Multitrophic interactions in soil
IOBC/wprs Bulletin Vol. 29 (2) 2006
pp. 157-161

Transcription factors in roots and shoots of *Arabidopsis* involved in rhizobacteria-induced systemic resistance

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Abstract: Plants possess inducible resistance mechanisms through which they can regulate their defense response to pathogen attack. Colonization of *Arabidopsis thaliana* roots by non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria triggers a jasmonate- and ethylene-dependent induced systemic resistance (ISR) that is effective against a broad range of foliar pathogens. In the roots, the transcriptional activity of a large number of genes is altered upon colonization by WCS417r. To investigate the role of WCS417r-responsive, root specific genes in ISR signaling, we screened T-DNA insertion lines of a subset of these genes. Bioassays revealed that *AtMYB72*, a transcription factor gene specifically induced in the roots upon colonization by WCS417r, is essential for activation of ISR. The *myb72* knockout mutant was incapable of mounting WCS417r-mediated ISR against the challenging pathogens *Pseudomonas syringae* pv. *tomato* DC3000 and *Hyaloperonospora parasitica*. Analysis of *AtMYB72* gene expression revealed that ethylene is an important regulator of *AtMYB72*. This was supported by the finding that AtMYB72 was found to physically interact with the ethylene-regulatory protein EIL3 in a yeast two-hybrid assay. Transcript profiling revealed that ISR-expressing leaves are primed for augmented expression of predominantly jasmonate- and ethylene-responsive genes. Promoter analysis of these primed genes showed overrepresentation of an *AtMYC2* binding motif, suggesting a regulatory role for this transcription factor in ISR. Further evidence for the involvement of *AtMYC2* in ISR arose from bioassays showing that *AtMYC2* knockout mutants were not able to show ISR after root colonization by WCS417r, while their level of basal resistance was comparable to that of wild-type Col-0.

Key words: *Arabidopsis thaliana*, *Pseudomonas fluorescens*, induced systemic resistance, transcription factor, *AtMYB72*, *AtMYC2*

Introduction

The direct surrounding of plant roots, the rhizosphere, is a very nutrient-rich habitat that provides a niche to numerous micro-organisms. Next to pathogens, also many fungi and bacteria with properties beneficial for the plant are present. *Mycorrhiza* and *Rhizobia* are well known examples of organisms that stimulate plant growth, either by enlarging the surface for nutrient uptake, or by enhancing the availability of nitrogen, respectively. Other beneficial micro-organisms help the plants in their defense, either by hampering growth and development of pathogens due to e.g. competition for nutrients or the secretion of antibiotics, or by stimuling the resistance mechanism of the plants themselves (Van Loon et al., 1998). Selective, non-pathogenic, species of *Pseudomonas* are a well known example of a group of organisms that lead to this state of induced systemic resistance (ISR) of the plant after colonization of the roots.

Research on mutants of *Arabidopsis thaliana* showed that responsiveness to the plant hormones ethylene (ET) and jasmonic acid (JA), and a functional NPR1 protein (non-expressor of PR-proteins) are essential for ISR (Pieterse et al., 2002). To identify genes that

are associated with ISR, transcriptome analysis of both root and foliar tissues of plants of which the roots were colonized by *Pseudomonas fluorescens* WCS417r was performed using Affymetrix GeneChips and compared to tissues of control-treated plants (Verhagen et al., 2004). This approach led to the identification of a MYB transcription factor in the roots and a MYC transcription factor in the systemic tissue that both seem to play a key role in the transduction of the ISR signal that is induced upon root colonization.

Material and methods

Cultivation of plants and induction of ISR

Cultivation of *Arabidopsis thaliana* accession Col-0, the T-DNA-insertion knockout *myb72* and the *AtMYC2* mutant *jin1-2* (Berger et al., 1996), and induction of ISR by non-pathogenic *Pseudomonas fluorescens* WCS417r bacteria was performed as described by Pieterse et al. (1996).

