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Response of *Sclerotinia sclerotiorum* to long exposure of isothiocyanates by transcriptomics approach

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Abstract

White mold disease, caused by the necrotrophic fungus *Sclerotinia sclerotiorum* affects *Brassica* crops. *Brassica* crops produce a broad array of compounds such as glucosinolates that contribute to defense against pathogens. From their hydrolysis arise several products with antimicrobial activity called isothiocyanates (ITCs) which toxicity is structure-dependent. It is recognized that *S. sclerotiorum* is able to overcome the toxic effect of moderate concentrations of ITCs after a prolonged exposure to their action. Our objective was to identify the molecular mechanism underlying response of *S. sclerotiorum* to long exposure of two chemically diverse ITCs: the aliphatic allyl-ITC (AITC) and the indolic indol-3-carbinol (I3C). We found, with a transcriptomic approach, that the response is dependent of the type of ITC and to their initial target, involving cell membranes in the case of AITC or DNA in the case of I3C. Mechanisms of response include the reorganization of chromatin, mediated by histones chaperones hip4 and cial, synthesis of ribosomes controlled by the pair kinase-phosphatase aps1-ppn1, catabolism of proteins, synthesis of ergosterol and induction of efflux-pumpers and glutathione transferases. These mechanisms probably help *S. sclerotiorum* to grow and survive in an environment where ITCs are constantly produced by *Brassica* plants upon breakdown of glucosinolates.

Introduction

Sclerotinia sclerotiorum is a necrotrophic fungal pathogen that attacks at least 408 species of plants from 278 genera, belonging to 75 families (Wang et al., 2019). *S. sclerotiorum* produces the so-called *Sclerotinia* stem rot or white mold in *Brassica* crops with symptoms that include rotting of leaves, stems and pods, causing important economic losses, especially in oil crops (Zhang et al., 2015).

Brassica plants are able to produce a broad array of compounds that contribute to defense against *S. sclerotiorum*. This includes regulatory compounds such as jasmonic, salicylic acid and ethylene as well as defensive compounds such as glucosinolates (Zhang et al., 2015). These are secondary metabolites, present in the Brassicaceae family, derived from amino acids. Depending on the side chain of the amino acid, glucosinolates are classified as: aliphatic (derived from methionine, alanine, valine, leucine, and isoleucine), aromatic (derived from phenylalanine or tyrosine) and indolic (derived from tryptophan) (Zukalova & Vasak, 2002). Glucosinolates are hydrolyzed by myrosinases enzymes into several chemically diverse products. Hydrolysis occurs upon tissue breakdown produced by the attack of herbivores or necrotrophic pathogens (Calmes et al., 2015). These products showed antimicrobial effects *in vitro* against a broad range of fungal and bacterial plant pathogens (Sotelo et al., 2015, Calmes et al., 2015). Among hydrolytic products, isothiocyanates (ITCs) are the most toxic ones, even at low concentrations (Pastorczyk & Bednarek, 2016).

Glucosinolates and their hydrolytic products have a role in the defense against *S. sclerotiorum* in planta (Madloo et al., 2019) and *in vitro* (Sotelo et al., 2015, Rahmanpour et al., 2009). The response of fungus seems to be dependent of the chemical structure of ITC (Madloo et al., 2019). However, to date, mechanisms through which ITCs exerts their toxicity and the ways fungus can overcome it are not

completely understood. Generally speaking, ITCs can act directly by covalent binding/modification of certain nucleophile-containing proteins referred as target or indirectly by activating gene transcription (Mi et al., 2011). *S. sclerotiorum* can overcome toxic effects driven by sub-lethal concentrations of ITCs after short exposure, basically by inducing antioxidant defenses and efflux pumpers (Rahmanpour et al., 2009), similarly to other necrotrophic fungi, such as *Alternaria brassicicola* (Sellam et al., 2007). This would allow the initial colonization of the plant by the fungus. However, as the disease progress, more glucosinolates are hydrolyzed, and therefore *S. sclerotiorum* is exposed during all the period of infection to the action of ITCs. Besides, in a plant there are at the same time glucosinolates of different chemical classes hydrolyzing to different ITCs which toxicity is structure-dependent (Li et al., 2013). In this line, this study aimed to identify molecular mechanism underlying response of *S. sclerotiorum* to long exposure of two chemically diverse ITCs. Identifying mechanisms of response will allow progressing our understanding on the interaction of *Brassica* plants with *S. sclerotiorum*.

