Review

The toolbox of Trichoderma spp. in the biocontrol of Botrytis cinerea disease

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SUMMARY

Botrytis cinerea is a necrotrophic fungal pathogen causing disease in many plant species, leading to economically important crop losses. So far, fungicides have been widely used to control this pathogen. However, in addition to their detrimental effects on the environment and potential risks for human health, increasing fungicide resistance has been observed in the B. cinerea population. Biological control, that is the application of microbial organisms to reduce disease, has gained importance as an alternative or complementary approach to fungicides. In this respect, the genus Trichoderma constitutes a promising pool of organisms with potential for B. cinerea control. In the first part of this article, we review the specific mechanisms involved in the direct interaction between the two fungi, including mycoparasitism, the production of antimicrobial compounds and enzymes (collectively called antagonism), and competition for nutrients and space. In addition, biocontrol has also been observed when Trichoderma is physically separated from the pathogen, thus implying an indirect systemic plant defence response. Therefore, in the second part, we describe the consecutive steps leading to induced systemic resistance (ISR), starting with the initial Trichoderma–plant interaction and followed by the activation of downstream signal transduction pathways and, ultimately, the defence response resulting in ISR (ISR-prime phase). Finally, we discuss the ISR-boost phase, representing the effect of ISR priming by Trichoderma spp. on plant responses after additional challenge with B. cinerea.

Keywords: biological control, grey mould, induced systemic resistance, transcriptomics.

INTRODUCTION

Plants are surrounded by a diverse range of organisms in their environment, including bacteria, fungi, oomycetes, nematodes, insects and viruses. Although some of these organisms may have a negative impact on the plant, others may exert beneficial effects by enhancing the general fitness of the plant and/or by suppressing plant disease. Research on such biocontrol organisms (BCOs) has intensified during recent decades and their importance has increased as a part of integrated management practices to reduce chemical pesticide use (Glare et al., 2012). Early biocontrol research mainly focused on BCOs exerting a protective effect by direct interaction with pathogens. Interestingly, some BCOs were later found to be effective against pathogens through indirect interactions mediated by the plant, as the BCOs and pathogen were spatially separated. In this case, the BCO causes a reprogramming of the plant’s gene expression, leading to induced systemic resistance (ISR; Shores et al., 2010; Van Wees et al., 2008).

The genus Trichoderma comprises a great number of fungal strains with BCO capacity. They have adapted to diverse environmental conditions and are often the most frequently isolated fungi from soil (Harman et al., 2004). They are prolific producers of extracellular proteins, including enzymes that degrade cellulose and chitin, which are widely used in industrial applications (Nagy et al., 2007). Moreover, their capacity to reduce plant disease has been studied intensively, with both direct and indirect effects on plant pathogens (Lorito et al., 2010). Their high reproductive capacity, ability to survive under unfavourable conditions and high nutrient utilization efficiency contribute to their success as BCOs (Benitez et al., 2004). Many Trichoderma strains are able to colonize plant roots of both dicots and monocots, and trigger ISR, which is effective against a wide range of pathogens. Given that Trichoderma spp. are also capable of living freely in soil, they are considered to be opportunistic plant symbionts (Harman et al., 2004).

The application of Trichoderma spp. against one of the economically most important pathogens, the necrotrophic fungus B. cinerea, offers interesting perspectives. This pathogen causes disease in more than 200 plant species, including numerous crops, attacking organs such as leaves, stems, fruits and flowers, both pre- and post-harvest (Elad et al., 2004). The occurrence of soft rot, accompanied by collapse and water soaking of parenchyma tissues, and the subsequent rapid appearance of grey conidial masses,
represent the most typical symptoms on leaves and fruits, referred to as grey mould (Williamson et al., 2007). The fungus has a predominant necrotrophic lifestyle, implying that it kills plant host cells by producing diverse phytotoxic compounds and cell wall-degrading enzymes (CWDEs), after which it extracts nutrients from the dead cells (Łazniowska et al., 2010). Botrytis cinerea is difficult to control because of its different attacking modes, survival under unfavourable conditions for extended periods as sclerotia in crop debris and development of fungicide-resistant strains (Williamson et al., 2007). The global expenses for B. cinerea control easily surmount €1 billion per annum; however, the impact of product and quality loss occurring despite all control measures is expected to be much higher (Dean et al., 2012). In addition, potentially harmful consequences associated with the use of fungicides for both humans and the environment are an incentive to develop alternative and/or complementary management approaches. This has led to the evaluation of potential BCOs capable of substantial disease suppression in a commercial context and within integrated crop management systems (Elad et al., 2004). One of the key microbial genera to show great potential for the control of B. cinerea disease is the above-mentioned genus Trichoderma (some recent reports include Martínez-Medina et al., 2013; Mathys et al., 2012; Tucci et al., 2011). This review elaborates on the different modes of action in the control of B. cinerea by Trichoderma spp., thereby discussing both direct and indirect interactions.

DIRECT INTERACTIONS

Several Trichoderma strains have been reported to inhibit B. cinerea directly, in both soil and on plant surfaces. The specific mechanisms involved in direct interactions comprise mycoparasitism, antibiosis and competition. The different modes of action are described here, specifically for the Trichoderma spp.–B. cinerea interaction.

Mycoparasitism

Mycoparasitism is an important biocontrol trait of the Trichoderma genus. A recent survey of over 1100 Trichoderma Hypocrea strains from 75 molecularly defined species revealed that all the tested species possessed mycoparasitic potential against B. cinerea (Druzhinina et al., 2011). Mycoparasitism is a multi-step process in which physical contact between two microorganisms is preceded by an early recognition stage. Trichoderma spp. are believed to constitutively secrete CWDEs at low levels in an attempt to locate potential prey. When fungal cell walls are encountered and degraded by these enzymes, oligomers are released, causing the induction of further Trichoderma CWDEs and directional growth towards the prey, in order to enable physical attack (Mukherjee et al., 2012). Evidence for this recognition comes, for example, from transcriptomic studies showing the induction of CWDE genes before actual contact with B. cinerea (Seidl et al., 2009). Once the fungi come into contact, Trichoderma spp. attach to the prey, coil around it and form appressoria on the host surface. During this process, Trichoderma spp. continue to produce CWDEs and probably also antibiotic compounds in order to further degrade their prey (Shoresh et al., 2010). For example, microscopic observations have revealed that T. atroviride LU132 grows alongside hyphae of B. cinerea and tightly coils around it. Although penetration was not observed after this initial interaction, B. cinerea hyphae rapidly collapsed and died within 4 days after inoculation (Card et al., 2009).

