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RESEARCH PAPER

MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe

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ABSTRACT

Colonisation of plant roots by selected beneficial *Trichoderma* fungi or *Pseudomonas* bacteria can result in the activation of a systemic defence response that is effective against a broad spectrum of pathogens. In *Arabidopsis thaliana*, induced systemic resistance (ISR) triggered by the rhizobacterial strain *Pseudomonas fluorescens* WCS417r is regulated by a jasmonic acid- and ethylene-dependent defence signalling pathway. Jasmonic acid and ethylene also play a role in *Trichoderma*-induced resistance. To further investigate the similarities between rhizobacteria- and *Trichoderma*-induced resistance, we studied the response of *Arabidopsis* to root colonisation by *Trichoderma asperellum* T34. In many aspects T34-ISR was similar to WCS417r-ISR. First, colonisation of the roots by T34 rendered the leaves more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato*, the biotrophic oomycete *Hyaloperonospora parasitica* and the necrotrophic fungus *Plectosphaerella cucumerina*. Second, treatment of the roots with T34 primed the leaf tissue for enhanced jasmonic acid-responsive gene expression and increased formation of callose-containing papillae upon pathogen attack. Third, T34-ISR was fully expressed in the salicylic acid impaired mutant *sid2*, but blocked in the defence regulatory mutant *npr1*. Finally, we show that the root-specific transcription factor MYB72, which is essential in early signalling steps of WCS417r-ISR, is also required for T34-ISR. Together, these results indicate that the defence pathways triggered by beneficial *Trichoderma* and *Pseudomonas* spp. strains are highly similar and that MYB72 functions as an early node of convergence in the signalling pathways that are induced by these different beneficial microorganisms.

INTRODUCTION

For health and environmental reasons, there is increasing legislative pressure to reduce reliance on pesticides for disease control in agriculture. This has raised the need to study alternatives, such as biological control agents. *Trichoderma* spp. are cosmopolitan soil fungi, widely used to interfere with plant pathogens and pests. It is well established that their effectiveness results from different modes of action, such as competition with other soil micro-organisms for nutrients (Chet 1987), or production of antibiotics and lytic enzymes to parasitise or inhibit other fungi (Harman *et al.* 1981; Schirmböck *et al.* 1994; Lorito *et al.* 1996; Zimand *et al.* 1996; Woo *et al.* 1999). Apart from these direct effects on plant pathogens, recent findings suggest that *Trichoderma* spp. can systemically elevate resistance against different types of pathogens in various plant species (De Meyer *et al.* 1998; Harman *et al.* 2004; Shoresh *et al.* 2005; Djonovic *et al.* 2006, 2007; Korolev *et al.* 2008).

Enhancement of basal resistance levels is a common reaction of plants to biotic and abiotic stresses (Van Loon 2000), and is commonly referred to as induced resistance. The classic example is that of systemic acquired resistance (SAR) (Durrant & Dong 2004) in which initial attack by a pathogen not only triggers local defence responses, but also leads to the generation of a signal that is systemically spread throughout the plant. Upon perception of this signal, the distal plant parts become more resistant to subsequent attack by a broad range of pathogens. SAR depends on the production of, and responsiveness to, salicylic acid (SA) (Delaney *et al.* 1994; Mauch-Mani & Métraux 1998; Nawrath & Métraux 1999). In *Arabidopsis*, pathogen-induced SA is synthesised from isochorismate by the enzyme isochorismate synthase, which is encoded by the *SID2* (*SALICYLIC ACID DEFICIENT2*) gene (Wildermuth *et al.* 2001). Furthermore, SAR is associated with the induction of novel PATHOGENESIS-RELATED (PR) proteins (Van Loon *et al.* 2006) and depends on the transcriptional regulator NONEXPRESSOR OF PR GENES1 (NPR1: Dong 2004).

