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The enhanced disease susceptibility phenotype of ethylene-insensitive tobacco cannot be counteracted by inducing resistance or application of bacterial antagonists

Bart P.J. Geraats[a,1], Peter A.H.M. Bakker[a,*], Huub J.M. Linthorst[b], Jan Hoekstra[b], L.C. van Loon[a]

[a]Plant-Microbe Interactions, Institute of Environmental Biology, Faculty of Science, Utrecht University, P.O. Box 800.84, 3508 TB, Utrecht, The Netherlands
[b]bInstitute of Biology, Clusius Laboratory, Leiden University, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

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Abstract

In an attempt to overcome the enhanced disease susceptibility phenotype that is typical for transgenic ethylene-insensitive tobacco (Tetr), Tetr plants were treated with chemical agents that induce resistance or with antagonistic rhizobacteria. Treatments with β-aminobutyric acid (BABA), benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), methyl jasmonate (MeJA), or salicylic acid (SA) induced *PR*-genes generally to a lesser extent than in non-transformed plants and did not reduce wilting symptoms upon infection with *Pythium* sp., except for a marginal effect of SA. In Tetr lines overexpressing *PR-1g*, *PR-5c*, or both, no significant reduction in disease development was apparent. Also treatment of Tetr plants with the antagonistic rhizobacteria *Bacillus cereus* UW85, *Pseudomonas aeruginosa* 7NSK2, *Pseudomonas fluorescens* WCS417r or Q8r-196, *Pseudomonas putida* WCS358r, or antibiotic-producing derivatives of WCS358r, did not reduce symptoms caused by *Pythium*.
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1. Introduction

The role of ethylene signalling in plant disease resistance appears to be diverse. Enhanced ethylene levels can either increase or reduce disease severity, depending on the plant and the pathogen [1–3]. In order to study the role of ethylene signalling in pathogenic interactions, transgenic tobacco (Tetr) plants with impaired ethylene perception were created [4]. These Tetr plants lack non-host resistance to *Pythium sylvaticum* and are hypersusceptible to various necrotrophic pathogens [5,6]. In contrast to non-transformed plants, Tetr plants that were grown in non-autoclaved soil developed symptoms of wilting and stem rot due to infection by soil-borne microorganisms. Inoculation studies revealed that Tetr plants were less resistant to *Botrytis cinerea*, *Cercospora nicotianae*, *Erwinia carotovora*, *Fusarium oxysporum*, *Fusarium solani*, *Pythium* spp., and *Thielaviopsis basicola* [6]. Resistance to the biotrophic pathogens *Oidium neolycopersici* and *Peronospora tabacina* was not reduced. This indicates that resistance to some pathogens is impaired in ethylene-insensitive Tetr tobacco, whereas defence against other pathogens is still fully functional.

In *Arabidopsis*, resistance to biotrophic and viral pathogens has been demonstrated to depend on salicylic acid (SA) signalling [7–9], whereas resistance to several necrotrophic pathogens requires jasmonic acid (JA) and

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; BABA, β-aminobutyric acid; BTH, benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester; DAPG, 2,4-diacetylphloroglucinol; MeJA, methyl jasmonate; PCA, phenazine-1-carboxylic acid; SA, salicylic acid; Tetr, Tobacco transformed with mutant *AtEtr1-1*

*Corresponding author. Tel.: +31 302536861; fax: +31 302518366.
E-mail address: p.a.h.m.bakker@uu.nl (P.A.H.M. Bakker).
[1]Present address: Nunhems BV, P.O. Box 4005, 6080 AA Haelen, The Netherlands

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ethylene signalling [10–12]. Moreover, treatment of *Arabidopsis* with either SA, methyl jasmonate (MeJA), or ethylene, increased resistance to selected pathogens. Because SA and JA could also contribute to the protection of Tetr tobacco plants against soil-borne pathogens through the activation of defence pathways that do not depend on ethylene, the possibility was considered to overcome the enhanced disease susceptibility phenotype by inducing resistance through application of these chemicals.

Systemic acquired resistance (SAR) is a SA-dependent mechanism that is triggered upon infection by necrotizing pathogens and enhances the plants defensive capacity against a broad spectrum of pathogens [13]. The induction of SAR is associated with the accumulation of pathogenesis-related (PR) proteins [14]. In tobacco, exogenous treatment with SA induced the expression of *PR* genes [15], as well as resistance to tobacco mosaic virus (TMV) [16,17], the fungus *B. cinerea* [18], the bacterium *E. carotovora* [19], and the oomycete *Pythium* sp. [20]. In addition, treatments with the SA-analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) or with β-aminobutyric acid (BABA) also triggered *PR*-gene expression and SAR to TMV in tobacco [21]. In contrast, culture filtrates of *E. carotovora* induced resistance to this pathogenic bacterium in a SA-independent manner [22]. Apparently, in tobacco resistance to *E. carotovora* can be induced through SA-dependent, as well as SA-independent defence signalling pathways.