The recently published genome sequences of three Trichoderma spp. confirm that CWDEs play an important role in Trichoderma spp. biocontrol, as genes encoding the CWDEs β-1,3-glucanases were over-represented in their genomes compared with those of other related fungi (Kubicek et al., 2011; Mukherjee et al., 2013). In addition, the genomes of two highly mycoparasitic species (T. virens and T. atroviride) were enriched in secondary metabolism-related genes compared with the weakly mycoparasitic T. reesei (Kubicek et al., 2011). Yang et al. (2009) reported that the presence of B. cinerea can specifically elicit the production of CWDEs in T. harzianum ETS323. The authors compared the secreted protein patterns of this strain grown in the presence or absence of B. cinerea, and found that two endochitinases were uniquely induced in the presence of B. cinerea, which also led to higher β-1,3-glucanase, β-1,6-glucanase and protease activity than in the absence of the pathogen. Similarly, the expression of α-1,3-glucanase was induced in T. asperellum T32 in direct confrontation assays with B. cinerea (Sanz et al., 2005).

Trichoderma spp. thus seem to have the perfect enzyme arsenal to target B. cinerea cell walls, in which chitin and non-cellulosic β-glucan are the main skeletal or microfibrillar polysaccharides, with proteins and α-glucans as the main cementing substances binding together the different structural cell wall components into macromolecular complexes (Cantu et al., 2009b). In addition, substantial amounts of dark pigment, melanin, can also be present in the B. cinerea cell wall and extracellular matrix of B. cinerea germlings and sclerotia (Cantu et al., 2009b). Melanin is formed by oxidative polymerization of various phenolic compounds and, as such, can be degraded by phenol-oxidases, such as laccase (Giardina et al., 2010). Catalano et al. (2011) found that the laccase gene Lcc1 was specifically highly expressed in T. virens during the early phases of contact with B. cinerea sclerotia, and that the T. virens deletion mutant Δlcc1 showed a significantly decreased ability to decay these sclerotia.

Antibiosis

Trichoderma spp. also produce a plethora of secondary metabolites with inhibitory activity against a diverse range of
microorganisms. The production of secondary metabolites in *Trichoderma* spp. can be strain dependent, and includes both volatile and non-volatile antimicrobial substances belonging to a variety of chemical compound classes (Reino *et al*., 2008). These include low-molecular-weight non-polar compounds, such as pyrones, butenolides, azaphylnones, anthraquinones, trichothecones, terpenoids and steroids, as well as non-ribosomal peptides, such as siderophores and peptaibols (Mukherjee *et al*., 2012).

One of the best-studied secondary metabolites from a biocontrol perspective is the ‘coconut aroma’ volatile pyrone 6-pentyl-2H-pyran-2-one, commonly produced by *Trichoderma* spp. (Vinale *et al*., 2008). Its antifungal activity against *B. cinerea* has been demonstrated *in vitro* (Pezet *et al*., 1999) and *in vivo*, e.g. on naturally infected kiwi fruit (Poole *et al*., 1998). Other secondary metabolites with proven *in vivo* anticomibial activity against *B. cinerea* include the butenolides T39 butenolide and harzianolide, secreted by *T. harzianum* T39, as well as 12azaphilone and an *N*-heterocyclic compound (harzianopryridone), secreted by *T. harzianum* T22 (Vinale *et al*., 2009). Another important group of compounds with antifungal activity produced by *Trichoderma* spp. are the anthraquinones. Liu *et al*., (2009) purified six compounds of this group from *T. harzianum* A6, all reducing *B. cinerea* disease incidence *in vitro*. Malmierca *et al*., (2012) showed that mutants defective in the formation of trichothecene mycotoxins (e.g. harzianum A) had reduced antifungal activity against *B. cinerea*. A novel hydroxyl-lactone, trivially named cerinolactone, was recently isolated from culture filtrates of *T. cerinum* and possesses *in vitro* antifungal activity against *B. cinerea* (Vinale *et al*., 2012).

Peptaibols represent another structurally different group of metabolites exerting antifungal activity. They constitute a class of linear short-chain-length (≤25 residues) non-ribosomal peptides of fungal origin. Their amphipathic nature allows them to self-associate into oligomeric ion channels which span the width of lipid bilayer membranes, through which the leakage of cytoplasmic material can occur, leading to cell death (Chugh and Wallace, 2001). Schirmbock *et al*., (1994) showed that the synthesis of *T. harzianum* peptaibols trichorzianine A1 and B1 was triggered by *B. cinerea* cell walls, and that they acted synergistically with chitinases and β-1,3-glucanases in inhibiting *B. cinerea* spore germination and hyphal elongation.

Enzymes with activities other than cell wall degradation have also been reported to contribute to the direct biocontrol potential of *Trichoderma* spp. against *B. cinerea*. For example, Yang *et al*., (2011) identified homodimeric L-amino acid oxidases (LAAOs) among the secreted proteins of *T. harzianum* ETS323. LAAOs are flavoenzymes that catalyse the stereospecific oxidative deamination of L-amino acid substrates to the corresponding α-ketoacids, with the release of hydrogen peroxide and ammonia (Zhang *et al*., 2003). Recently, it was shown that LAAO derived from *T. harzianum* ETS323 was able to lyse *B. cinerea* hyphae, which was accompanied by apoptosis-related events, such as DNA fragmentation, caspase pathway activation (e.g. the induction of cytochrome c, caspase 3 and caspase 9) and reactive oxygen species (ROS) generation (Cheng *et al*., 2012a, b).

