) with the neomycin resistance cassettes following the protocol previously described (Rispail and Di Pietro, 2009). For the complementation of the \( \Delta clr1 \) deletion mutant, obtained as described before, a co-transformation with the phleomycin resistance cassette was performed as reported (Manuel S. López-Berges et al. 2010). The oligonucleotides used to generate PCR fragments for gene replacement, complementation or identification of mutants are listed in Table X. PCRs were routinely performed with the High-Fidelity Template PCR system (Roche Diagnostics, Barcelona, Spain) using an MJ Mini personal thermal cycler (Bio-Rad, Alcobendas, Spain). The amplified flanking sequences were PCR fused with partially overlapping truncated versions of the neomycin (Neo\(^{+}\)). Transformants were purified by monoconidial isolation (Di Pietro and Roncero, 1998).

**Arabidopsis plant infection assays.**

Arabidopsis infection assays were performed as described previously (Huerta et al., 2020; Kesten et al., 2019). In summary, 8 days old plants were transplanted from plates without fungus to plates with 100 μl of 1 x \( 10^7 \) microconidia/ml of Fo5176-pSIX1. The number of vascular penetrations per root was analyzed using the signal from the GFP, when it showed a clear, linear and root central pattern. The experiment in which the plants were pretreated with cellobiose 100 μM or chitin 100μg/ml were based on (Souza et al., 2017). 10 days old roots treated with cellobiose or chitin during 25 minutes, hereafter they were transferred on a plate with mycelia already growing for three days. The number of vascular penetrations were monitored as it is explained before.

**Fusarium fitness.**

We performed plate infection assays as indicated above. Once the plant was killed by the fungus, we waited for five days. Then, we collected the plant, we weighted it and we added 1.2 ml of \( H_2O \). The samples were shaken gently and the plant material removed. Later the number of microconidia was quantified.

**Real-time quantitative PCR.**

For the analysis of fungal and plant gene expression in different conditions (indicated in each figure legend) the material was collected and immediately frozen in liquid nitrogen. Samples were grinded using a TissueLyser II (Quiagen) and glass beads. Isol-RNA lysis reagent (5 PRIME) was used to extract total RNA, and subsequently 1 μg of total RNA per reaction was used to generate first-strand cDNA using Thermo Scientific Maxima\(^{TM} \) H Minus cDNA Synthesis Master Mix with dsDNase (Thermofisher), following manufacturer’s instructions. Quantitative PCR reactions were performed in a LightCycler 480II apparatus (Roche) using Fast SYBR Green Master Mix (Thermofisher) in a 10 μl reaction. Relative transcript levels were quantified with respect to a reference gene (\( GAPDH60OB \) for plants (Czechowski et al. 2005) or \( Fo\beta TUB \) (FOXG_06228) for Fusarium). The 2\(^{A ΔCt} \) method was used to quantify the relative expression of each gene. Primers are indicated in table x.

**Secretome.**

10-day old hydroponically-grown seedlings were infected with 20 μl of a solution containing \( 10^7 \) microconidia/ml of either Fo5176 WT or \( clr1 \), as described before (Menna/Dora/Sancho-Andres et al, resubmitted to BMC Bio). All pots were incubated for 30 minutes at 100 rpm on a shaker. The media of the infected plants was then replaced with fresh ½ MS media. The plants were further grown under the same conditions until the roots were harvested. At 3 days after the infections, the roots were removed and the liquid media was collected. First, to remove big particles present in the media, we filtered it with a 45
microns sterile filter (Starlab) to remove big particles present in the media. Then, the filtered media was concentrated. Subsequently, by using centricons (Amicon Ultra 3k, Merck Millipore) the media was concentrated to until 1.5 ml volume. 150 microliters of these 1.5 ml were sent to proteomic analysis.

Protein digestion.

For each sample, proteins were precipitated with trichloroacetic acid (TCA; Sigma-Aldrich) at a final concentration of 5% and washed twice with cold acetone. The dry pellets were dissolved in 45 μl buffer (10 mM Tris + 2 mM CaCl2, pH 8.2). Reduction and alkylation of the proteins was performed by adding 2 mM of Tris(2-carboxyethyl)phosphin –hydrochlorid (TCEP) and 15 mM of iodoacetamine (IAA). After 30 min at 60°C the samples were cooled to room temperature and 4 μg of Sequencing Grade Trypsin (Promega) for digestion were added. The digestion was carried out at 37°C for 4 hours. The samples were dried to completeness and re-solubilized in 20 μl of 3% acetonitrile, 0.1% formic acid for LC-MS/MS analysis. Before injection the samples were diluted 1:20 in the same solvent.

