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ORIGINAL PAPER

Induction of defense-related genes in tomato plants after treatments with the biocontrol agents *Pseudomonas chlororaphis* ToZa7 and *Clonostachys rosea* IK726

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Abstract
Pseudomonas chlororaphis ToZa7 is a promising biocontrol agent possessing valuable characteristics and reducing disease severity caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) in tomato. In this study, the strain's ability to induce three pathogenesis-related (PR) genes (*PR-1a*, *GLUA*, and *CHI3*) in tomato, was studied using quantitative reverse transcription PCR. The genes *PR-1a* and *GLUA* were up-regulated after 120 h exposure to *P. chororaphis* ToZa7 (15.22- and 13.11-fold, respectively), as compared to the untreated control, without challenge inoculation by the pathogen. To study the effects of individual or combined application of *P. chororaphis* ToZa7 and the compatible biocontrol fungus *Clonostachys rosea* IK726, challenged with the pathogen, the expression patterns of the above three PR genes were monitored, in tomato roots. Expression of *PR1-a* was noteworthy, especially 48 h after challenge inoculation, when *C. rosea* IK726 alone or in combination with *P. chororaphis*, ToZa7 was pre-inoculated on tomato roots (38.53-fold and 53.74-fold, respectively). Expression of *PR1-a,* 72 h after challenge inoculation, was the highest in *P. chororaphis* ToZa7, among biocontrol treatments. Expression of *CHI3* was much lower, while up-regulation of *GLUA* was overall not observed. Confocal laser scanning microscopy of intact tomato roots and bacterial counts of superficially disinfected roots revealed, for the first time, that *P. chororaphis* ToZa7 colonizes the exterior as well as the internal tissues.

Keywords Endophytic colonization · *Pseudomonas chlororaphis* · Defense-related proteins · Biocontrol · *Clonostachys rosea*

Introduction

The current conventional agricultural practices involve effective and fast acting agrochemicals, worldwide, especially for the elimination of plant pathogens. However, besides being cost-effective, agrochemicals comprise environment aggravating methods that could pollute the plant tissues and the environment itself with residues. In addition, several soil-borne diseases are impossible to control with fungicides. An alternative to chemical treatments is the exploitation of plant-beneficial microbes, as biological control agents (BCAs), since they can promote plant growth and tolerance to diseases (Lugtenberg and Kamilova 2009). Several microorganisms have been previously described as potential BCAs. Within the bacterial BCAs, the genus *Pseudomonas* is one of the most studied (Weller 2007), because rhizospheric *Pseudomonas* spp. have many properties that make them well suited as biological control and growth-promoting agents. Usually, such properties include production of antifungal metabolites or efficient root colonization traits (Lugtenberg and Kamilova 2009). Many fungal BCAs have also been described to manage soil-borne diseases, using different modes of action, such as mycoparasitism among the most important. Some representative species of fungal

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BCAs belong to the genera *Trichoderma* and *Clonostachys* (Barea et al. 2005; Jensen et al. 2007).

During the interaction of soil-borne beneficial microbes with the plant root, colonization is generally recognized as a prerequisite for biocontrol ability (Lugtenberg and Kamilova 2009). Interestingly, some beneficial microorganisms could have endophytic behavior, leading to positive effects to both the host and the colonizing microorganism itself (Rosenblueth and Martínez-Romero 2006; Mercado-Blanco and Prieto 2013). Bacteria that colonize plant roots and promote plant growth are known as plant growth-promoting rhizobacteria (PGPR), but many fungi can also elicit plant-growth promotion (PGP). Effects of efficient root colonizers can occur via local antagonism with, or parasitism on soil-borne pathogens, or by induction of plant systemic resistance, leading to faster defense capacity towards subsequent pathogen attack (Zipfel 2014; Trda et al. 2015).

Salicylic acid (SA) accumulation in plants, triggered by microbe-associated molecular patterns (MAMPs), plays a crucial role in defense gene regulation (Robert-Seilaniantz et al. 2011; Pieterse et al. 2012; Zamioudis and Pieterse 2012). Hence, investigating the regulation of genes related to the SA signaling pathway, such as *PR-1a*, which encodes for an acidic type of pathogenesis-related protein-1 (PR-1a) and *GLUA,* which encodes for an extracellular β-1,3-glucanase, has been considered important in characterizing different BCAs' ability to reduce disease (Aimé et al. 2013). While accumulation of SA was until recently, directly correlated to a challenge inoculation with a plant pathogenic strain, numerous studies have shown that it could also be triggered by inoculation with non-pathogenic beneficial fungal strains (He and Wolyn 2005; Paparu et al. 2007; Veloso and Díaz 2012). Nonpathogenic strains of *F. oxysporum* protect *Asparagus officinalis* from pathogenic strains of *Fusarium* spp. and cause an accumulation in the inoculated roots of defense-related enzymes, such as peroxidase and phenylalanine ammonia-lyase (PAL; He et al. 2002). Similar effects were observed by Paparu et al. (2007) when increased expression of catalase and PR-1 protein was detected in banana roots treated with non-pathogenic *Fusarium oxysporum* endophytes (Paparu et al. 2007). Moreover, in green pepper roots, pre-inoculated with the non-pathogenic strain *F. oxysporum* Fo47, an up-regulation of three genes encoding a PR-1 protein (basic type), a type II chitinase, and a cyclase, was observed after challenge inoculation with *Verticillium dalhiae* (Veloso and Díaz 2012).