**Competition**

Necrotrophic pathogens, such as *B. cinerea*, often rely on exogenous nutrients in order to germinate and grow on a plant surface before penetration (Benitez *et al*., 2004). A reduction in nutrient concentration thus generally results in reduced conidial germination and slower germ tube growth of the pathogen, thereby reducing the number of infection sites and the extent of subsequent necrosis caused by the pathogen (Nassr and Barakat, 2013). The point of entry for *B. cinerea* into the plant tissue comprises wounds, senescing host tissues or natural openings, such as stomata and lenticels, which are generally nutrient-rich areas owing to the exudation of sugars and amino acids. Therefore, by colonizing wounds or senescing tissue, *Trichoderma* spp. can compete for nutrients with *B. cinerea*, thereby preventing infection. Card *et al*., (2009) demonstrated that *T. atroviride* inhibits *B. cinerea* on strawberry leaves through different mechanisms, including competition for nutrients. Indeed, they observed a 25% reduction in the length of *B. cinerea* germ tubes present on *Trichoderma*-treated leaves relative to untreated leaves when glucose was limited. This difference was negligible when the glucose concentration was increased. In the presence of other sugars, such as sucrose and fructose, however, a significant inhibitory effect of *T. atroviride* was noticed at all tested concentrations of the sugar.

**INDIRECT INTERACTIONS**

In some cases, a biocontrol effect of *Trichoderma* spp. on *B. cinerea* disease has been observed, even though both fungi are physically separated from each other, indicating the involvement of a plant-mediated systemic response (De Meyer *et al*., 1998; Horst *et al*., 2005; Olson and Benson, 2007; Tucci *et al*., 2011). The consecutive steps in setting up a tripartite *Trichoderma* spp.–plant–*B. cinerea* interaction, resulting in the biocontrol of the disease, are further discussed in more detail in this section, covering the initial *Trichoderma*–plant interaction, the activation of downstream signal transduction pathways and defence responses resulting in ISR, and, finally, the effect of ISR on plant responses after *B. cinerea* challenge.

**Initiation of the *Trichoderma*–plant interaction and recognition of *Trichoderma* by the plant**

Some *Trichoderma* strains colonize only local sites on roots, whereas rhizosphere-competent strains colonize large root
surfaces (Harman and Shoresh, 2007). During this process, the hyphae of *Trichoderma* spp. coil around the roots, form appressoria-like structures and, finally, penetrate the root cortex, with morphological features reminiscent of those seen during mycoparasitism (Shoresh et al., 2010). The *Trichoderma* spp. then often grow intercellularly in the root epidermis and cortex, albeit usually limited to the first or second cell layers (Hohmann et al., 2012), inducing the deposition of cell wall material and the production of phenolics in surrounding cells, which limits their further ingress inside the root (Mandal and Mitra, 2007; Yedidia et al., 1999).

The induction of a plant-mediated ISR response starts with the recognition of *Trichoderma* by the plant, in a similar manner as for pathogens. Collectively, the general determinants of microbes that are recognized by the plant are referred to as microbe-associated molecular patterns (MAMPs) (Boller and Felix, 2009). A diversity of MAMPs of beneficial microorganisms have been shown to be implicated in the onset of ISR (Van der Ent et al., 2009), and this is also an active research domain for *Trichoderma* spp. in particular. In the interaction zone with the plant, *Trichoderma* spp. release many compounds that can induce ISR in plants, similar to those involved in the direct interaction with fungal pathogens (Hermosa et al., 2012, 2013). For example, *Trichoderma*-secreted proteins with enzymatic activity and other proteins and peptides with hydrophobin-like properties can enhance disease resistance (Djonovic et al., 2006; Martínez et al., 2001; Rotblat et al., 2002; Seidl et al., 2006). A number of reports have indicated that also peptaibols and other secondary metabolites produced by *Trichoderma* spp. can elicit plant defence responses, in addition to their direct antifungal activity (Engelberth et al., 2001; Vinale et al., 2008; Viterbo et al., 2007). A different class of plant defence elicitors includes oligosaccharides and low-molecular-weight compounds which are released from fungal or plant cell walls as a consequence of the activity of *Trichoderma* enzymes (Harman et al., 2004; Woo and Lorito, 2007; Woo et al., 2006).

Jones and Dangl (2006) comprehensively described the two-branched immune system involved in the plant response to pathogens as the so-called ‘zig-zag model’. Interaction of a pathogen-derived MAMP with the corresponding transmembrane pattern recognition receptor (PRR) of the plant activates a primary defence response, called MAMP-triggered immunity (MTI). In a second phase, successful pathogens deploy effectors that contribute to pathogen virulence and interfere with MTI, resulting in effector-triggered susceptibility (ETS). Effector-triggered immunity (ETI), however, is based on the highly specific, direct or indirect interaction of these pathogen effectors and the products of plant resistance (R) genes, according to the gene-for-gene resistance theory. The R genes usually encode proteins of the nucleotide binding-leucine-rich repeat (NB-LRR) class, cytoplasmic proteins with a nucleotide binding site (NBS) in front of a series of leucine-rich repeats (LRRs) (Boller and Felix, 2009; Jones and Dangl, 2006; Monaghan and Zipfel, 2012). *Trichoderma* spp. may affect the plant response by increasing its basic immunity or MTI (Lorito et al., 2010). Indeed, using a transcriptomic approach and subsequent gene ontology (GO) analysis, we observed the induction of the MTI response in *Arabidopsis thaliana* leaves after inoculation with *T. hamatum* T382, which resulted in the suppression of subsequent infection by *B. cinerea* (Mathys et al., 2012).