Liquid chromatography-mass spectrometry analysis.

Mass spectrometry analysis was performed on an Orbitrap Fusion Lumos (Thermo Scientific) equipped with a Digital PicoView source (New Objective) and coupled to a M-Class UPLC (Waters). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. For each sample 1 μL of diluted peptides were loaded on a commercial MZ Symmetry C18 Trap Column (100Å, 5 μm, 180 μm x 20 mm, Waters) followed by nanoEase MZ C18 HSS T3 Column (100Å, 1.8 μm, 75 μm x 250 mm, Waters). The peptides were eluted at a flow rate of 300 nL/min by a gradient from 5 to 22% B in 80 min, 32% B in 10 min and 95% B for 10 min. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA) acquiring a full-scan MS spectra (300–1’500 m/z) at a resolution of 120’000 at 200 m/z after accumulation to a target value of 500'000. Data-dependent MS/MS were recorded in the linear ion trap using quadrupole isolation with a window of 0.8 Da and HCD fragmentation with 35% fragmentation energy. The ion trap was operated in rapid scan mode with a target value of 10’000 and a maximum injection time of 50 ms. Only precursors with intensity above 5’000 were selected for MS/MS and the maximum cycle time was set to 3 s. Charge state screening was enabled. Singly, unassigned, and charge states higher than seven were rejected. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 20 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200. The mass spectrometry proteomics data were handled using the local laboratory information management system (LIMS) (Türker et al., 2010).

Protein identification and label free protein quantification.

The acquired raw MS data were processed by MaxQuant (version 1.6.2.3), followed by protein identification using the integrated Andromeda search engine (Cox and Mann, 2008). Spectra were searched against a provided Fusarium oxysporum database concatenated to the Arabidopsis protein database (https://www.arabidopsis.org/download/index-auto.jsp?dir=%2Fdownload_files%2FSequences%2Fraport11_blastsets, version 2020-06-18), concatenated to its reversed decoy fasta database and common protein contaminants. Carbamidomethylation of cysteine was set as fixed modification, while methionine oxidation and N-terminal protein acetylation were set as variables. Enzyme specificity was set to trypsin/P allowing a minimal peptide length of 7 amino acids and a maximum of two missed-cleavages. MaxQuant Orbitrap default search settings were used. The maximum false discovery rate (FDR) was set to 0.01 for peptides and 0.05 for proteins. Label free quantification was enabled and a 2 minutes window for
match between runs was applied. In the MaxQuant experimental design template, each file is kept separate in the experimental design to obtain individual quantitative values. Protein fold changes were computed based on Intensity values reported in the proteinGroups.txt file. A set of functions implemented in the R package SRMSuite (Wolski, Witold, Jonas Grossmann, and Christian Panse., 2018) was used to filter for proteins with 2 or more peptides allowing for a maximum of 4 missing values, and to normalize the data with a modified robust z-score transformation and to compute p-values using the t-test with pooled variance. If all measurements of a protein are missing in one of the conditions, a pseudo fold change was computed replacing the missing group average by the mean of 10% smallest protein intensities in that condition.

OG characterization and quantification

Plates infection assays were performed as indicated above. The roots were collected different days post transfer to plates with microconidia. The fresh weight was measured and the roots were covered with 100% ethanol. After several days the roots were dried using a freeze-dryer (Christ, Alpha 2-4). Lyophilized material was analyzed via HP-SEC-MS.

High-performance size-exclusion chromatography (HP-SEC). Samples were diluted at 1 mg/ml in ammonium formate 50 mM, formic acid 0.1%. Chromatographic separation was performed on an ACQUITY UPLC Protein BEH SEC Column (125A, 1.7 µm, 4.6 mm X 300 mm, Waters Corporation, Milford, MA, USA). Elution was performed in 50 mM ammonium formate, formic acid 0.1% at a flow rate of 400 l/min and a column oven temperature of 40 °C. The injection volume was set to 10 l. MS-detection was performed in negative mode with the end plate offset set voltage to 500 V, capillary voltage to 4000 V, Nebulizer 40 psi, dry gas 8 l/min and dry temperature 180 °C.

Data analysis

Major peaks were annotated following accurate mass annotation, isotopic pattern and MS/MS analysis, as previously described (Voxeur et al., 2019).

Statistical analysis.

Statistical analyses were performed using GraphPad Prims 9.0.0 (GraphPad Software, Inc). In each figure legend is indicated the statistical analysis which was performed and the level of significance.

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