Phenotypically similar induced systemic resistance (ISR) that is triggered upon root colonisation by specific non-pathogenic rhizobacteria is regulated by a different signal transduction cascade (Pieterse *et al.* 1996). Using *Arabidopsis thaliana* as a model plant species and rhizobacterial strain *Pseudomonas fluorescens* WCS417r as ISR inducer, it was demonstrated that, similar to SAR, ISR is dependent on NPR1, but requires responsiveness to jasmonic acid (JA) and ethylene (ET), rather than SA signalling (Pieterse *et al.* 1998). Even though colonisation of the roots by ISR-inducing bacteria enhances the plant's defensive capacity, no significant changes in defence-related gene expression could be observed in the above-ground plant tissues (Van Wees *et al.* 1999; Verhagen *et al.* 2004; Pozo *et al.* 2008). Yet these tissues responded faster and more strongly to pathogen attack (Van Wees *et al.* 1999; Hase *et al.* 2003; Verhagen *et al.* 2004; Pozo *et al.* 2008; Van der Ent *et al.* 2008), a phenomenon known as priming (Conrath *et al.* 2006). Microarray analysis revealed that the R2R3-MYB-like transcription factor gene *MYB72* is specifically activated in the roots upon colonisation by WCS417r (Verhagen *et al.* 2004; Van der Ent *et al.* 2008). Knockout mutant analysis showed that *myb72* mutants are impaired in WCS417r-ISR against a broad spectrum of pathogens, indicating that MYB72 is required in the roots during early signalling steps of rhizobacteria-mediated ISR (Van der Ent *et al.* 2008).

Trichoderma asperellum isolate T203 has been shown to induce resistance in cucumber to the bacterial leaf pathogen *Pseudomonas syringae* pv. *lachrymans* (Shoresh *et al.* 2005). Upon colonisation of cucumber roots by T203, no differences in host SA or ET production could be observed (Shoresh *et al.* 2005). However, blocking the action of ET or the synthesis of JA with the chemical inhibitors silver thiosulfate and diethyldithiocarbamate, respectively, diminished the enhanced protective effects (Shoresh *et al.* 2005). This suggests a similar signalling role for these hormones in *Trichoderma*- and rhizobacteria-mediated systemic resistance. Further evidence for a role of JA and ET in *Trichoderma*-induced resistance came from a study by Korolev *et al.* (2008), who demonstrated that mutants of *Arabidopsis* with a defect in ET or JA signalling were unable to elevate their systemic resistance levels to *Botrytis cinerea* after colonisation of the roots by *Trichoderma harzianum* Rifai T39.

Trichoderma asperellum isolate T34 is a biocontrol agent that is effective against diseases caused by soil-borne pathogens such as *Fusarium oxysporum* (Cotxarrera *et al.* 2002) and *Rhizoctonia solani* (Trillas *et al.* 2006). In cucumber plants, T34 is also able to induce systemic resistance against *P. syringae* pv. *lachrymans* (Segarra *et al.* 2007). This *T. asperellum* ISR is associated with increased peroxidase and chitinase activity in local and systemic cucumber tissues (Yedidia *et al.* 2003; Segarra *et al.* 2007). Moreover, application of high inoculum densities of T34 spores at the roots resulted in a systemic increase in levels of SA and JA (Segarra *et al.* 2007). Hence, both similarities and differences seem to exist between the signalling pathways controlling rhizobacteria- and *Trichoderma*-induced resistance. However, the results are difficult to compare because they were obtained using different plant species. In order to investigate to what extent the *Trichoderma*-induced defence pathway is similar to that induced by beneficial rhizobacteria, we tested the ability of *T. asperellum* T34 to induce systemic resistance in *Arabidopsis* wild-type plants and various ISR and SAR signalling mutants.