Only a limited number of MAMP–PRR pairs have been characterized (Boller and Felix, 2009; Monaghan and Zipfel, 2012), including one example of a specific *Trichoderma* MAMP–PRR pair. A xylanase from *T. viride*, called the ethylene-inducing xylanase (Xyn2 or Eix), is a potent elicitor in tomato and tobacco (Rotblat et al., 2002) for which the corresponding receptor, EIX2, has been identified as a receptor-like protein with an extracellular LRR domain, a transmembrane domain and a short cytoplasmic tail without kinase domain (Ron and Avni, 2004). Another recently characterized MAMP–PRR pair possibly involved in the recognition of *Trichoderma* spp. by the plant is the *A. thaliana* lysine motif (LysM) receptor-like kinase CERK1 (chitin elicitor receptor-like kinase 1), which binds chitin (Iizasa et al., 2010; Petutschnig et al., 2010). It has been established that perception of chitin oligosaccharides through such LysM receptors contributes to disease resistance in *A. thaliana* (Wan et al., 2008). The recognition of chitin is also supported by our above-mentioned transcriptome study of the *A. thaliana–T. hamatum* T382 interaction (Mathys et al., 2012), indicating that the response to chitin is one of the most significantly induced biological processes in *A. thaliana* leaves in response to *T. hamatum* T382. Brotman et al. (2013) found the same GO process to be significantly enriched in *A. thaliana* roots, 24 h after inoculation with *T. asperelloides* T203. Hermosa et al. (2012) also proposed that chitin oligosaccharides are indirect inducers of MTI, as they can be released by the activity of both plant and *Trichoderma* chitinases. In addition, several proteomic and transcriptomic studies have shown that plant interactions with various *Trichoderma* spp. result in the induction of cytoplasmic NB-LRRs (Marra et al., 2006; Palmieri et al., 2012; Perazzolli et al., 2012; Shoresh and Harman, 2008), suggesting that *Trichoderma* spp., apart from increasing MTI, can also have an effect on the plant response by increasing ETI.

**Plant responses induced by *Trichoderma* spp.: activation of signal transduction pathways**

On MAMP recognition, mitogen-activated protein kinase (MAPK) cascades are activated as an early plant response (Boller and Felix, 2009). MAPK cascades convert extracellular stimuli into intracellular responses, amplifying, at the same time, the transducing signal via three reversibly phosphorylated kinases (Ichimura et al., 2002; Opdenakker et al., 2012; Rodriguez et al., 2010). Shoresh et al. (2006) demonstrated that a cucumber MAPK homologous to
A. thaliana MPK3 is activated by the inoculation of roots with T. asperellum T203. Moreover, induction of this Trichoderma-induced protein kinase (TIPK) appeared to be necessary for ISR against bacterial pathogens, whereas silencing of TIPK completely eliminated this protection (Shoresh et al., 2006). The homologous A. thaliana MPK3 and a MAPK-kinase encoding gene (MKK4) were also induced in A. thaliana leaves after treatment of the roots with T. hamatum T382, resulting in reduced disease symptoms caused by B. cinerea (Mathys et al., 2012). Activation of MAPK cascades leads to the phosphorylation of substrate proteins, whose altered activities mediate a wide array of responses which are interconnected to other signalling molecules, such as plant hormones (Rodriguez et al., 2010).

Plant responses induced by Trichoderma spp.: plant hormone signalling

The action of plant hormones regulates the defence network that translates Trichoderma spp.-induced early signalling events into the activation of effective defence responses. Two main mechanisms are recognized: systemic acquired resistance (SAR) and ISR. SAR is traditionally defined as being triggered by pathogen infection, providing long-term systemic resistance to subsequent pathogen attack, which is correlated with the activation of pathogenesis-related (PR) proteins and requires the involvement of the signal molecule salicylic acid (SA) and subsequent mediation of the positive regulator protein NPR1 (Durrant and Dong, 2004). Early research on ISR in A. thaliana mainly focused on rhizobacteria-ISR (van Loon et al., 1998), which is thought to be regulated by ethylene (Et)- and jasmonic acid (JA)-mediated signalling pathways (Pieterse et al., 1998), with mediation of NPR1, but without induction of the SA-responsive genes PR1, PR2 and PR5 (Pieterse et al., 1996). However, this traditional view on ISR appears to be more complex. In recent years, it has become clear that an intensive interplay between hormone signalling pathways exists, the outcome of which determines the effectiveness of the immune response to a specific type of invader (Zamioudis and Pieterse, 2012).

Trichoderma spp. seem to be able to activate both the SA- and JAEt-mediated signal transduction pathways, although the pattern varies depending on the experimental conditions and organisms involved (Contreras-Cornejo et al., 2011; Salas-Marina et al., 2011; Velazquez-Robledo et al., 2011). This may be an important trait for B. cinerea management, as resistance to this pathogen is largely dependent on complex overlapping signalling pathways, involving both SA- and JAEt-mediated signal transduction pathways (Audenaert et al., 2002; Diaz et al., 2002; El Oirdi et al., 2011; Windram et al., 2012). Transcriptomic studies focusing on the plant response after inoculation with Trichoderma spp. are generally in line with this, although, depending on the study, more weight can be attributed to one of the two pathways.

We recently investigated the response of A. thaliana leaves at the transcriptome level, 48 h after root inoculation with T. hamatum T382, referred to as the ‘ISR-prime’ phase, to indicate that pathogen infection had not yet occurred. GO analysis revealed a significant induction of the response to SA, whereas responses to JA and Et were not altered significantly (Mathys et al., 2012). We furthermore found a striking resemblance between the ISR-prime response and SAR response. Indeed, GO analysis revealed a strong induction of the biological process ‘SAR’ and ‘regulation of SAR’ during the ISR-prime phase, which was further highlighted by the induction of genes belonging to the SA pathway. This included, for example, the induced expression of WRKY6, WRKY53, PR1, PR2 and PR5, and was also confirmed by the analysis of A. thaliana NahG, npr1 and sid2 mutants. Despite the similarities with the defence response induced by B. cinerea, the ISR-prime phase could be distinguished by the much more pronounced induction of the SA pathway, the production of SA via isochorismate instead of phenylalanine and the absence of involvement of the JA and Et pathways (Mathys et al., 2012). Moran-Diez et al. (2012) performed a transcriptome analysis of A. thaliana leaves, 24 h after incubation with T. harzianum T34 in liquid culture. A very limited number of differentially expressed genes could be detected, and genes involved in both SA and JA/Et signalling pathways were mainly down-regulated, although further analysis of marker genes for both pathways 48 h after incubation revealed an increased expression (Moran-Diez et al., 2012). These findings could suggest that T. harzianum T34 suppresses the initial plant defence response in order to allow root colonization, which is in contrast with another recently performed transcriptome analysis of A. thaliana roots in the interaction with T. asperelloides T203, which did not indicate major down-regulation of defence-related processes at the same time point in the roots (Brotman et al., 2013). This study revealed a more central role for JA, with the enhanced expression of several genes related to JA biosynthesis and JA signalling in the roots 24 h after T. asperelloides inoculation. For example, the authors reported the enhanced expression of the transcription factors WKRKY18 and WKRKY40, which stimulate JA signalling via suppression of JAZ repressors. In addition, GO analysis indicated the significant enrichment of JA biosynthesis.