Regarding bacterial BCAs, earlier reports on model plants describe that different strains could have different effects in defense induction. For example, induction of systemic resistance by a strain may be correlated with three different signal molecules, SA, Jasmonic acid (JA), and Ethylene (ET) (Timmusk and Wagner 1999). Typically, JA and ETH-dependent ISR induction may not be accompanied by PR-protein activation (Pieterse et al. 1996, 2000; Van Wees et al. 1997) or SA accumulation (Iavicoli et al. 2003). While in other cases, SA-dependent induction of resistance does not include the typical expression of *PR-1a* (De Meyer et al. 1999). Therefore, it is important to study the expression of genes that are related to both signaling pathways, SA and JA/ETH, to determine the systemic resistance triggering effects of a single BCA under study (Pieterse et al. 2012; Zamioudis and Pieterse 2012; Aimé et al. 2013).

Indicative genes related to induction of resistance in tomato, which is an agronomically important plant, are *CHI3* and *CHI9,* encoding an acidic and a basic chitinase, respectively, *GLUA* and *GLUB,* encoding an acidic and a basic extracellular β-1,3-glucanase, *LOXD* encoding a lipoxygenase, and *PR-1a* encoding an acidic type of PR-1 (Kavroulakis et al. 2006; Aimé et al. 2013).

In the present work, two beneficial microorganisms were studied. First, *P. chlororaphis* ToZa7, a rhizobacterium isolated in Greece, from tomato roots, and reported to decrease tomato foot and root rot severity, caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl) (Kamou et al. 2015). This strain produces the broad-spectrum antibiotic phenazine-1-carboxamide (PCN), proteases, siderophores, and hydrogen cyanide (HCN) (Kamou et al. 2015). Second, strain IK726 of the common soil fungus *Clonostachys rosea*, isolated from barley rhizosphere, in Denmark, and proven to have growth-promoting ability, as well as biocontrol ability, against several important plant pathogens (Jensen et al. 2000). Both strains possess interesting traits making them efficient BCAs (Kamou et al. 2015, 2016; Table 1). Karlsson et al. (2015) demonstrated that a consortium of *C. rosea* IK726 with other *P. chlororaphis* strains is possible. In the same study, the colonization ability of *C. rosea* IK726, on tomato roots was confirmed and the colonization pattern was monitored. In addition, a detailed study regarding the compatibility between *P. chlororaphis* ToZa7 and *C. rosea* IK726 was previously reported (Kamou et al. 2016). *In planta*, experiments have demonstrated that combined treatment of *C. rosea* IK726 and *P. chlororaphis* ToZa7, against Forl, effectively reduce disease severity to a higher degree, compared to *C. rosea* IK726 alone or to its combination with other bacterial strains (Kamou et al. 2016). Due to the aforementioned results, it was considered useful to continue our research using the tomato—Forl pathosystem, to unravel more aspects of the mode of action of these two BCAs, specifically their ability to induce defense responses in tomato.

Since *P. chororaphis* ToZa7 has demonstrated attractive biocontrol traits, it could be used as a pre-transplanting inoculant to prime tomato plants against Forl, or other soil-borne pathogens. Moreover, combination of *P. chororaphis* ToZa7 and *C. rosea* IK726, against Forl, seems promising, and new evidence to support further the perspective of their successful application would be advantageous.

Table 1 Characteristics of the fungal and bacterial strains used in this study

Strains	Mode of action	Target pathogen	References
Bacterial			
Pseudomonas chlororaphis ToZa7	Promotes growth of tomato plants, produces proteases, siderophores, hydrogen cyanide (HCN) and the broad-spectrum antibiotic phenazine-1-carboxamide (PCN)	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Kamou et al. (2015, 2016)
Pseudomonas chlororaphis ToZa7-GFP	Wild-type ToZa7 gfp-expressing mutant by bacterial conjugation, colonizes tomato root system, presenting epiphytic and endophytic growth		This study
Fungal			
Clonostachys rosea IK726	Promotes plant growth, up-regulates the expression of defense-related genes, production of secondary metabolites	*Bipolaris sorokiniana, Fusarium culmorum, Pythium tracheiphylum, Alternaria* spp., *Botrytis cinereal, Fusarium* spp., *Plasmodiophora brassicae*	Jensen et al. (2007), Li et al. (2004), Luongo et al. (2005), Roberti et al. (2008), Lahlali and Peng (2013)
Fusarium oxysporum f. sp. *radicis-lycopersici ZUM 2407*	Wild-type plant pathogenic fungus. Produces tomato crown and root rot		Lagopodi et al. (2002)