MATERIALS AND METHODS

Plant growth conditions

Seeds of wild-type *Arabidopsis thaliana* accession Col-0 and mutants *myb72-1*, which is impaired in WCS417r-ISR (Van der Ent *et al.* 2008), *npr1-1*, which is impaired in both SAR and ISR (Cao *et al.* 1994; Pieterse *et al.* 1998), and *sid2-1*, which is impaired in SA biosynthesis (Nawrath & Métraux 1999), were sown in quartz sand. Two-week-old seedlings were transferred to 60-mL pots containing a sand–potting soil mixture that had been autoclaved for 20 min at 120 °C twice on consecutive days. Plants were cultivated as described by Pieterse *et al.* (1996).

Cultivation and application of biocontrol agents

Trichoderma asperellum strain T34 (Trillas & Cotxarrera 2002) was grown on 10 $g \cdot l^{-1}$ malt agar plates for 5 days at 22 °C. Conidia were collected in distilled water, resuspended to a density of 5×10^6 colony-forming units (cfu) ml^{-1} and mixed through the soil to a final density of 10^5 $cfu \cdot g^{-1}$, before transplanting the *Arabidopsis* seedlings.

For the induction of ISR, non-pathogenic, rifampicin-resistant *Pseudomonas fluorescens* WCS417r bacteria were cultivated and mixed through the soil as described previously (Pieterse *et al.* 1996).

Pseudomonas syringae pv. *tomato* DC3000 bioassays

Plants were challenged when 5 weeks old by dipping the leaves for 2 s in a solution of 10 mM $MgSO_4$, 0.015% (v/v) Silwet L-77, containing 2.5×10^7 cfu·ml^{-1} *Pseudomonas syringae* pv. *tomato* DC3000 (Whalen *et al.* 1991) bacteria that were cultivated as described (Pieterse *et al.* 1996). One day before challenge inoculation, the plants were placed at 100% relative humidity. Four days after challenge, disease severity was assessed by determining the percentage of diseased leaves per plant (n = 20) as described previously (Pieterse *et al.* 1996). Experiments were repeated with similar results.

Hyaloperonospora parasitica bioassays

Three-week-old plants were misted with a *Hyaloperonospora parasitica* WACO9 spore suspension containing 5×10^4 spores ml^{-1} that was generated as described by Koch & Slusarenko (1990). To stimulate symptom development, plants were further cultivated as described by Van der Ent *et al.* (2008). Disease symptoms were scored 9 days after inoculation on about 250 leaves (from about 40 plants) per treatment. Disease ratings were expressed as severity of disease symptoms and pathogen sporulation on each leaf: I, no sporulation; II, trailing necrosis; III, <50% of the leaf area covered by sporangia; IV, >50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. Quantification of callose deposition was performed as described by Ton *et al.* (2005). Experiments were performed at least twice leading to similar results.

Plectosphaerella cucumerina bioassays

Five-week-old plants (n = 20) were challenge-inoculated with *Plectosphaerella cucumerina* (Palm *et al.* 1995) by applying 6-μl droplets containing 5×10^6 spores ml^{-1} to five fully expanded leaves, as described previously (Ton & Mauch-Mani 2004). Inoculated plants were kept at 100% relative humidity. At 7 days after challenge, disease severity was determined. Disease ratings were expressed as severity of disease symptoms on each leaf: I, no symptoms; II, lesion diameter ≤2 mm; III, lesion diameter ≥2 mm. Experiments were repeated with similar results.

Detection of *Trichoderma* in plant tissue

Sections of 5 *Arabidopsis* stems were taken 2 and 4 weeks after transplanting seedlings into soil containing T34. The pieces were surface-sterilized by soaking them in 96% ethanol for 1 min, then in 70% ethanol for 2 min, and finally 2 min in sterile distilled water, before placing them on *Trichoderma*-specific agar medium as described (Chung & Hoitink 1990), to monitor outgrowth. Rhizosphere colonisation was assessed 4 weeks after transplanting seedlings into soil containing T34. Roots of 5 randomly selected plants were harvested and weighed. The samples were homogenised in 10 mM $MgSO_4$, serially diluted and plated on *Trichoderma* selective medium (Chung & Hoitink 1990).