A few transcriptomic studies have also been performed with other plants. Concerning the role of the main hormonal pathways in these studies, the transcriptome analysis of tomato leaves after root inoculation with T. hamatum T382 only revealed the induced expression of PR5 (Alfano et al., 2007), whereas the transcriptome analysis of grapevine leaves after leaf treatment with T. harzianum T39 mainly pointed to the activation of Et metabolism (Perazzolli et al., 2012). Activated Et metabolism was also found in our very recently performed detailed transcriptome analysis of the tomato–T. hamatum T382 interaction, in addition to a clear up-regulation of many JA biosynthesis-related genes (Yang, 2013). The comparison of the tomato transcriptomic study with
that in A. thaliana (Mathys et al., 2012) enabled us to determine to what extent the ISR mechanisms of T. hamatum T382 can be extrapolated from one experimental system to another. Changing the model plant A. thaliana for the crop plant tomato revealed significant overlap in the ISR-prime mechanisms, as well as specificities for each interaction. For example, the JA biosynthesis pathway was found to be most prominently induced in tomato, whereas the induction of the SA signalling pathway appeared mainly in A. thaliana.

The involvement of JA, Et and SA in Trichoderma spp.–plant interactions is also often deduced from expression studies on marker genes linked to these signalling pathways. For example, up-regulation of Lox1 (lipoxygenase 1), which encodes a lipoxygenase involved in JA synthesis, was observed after inoculation with T. atroviride IMI206040 in both the roots and leaves of A. thaliana (Salas-Marina et al., 2011). Expression studies with transgenic A. thaliana reporter plants portraying activity of the Lox2 promoter showed the induction of this gene after inoculation with T. virens Tv29-8 and T. atroviride IMI206040 (Contreras-Cornejo et al., 2011). A marker gene for JA/Et-mediated signalling in A. thaliana, PDF1.2a (encoding plant defensin 1.2; Thomma et al., 1998), was also up-regulated in both roots and leaves after T. atroviride treatment (Salas-Marina et al., 2011). The same study also showed increased expression of SA-inducible PR genes (PR1 and PR2) after treatment with T. atroviride, both locally in roots and systemically in leaves. A similar induction of PR1 by T. virens Tv29.8 was detected by Velazquez-Robledo et al. (2011) using transgenic A. thaliana reporter studies. The Lox2 and PR1 responses were absent in reporter plants co-cultivated with a mutant of T. virens which was unable to induce resistance against B. cinerea, suggesting that these genes are involved in resistance against B. cinerea, as the mutant colonized the root system as well as the wild-type strain (Velazquez-Robledo et al., 2011). In tomato, Tucci et al. (2011) detected the transcriptional activation of several PR genes in leaves after the addition of T. harzianum T22 and T. atroviride P1. For example, the PR1 family gene PR1b1 (Tornero et al., 1997) and the PR4 family gene PR-P2, mainly induced by SA in tomato (Van Kan et al., 1995), showed induced gene expression after Trichoderma inoculation. However, Martinez-Medina et al. (2013) did not observe an increased expression of the SA-inducible gene PR1a in tomato leaves after treatment with T. harzianum T78. By contrast, the JA-responsive genes PI II, MC and PS, coding for proteinase inhibitor II, multicystatin and prosystemin, respectively, showed increased expression (Martinez-Medina et al., 2013). Yoshioka et al. (2012) reported a significant increase in expression of both the SA-inducible genes PR1, PR2 and PR5 and JA/Et-inducible genes, such as PDF1.2a, in A. thaliana leaves after root treatment with T. asperellum SKT-1. Remarkably, even the application of the cell-free culture filtrate of this strain led to an increased expression of the same marker genes (Yoshioka et al., 2012). Further evidence for the induction of JA and SA by Trichoderma spp. comes from metabolic studies. Levels of JA and SA were shown to accumulate in above-ground plant parts of cucumber and A. thaliana after the application of T. asperellum T34 or T. virens and T. atroviride, respectively (Contreras-Cornejo et al., 2011; Segarra et al., 2007). In cucumber, a proteomic study also revealed that T. asperellum T34 up-regulates the Et biosynthesis enzyme aminocyclopropane-1-carboxylate oxidase 1 (Segarra et al., 2007). The reported induction of PR gene expression described above is in agreement with proteomic analyses of bean and maize interacting with T. atroviride P1 (Marra et al., 2006) and T. harzianum T22 (Shoresh and Harman, 2008), respectively.

However, the impact of Trichoderma colonization on plant hormone signalling is not limited to the typical defence-related hormonal pathways of JA, Et and SA alone. For example, GO analysis also revealed the activation of the response to abscisic acid (ABA) in the interaction of T. hamatum T382 with either A. thaliana or tomato (Mathys et al., 2012; Yang et al., 2013), and Martinez-Medina et al. (2011) found that the ABA level in melon shoots was increased significantly after root treatment with T. harzianum T-78. Recently, the same authors also reported an up-regulation of the ABA-related marker gene Le4, encoding a desiccation protective protein, in tomato leaves after pre-inoculation of the roots with the T-78 strain (Martinez-Medina et al., 2013). ABA is an important regulator that balances responses to abiotic and biotic stresses in plants, but its exact role in plant–Trichoderma interactions is not yet clear. The same holds true for another class of hormones, the gibberellins (GA). GA can control the onset of JA- and SA-dependent defence responses through the regulation of DELLA protein degradation (Hermosa et al., 2012), but, so far, only a few papers have reported the involvement of the GA-associated pathway in Trichoderma-induced ISR. For example, the ISR phenotype was disrupted in the GA-impaired A. thaliana mutant spy3, despite treatment with T. harzianum T39 (Korolev et al., 2008), and an increase in GA levels in tomato roots was found after the application of T. harzianum OTPB3 to tomato seeds (Chowdappa et al., 2013).