Hence, the aims of the present study were: (1) to investigate the expression of *PR-1a*, *GLUA*, and *CHI3* in tomato plants, after treatment with *P. chlororaphis* ToZa7, in the absence of the pathogen, to prove the strain's priming ability, (2) to visualize the colonization pattern of *P. chororaphis* ToZa7 on tomato roots, and investigate possible endophytic growth, to expose its rhizosphere colonizing traits and unravel further interactions with tomato, and (3) to study the expression of the above genes in tomato after combined application of *C. rosea* IK726 and *P. chlororaphis* ToZa7, in the presence of the pathogen, to strengthen their value as effective biocontrol pair.

Materials and methods

Strains, cultural practices, and *gfp*-tagging of *Pseudomonas chlororaphis* ToZa7

Clonostachys rosea strain IK726, and *F. oxysporum* f. sp. *radicis-lycopersici* (Forl), strain ZUM 2407 (IPO-DLO), were kindly provided by professors D.F. Jensen (Swedish University of Agricultural Sciences, Sweden; SLU), and B.J.J. Lugtenberg (Leiden University, The Netherlands), respectively. Fungi were routinely kept on potato dextrose agar (PDA, LAB M, U.K.) plates, at 25 °C. *C. rosea* conidia were harvested from 10-day PDA cultures, as a sterile aqueous suspension, passed through glass wool filters to exclude mycelium. Forl was grown in Czapek Dox Broth (Duchefa Biochemie, Haarlem, The Netherlands), for 5–7 days, at 25 °C, on a rotating incubator, at 150 rpm, and conidia were separated from mycelium by filtration through Miracloth (Calbiochem, USA). The conidial concentration of the two fungi was determined each time using a hemocytometer, and was adjusted to 10^4 spores ml^{-1} (Thoma, Blaubrand GmbH, Germany, 0.1 mm × 0.0025 mm^2).

Stock cultures of *P. chlororaphis* ToZa7 were grown on Luria–Bertani (LB; Bertani 1951) agar plates, at 25 °C. To visualize the bacterial colonization on the tomato roots, the wild-type strain was derivative chromosomally tagged with a mini-*Tn7* site-specific construct, bearing the green fluorescent protein (GFP) to facilitate microscopy (Lambertsen et al. 2004). The gentamycin-resistant mini-*Tn7* transposon (mini-*Tn7*(Gm)PAI/04/03 gfp.ASV-a; Lambertsen et al. 2004) was used to mark the wild-type *P. chlororaphis* ToZa7 strain. To produce a *gfp*-expressing *P. chlororaphis* ToZa7, a GFP-tagged derivative strain was obtained by integration into the unique *att* site in *glmS*. In this derivative strain, GFP is constitutively expressed from a *lac*-derived promoter. Bacterial strains were cultured on LB-agar plates and cultures were cryopreserved in 50% glycerol, at − 80 °C.

Colonization of tomato roots by *Pseudomonas chlororaphis* ToZa7

Biological control ability of *gfp*-expressing *P. chlororaphis* ToZa7, against Forl, was confirmed *in planta*, as described previously for wild-type strain *P. chororaphis* ToZa7 (Kamou et al. 2015). The gnotobiotic system described by Simons et al. (1996) was used with minor modifications (Kamou et al. 2015), to grow tomato plantlets cv. 'ACE55' for 2 weeks, and study the colonization ability of the *gfp*-expressing *P. chororaphis* ToZa7 strain. Pre-germinated tomato seeds were inoculated with the transformed strain and seedlings were examined every 24 h, for 2 weeks, starting 2 days after inoculation. Plantlets were gently removed from the glass tubes and washed carefully to remove sand particles, and the whole root system was directly placed under the microscope. Colonization of tomato roots was monitored using a Nikon D-Eclipse C1 confocal microscope, using the default filter set. Digital images were acquired with the manufacturer's software.

To confirm endophytic growth of *P. chlororaphis* ToZa7, tomato roots previously inoculated with the bacterium were surfaced sterilized by immersion in 5% NaOCl solution, as described by Devi et al. (2017), with minor adjustments. Root system of tomato plants, grown for 4 weeks, was soaked in bacterial cell suspension ($OD_{625} = 0.7$), for 0.5, 1, and 2 h. Each timepoint served as a different treatment and each treatment consisted of two plants, as biological replicates. Inoculated plants were transplanted in pots containing 100 g of peat, and were grown for 1 week, under controlled conditions, with 16 h photoperiod, at 24 °C. After discarding the stem and leaves, roots were thoroughly washed and successively placed in Falcon tubes, containing 70% ethanol, for 1 min, and then in tubes containing 5% NaOCl, for 3 min. After five successive washes, with sterile distilled water, 100 µl from the final wash were coated on tryptone glucose yeast (TGY) agar plates. Root tissue was then removed from the tubes and macerated with sterilized mortar and pestle, in sterilized distilled water, and 100 µl were transferred on TGY agar plates and subsequently incubated at 25 °C. Plates were visually examined after 3 days for colony formation. *P. chlororaphis* ToZa7 colonies were identified through colony observation and 16S rRNA sequencing, as described previously (Kamou et al. 2015). Non-bacterized tomato plantlets were used as controls, at all steps. All treatments were aseptically performed, in a laminar flow and the experiment was repeated twice.