RNA extraction and Northern blot analysis

RNA extraction and Northern blot analysis was performed as described (Van der Ent *et al.* 2008). The *LOX2* probe was generated through PCR on *A. thaliana* cDNA using gene-specific primers (At3g45140; 5′-GCA TCC TCA TTT CCG CTA CAC CA-3′ and 5′-TCC GCA CTT CAC TCC ACC ATC CT-3′). A gene-specific probe for 18S rRNA was used to check for equal loading.

RESULTS AND DISCUSSION

To determine whether root colonisation by *T. asperellum* T34 (T34) can enhance resistance in *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), the severity of bacterial speck disease inflicted by this pathogen was quantified for control and T34-treated plants. As shown in Fig. 1, plants of which the roots were treated with T34 showed significantly fewer disease symptoms than uninduced control plants. The extent of the T34-mediated reduction in disease severity was larger than that conferred by the WCS417r-ISR. The difference in effectiveness may be caused by local effects if T34 colonised *Arabidopsis* shoots endophytically, or may be truly systemic if T34 remained confined to the roots. To verify that T34 remained confined to the roots, externally

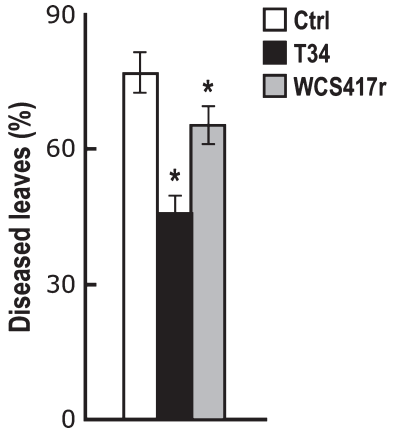

Fig. 1. *Trichoderma* triggers systemic protection against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*. Levels of T34- and WCS417r-induced protection against *Pst* DC3000 in *Arabidopsis* Col-0. Resistance was induced by growing the plants for 3 weeks in soil containing either T34 conidia or ISR-inducing WCS417r bacteria. Five-week-old plants were challenge-inoculated with a bacterial suspension of virulent *Pst* DC3000. Four days after challenge inoculation, the percentage of diseased leaves was assessed. Asterisks indicate statistically significant differences compared to non-induced control plants (Student's *t*-test, $\alpha < 0.05$, n = 20). The experiment was repeated with similar results.

sterilized sections of *Arabidopsis* stems were harvested and placed on *Trichoderma*-specific agar medium. None of the stem sections gave rise to outgrowth of *Trichoderma* mycelium. However, when root tissue with adhering rhizosphere of 5-week-old T34-treated plants was plated on the *Trichoderma*-specific medium, massive outgrowth of mycelium was detected ($16.5 \pm 7.6 \times 10^5$ cfu·g^{-1} root). These results demonstrate that T34 colonised the *Arabidopsis* rhizosphere and/or root tissue, but did not spread into the aboveground parts. It can thus be concluded that T34 induced a plant-mediated systemic resistance response in *Arabidopsis* that was effective against *Pst* DC3000.

WCS417r-ISR is effective against a broad range of pathogens, including obligate biotrophs and necrotrophic fungi (Ton *et al.* 2002). To investigate whether T34-ISR is similarly effective against these types of pathogens, the level of T34-induced protection against the biotrophic oomycete *Hyaloperonospora parasitica* and the necrotrophic fungus *Plectosphaerella cucumerina* was assessed. Fig. 2A shows that T34-treated plants developed less *H. parasitica*-inflicted downy mildew symptoms compared to the uninduced control plants. Similarly, root colonisation by T34 resulted in a significant reduction in disease symptoms when *Plectosphaerella cucumerina* was used as the challenging pathogen (Fig. 2B). Together, these results demonstrate that colonisation of *Arabidopsis* roots by T34 triggers a systemic resistance response that is effective against different types of foliar pathogens.