**Plant responses induced by Trichoderma spp.: production of secondary metabolites**

Together with other plant defence strategies, the production of phytoalexins plays an important role in defence against B. cinerea. The phenylpropanoid pathway, with phenylalanine ammonium lyase (PAL) as the first enzyme, is a major source for the production of antimicrobial phenolic compounds, as well as SA precursors (Huang et al., 2010; Mauch-Mani and Slusarenko, 1996). Brotman et al. (2013) found an increased expression of several genes involved in the phenylpropanoid pathway, such as PAL1, PAL2 and 4CL, in A. thaliana roots after inoculation with T. asperelloides T203, but Martinez-Medina et al. (2013) did not observe an increased expression of PAL in tomato leaves after colonization
with *T. harzianum* T78. Calderon *et al.* (1993) demonstrated that cell suspension cultures of grapevine treated with an elicitor from *T. viride* produced increased amounts of resveratrol, a phytoalexin with growth inhibitory activity towards *B. cinerea* (Ali *et al.*, 2003). Later studies also proved that phytoalexins from other plant species can effectively inhibit the proliferation of *B. cinerea* (Chassot *et al.*, 2008; Denby *et al.*, 2004; Ferrari *et al.*, 2003). More extensive phytoalexin research has been performed in the model plant *A. thaliana*. The last step in the biosynthesis of its most abundant phytoalexin, camalexin, is catalysed by a cytochrome P-450 enzyme CYP71B15 (or PAD3). Mutants deficient in camalexin production, such as pad3 (phytoalexin deficient 3), display enhanced susceptibility to *B. cinerea* (Ferrari *et al.*, 2003). Interestingly, PAD3 expression was found to be up-regulated in both roots and leaves after treatment of *A. thaliana* with *T. atroviride* (Salas-Marina *et al.*, 2011). Similarly, *A. thaliana* seedlings colonized with wild-type *T. virens* accumulated higher levels of camalexin than axenically grown seedlings or plants co-cultivated with a *T. virens* mutant unable to trigger ISR (Velazquez-Robledo *et al.*, 2011). Such induction of camalexin levels in *A. thaliana* seedlings was confirmed in another study using both *T. virens* and *T. atroviride* (Contreras-Conrero *et al.*, 2011). In addition, the authors observed that *T. virens* produces indole-3-carboxaldehyde, a possible precursor of camalexin (Devys and Barbier, 1991; Zook and Hammerschmidt, 1997). Moreover, this compound induces camalexin accumulation when externally applied to *A. thaliana* seedlings, suggesting that the plant might use this compound for camalexin production by either direct or indirect means (Contreras-Conrero *et al.*, 2011). Furthermore, the same authors observed induced levels of anthocyanin, which corresponds to our recent observations of a very pronounced induction of anthocyanin biosynthesis after inoculation of *A. thaliana* roots with *T. hamatum* T382 via both transcriptomic analysis and the chemical quantification of anthocyanin levels (Mathys *et al.*, 2012). Moreover, we showed that *T. hamatum* T382 was unable to induce ISR in *A. thaliana* mutant plants affected in different parts of the phenylpropanoid pathway. However, we did not observe a significant up-regulation of the genes involved in camalexin biosynthesis in the ISR-prime phase. The induction of the phenylpropanoid pathway instead of the camalexin pathway as the main source of secondary metabolites in the ISR-prime phase induced by *T. hamatum* T382 was proposed to be one of the characteristic differences to distinguish the ISR-prime phase from the plant response to *B. cinerea* (Mathys *et al.*, 2012). In addition, we found an apparent overlap in the induction of the phenylpropanoid pathway in the ISR-prime phase in both *A. thaliana* and tomato (Yang, 2013).

In addition to the involvement of the phenylpropanoid pathway, the expression of genes encoding lipid transfer proteins (LTPs) (PR-14) is also often reported to be influenced by *Trichoderma* colonization. Many biological roles have been suggested for LTPs, including a role in plant defence. Antimicrobial activity has been demonstrated for various LTPs against several fungi and bacteria, probably resulting from their capacity to interact with biological membranes, which may lead to membrane permeabilization (reviewed by Sels *et al.*, 2008). Proteome analysis revealed that the spraying of grapevine leaves with *T. harzianum* T39 increased the abundance of an LTP in the leaves (Palieri *et al.*, 2012), and various transcriptome studies have also reported an altered expression of LTPs. For example, Moran-Diez *et al.* (2009) compared the differential expression of *A. thaliana* genes during interaction with either a wild-type *T. harzianum* T34 or an endopolygalacturonase-silenced strain. One of the 10 genes that differed significantly in expression between both interactions was an LTP, with a reduced expression level in the presence of the silenced strain. Multiple LTPs have been reported to be up-regulated after root colonization with various *Trichoderma* strains in *A. thaliana*, pepper and cocoa (Bae *et al.*, 2011; Bailey *et al.*, 2006; Moran-Diez *et al.*, 2012), as well as in the ISR-boost phase of the *A. thaliana*- *B. cinerea*- *T. hamatum* T382 interaction (Mathys *et al.*, 2012). The possible importance of LTPs in defence against *B. cinerea* has been demonstrated, for example, by Chassot *et al.* (2007), who showed that endogenous over-expression of three LTP-like genes in *A. thaliana* resulted in enhanced tolerance to this pathogen.