Treatments and gene expression analysis

Tomato plants, cv. 'ACE55', grown as described previously (Kamou et al. 2016), were used to study the expression of *PR-1a*, *GLUA*, and *CHI3* genes, after treatment with *P. chlororaphis* ToZa7. Gene expression was monitored 48, 72, and 120 h after treatment, without challenge inoculation by the pathogen, and was compared with the same genes' expression in untreated plants.

To investigate induction of *PR-1a*, *GLUA* and *CHI3* expression in tomato plants, after pre-inoculation (induction inoculation) with the two BCA's and challenge inoculation with the pathogen, *C. rosea* IK726, and *P. chlororaphis* ToZa7 were inoculated individually, or in combination, on tomato plants, 72 h before challenge inoculation with Forl. In all above cases, inoculation of microorganisms was applied by root drenching with a suitable inoculum suspension. Forl and *C. rosea* IK726 were inoculated as a 1:1 (v:v) mixture of conidia in water (10^4 spores ml^{-1}), with a 4% methyl cellulose aqueous solution. Inoculation of the combined BCAs was performed using a mixture 1:1:2 (v:v:v) of: (1) bacterial cell suspension in PBS ($O.D._{620} = 0.7$), (2) conidial suspension in water (10^4 spores ml^{-1}), and (3) methyl cellulose aqueous solution. The following treatments were included: (1) Forl, (2) untreated control, (3) *P. chlororaphis* ToZa7, (4) *P. chlororaphis* ToZa7 + Forl, (5) *C. rosea* IK726, (6) *C. rosea* IK726 + Forl, (7) *C. rosea* IK726 + *P. chlororaphis* ToZa7, and (8) *C. rosea* IK726 + *P. chlororaphis* ToZa7 + Forl.

Gene expression analysis was performed 48 and 72 h after challenge inoculation with Forl. According to earlier studies (Lagopodi et al. 2002), the time between 48 and 72 h after inoculation is crucial for Forl establishment within the root tissues. For this reason, induction of resistance after the 72 h is considered as of low value. Six plants grown for 6 weeks before induction inoculations were used per treatment. Plants were placed under controlled conditions, at 22 °C, and photoperiod of 14 h light/10 h darkness.

Regarding transcription analysis of the three PR genes, total RNA from tomato roots was extracted using the Qiagen RNeasy kit (Qiagen, Hilden, Germany). RNA was harvested at 48 and 72 h post-challenge inoculation (hereafter mentioned as hpi), in all treatments. Regarding the treatment of *P. chlororaphis* ToZa7 alone, without challenge inoculation with Forl, and its untreated tomato control, RNA was additionally harvested at 96 and 120 h after inoculation. RNA concentration was determined with a P330 nanophotometer (Implen GmbH, Germany). Residual traces of DNA were removed by treatment with RNase-free DNase I (Qiagen, Hilden, Germany), and a one-tube real-time qRT-PCR was performed (Pappi et al. 2015). The total volume of the RT mix was 25 µl per reaction and the content of the master mix as well as the thermal cycling conditions were performed according to Pappi et al. (2015). Gene-specific primers (Table 2) were used for the transcription analysis, and the genes encoding actin (*ACTIN*) and an internal control (*CyOXID*) of mitochondrion cytochrome oxidase subunit I (*mtCOXI*) gene were used as reference genes for

Table 2 Primers used in RT-qPCR in tomato experiments

Gene name	Primers	Encoding protein	Defense pathway	References
PR1-a	For: TCTTGTGAGGCCCAAAATTC Rev: TAGTCTGGCCTCTCGGACA	PR-1 (acidic type)	SA signaling pathway	Aimé et al. (2013)
GLUA	For: GTCTCAACCGCGACATATT Rev: CACAAGGGCATCGAAAAG AT	PR-2 (β-1,3 glucanase, basic type)	SA signaling pathway	Aimé et al. (2013)
CHI3	For: TGCAGGAACATTCACTGGAG Rev: TAACGTTGTGGCATGATGGT	PR-3 (Chitinase)	JA/ETH signalling pathway	Aimé et al. (2013)
ACTIN	For: GAAATAGCATAAGATGGC AGACG Rev: ATACCCACCATCACACCA GTAT	Actin	Reference gene	Aimé et al. (2013)
CyOXID	For: TGGTAATTGGTCTGTTCC GATT Rev: TGGAGGCAACAACCAGAA TG	Cytochrome oxidase subunit I	Reference gene	Papayiannis et al. (2011)

normalization in tomato (Papayiannis et al. 2011; Aimé et al. 2013). Expression of *PR*-1a *GLUA* and *CHI3* genes was measured by quantitative reverse transcriptase PCR (RT-qPCR), using a Stratagene Mx3005P™ and melt curve analysis that was conducted to assess specific amplification. Transcript levels were quantified in three pooled samples, each one produced by mixing equal quantities of six independent biological replicates. Data analysis was carried out with relative quantification, using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), and data normalization was achieved using the expression levels of the reference genes.