Results

Phenotypical effect of ITC treatments on *S. sclerotiorum*

Mycelium of *S. sclerotiorum* spent more time to reach the end of Petri dish when it was treated with ITCs. This effect was more evident when samples were treated with I3C (Figure 1A). Growth rate reached its maximum in the first day under control conditions and in the second day in treated samples, then it decay progressively in control and AITC treated samples (Figure 1B). However, growth rate of fungus treated with I3C stabilized in the period of 3-6 days and then it decayed again. Once mycelium of AITC and control samples reached the end of petri dishes they start producing sclerotia in the border. However, sclerotia of I3C treated samples was produced also in a middle point of petri dishes (Figure 1E). 100 μ M AITC provoked permeabilization of membrane and it was half of that by 70% ethanol (Figure 1C). No permeabilization effect was observed for I3C (Figure 1D).

Transcriptomic analysis

An average of 14960380, 16415912 and 10776800 tag sequences were obtained from controls and treated samples with AITC and I3C, respectively. To have a general overview of the results, GO analysis with all the annotated transcripts was done for each comparison (ITC vs control and AITC vs I3C) with GO-Tool2. We give a description of the most important features found in the category of biological process (BP) (Supplementary Table S1).

AITC vs Control

Analysis of the whole transcripts resulted in 212 significantly enriched GO terms ($p \leq 0.05$ and $-1 \leq \log_2 \text{fold change} \leq 1$), from which 181 were induced and 31 repressed. The most significant induced GO terms were related to mitosis and cell division (i.e., GO:1901989, GO:1901992, GO:1903047, GO:0051301). Other categories were related to organonitrogen compound metabolic process (GO:1901564) and small molecule metabolic process (GO:0044281). Besides, there is a large set of significantly enriched terms associated with positive regulation of proteasomal protein catabolic process (GO:1901800, GO:0032436). The first six most significantly repressed GO terms are related to toxin biosynthetic pathways (GO:1901376; GO:1901378; GO:0045461; GO:0045460; GO:0009403, GO:0009404). Several terms were related to apoptosis and cell death (GO:0006915, GO:0012501, GO:0008219; GO:0016265). Other significant terms included processes related to mitochondrial transmembrane transport (GO:1990542, GO:0045041), mitochondrial DNA repair (GO:0043504), and maturation of LSU-rRNA (GO:0000463, GO:0000470).

I3C vs Control

GO analysis of I3C vs control rendered 197 significant GO terms, from which, 190 were repressed and 7 induced. Significantly induced GO terms were mostly related to synthesis of aminoacids, including serine, (GO:0009070, GO:0009092), threonine (GO:0009088) and sulfur amino acids (GO:0000097). Besides, ubiquitin-dependent protein catabolic process via the multivesicular body sorting pathway (GO:0043162)

and glyoxylate metabolic process (GO:0046487) were also significant. A large part of significant GO repressed terms were related to translation and to ribosome biogenesis and assembly (GO:0002181, GO:0042273, GO:0042255). Among the most significant GO down-regulated terms we could find several related to stress and stimulus response (GO:1901700, GO:0042221, GO:0006970, GO:0009408, GO:0010035). Other significantly repressed terms were implicated in small molecule metabolic process and nucleobase-containing small molecule metabolic process (GO:0044281, GO:0055086) and toxin biosynthetic process (GO:0009403).

AITC vs I3C

Expression of 296 GOs was higher in mycelium treated with AITC than in mycelium treated with I3C. Among them we can find terms related to cell division, such as: positive regulation of metaphase/anaphase transition of cell cycle (GO: 1902101), positive regulation of cell cycle phase transition (GO: 1901989) or actin polymerization or depolymerization (GO:0008154). Other terms are related to catabolic processes such as: positive regulation of proteosomal protein catabolic process (GO:1901800), positive regulation of proteolysis (GO:0045862). Translation (GO:0006412), ribonucleoprotein complex assembly (GO:0022618), ER-nucleus signaling pathway (GO:0006984), cellular response to sterol depletion (GO:0071501) and fungal-type cell wall biogenesis (GO:0009272) were also up-regulated by AITC. The expression of four GO terms was higher in the treatment with I3C compared to AITC: mitochondrial DNA repair (GO:0043504), cellular response to light stimulus (GO:0071482), mitochondrial DNA metabolic process (GO:0032042) and response to light stimulus (GO:0009416).

Differential expressed genes

There was higher number of DEGs in response to AITC than in response to I3C compared to control. Thirty genes were commonly induced by both treatments, and 98 were repressed (Figure 2A, B). In the comparison AITC *vs* I3C, 291 DEGs were induced in response to AITC and 427 were induced in response to I3C (figure 2C). The relationships among DEGS from each comparison were deduced by using the web-tool STRING (v10.5). One hundred twenty-three DEGS from AITC *vs* Control comparison interact in one single network (Figure 3). Confirming results from GO analysis, DEGs interact in several clusters related to sterol and lipid synthesis, proteasome, translation, cell division and DNA replication (Figure 3, Supplementary Table S2). Twenty-one DEGs interact in two different networks in the comparison I3C *vs* Control (Figure 4, Supplementary Table S2). Part of the structure of network 1 (including DEGs *mad2*, *k1p9*, *mug190*, *ish1*, *hsp9* and *SPAC32A11*) is shared with the network of the comparison AITC *vs* Control. In both comparisons, *mad2* and *k1p9* are induced and *mug190*, *ish1*, *hsp9* and *SPAC32A11* are repressed.