**Plant responses induced by *Trichoderma* spp.: control of ROS damage**

Although phytoalexins can have a direct inhibitory effect on *B. cinerea*, inoculation with *Trichoderma* spp. can also induce plant responses that are more indirectly involved in the control of *B. cinerea* disease, by interfering with the pathogen’s infection strategy. The oxidative burst, characterized by the rapid generation of ROS and the accumulation of H$_2$O$_2$ (Lamb and Dixon, 1997), belongs to the early events of both resistant and susceptible plant responses to *B. cinerea* (Govrin and Levine, 2000). It was demonstrated that *B. cinerea* actively contributes to the generation of an oxidative burst during the plant–pathogen interaction, by both direct generation of ROS and the secretion of enzymes and metabolites that influence the oxidative burst in the plant (Schouten *et al.*, 2002; Temme and Tudzynski, 2009). The production of ROS and concomitant host cell death occurring early after infection by *B. cinerea* are indicative of a successful infection, as *B. cinerea*, a necrotroph, benefits from dead tissue (Govrin *et al.*, 2006). In addition, it has been established that, during plant–pathogen interactions, ROS can also act as signalling molecules, inducing a MAPK cascade and eliciting a defence response (Hancock *et al.*, 2002). In this respect, our transcriptome analysis of the interaction between *T. hamatum* T382 and *A. thaliana* revealed a pronounced induction of ROS-inducible genes in the leaves, supposedly followed by the induction of a MAPK cascade (Mathys *et al.*, 2012).
Because of the dual functions of ROS, controlled generation is of tremendous importance for the outcome of B. cinerea infection. Positive interference of Trichoderma spp. with ROS production, e.g. through an increase in the plant’s ROS-scavenging abilities at future infection sites, can potentially reduce the damaging levels of ROS in leaves, thus limiting B. cinerea infection. In this context, our transcriptome analysis of the ISR-prime phase in the A. thaliana–T. hamatum T382 interaction showed the induction of multiple peroxidases, glutathione-reductases, glutathione-S-transferases and other detoxifying enzymes in A. thaliana leaves (Mathys et al., 2012); comparable results were also obtained in our tomato–T. hamatum T382 ISR-prime study (Yang, 2013), as well as by Perazzolli et al. (2012) for grapevine leaves after leaf treatment with T. harzianum T39. Similarly, in another study, the expression level of a peroxidase-encoding gene was demonstrated to increase in both roots and leaves of A. thaliana after inoculation with T. atroviride (Salas-Marina et al., 2011). This was confirmed by a proteomic study on maize seedlings root inoculated with T. harzianum, which demonstrated increased levels of superoxide dismutase, peroxidase, glutathione-reductase, glutathione-S-transferase and other ROS-scavenging enzymes in leaves (Shoresh and Harman, 2010). Recently, Brotman et al. (2013) also reported the increased expression of genes encoding antioxidant enzymes in roots of both A. thaliana and cucumber after inoculation with T. asperelloides T203. For example, the MDAR gene, encoding monodehydroascorbate reductase, was significantly up-regulated in both plants.

Responses induced by B. cinerea in Trichoderma-triggered plants

The activation of plant signalling pathways and the reprogramming of plant gene expression by Trichoderma spp., as described in the previous section, are responsible for the establishment of a unique physiological situation, called the ‘primed’ state of the plant (Conrath, 2009; Shoresh et al., 2010). This primed condition can also be achieved after the colonization of the roots by other beneficial microbes, infection by a pathogen, treatment with various chemicals or wounding (Conrath, 2009). Primed plants respond more rapidly and/or more strongly through the activation of defence responses when subsequently challenged by microbial pathogens, herbivorous insects or abiotic stresses (Conrath, 2009, 2011). To demonstrate this change in (defence) response on pathogen challenge in primed plants, an examination of the three-player interaction (BCO–plant–pathogen) is necessary. Evidence has been provided for a priming effect induced by Trichoderma spp. against diverse types of pathogens (Brotman et al., 2012; Gallou et al., 2009; Perazzolli et al., 2011; Shoresh et al., 2005; Yedidia et al., 2003), but data are limited and mostly fragmentary. Moreover, the different studies applied different experimental set-ups, making comparison of the data obtained difficult. With respect to B. cinerea, to our knowledge, we were the first to report a transcriptome analysis on such a three-player interaction, in which the gene expression profiles induced by inoculation with B. cinerea in A. thaliana plants, pre-inoculated with T. hamatum T382, were compared with those in B. cinerea only-infected plants (Mathys et al., 2012). We termed this phase the ‘ISR-boost phase’ to distinguish it from the ISR-prime phase before B. cinerea infection. Pre-inoculation with T. hamatum T382 appeared to prime the plant to respond more quickly to B. cinerea infection, as GO analysis revealed a transient activation of the JA biosynthesis process, which was not observed in plants without T. hamatum T382 pre-inoculation. The responses to JA and wounding, both linked to the JA pathway, were also reinforced in the T. hamatum T382-treated plants. Furthermore, genes involved in the biosynthesis of secondary metabolites, such as anthocyanins, flavonoids and galactolipids, were induced, which was not observed in B. cinerea only-infected plants. The former was also confirmed via chemical analysis of anthocyanin content (Mathys et al., 2012). However, a clear moderation of transcriptional changes induced by B. cinerea (including GO terms dealing with defence responses, hormone pathways and camalexin production) was also observed, possibly resulting from the priming effect and subsequent inhibition of B. cinerea proliferation. In addition, an impaired ISR phenotype against B. cinerea was observed in both JA and SA mutants of A. thaliana, but not in Et mutants (Mathys et al., 2012).

More evidence on the involvement of JA- and Et-mediated pathways in Trichoderma spp.-induced ISR against B. cinerea comes from a phenotypic study by Korolev et al. (2008) on a series of A. thaliana mutants with impaired JA/Et-mediated responses. In contrast with their wild-type plants, none of the mutants developed ISR against B. cinerea in response to the treatment of roots with T. harzianum T39 (Korolev et al., 2008). Interestingly, four mutants impaired in SA-mediated signalling were also analysed, but the ability of T. harzianum T39 to induce ISR against B. cinerea was not affected in these plants (Korolev et al., 2008). The results obtained with tomato mutants confirm the role of the main plant hormones in the ISR response against B. cinerea (Martínez-Medina et al., 2013). Root colonization by T. harzianum T78 significantly reduced B. cinerea infection in tomato wild-types, but not in mutants impaired in the biosynthesis of JA or in the accumulation of SA or ABA, thus indicating a role for these plant hormones in the ISR response. Further expression analysis also revealed that the JA-responsive genes PI II, MC and PS, which were already slightly induced in the ISR-prime phase, were induced to much higher levels in the ISR-boost phase, thus indicating the importance of the priming of JA-dependent defence responses in the T. harzianum-induced biocontrol against B. cinerea in tomato (Martínez-Medina et al., 2013). In addition, using tomato mutants, we recently observed the need for a functional JA and phenylpropanoid biosynthesis pathway for the ISR induced by T. hamatum T382 against B. cinerea (Yang et al.,
Bipartite interactions: A small transcriptional study in tomato performed by Tucci et al. (2011) demonstrated that the expression of two JA/Et-responsive genes was induced and maintained at a higher level during B. cinerea infection in Trichoderma-treated versus untreated plants. This priming effect generally correlated with increased pathogen tolerance, although the authors observed that this response was dependent on the plant genotype and Trichoderma strains used. The opposite was true for two SA-responsive PR genes (of PR family 1 and 2), for which the expression in tomato plants infected by B. cinerea was lower in T. harzianum-treated relative to untreated plants. However, Marra et al. (2006) observed enhanced induction of a thaumatin-like protein (PR5b), which is also SA responsive, in a proteomic analysis of the tripartite interaction T. atroviride–bean–B. cinerea.