Statistical analysis

Data from gene expression analysis were analyzed by analysis of variance (ANOVA), based on the completely randomized design (CRD), and mean values were computed from three replicates. Following a significant ANOVA *F* test, the differences between treatments' mean values were compared using Tukey's test, and comparisons were made between treatments and the untreated controls. The significance level in all hypothesis testing procedures was predetermined at $P \leq 0.05$. All statistical analyses were performed with the SPSS v 19.0 software (SPSS Inc. Chicago, IL).

Results

Colonization of tomato roots by *gfp*-expressing *Pseudomonas chlororaphis* ToZa7

Pseudomonas chlororaphis ToZa7 efficiently colonized the tomato roots by rapidly growing along the junctions of epidermal cells, forming microcolonies (Fig. 1b). Root hairs were also heavily colonized (Fig. 1c, d). Dense microcolonies on the root surface were visible 4 days post-inoculation (hereafter mentioned as dpi). Interestingly, *P. chlororaphis* ToZa7 seem to grow endophytically, at 6 dpi, as it could be pointed out by bacterial cells looking to be allocated inside epidermal plant cells (Fig. 1e, f). Endophytic behavior was confirmed by isolating the bacterium from the inner parts of surface-sterilized tomato root tissue. After tomato root disinfection, *P. chlororaphis*-like colonies were re-isolated on LB plates, only from the surface-disinfected root tissue. After 3 days, characteristic creamy colonies, showing a bright yellow coloration on LB, and dark green color pigment in the colony center, confirming the production of phenazine, were observed. 16SrRNA sequencing confirmed the presence of *P. chlororaphis*.

Induction of defense-related genes in tomato treated with *Pseudomonas chlororaphis* ToZa7 in the absence of the pathogen

Application of *P. chlororaphis* ToZa7 promoted the expression of defense-related genes in tomato, without challenge inoculation by the pathogen, as compared to the untreated control. The highest expression level was recorded 120 h after inoculation. Specifically, mean transcript levels of genes *PR-1a* and *GLUA* were significantly higher (15.22-fold and 13.11-fold, respectively) after exposure to *P. chlororaphis* ToZa7 alone, in the absence of Forl (Fig. 2).

Induction of defense-related genes in tomato challenged with Forl after treatment with *Pseudomonas chlororaphis* ToZa7 and *Clonostachys rosea* IK726

Biological control ability of the two tested BCAs, applied individually or in combination, was confirmed by estimating