Seventy-four DEGs are interacting in the comparison AITC *vs* I3C (Figure 5, Supplementary Table S2) in two different networks. In network 1 there is a cluster of structural proteins related to ribosome synthesis, which are higher induced by AITC than by I3C. All these structural proteins are related to protein *aps1*, which is repressed by I3C and induced by AITC. In this network a cluster of DEGs related to ergosterol synthesis (induced by AITC) and leucine synthesis (induced by I3C) also appear, interacting by protein *SPAC22A12.16.1*. In network 2 several DEGs: *hip4*, *hhf1*, *mst1*, *ngg1*, are induced by AITC, whereas DEGS *rhp6*, *ubc7*, *rev1* and *rhp14*, are induced by

182 I3C. Both branches are joined by DEG *cia1*, which is induced by I3C and repressed by
183 AITC.

184 DEGs in the three comparisons that were related to xenobiotic transport, transfer
185 of glutathione and toxin biosynthesis are shown in Table 1.

186

Discussion

ITCs alter fungus growth and cell cycle

Treatment with both ITCs produced a slower growth of mycelium compared to control conditions. This effect was more evident in fungus treated with I3C than with AITC. Besides, in I3C treated samples number of transcripts found was lower than in AITC samples or control, indicating that at the same concentrations, I3C is more toxic than AITC on *S. sclerotiorum*.

Both treatments provoked an anti-apoptotic response. Necrotrophic fungi reduce growth and blocks host-induced programmed cell death to maintain at least few hypha bodies to survive in early stages of infection. In the second phase of infection, surviving hypha will start growing to produce new mycelium that will invade plant tissues producing lesions in the plant (Goncalves et al., 2017). Several common transcripts in both treatments have anti-apoptotic function, of which only *casA* and *crz1* were significantly repressed by both ITCs and *ppk31* was significantly repressed by AITC. *CasA* is a metacaspase-1, which in other fungi (*Aspergillus nidulans* and *A. fumigatus*) is a positive regulator of endoplasmatic reticulum (ER) stress induced cell death (Tsiatsiani et al., 2011). *Crz1* is a target of the calcineurim protein that is related to resistance to antifungal molecules and cations homeostasis in *Saccharomyces cerevisiae* (Karababa et al., 2006).

Ppk31 is involved in the induction of autophagy and in the regulation of lifespan. *Ppk31* increases of chronological lifespan of the *S. pombe* DY8531 strain (Ellis et al., 2018). Lifespan can be modified by changing the number of mitotic divisions that a cell can undergo during its lifetime, or by modifying the time, a cell

remains viable. In the first case, the term employed is replicative lifespan and chronological lifespan in the second case (Ellis et al., 2018). Therefore, by inhibiting ppk31, fungus treated by AITC may stop chronological lifespan to increase replicative lifespan.

Unlike AITC, there appeared to be no increase in expression of GOs associated with mitosis in mycelium treated with I3C. Chronological lifespan in *S. cerevisiae* is related to amino acid availability. The addition of isoleucine, valine, and leucine extended chronological lifespan in *S. cerevisiae* on media with limitation of nitrogen and carbon sources (Alvers et al., 2009). Accordingly, in the comparison AITC vs I3C, DEGs *leu1* and *leu2*, key genes in the synthesis of leucine were induced by I3C. Besides, this DEGS co-expressed with *oac1*. *Oac1* exchanges isopropylmalate by oxalacetate from mitochondria to cytosol. Leucine synthesis is related to this transporter, since its precursor pyruvate can be obtained by oxaloacetate, once this is in the mitochondria (Marobbio et al., 2008). (Tsang et al., 2015) proved that *oac1* is related to life span extension in *S. cerevisiae* through regulation of NAD⁺. In the GO analysis, glyoxylate cycle was up-regulated by I3C compared to control. When simple sugars are scarce, lower eukaryotes employ glyoxylate cycle to obtain energy from two-carbon compounds (Liberti et al., 2013). There is a link between glyoxylate cycle and chronological lifespan in *S. cerevisiae*. *Icl1* is a key gene in glyoxylate cycle and it was induced by I3C. Deletion of this gene in *S. cerevisiae* under calorie restriction provokes a decrease in chronological lifespan (Orlandi et al., 2013). Following (Orlandi et al., 2013), chronological lifespan is favored by glyoxylate/gluconeogenesis flux contrarily to the flux of ethanol and acetate via Krebs cycle and mitochondrial respiration. One of the responses of fungus treated with I3C may be to increase the chronological lifespan

on mycelium cells. Therefore, it seems that fungus is following two different growth strategies to cope with the toxic effects of both ITCs.