CONCLUSIONS AND PERSPECTIVES
Considering the potential direct effects of Trichoderma spp. on B. cinerea, as well as their ability to induce systemic resistance in the plant, they are very well suited to complement the current measures for the control of B. cinerea disease. Different mechanisms regarding the direct inhibition of B. cinerea by Trichoderma spp. have been described, often probably acting in combination. In the case of ISR against B. cinerea, the specific pathways involved also seem to vary according to the Trichoderma species under study. Furthermore, it has been shown that the plant genotype also plays an important role in the ISR-inducing ability of a Trichoderma strain (Korolev et al., 2008; Tucci et al., 2011; Yang, 2013). Moreover, the concentration of the Trichoderma spp.

Table 1 Overview of transcriptomic studies performed on bipartite and tripartite plant interactions with Trichoderma spp. and Botrytis cinerea.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Plant tissue</th>
<th>Trichoderma species</th>
<th>Time point(s) of analysis</th>
<th>Remarks</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>Aerial parts</td>
<td>T. harzianum T34</td>
<td>1 dpTi</td>
<td>Microarray</td>
<td>Moran-Diez et al. (2012)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Leaves</td>
<td>T. hamatum T382</td>
<td>2 dpTi</td>
<td>Microarray</td>
<td>Mathys et al. (2012)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Roots</td>
<td>T. asperelloides T203</td>
<td>1 dpTi</td>
<td>Microarray; salt stress applied</td>
<td>Brotman et al. (2013)</td>
</tr>
<tr>
<td>Vitis vinifera</td>
<td>Leaves</td>
<td>T. hamatum T382</td>
<td>2 dpTi</td>
<td>Microarray; very low number of DE genes</td>
<td>Alfano et al. (2007)</td>
</tr>
<tr>
<td>Solanum lycopersicum</td>
<td>Leaves</td>
<td>T. hamatum T382</td>
<td>5 wpTi</td>
<td>Microarray; RNA-seq, interaction with Plasmopara viticola; T39 applied to leaves</td>
<td>Yang (2013)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Aerial parts</td>
<td>–</td>
<td>0, 24, 36, 60 hpBi</td>
<td>Microarray, comparison with mutant plants</td>
<td>AbuQamar et al. (2006)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Leaves</td>
<td>–</td>
<td>18, 48 hpBi</td>
<td>Microarray, comparison with elicitor treatment</td>
<td>Ferrari et al. (2007)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Leaves</td>
<td>–</td>
<td>14 hpBi</td>
<td>Microarray, comparison with mutant plants</td>
<td>Birkenbihl et al. (2012)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Systemic leaves</td>
<td>–</td>
<td>1, 2 dpBi</td>
<td>Microarray</td>
<td>Mathys et al. (2012)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Leaves/leaf discs</td>
<td>–</td>
<td>12, 24, 48 hpBi</td>
<td>Microarray, also spatial analysis of leaf response</td>
<td>Mulema and Denby (2012)</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Detached leaves</td>
<td>–</td>
<td>Every 2 h up to 48 hpBi</td>
<td>High-resolution microarray</td>
<td>Windram et al. (2012)</td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td>Leaves</td>
<td>–</td>
<td>12, 24, 48 hpBi</td>
<td>RNA-seq</td>
<td>De Cremer et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>48, 72, 96 hpBi</td>
<td>(systemic leaves)</td>
<td></td>
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<tr>
<td>Solanum lycopersicum</td>
<td>Detached leaves</td>
<td>–</td>
<td>0, 8 hpBi</td>
<td>Microarray, comparison with mutant plants</td>
<td>Asselbergh et al. (2007)</td>
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<tr>
<td>Solanum lycopersicum</td>
<td>Fruits</td>
<td>–</td>
<td>1 dpBi</td>
<td>Microarray, comparison of ripening stages</td>
<td>Cantu et al. (2009a)</td>
</tr>
<tr>
<td>Solanum lycopersicum</td>
<td>Fruits</td>
<td>–</td>
<td>1 dpBi</td>
<td>Transcriptome analysis of hormone-related genes</td>
<td>Blanco-Ulate et al. (2013)</td>
</tr>
</tbody>
</table>

dpBi, days post-B. cinerea inoculation; dpTi, days post-Trichoderma spp. inoculation; hpBi, days post-B. cinerea inoculation; wpTi, weeks post-Trichoderma spp. inoculation; ISR, induced systemic resistance; DE, differentially expressed.
inoculum, the developmental stage of the plant and the timing of the interaction have also been shown to affect the Trichoderma spp.–plant interaction (Contreras-Cornejo et al., 2011; Hermosa et al., 2012). Above all, the success of Trichoderma spp. as BCOs depends strongly on the challenging pathogen, which was focused in this review on B. cinerea, an important pathogen of current agricultural crops with one of the broadest host spectra.

So far, large-scale transcriptional profiling of the plant response following infection with B. cinerea has mainly been performed in A. thaliana, with a few studies carried out in tomato and recently also in lettuce (Table 1). Similarly, transcriptional profiling of the Trichoderma–plant interaction has only been reported in a few studies, focusing on different Trichoderma strains and plant species (Table 1). Concerning tripartite interactions, however, genome-wide analyses that could drastically advance our understanding of ISR mechanisms remain extremely scarce to date. For B. cinerea, our recent transcriptomic study on the tripartite interaction in the A. thaliana–T. hamatum T382 experimental system is, to our knowledge, the only one published so far (Mathys et al., 2012; Table 1). With the emergence of next-generation sequencing techniques and the increasing amount of available sequenced genomes of all players in the tripartite interactions, however, this is expected to change in the years ahead, which will undoubtedly result in a great leap forward in our understanding of tripartite Trichoderma–B. cinerea biocontrol interactions.

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