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Knocking down expression of the auxin-amidohydrolase *IAR3* alters defense responses in Solanaceae family plants

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ABSTRACT

In plants, indole-3-acetic acid (IAA) amido hydrolases (AHs) participate in auxin homeostasis by releasing free IAA from IAA-amino acid conjugates. We investigated the role of IAR3, a member of the IAA amido hydrolase family, in the response of Solanaceous plants challenged by biotrophic and hemibiotrophic pathogens. By means of genome inspection and phylogenic analysis we firstly identified IAA-AH sequences and putative IAR3 orthologs in *Nicotiana benthamiana*, tomato and potato. We evaluated the involvement of *IAR3* genes in defense responses by using virus-induced gene silencing. We observed that *N. benthamiana* and tomato plants with knocked-down expression of *IAR3* genes contained lower levels of free IAA and presented altered responses to pathogen attack, including enhanced basal defenses and higher tolerance to infection in susceptible plants. We showed that *IAR3* genes are consistently up-regulated in *N. benthamiana* and tomato upon inoculation with *Phytophthora infestans* and *Cladosporium fulvum* respectively. However, *IAR3* expression decreased significantly when hypersensitive response was triggered in transgenic tomato plants coexpressing the *Cf-4* resistance gene and the avirulence factor *Avr4*. Altogether, our results indicate that changes in *IAR3* expression lead to alteration in auxin homeostasis that ultimately affects plant defense responses.

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1. Introduction

The plant hormone indole-3-acetic acid (IAA) is the most common natural auxin. IAA plays essential roles throughout the plant life cycle participating in growth, development and responses against environmental stresses. Plants coordinate IAA homeostasis by regulating its biosynthesis, transport, oxidation, degradation and conjugation [1,2]. Auxin conjugates play important roles as storage forms for the active IAA. Auxin conjugates can be divided in low molecular weight conjugates (with sugars or aminoacid moieties, either via ester or amide bonds respectively) and high molecular weight conjugates, with peptides and proteins via amide bond. In particular, the formation of IAA-amido-conjugates through the conjugation of IAA with amino acids results in the hormone inactivation and also creates a reservoir of IAA that can be rapidly made available by IAA amido-hydrolase (IAA-AH) enzymes [3]. In Arabidopsis, seven genes encoding IAA-AHs have been identified [4]. Gene sequences for IAA-AHs have been also identified in other plant species, including *Triticum aestivum*, *Brassica rapa* and *Medicago truncatula* [5–7]. However, the role of the amido-hydrolase enzymes in the physiology of the plant has not been well determined yet. IAA amido-hydrolases could be promiscuous enzymes and their respective conjugated IAA substrates could have additional functions associated with plant defense mechanisms [7].

The ability of many plant pathogenic microorganisms to produce IAA during infection also points to the involvement of this hormone in plant disease development [8,9]. In particular, disruption of IAA-conjugate metabolism, leading to IAA-Asp accumulation, has been reported as part of a strategy to promote disease by the necrotroph *Botrytis cinerea* and the hemibiotroph *Pseudomonas syringae* infecting Arabidopsis *thaliana* [10]. Previously, we identified a potato transcript coding for a protein that is 67%

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identical to *At*IAR3 which was also up-regulated in potato tubers upon infection with *Fusarium solani* f. sp. *eumartii* [11].

In this work, we studied the involvement of *IAR3* gene in defense response in two plant-pathogen interactions; *Nicotiana benthamiana* inoculated with an avirulent strain of *Phytophthora infestans* (an incompatible interaction) and tomato (*Solanum lycopersicum*) inoculated with a virulent strain of *Cladosporium fulvum* (a compatible model). By means of virus induced gene silencing experiments and gene expression analysis we conclude that modifications in *IAR3* genes expression promote changes in auxin homeostasis that affect pathogen proliferation in plant tissues.

2. Material and methods

2.1. Plant material and growth conditions

Nicotiana benthamiana, Solanum lycopersicum "Money Maker"-Cf4 (MM-Cf4) and "Money Maker"-Cf0 (MM-Cf0) plants were grown on organic substrate and vermiculite (8:1) and placed in the greenhouse with the following settings: 25 °C with 60% relative humidity under 120 μmol photons/m^2 s^{-1} with 16:8 h light:dark cycles.

Tomato plants offspring expressing the resistance *Cf-4* (=*Hcr9-4D*) gene and the avirulence gene *Avr4* from *Cladosporium fulvum* were generated by crossing transgenic MM-Cf0 plants expressing *Avr4* ("Money Maker"-*Cf0*:*Avr4*) to transgenic "Money Maker"-Cf0:*Hcr9-4D* ("Money Maker"-Cf4) plants as described earlier [12,13]. Seeds of the obtained *Cf-4*/*Avr4* and parental lines were germinated and kept for 7 days under normal daylight conditions at room temperature as described by [14]. At day 8 after germination seedlings were incubated at 33 °C and 100% relative humidity (RH) under a 16:8 h light:dark regime for an additional 14 days. In order to promote the initiation of the response induced by Cf-4/Avr4 interaction, seedlings were shifted to 20 °C/70% RH [15].