In contrast to pathogen-induced SAR, WCS417r-ISR is not associated with major transcriptional reprogramming in distal plant parts, but rather with priming for enhanced JA-responsive gene expression upon subsequent pathogen attack (Van Wees *et al.* 1999; Verhagen *et al.* 2004; Pozo *et al.* 2008). Notably, expression of the JA-responsive gene *LOX2* (*LIPOXYGENASE2*) serves as a molecular marker for WCS417r-induced priming for defence in ISR (Conrath *et al.* 2006; Pozo *et al.* 2008). Similarly, treatment of *Arabidopsis* roots with T34 was not associated with direct transcriptional activation of SA- and JA-regulated genes in distal plant parts (data not shown), but did result in augmented *LOX2* gene expression (Fig. 3A). In addition, WCS417r-ISR is accompanied by priming for enhanced formation of callose-containing papillae at sites of attempted penetration by *H. parasitica* (Van der Ent *et al.* 2008). To investigate whether T34-ISR in *Arabidopsis* is also associated with priming for

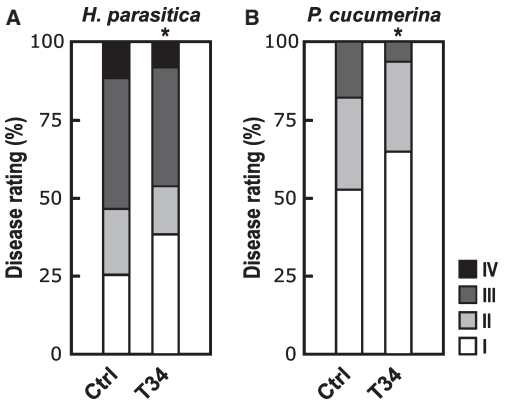

Fig. 2. *Trichoderma*-induced systemic resistance has a broad range of effectiveness. A: Quantification of T34-ISR in *Arabidopsis* Col-0 against *Hyaloperonospora parasitica*. Resistance was induced by growing the plants in soil containing T34 conidia. Plants were challenge-inoculated with *H. parasitica* when 3 weeks old. Disease severity was determined 9 days after challenge. Disease ratings are expressed as the percentage of leaves (n = ~250) in disease-severity classes: I, no sporulation; II, trailing necrosis; III, <50% of the leaf area covered with sporangia; IV, >50% of the leaf area covered with sporangia, with additional chlorosis and leaf collapse. B: Quantification of T34-ISR against *P. cucumerina*. Plants were inoculated with *P. cucumerina* when 5 weeks old. At 7 days after challenge, disease severity was determined. Disease ratings were expressed as severity of disease symptoms on each leaf: I, no symptom; II, lesion diameter ≤2 mm; III, lesion diameter ≥2 mm. Asterisks indicate statistically significantly different distributions of the disease severity classes compared with the non-induced control treatments (Chi-square, $\alpha < 0.05$, n = 120). Experiments were repeated with similar results.