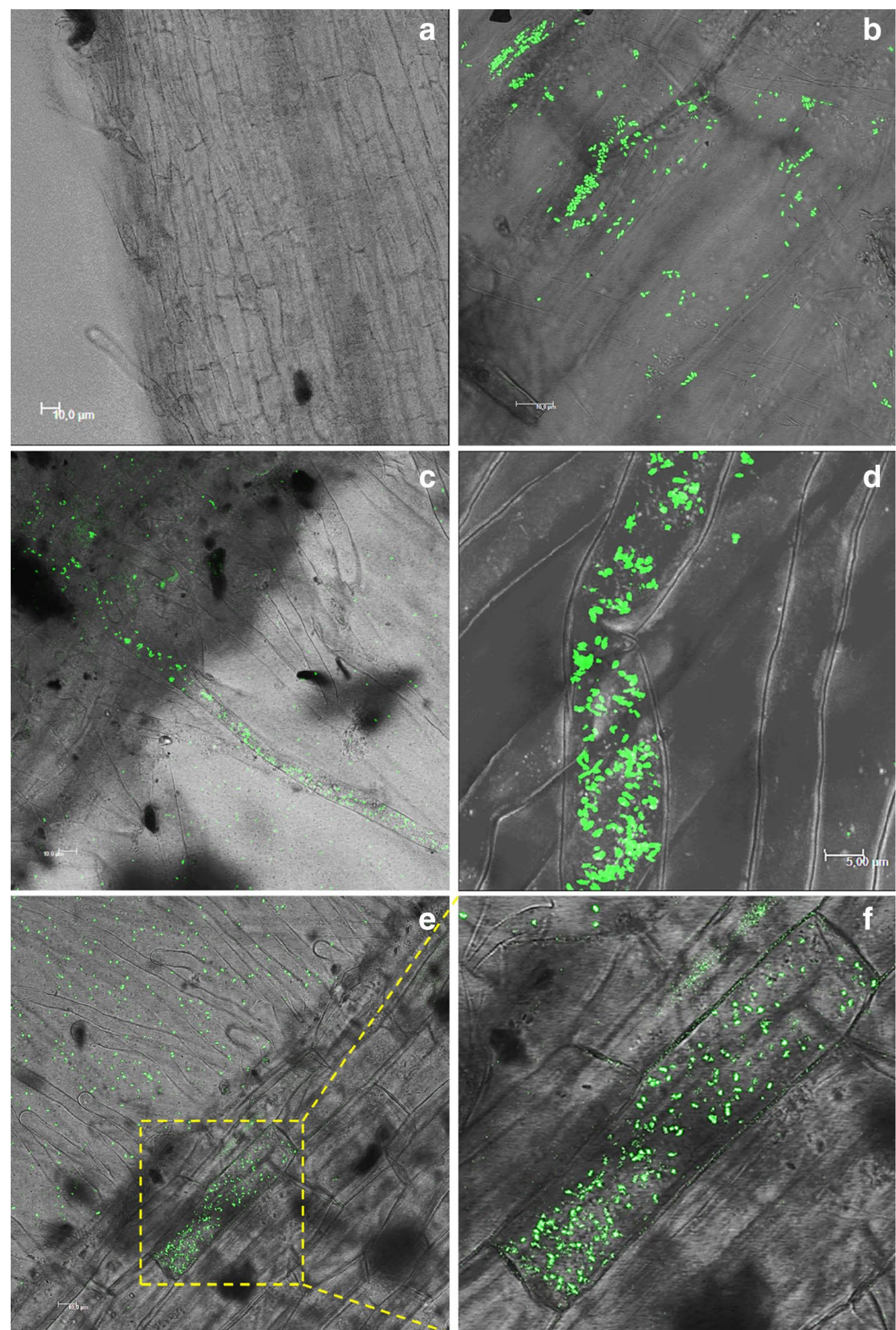

Fig. 1 Bacterial cells of the transformed strain *Pseudomonas chlororaphis* ToZa7, expressing the green fluorescent protein (gfp). Uncolonized tomato root showed an autofluorescence signal permitting observation of individual root cells (**a**); the beneficial rhizobacterium *P. chlororaphis* ToZa7 efficiently colonized the tomato roots by forming microcolonies visible 4 days post-inoculation, along the junctions of epidermal cells (**b**), and the root-hairs (**c**, **d**). Interestingly, *P. chlororaphis* ToZa7 showed endophytic behavior at 6 days post-inoculation (**e**, **f**). Colonization ability was monitored using the Multizoom Nikon, model AZ-100 fluorescence detecting microscope, with a detection range of 440–510 nm for the gfp. Digital images were acquired with the manufacturer's software

disease severity in plants challenged with Forl, and showing results very similar to those previously reported (Kamou et al. 2015, 2016). Results in gene expression 48 and 72 hpi are presented, for all treatments, in Figs. 3 and 4, respectively.

Overall, BCA control treatments did not induce gene expression either 48 or 72 hpi. Several positive effects were observed, such as the following: At 48 hpi, a 53.74-fold induction of *PR-1* gene was observed after challenge inoculation with Forl, when *C. rosea* IK726 and *P. chlororaphis* ToZa7 were applied in combination, as compared to the untreated control ($P<0.05$). Moreover, a 38.53-fold induction was observed in plants inoculated with *C. rosea,* 5.75-fold induction in the *P. chlororaphis* ToZa7 treatment, and 20.7-fold induction when plants were not inoculated with any of the BCAs.

Mean transcript levels of gene *CHI3* were higher (16.8-fold), 48 hpi regarding the *C. rosea* IK726 treatment, as compared to the untreated control (Fig. 3). Treatment with *P. chlororaphis* ToZa7 did not induce *CHI3* expression, at 48 hpi, but

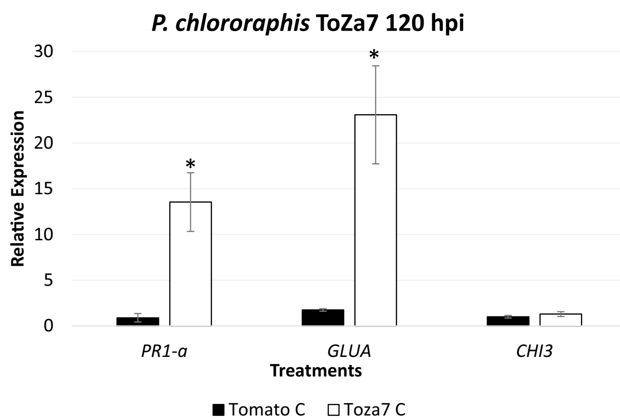

Fig. 2 Expression analyses of defense genes *PR-1a, GLUA* and *CHI3,* in tomato plants, 120 h after inoculation with *Pseudomonas chlororaphis* ToZa7 and without challenge inoculation by pathogen. Error bars represent the standard deviation based on six biological replicates. An asterisk indicates a significant difference of expression in comparison with control treatment, according to Tukey's test ($P<0.05$)