2.2. Generation of microbial inoculums

Phytophthora infestans (Mont.) de Bary (race R2 R3 R6 R7 R9, mating type A2) was isolated from infected leaflets of potato and routinely grown on potato slices for one week at 18 °C and 90% RH in the dark [16]. Sporulation was induced by incubation of mycelium over night in water at 4 °C. Sporangiospores were isolated by filtration through a 10-μm nylon mesh. Spores were counted under an optic microscope (Nikon Eclipse E200) by using a Neubauer chamber and inoculations were performed with a suspension of 2×10^4 spores/ml. *C. fulvum* race 5, that express *Avr4* and contains a *pGPD:GUS* transgene expressing the *GUS* gene under control of the constitutive glyceraldehyde-3-P dehydrogenase promoter (pGPD) was cultured on potato dextrose agar at room temperature in the dark. After 10 days, spores were isolated and tomato plants were inoculated at the lower side of the leaves, as described by [17].

2.3. Silencing of N. benthamiana and tomato plants

Construction of silencing vectors was performed as described (Liu et al., 2002a; Liu *et al.*, 2002b). Tobacco rattle virus (TRV)-based binary vectors pTRV1 and pTRV2 were obtained from Dr. S. P. Dinesh-Kumar (UC Davis, USA). For pTRV2:*IAR31* construction, a 339 bp fragment corresponding to *Solanum tuberosum IAR31* from position 316 to 654 with respect to the ATG initiation codon (see table S1 for primer sequences) was subcloned, digested with *BamHI*/*XhoI* and ligated into *BamHI*/*XhoI*-digested pTRV2. The resulting pTRV2:*IAR31* silencing vector was transformed to *Agrobacterium tumefaciens* strain GV3101 by electroporation [18]. A non-functional 396 bp fragment of the β-*glucuronidase* gene (*GUS*) of *E. coli*, with no significant homology with any gene in Solanaceous plants, was cloned into pTRV2 (pTRV2:*GUS*) and used as a control [19]. Constructions pTRV:*Cf-4* and pTRV:*PDS* have been previously described [20,21]. pTRV2:*PDS* was used as a control in VIGS assays; *phytoene desaturase* (*PDS*) silencing caused by pTRV2:PDS leads to the inhibition of carotenoid synthesis and a photo-bleached phenotype [22]. Equal aliquots of *A. tumefaciens* containing pTRV1 and pTRV2:*GUS*, pTRV2:*IAR31*, pTRV2:*PDS* or pTRV2:*Cf-4* were mixed to OD_{600} = 1.0 and used to infiltrate the abaxial side of the leaves of 3-week-old *N. benthamiana* plants or alternatively, 10-day-old tomato seedlings. Five plants were infiltrated with the mixed cultures containing pTRV1 and either pTRV2:*IAR3*, pTRV2:*GUS* or pTRV2:*PDS* in each experiment. Targeting of *IAR3* in MM-Cf4 and MM-Cf0 tomato plants was performed similarly using the same pTRV constructs. Additionally, MM-Cf4 tomato plants were inoculated with pTRV:*Cf-4* as control.

2.4. P. infestans inoculations and growth quantification

Three-week-old *N. benthamiana* plants were inoculated with *P. infestans* by infiltration of 100 μl of a 10 mM $MgCl_2$ solution containing 2×10^4 spores/ml on the abaxial and right sides of detached leaves. Control plants were mock-inoculated with 100 μl of 10 mM $MgCl_2$ solution on the left side of the leaf. VIGS-silenced plants were inoculated 21 days after infiltration with the recombinant virus targeting the gene to be silenced. Detached leaves were placed in Petri dishes with 16:8 h light:dark cycles at 18 °C under 120 μmol photons/m^2 s^{-1}. Hypersensitive response (HR) development was scored daily. Leaf disks of 1.5 cm diameter were dissected from the area surrounding the inoculated site at 0, 24 and 72 h post inoculation (hpi) and used for determination of viable *P. infestans* or frozen in liquid nitrogen and stored at −80 °C for RNA or protein extraction.

Determination of viable pathogen on inoculated leaves was assessed by a growth assay in fresh media plates. Leaf disks dissected from the inoculated area were incubated on potato dextrose agar by 48 h in darkness at 18 °C. Mycelium growth was photographed and an index of the oomycete growth was calculated by using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij).

2.5. C. fulvum inoculations and GUS measurements in MM-Cf0 and MM-Cf4 tomato plants

C. fulvum conidial suspensions (OD_{600} = 0.6) were used to inoculate MM-Cf0 and MM-Cf4 tomato plants by spraying 3 weeks after inoculation *IAR3* silencing. Thirteen days later, 2 to 4 leaflets of putatively silenced leaves (as determined by the observation of bleached phenotype occurrence in PDS silencing experiments) from at least 5 plants were harvested and vacuum-infiltrated with X-gluc buffer (0.1 M NaPO4, pH 7.0, 1% Triton X-100, 1% DMSO, 10 mM EDTA and 1 mg/ mL X-gluc). Leaflets were incubated overnight at 37 °C in the darkness, distained with 70% ethanol and photographed. Blue staining was quantified using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij). Leaflets were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C at the indicated times for RNA extraction.

2.6. Plant phenotypic analysis

Phenotypes of pTRV2:*IAR31*- and pTRV2:*GUS*-infiltrated *N. benthamiana* plants were analyzed 3 weeks post viral inoculation. Relative values of leaf area and plant height were calculated using ImageJ software (National Institutes of Health; http://rsb.info.nih.gov/ij).

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