Both ITC provoke a delay in growth of mycelium. However, once fungus treated with AITC start growing, the shape of the graph of growth on time is similar to that of control. Rate of growth decreases on time until fungus reaches its maximum size, coinciding with the end of Petri dish. Therefore, it seems that AITC is provoking a delay in the normal cell cycle of fungus. However, I3C treated samples shows a different growth pattern. Between days 3-6 samples the rate of growth stabilizes and then, on day 7 starts decaying again until reaching the end of Petri dish. Differential expression on genes related to cell cycle may underlie differences in growth patterns.

However, although fungus treated with both ITCs showed different growth pattern, there are several transcripts related to cell cycle that were commonly regulated in the comparisons AITC vs Control and I3C vs Control. DEGs *mad2* and *kfp9* were induced by both ITCs. They are related to spindle dynamics during mitosis (Fu et al., 2009, Moyle et al., 2014). *Ish1* and *hsp9* are part of the mitogen-activated protein kinase (MAPK) pathway and both are down-regulated by both ITCs. The expression of *ish1* increases after exposure of *S. pombe* to osmotic stress or glucose deprivation (Taricani et al., 2002). The function of *hsp9* is related to regulation of cell cycle by increasing the length of *S. pombe* cells and decreasing their growth rate (Ahn et al., 2012).

Other common DEGS were regulated differentially by both treatments in the comparison AITC vs I3C. DEGS of network 2 are mostly related to modification of histones and DNA repair. Synthesis of histone H4 (DEG *hhf1*) is induced by AITC. Histone chaperones modulates nucleosomes assembly and therefore, the structure of chromatin (Amin et al., 2012). *Hip4* and *cia1* in *S. pombe* (homologous to *hpc2* and *asf1* of *S. cerevisiae*, respectively) are chaperones acting on histones H3 and H4. *Hip4* is a

component of the HIR complex. Deletion of *hip4* provokes cycle delay (Anderson et al., 2010). This gene is necessary for accurate chromosome segregation. *Cia1* is a repressor of the HIR-dependent histone genes. Mutants in *cia1* causes double strand breaks in DNA, increase DNA sensitivity to nuclease, and subsequently activate the DNA damage checkpoint pathway. Consequently, lifespan is severely reduced (Tanae et al., 2012, Anderson et al., 2010). AITC induces *hip4* and I3C induces *cia1*.

The function of histone chaperones can be regulated by post-transcriptional modifications, leading to a regulation of different processes involving DNA, such as transcription, reparation or condensation (Zofall & Grewal, 2007). DNA replication is regulated by the acetylation of histones H3 and H4 (Sklenar & Parthun, 2004). This function is carried on by DEGS *ngg1* and *mst1* (Ruan et al., 2015), when fungus is treated with AITC. In the other hand, ubiquitination of DNA is induced by I3C, by means of *rhp6* and *rhp14*. Both DEGS, together with the DNA polymerase *rev1* act in DNA repair (Meyer et al., 2019, Cui et al., 2018, Hohl et al., 2001), contributing to genome stability.

Therefore, there is a regulation of chromatin structure dependent on ITCs. In the case of AITC treated samples, this reorganization seems to be involved in mitosis and DNA replication, whereas in the case of I3C it may be related to DNA repair. Then differences in growth performance between both samples can be due to DNA reparation, being DNA a possible direct or indirect target of I3C.

ITCs alter metabolism of proteins and lipids

Translation and ribosome synthesis were induced by AITC and repressed by I3C. Since mitosis of AITC treated cells is induced, they need to produce new proteins. However,

in the case of I3C cells, which growing rate is slower, and needs to repair its DNA, ribosome synthesis is a high-consuming energy process. Eukaryotic cells cease the production of ribosomes very rapidly upon unfavorable conditions in order to ensure survival (Drygin et al., 2010). In the network created with DEGS of the comparison AITC vs I3C, a big cluster of ribosome-associated proteins is induced by AITC. All these proteins are linked to *asp1*, which is induced by I3C.

The inositol pyrophosphates (PP-InsPs) play a central role in signaling and reprogramming cell metabolism through their phosphorylation/dephosphorylation. *Asp1* is a inositol kinase, which participates in the synthesis of the diphosphate group of PP-InsP5 and (PP)2-InsP4 (Wu et al., 2016). Wu et al., (2016) found that a large number of proteins isolated from *S. cerevisiae* that interact with 5PP-InsP5 are related to ribosome biogenesis and maturation. *Asp1* co-expresses with *ppn1*, which is an endopolyphosphatase and catalyzes the hydrolysis of polyphosphate chains. This last DEG was induced by AITC. Therefore, it seems that the system *aps1-ppn1* is controlling the synthesis of ribosomes in fungus treated with ITCs.