Pathogen bioassays

Bioassays in which the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was used for challenge inoculation, were performed as described by Pieterse et al. (1998). For *Hyaloperonospora parasitica* bioassays 3-week-old Arabidopsis Col-0 and *myb72* plants were misted with a conidiospore suspension of *H. parasitica* WACO9 containing 7.5 x 10^4 condiospores per ml. Inoculated plants were maintained at 17°C and 100% relative humidity for 24 hours. Subsequently, humidity was lowered to 70% to reduce direct effects on plant development and to reduce the chance of secondary infections by opportunistic pathogens. Seven days after challenge inoculation humidity once again was raised to 100% to enable *H. parasitica* to form sporangiophores. Disease symptoms were scored 9 days after inoculation. Quantification of callose deposition was performed following the method described by Ton et al. (2005).

Yeast two-hybrid screen

Constructs for yeast two-hybrid analysis were generated using vectors pDEST^{TM}32 and pDEST^{TM}22 (Invitrogen). Full-length cDNA inserts of *AtMYB72* and *AtEIL3* were introduced using GATEWAY^{TM} technology (Invitrogen), following manufacturers instructions. The yeast two-hybrid screen was performed essentially as described by James et al. (1996).

Results and discussion

AtMYB72, a WCS417- responsive, root-specific gene required for ISR

Analysis of changes in the transcriptome of Arabidopsis roots in response to colonization by ISR-inducing WCS417r bacteria revealed 97 genes that showed a locally altered expression in the roots (Verhagen et al., 2004). To investigate the possible involvement of these genes in ISR signaling, we started to systematically analyze knockout mutants of these genes for their ability to express WCS417r-mediated ISR. Out of 11 knockout mutants tested, one knockout mutant with a T-DNA insertion in the *AtMYB72* gene, which is significantly up-regulated in the roots upon colonization by WCS417r, was identified as unable to mount ISR against *Pst* DC3000 in response to colonization of the roots by WCS417r.

To investigate whether *AtMYB72* is involved in ISR against other pathogens as well, bioassays with the oomycete *H. parasitica* were performed. After germination from spores, this pathogen forms an apressorium from which a penetration hyphae invades the leaf tissue in between epidermal cells. Successful invasion allows *H. parasitica* to colonize the leaf tissue and eventually form sporangiophores that appear from the leaf surface. Table 1 shows that WCS417r-mediated ISR against *H. parasitica* significantly reduced disease symptoms in wild-type Col-0 plants. However, in *myb72* mutant plants, ISR against *H. parasitica* was

completely abolished. Induced resistance against *H. parasitica* has been shown to be associated with priming for enhanced callose deposition at the site of penetration (Kohler et al., 2002; Ton et al., 2005). ISR-expressing Col-0 plants also show an enhanced induction of callose formation. However, in mutant *myb72* plants, this enhanced callose formation could not be detected. Together these results indicate that AtMYB72 is a component of the ISR signaling pathway that is essential for the induction of ISR against different pathogens.

Table 1. WCS417r-mediated ISR against *H. parasitica* is abolished in *myb72*.

	Increasing disease severity class	*Relative disease rating*[1] **Ctrl**	**WCS417r**
Col-0	1	60.5	70.1
	2	10.8	6.0
	3	23.3	22.2
	4	5.5	1.7
myb72	1	53.6	52.9
	2	7.8	11.3
	3	27.5	27.4
	4	11.0	8.4

[1] The distribution over the disease severity classes was significantly different from the control in Col-0, but not in *myb72* (Chi-square, $\alpha = 0{,}05$).