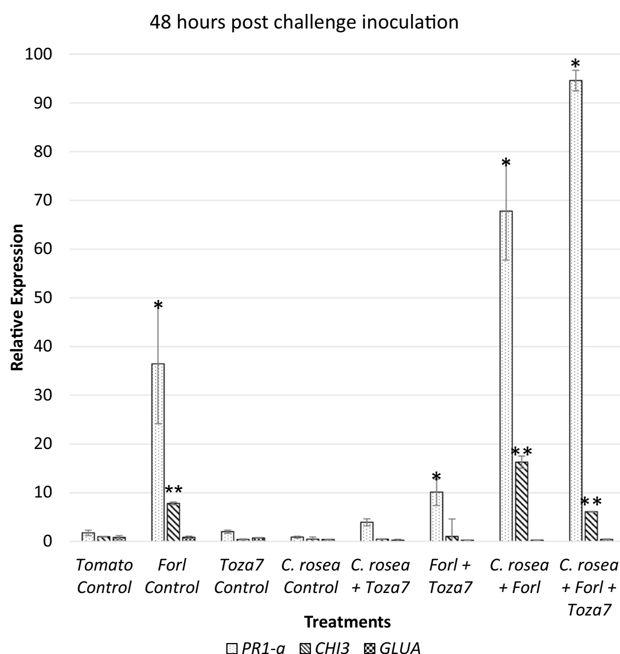

Fig. 3 Expression analyses of SA-related defense genes *PR-1a* and *GLUA* and JA/ET-related defense gene *CHI3,* in tomato plants, challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl), 48 h after induction inoculation with *Clonostachys rosea* IK726, *Pseudomonas chlororaphis* ToZa7 and their combination. Error bars represent the standard deviation based on six biological replicates. An asterisk indicates a significant difference of expression of *PR-1a,* in comparison with the untreated control, according to Tukey's test ($P<0.05$). Two asterisks indicate a significant difference of expression of *CHI3,* in comparison with the untreated control, according to Tukey's test ($P<0.05$)

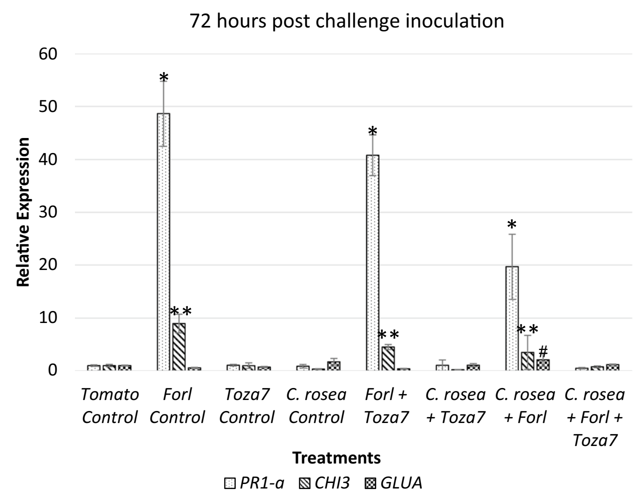

Fig. 4 Expression analyses of SA-related defense genes *PR-1a* and *GLUA* and JA/ET-related defense gene *CHI3,* in tomato plants, challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Forl), 72 h after induction inoculation with *Clonostachys rosea* IK726, *Pseudomonas chlororaphis* ToZa7 and their combination. Error bars represent the standard deviation based on six biological replicates. An asterisk and a diesi, respectively, indicates a significant difference of expression of *PR-1a,* in comparison with the untreated control, according to Tukey's test ($P<0.05$). Two asterisks indicate a significant difference of expression of *CHI3,* in comparison with the untreated control, according to Tukey's test ($P<0.05$)

in combination with *C. rosea* IK726, it resulted in a (6.3-fold) up-regulation in the expression of *CHI3* when compared with untreated tomato plants (Fig. 3). Besides, when plants were not inoculated with any of the BCAs, a 8.05-fold expression of *CHI3* was recorded.

However, at 72 hpi, the inducing effect of *C. rosea* IK726 on *CHI3* expression was −3.46-fold, as compared to the untreated control (Fig. 4), whereas a 8.79-fold induction was observed in plants not treated with any of the BCAs. A 4.53-fold expression was observed in the *P. chororaphis* ToZa7 treatment and no induction was observed in the combined treatment. As for *PR-1* gene expression, 72 hpi, treatments of *P. chlororaphis* alone and in combination with *C. rosea* caused a 40.41 and 19.5-fold up-regulation, respectively, as compared with the untreated control (Fig. 4). However, induction of *PR-1* was highest in plants not treated with any of the BCAs.