Both treatments induced catabolism of proteins. AITC induced the ubiquitin-proteasomal system, whereas I3C induced the multivesicular (MVB) body sorting pathway. The ubiquitin-proteasome system has an essential role in cell growth and differentiation, survival, stress tolerance and adaptation to environmental changes (Mi et al., 2011, Piper & Katzmann, 2007). This mechanism is critical for cell survival (Huang et al., 2001). Formation of MVB takes place by invagination of the membrane of an endosome. MVB are degraded by fusing to lysosomes (Piper & Katzmann, 2007). Both mechanisms serve to regulate the turnover of proteins and to degrade the damaged ones. Proteins to be degraded by both systems are joined to ubiquitin as a signal (Piper & Katzmann, 2007). In the comparison AITC vs I3C, two ubiquitination-related DEGS

were significantly expressed. Ubc11 was induced by AITC, whereas, Ubc7 was induced by I3C.

Catabolism of proteins can be associated to mitosis and also to damage caused by ITC treatments. Secretory proteins are primarily assembled and folded in the endoplasmic reticulum (ER). Increment in misfolded proteins inside ER lumen can be provoked by and increment in the protein load but also it can respond to oxidative stress and to modifications in lipids and calcium (Volmer & Ron, 2015). Lipid perturbation seems to happen in cells treated with AITC because this treatment diminished permeabilization of *S. sclerotiorum* cells, whereas I3C had no effect on it. Sterol biosynthetic process was induced in fungus treated with AITC in comparison to I3C. Sterols play an important structural role to regulate membrane fluidity and permeability (Parks & Casey, 1995). Besides, DEG *wA* was induced by AITC while repressed by I3C. This gene is related to the synthesis of melanin in fungi, which gives strength and rigidity to the cell wall and consequently it is related to cell permeability and turgor (Cordero & Casadevall, 2017). In the comparison AITC vs I3C DEG *plh1* was induced by I3C. This DEG codifies for a phospholipid: diacylglycerol acyltransferase, related to the synthesis of triacylglycerols (Feng et al., 2019). Fungus treated with AITC probably modify the composition of cellular membranes by increasing the level of sterols in comparison with control and I3C treatments and decreasing tryacylglycerols in order to increase their stability. Similarly, ergosterol synthesis pathway and specifically, gene *erg11* are a target of azol and non-azol fungicides in different fungal species (Sellam et al., 2007, Somani et al., 2019, Bastos et al., 2019).

Other response mechanisms

Exposure to AITCs causes oxidative damage in fungi (Rahmanpour et al., 2009, Calmes et al., 2015, Sellam et al., 2007). One of the possible explanations is that conjugation of glutathione with ITCs may cause a drop in the free glutathione and the inhibition of glutathione reductase. Conjugation of glutathione to ITCs is catalyzed by glutathione-S-transferases. As a mechanism of detoxification, glutathione transferases were induced by *S. sclerotiorum* (Rahmanpour et al., 2009), and *A. brassicicola* (Sellam et al., 2006, Calmes et al., 2015) after exposure to several ITCs. In line with this, several glutathione transferases were induced by both treatments, coincidentally, SS1G_10295 was also induced after treatment of *S. sclerotiorum* with AITC (Rahmanpour et al., 2009).

Hog1 is a MAP kinase related to the regulation of the response to oxidative stress. Hog1 was induced after exposure of *A. brassicicola* to AITC (Calmes et al., 2015, Joubert et al., 2011). Hog1 and ras1 controlled the response of osmotic stress in *Cryptococcus neoformans* exposed to amphotericin B (Sun et al., 2013). Signal transduction pathways involving MAP kinases are one of the major mechanisms that fungus employ to sense and response to environmental changes. However, in this work, sty1 (homologous to hog1) and ras1 were repressed after AITC treatment, probably because at the time point when samples were taken, osmotic and/or oxidative stress caused by AITC were reduced after several days of adaptation.

Induction of MFS and ABC transporters is a common mechanism of defense in order to detoxify fungi cells (Somani et al., 2019, Vela-Corcia et al., 2019, Sellam et al., 2007). In this case, several MFS and one ABC transporters were induced by ITC treatments. Induced transporters were not coincident between treatments, indicating

specificity of substrate. MFS transporter SS1G_12101 was also induced in *S. sclerotiorum* after invasion of *B. napus* plants (Seifbarghi et al., 2017).

In GO analysis, toxin biosynthetic process was down-regulated by both ITCs. This GO term include genes similar with sterigmatocystin coding genes. Sterigmatocystin is a polyketide compound that is produced by several *Aspergillus* species and is a precursor of aflatoxin. Repression of toxin related genes by fungi in the presence of fungicides has previously been described (Somani et al., 2019) and it may respond to a need of derive energy to primer metabolism.