Selected non-pathogenic, root-colonizing bacteria can also induce disease resistance through either SA-dependent or -independent pathways. In tobacco, the rhizobacterium *Pseudomonas aeruginosa* strain 7NSK2 was demonstrated to induce resistance to TMV in a SA-dependent fashion [23], whereas induced resistance against *Pseudomonas syringae* pv. *tabaci* by *Serratia marcescens* operated through a SA-independent pathway [24]. In *Arabidopsis*, root colonization by *Pseudomonas fluorescens* strain WCS417r or *Pseudomonas putida* strain WCS358r induced resistance to *P. syringae* pv. *tomato* independently of SA accumulation, but dependent on JA and ethylene signalling [25,26].

Besides inducing systemic resistance, rhizobacteria can act antagonistically against pathogens by competing for iron through the production of siderophores or by secreting antibiotics [27,28], thereby protecting plants against soil-borne diseases. Selected strains of rhizobacteria have biocontrol activity against *Pythium* and for some the mode of action of suppression of *Pythium* has been studied. Strain 7NSK2 protected tomato against *Pythium* damping-off, whereas its non-siderophore-producing mutant KMPCH had lost most of its biocontrol activity [29]. *Bacillus cereus* UW85 produces at least two antibiotics, i.e. zwittermicin A and antibiotic B [30], and reduced damping-off of tobacco seedlings caused by *Pythium aphanidermatum*, *Pythium torulosum* and *Phytophthora parasitica* [20]. *P. fluorescens* Q8r1-96 produces the antibiotic 2,4-diacetylphloroglucinol (DAPG), a compound that was shown to reduce *Pythium* diseases in several plant species, and antagonized the root pathogen *Gaeumannomyces graminis* in wheat [31]. Phenazine-1-carboxylic acid (PCA) production by *P. aeruginosa* strain PNA1 contributed to the suppression of *Pythium* damping-off in bean [32].

In this study it was investigated whether the increased disease susceptibility of Tetr plants could be counteracted either by applying chemicals that induce resistance, or by growing the plants in soil containing root-colonizing bacteria that were demonstrated to possess antagonistic and resistance-inducing properties.

2. Materials and methods

2.1. Plant material

T_2 seeds of transgenic, ethylene-insensitive Tetr18-5 tobacco [4], homozygous for the *Arabidopsis etr1-1* transgene, and seeds of corresponding non-transformed tobacco (*Nicotiana tabacum* cv. Samsun NN) were sown on either autoclaved (2×20 min with a 24 h interval) or non-autoclaved potting soil. The seedlings were grown for two weeks at RH 100% in a temperature-regulated greenhouse, at 24 °C during the day and 21 °C at night, and a photoperiod of 16 h.

All *PR*-overexpressing lines were developed in the Tetr18-5 genomic background. The cDNAs corresponding to tobacco *PR-1g* and *PR-5c* were obtained by RT-PCR, using upstream and downstream primers containing KpnI and HindIII sites. After digestion with KpnI and HindIII, the resulting PCR fragments were cloned between the cauliflower mosaic virus 35S promoter/alfalfa mosaic virus RNA4 leader (35S) and potato inhibitor terminator (Term) of pMOG843. The correctness of the cDNAs was confirmed by sequencing. The 35S–PR-cDNA–Term cassettes from the pMOG843 constructs were obtained by digestion with EcoRI and XbaI, and cloned in the T-region of the pMOG22 transformation vector (hygromycin-resistance). The resulting transformation plasmids were named pMOGPRb1 and pMOGPRb5. A double construct containing both the *PR-1g* and the *PR-5c* cDNAs was obtained by ligation of an XbaI fragment with the 35S–PR-5c-cDNA–Term cassette to XbaI-digested pMOGPRb1. This resulted in transformation plasmid pMOGPRb15. All transformation plasmids were electroporated into *Agrobacterium tumefaciens* LBA4404. *Agrobacterium*-mediated leaf disc transformation was performed as described previously [33]. Primary transformants were allowed to self-pollinate. The *PR* overexpression lines were selected on the basis of high PR mRNA expression levels.

Seeds of Tetr18-5 lines overexpressing *PR-1g* (designated EPRO1-16 and EPRO1-31), *PR-5c* (EPRO5-7 and EPRO5-12), both *PR-1g* and *PR-5c* (EPRO15-17), or no *PR* genes (22-11; empty vector), were sterilized by immersion in 1% NaClO for 20 min, washed three times with sterile water, and sown on Murashige & Skoog agar medium containing 1% (w/v) glucose and 20 $\mu g\,ml^{-1}$ hygromycin. The seedlings were grown with an

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