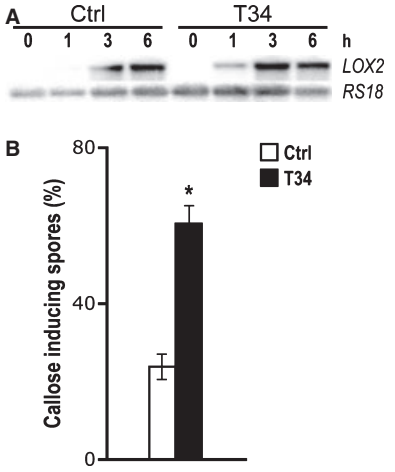

Fig. 3. *Trichoderma* primes for enhanced defence. A: *LOX2* expression in 5-week-old control and T34-treated plants at different times after treatment with 100 μM MeJA. Equal loading of RNA samples was checked using a probe for the constitutively expressed 18S rRNA (*RS18*). B: Induced resistance against *Hyaloperonospora parasitica* is associated with enhanced deposition of callose-containing papillae at sites of attempted penetration, resulting in a reduction in the number of spores that lead to successful penetration into *Arabidopsis* leaves. Two days after challenge with *H. parasitica*, leaves of plants in which the roots were pre-treated with water (Ctrl) or T34 were stained with calcofluor/aniline blue and inspected by epifluorescence microscopy (UV) to assess the percentage of germinating spores that induced callose deposition in the epidermal cell layer. The asterisk indicates a statistically significantly different distribution of callose-inducing compared to non-callose-inducing *H. parasitica* spores between the two treatments (chi-square, $\alpha < 0.05$, n = 250). The experiment was repeated with similar results.

enhanced callose deposition, we assessed the percentage of germinating *H. parasitica* spores being blocked by the formation of callose-containing papillae. Figure 3B shows that colonisation of the roots by T34 indeed primes the aboveground plant tissue for enhanced deposition of callose at the sites of attempted pathogen entry. Together, these results indicated that, like WCS417r-ISR, T34-ISR is associated with priming for enhanced systemic defence responses, confirming previous findings in cucumber plants (Zhang *et al.* 1998; Shoresh *et al.* 2005).

Since T34-ISR displays hallmarks of WCS417r-ISR, such as broad-spectrum effectiveness and priming, we investigated whether T34-ISR is regulated similarly to WCS417r-ISR. To test this, we assessed T34-induced resistance in *myb72-1*, a representative mutant of the ISR signalling pathway (Van der Ent *et al.* 2008). In contrast to other ISR mutants, *myb72-1* is not disrupted in a component required for general ET or JA signalling, but specifically affected in early steps of ISR signalling (Pieterse *et al.* 2002; Van der Ent *et al.* 2008). As controls, we included the SA biosynthesis mutants, *sid2-1* and *npr1-1*, which are disrupted in SAR and both SAR and ISR, respectively. Fig. 4 shows that *sid2-1* developed a similar level of resistance against *Pst* DC3000 upon colonisation of the roots by T34, indicating that, like WCS417r-ISR, T34-ISR functions independently of SA. Furthermore, the WCS417r-ISR minus mutant *npr1-1* was blocked in its ability to mount T34-ISR, indicating that the regulatory protein NPR1 is required for expression of this type of *Trichoderma*-induced resistance. Recently, we demonstrated that the root-specific transcription factor MYB72 plays an important role in early signalling steps of WCS417r-ISR (Van der Ent *et al.* 2008). Fig. 4 shows that MYB72 is also required for T34-ISR. Since MYB72 is a root-specific transcription factor (Van der Ent *et al.* 2008), we postulate that it is required in early signalling steps that eventually lead to systemic resistance in aboveground plant parts. Since MYB72 is required for both WCS417r-ISR and T34-ISR, we conclude that MYB72 functions as an early signalling node that is required for the expression of ISR triggered by different beneficial soil microorganisms.

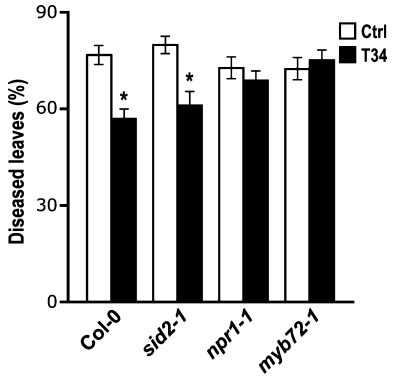

Fig. 4. *Trichoderma*-induced systemic resistance functions independently of SA, but requires NPR1 and MYB72. Levels of T34-induced protection against *Pst* DC3000 in wild-type Col-0 and *sid2-1*, *npr1-1* and *myb72-1* mutant *Arabidopsis* plants. Induction of resistance, pathogen challenge and disease assessment were performed as described in the legend to Fig. 1. Asterisks indicate statistically significant differences compared to non-induced control plants (Student's *t*-test, $\alpha < 0.05$, n = 20). The experiment was repeated with similar results.

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