Conclusions

Moderate concentrations of ITCs inhibit growth of *S. sclerotiorum* *in vitro*. However, after long-exposure fungus is able to overcome their toxic effects and growth at the same level as non-treated fungus. We give an overview of the mechanisms of adaptation of *S. sclerotiorum* to two chemically diverse ITCs, the aliphatic AITC and the indolic I3C. We show that mechanisms of adaptation are dependent of the type of ITC and to their initial target, involving cell membranes in the case of AITC or DNA in the case of I3C. Mechanisms of adaptation include the reorganization of chromatin, mediated by histones chaperones *hip4* and *cia1*, synthesis of ribosomes controlled by the pair kinase-phosphatase *aps1-ppn1*, catabolism of proteins, synthesis of ergosterol and induction of efflux-pumpers and glutathione transferases. These mechanisms probably help *S. sclerotiorum* to grow and survive in an environment where ITCs are being produced by *Brassica* plants upon breakdown of glucosinolates.

Material and methods

Fungus growth and treatment with ITCs

We collected the *S. sclerotiorum* isolate MBG-Ss2 employed in the experiments on *B. napus* in Spain and it is currently maintained at MBG-CSIC. In order to provide fresh colonies for experiments, surface sterilized sclerotia were cultivated on potato dextrose agar (PDA, Difco) plates and incubated at 24 °C in the dark for 4 days. Fresh colonies were obtained through routinely growth of mycelium-agar plugs from the margin of the fungus colony on PDA and incubated at 24°C for 72 h. ITCs were purchased from SIGMA-ALDRICH including allyl-ITC (AITC) (36682) and indole-3-carbinol (I3C) (I7256-5G). Minimal inhibitory concentration which resulted in complete growth inhibition of fungus was tested for each ITC. The growth of fungus in 2x YPG media was monitored spectrophotometrically for 4 days at 25 °C with the addition of each ITC in concentrations ranging from 0 to 250 µM. Then a sub-lethal concentration of 100 µM for both compounds was selected to use in transcriptomic analysis.

Fungus was grown in PDA medium supplemented with each ITC, which was added to the medium before solidification to a concentration of 100 µM. Petri dishes were coated with one layer of cellophane membrane (Bio-Rad Catalog No. 165–0963). Then, agar plugs of 2.5 mm diameter containing fresh fungus colonies were transferred to petri dishes and incubated at 24°C in darkness. After 5 days mycelium mass was harvested from cellophane membranes and homogenized in liquid nitrogen. The resultant material was used immediately for isolation of total RNAs. Two replicates per treatment and control samples were carried out.

In other experiment, diameter of fungal mycelium growing radially from the center was measured during 8 days, until reaching the end of petri dishes. Growth rate was computed between time points as follows: $(\text{growth } t_{i+1} - \text{growth } t_i) / \text{growth } t_{i+1}$, varying i from 1 to 7. Experimental conditions were the same as in RNA experiment. Three replicates were done for each treatment.

RNA isolation, library preparation and sequencing

RNA was extracted with Spectrum™ Plant Total RNA Kit (Sigma MO, USA) according to the manufacturer's protocol. To remove any traces of genomic DNA, the RNA was treated with DNase by following the manufacturer's instructions. Massive Analysis of 3'-cDNA Ends (MACE) libraries were generated using GenXPro's MACE kit (GenXPro GmbH, Frankfurt, Germany) as described by Zawada et al. (2014). Briefly, cDNA from 2 µg of total RNA was randomly fragmented and biotinylated 3' ends were captured after binding to a streptavidin matrix. A library ready for high-throughput sequencing was prepared using TrueQuant adapters included in the kit. The library consisted of 50–700 bp-long fragments derived from the 3'-end of the cDNAs. The 5'-ends of the libraries were sequenced using an Illumina Hiseq 2000 version 4 chemistry (Illumina, Inc., San Diego, CA, USA).

NextSeq -derived sequence reads were processed with GenXPro's online in-house MACE analysis pipeline. Briefly, libraries were sorted according to their respective index and all duplicate reads detected by the TrueQuant technology were removed from the raw datasets. Additionally, quality filtering (by software Cutadapt ([https:// github.com/marcelm/cutadapt/](https://github.com/marcelm/cutadapt/))), and poly(A)-tails were clipped by an in-house python-script. The reads were aligned to reference sequences using "bowtie2". On this

genome, as the functional annotation was poor, a "cluster_bed" step was performed. Reads that map successfully to the genomic regions with no annotation (feature) were clustered. The clusters (consensus sequences) were annotated via BLASTX to the Uniprot database (Swiss-Prot and TrEMBL). Reads that did not match the genome were taken into a *de novo* assembly step and annotated via BLASTX to the Uniprot database (Swiss-Prot and TrEMBL). The assembly was done with Trinity RNA-Seq (<https://github.com/trinityrnaseq/trinityrnaseq/wiki>). For the functional annotation Uniprot (<http://www.uniprot.org/>) and NCBI fungal sequences were employed

Bioinformatic analysis

To have a general overview of the results, GO analysis with all the annotated transcripts was done for each comparison (ITC *vs* control and AITC *vs* I3C) with GO-Tool2. The likelihood of a specific GO term was calculated using the Fisher's exact test.