AtMYB72 expression is regulated by ET

Using the ET-insensitive mutant *ein2-1* (Guzmán and Ecker, 1990), Knoester et al. (1999) demonstrated that the induction of WCS417r-mediated ISR requires ET-signaling at the site of application of the inducer. To investigate whether WCS417r-induced expression of *AtMYB72* in the roots is also regulated by ET, transcript accumulation of this gene was studied in WCS417r-treated *ein2-1* plants. The absence of induced *AtMYB72* mRNA levels indicated that ET indeed has a regulatory function in *AtMYB72* induction. Further evidence for the involvement of this hormone in *AtMYB72* expression came from the observation that *AtMYB72* transcript levels accumulate after treatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

AtMYB72 interacts with EIL3 in vitro

The yeast two-hybrid system is a frequently used molecular genetic tool to study *in vitro* protein-protein interactions. The set-up of this system enables yeast to grow on selective medium only if reporter-genes are activated by the transcription regulation protein GAL4. To do this, the DNA-binding domain (BD) and the activation domain (AD) of GAL4 are needed in close proximity of each other. Fusing two proteins of interest each to a different domain of GAL4 will lead to expression of downstream reporter genes only if the two domains are brought together due to interaction of the two proteins to which they are fused. Using this technique AtMYB72 was found to physically interact with the EIN3-like protein EIL3. EIN3 and its paralogs, the EIN3-like proteins EIL1, EIL2 and EIL3, are nuclear transcription factors that bind to the promoters of ET-responsive genes, such as *ERF1*, and initiate a transcriptional cascade leading to the regulation of ET target genes (Solano et al., 1998). These results again suggest a role of the ET signaling pathway in the regulation of AtMYB72 function.

Priming of JA-dependent responses during ISR is regulated by AtMYC2

Transcript profiling of ISR-expressing *Arabidopsis* leaves revealed that the onset of ISR is not associated with detectable changes in gene expression. However, upon pathogen attack, a large set of predominantly JA/ET-regulated genes showed a potentiated expression pattern. Evidently, ISR-expressing plants are primed for augmented expression of pathogen-inducible genes, which might allow the plant to react more effectively to a broad spectrum of pathogens (Verhagen et al., 2004). To further elucidate this WCS417r-induced priming-phenomenon the promoter regions of genes that showed an augmented expression in response to treatment with methyl jasmonate (MeJA) were scanned for the presence of common binding sites of regulating transcription factors. This analysis led to the finding that binding motifs for AtMYC2, a transcription factor protein known to be responsive to JA (Lorenzo et al., 2004) were overrepresented, suggesting that AtMYC2 plays an important role in ISR signaling.

To further investigate the role of AtMYC2 in ISR, a mutant of AtMYC2, *jin1-2* (Berger et al., 1996) was tested for its ability to show WCS417r-mediated ISR against *Pst* DC3000. While WCS417r-treated Col-0 plants showed a significant reduction in the percentage of leaves with symptoms, *jin1-2* did not, indicating that *jin1-2* is impaired in its ability to express ISR. Hence, AtMYC2 is of crucial importance for ISR. Detailed analysis of *AtMYC2* expression in ISR-expressing tissues revealed that *AtMYC2* mRNA levels are significantly higher in systemic tissues of WCS417r-induced plants, suggesting that enhanced levels of the AtMYC2 transcription factor are responsible for the augmented expression of JA-responsive genes as observed in ISR-expressing plants.

Acknowledgements

This project was (co)financed by the Centre for BioSystems Genomics (CBSG), which is part of the Netherlands Genomics Initiative/Netherlands Organisation for Scientific Research, grant 865.04.002 of the Earth and Life Sciences Foundation (ALW), which is subsidized by the Netherlands Organization of Scientific Research (NWO), and grant QLK5-CT-2001-52136 of the Marie Curie Individual Fellowship programme of the European Commission.

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IOBC / WPRS

Working group
"Multitrophic Interactions in Soil and Integrated Control"

Proceedings of the meeting

at

Wageningen (The Netherlands)

5 - 8 June, 2005

Edited by:
Jos M. Raaijmakers & Richard A. Sikora

IOBC wprs Bulletin
Bulletin OILB srop **Vol. 29 (2), 2006**

WPRS / SROP

Multitrophic Interactions in Soil and Integrated Control

editors:

Jos M. Raaijmakers & Richard A. Sikora

IOBC wprs Bulletin
Bulletin OILB srop

Vol. 29(2)2006

International Organization for Biological and Integrated Control of Noxious Animals and Plants
Organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles
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