Those transcripts with a false discovery rate (FDR) ≤ 0.05 and $-1 \leq \log_2$ fold change (FC) ≥ 1 were considered to be differentially expressed genes (DEGs) between treatments. The web-tool STRING (v. 10.5) was used in order to study the interconnections between DEGs. This resource, in addition to well-supported protein–protein interactions experimentally observed, includes indirect and predicted interactions on top (Szkłarczyk et al., 2017). However, there is little information about interactions among proteins in *S. sclerotinia* genome. Relationships among proteins were deduced employing the genome of *Schizosaccharomyces pombe* as reference, since 10% of annotations were found in this genome and there is much more experimental evidence of interactions among proteins than in *S. sclerotiorum*.

Determination of membrane permeabilizing potential in *S. sclerotiorum* treated with ITCs

The SYTOX®-Green uptake assay was carried out following Theis *et al.* (2003). When plasma membrane integrity is compromised, influx of the dye and subsequent binding to DNA causes a large increase in fluorescence. In brief, 10 µl of fungus mycelium suspension (OD 0.37) was added to 100 µl of 2×YPG liquid medium (pH 4.5) and incubated for 48 h at 27 °C. Then, AITC or I3C was added to reach the final concentrations of 50, 75 and 100 µM. SYTOX-Green was added to a concentration of 0.2 µM. Samples showing 100% permeabilization were prepared by degrading cells with 70% ethanol. Fluorescence reads were carried out over a time course of t = 0 to t = 210 min using a Promega fluorescence measurement system (GloMax) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence values were corrected by absorbance of samples measured at 600 nm. Measurements were carried out in triplicate.

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Author contributions

PM did the experimental work. ML and PS designed the experiments. PM, ML, MEC and PS contributed to the discussion of the results. PM and PS wrote the manuscript.

Data Availability Statement

The data that support the findings of this study are openly available in GeneExpression Omnibus repository at <https://www.ncbi.nlm.nih.gov/geo/>, reference number GSE144344.

Figure legends

Figure 1. Radial growth A) and growth rate B) of *Sclerotinia sclerotiorum* in PDA medium with 100 μ M of allyl-isothiocyanate (AITC) or indol-3-carbinol (I3C) measured during eight consecutive days. Cell permeability assay of *Sclerotinia sclerotiorum* treated with AITC C) and I3C D) 40 minutes after the addition of SYTOX®-Green. Letters in the top of the bars indicate separation of means by least

significant differences at $p \leq 0.05$. Production of sclerotia of *Sclerotinia sclerotiorum* grown in PDA medium under three different treatments (from left to the right): Control, AITC and I3C.

Figure 2. Venn graph showing overlapping A) up-differentially expressed genes (DEGs) and B) down-DEGs between the comparisons AITC vs Control and I3C vs Control. C) Number of DEGs up-regulated by AITC or I3C in the comparison AITC vs I3C. Identification of DEGs was performed based on a false discovery rate (FDR) ≤ 0.05 and $-1 \leq \log_2$ fold change (FC) $\geq 1 \log_2$.

Figure 3. Protein association network built with all DEGs of the comparison AITC vs Control. Line thickness indicates the strength of data support, which is based on experimental results and co-expression analysis in *Schizosaccharomyces pombe*. Blue and yellow nodes indicate up or down-regulation of DEGs, respectively. Name of DEGs cited in the text are written in green. The name of DEGS shared with the comparison I3C vs Control are shown in red. Description of DEGs is given in Supplementary Table S2.

Figure 4. Protein association network built with all DEGs of the comparison I3C vs Control. Line thickness indicates the strength of data support, which is based on experimental results and co-expression analysis in *Schizosaccharomyces pombe*. Blue and yellow nodes indicate up or down-regulation of DEGs, respectively. Name of DEGs cited in the text are written in green. The name of DEGS shared with the comparison I3C vs Control are shown in red. Description of DEGs is included Supplementary Table S2.

Figure 5. Protein association network built with all DEGs of the comparison AITC vs I3C. Line thickness indicates the strength of data support, which is based on experimental results and co-expression analysis in *Schizosaccharomyces pombe*. Blue nodes indicate up-regulated DEGs by AITC and yellow nodes indicate up-regulated DEGs by I3C, respectively. Description of DEGs is included in Supplementary Table S2.

Supplementary information legends

Supplementary Table S1: GO terms of Biological Processes with p value lower than 0.05 and $\log_2 > 1$ in the comparisons AITC vs control, I3C vs Control and AITC vs I3C.

Supplementary Table S2: List of DEGs which show protein-protein interaction forming networks in the comparisons AITC vs Control I3C vs Control and AITC vs I3C.

706 Table 1. List of differentially expressed genes (DEGs) related to transport of genotoxics, transfer of glutathione and toxin biosynthesis.
 707 Annotations of DEGs were performed via BLASTX to the Uniprot database (Swiss-Prot and TrEMBL) and TrinityRNA-Seq. The false discovery
 708 rate (FDR) of each DEG and its log₂ fold change are shown for each one of the comparisons where they are significant.

Gene name	Description	Species	AITC <i>vs</i> Control		AITC <i>vs</i> I3C		I3c <i>vs</i> Control	
<i>Transporters</i>			FDR	log ₂	FDR	log ₂	FDR	log ₂
BcDW1_206	Putative mfs transporter protein	<i>Botryotinia fuckeliana</i>	0.042	1.038				
BcDW1_3316	Putative mfs multidrug transporter protein	<i>Botryotinia fuckeliana</i>					0.009	2.510
BcDW1_10619	Putative mfs monocarboxylate transporter protein	<i>Botryotinia fuckeliana</i>			0.025	-2.977	0.019	3.416
SPAC1399.02	Uncharacterized mfs-type transporter	<i>Schizosaccharomyces pombe</i>	0.004	1.335	0.000	1.279		
SS1G_12101	Putative mfs transporter protein	<i>Sclerotinia sclerotiorum</i>					0.000	1.257
cdr1	Pleiotropic ABC efflux transporter of multiple drugs	<i>Candida albicans</i>					0.038	1.216
<i>Glutathione transferases</i>								

SS1G_10295	Putative glutathione S-transferase	<i>Sclerotinia sclerotiorum</i>	0.001	2.466	0.032	1.210		
SS1G_14440	Putative glutathione S-transferase	<i>Sclerotinia sclerotiorum</i>	0.002	1.250			0.001	1.840
gst1	Glutathione S-transferase	<i>Schizosaccharomyces pombe</i>	0.000	1.343	0.000	1.341		
<i>Toxin biosynthetic process</i>								
SS1G_04352	Putative uncharacterized protein	<i>Sclerotinia sclerotiorum</i>	0.000	-1.985			0.026	-1.002
SS1G_05105	Putative uncharacterized protein	<i>Sclerotinia sclerotiorum</i>	0.000	-4.490	0.001	-2.288	0.000	-2.203
stcC	Putative sterigmatocystin biosynthesis peroxidase	<i>Emericella nidulans</i>	0.004	-4.080				
tcmN	Multifunctional cyclase-dehydratase-3-O- methyl transferase	<i>Streptomyces glaucescens</i>			0.000	-3.530	0.000	2.798
ccsA	Polyketide synthase-nonribosomal peptide synthetase	<i>Aspergillus clavatus</i>	0.000	1.656	0.000	2.230		

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711

Figures

Figure 1

Radial growth A) and growth rate B) of *Sclerotinia sclerotiorum* in PDA medium with 100 μM of allyl-isothiocyanate (AITC) or indol-3-carbinol (I3C) measured during eight consecutive days. Cell permeability assay of *Sclerotinia sclerotiorum* treated with AITC C) and I3C D) 40 minutes after the addition of SYTOX®-Green. Letters in the top of the bars indicate separation of means by least significant differences at $p \leq 0.05$. Production of sclerotia of *Sclerotinia sclerotiorum* grown in PDA medium under three different treatments (from left to the right): Control, AITC and I3C.

Figure 2

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Figure 4

Protein association network built with all DEGs of the comparison I3C vs Control. Line thickness indicates the strength of data support, which is based on experimental results and co-expression analysis in *Schizosaccharomyces pombe*. Blue and yellow nodes indicate up or down-regulation of DEGs, respectively. Name of DEGs cited in the text are written in green. The name of DEGS shared with the comparison I3C vs Control are shown in red. Description of DEGs is included Supplementary Table S2.

Figure 5

Protein association network built with all DEGs of the comparison AITC vs I3C. Line thickness indicates the strength of data support, which is based on experimental results and co-expression analysis in *Schizosaccharomyces pombe*. Blue nodes indicate up-regulated DEGs by AITC and yellow nodes indicate up-regulated DEGs by I3C, respectively. Description of DEGs is included in Supplementary Table S2.

Supplementary Files

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