Regarding the SA-related gene *GLUA*, induction was slightly higher (2.07-fold), at plants treated with *C. rosea* and challenged with Forl, 72 hpi ($P<0.001$), as compared to the untreated control (Fig. 4).

Discussion

Increase of mean transcript levels of *PR-1a*, and *GLUA* genes after exposure to *P. chlororaphis* ToZa7, as compared to the untreated control, in the absence of the Forl, suggests induction of systemic resistance in tomato. It is one of the very few examples reported in the literature (Li et al. 2015; Aime et al. 2013), of PR-protein induction by a BCA, in the absence of a pathogen. A possible explanation for these results could be that the beneficial microorganisms are initially recognized as a plant invaders, and therefore, defense-related protein expression is elicited (Yedidia et al. 2003; Salas-Marina et al. 2011; Alonso-Ramírez et al. 2014). *GLUA* has been considered important in characterizing a BCAs' ability to reduce disease (Aimé et al. 2013). Such biological control agents can be valuable and could be practically used as root inoculants to protect transplants before transplanting in infested soils. Our results suggest that *P. chlororaphis* ToZa7 could be used as a tomato transplant protectant 120 h prior to transplanting.

The interest of the scientific community for exploration of defense-related protein expression triggered by BCAs is rising, since it is a very strong aspect in the direction of BCAs' use for a sustainable agriculture. Li et al. (2015) confirmed the up-regulation of *PR-1* gene in cucumber leaves, after exposure to *Bacillus amyloliquefaciens* LJ02. Aime et al. (2013) reported up-regulation of various defense-related genes in tomato, after inoculation with fungal biocontrol agent *F. oxysporum* Fo47. These findings suggest that SA-related SAR response has been induced by the presence of these BCAs (Li et al. 2015). It has been suggested that just alike in the case of plant intruders, pattern-recognition receptors (PRRs) which recognize MAMPs/PAMPs are activated leading to a cascade of defense responses (Trda et al. 2015).

In the presence of the pathogen, induction of the SA-related *PR-1a* gene, in tomato, was higher when plants were not treated with *P. chlororaphis* ToZa7. *Pseudomonas* species have often been reported in the literature for not eliciting, or even down-regulating PR-protein activation (Verhagen et al. 2004; Pieterse et al. 1996; De Meyer et al. 1999). In addition, lower level activation of defense-related genes has been sometimes observed in pathogen-challenged plants, treated with a biological control agent, as compared to pathogen-treated control (Aimé et al. 2013).

Transcript-level profiles of *CHI3* were lower, compared to *PR-1a*, in all cases, however, induction where observed indicates its potential role as part of the defense mechanisms triggered by the two BCAs in tomato plants. It has been reported that a higher expression of genes encoding chitinase, glucanase, and peroxidase was induced in cucumber after pre-inoculation of plants with the fungal BCA *Trichoderma* sp. (Shoresh et al. 2005). Overall, lower expression profiles of both *PR-1a* and *CHI3* from 48 to 72 h, as compared to plants treated with the pathogen alone, may be due to advanced invasion of tissues by the pathogen at 72 hpi (Lagopodi et al. 2002).

Clonostachys rosea can endophytically colonize cucumber plants (Chatterton et al. 2008) and can also induce expression of defense-related genes in wheat and canola (Roberti et al. 2008; Lahlali and Peng 2013). Roberti et al. (2008) demonstrated that treatment with *C. rosea* caused a rapid increase of PR-4 defense-related proteins, in wheat plants, as compared to treatment with the pathogen. The results of the current study strengthen the hypothesis that *C. rosea* IK726 can induce systemic defense responses through both SA and JA/ETH signaling pathways, providing protection to the host from the early growth stages. According to the present study, it is shown for the first time that treatment with *C. rosea* IK726 causes an induction of the SA-related *PR-1a* gene also in the tomato-Forl pathosystem.

The successful combination of *P. chlororaphis* strains with fungal BCAs has been previously described (Duffy et al. 1996; Karlsson et al. 2015). *C. rosea* IK726 has been successfully combined with other *P. chlororaphis* strains (Karlsson et al, 2015; Tzelepis and Lagopodi 2011). Our previous studies showed that *C. rosea* IK726 and *P. chlororaphis* ToZa7, in combination, significantly reduced tomato foot and root rot severity (Kamou et al. 2016). Recent observations indicate the ability of BCAs to trigger expression of defense proteins in the tomato plant, individually and/or in consortia (Srivastava et al. 2010). The regulation of genes related to the SA signaling pathway, such as *PR-1a* and *GLUA*, has been considered as an important trait when characterizing BCAs as efficient (Aimé et al. 2013).

Combination of *C. rosea* IK726 with *P. chlororaphis* ToZa7 caused a more intense positive response, after challenge inoculation with the pathogen, regarding the *PR-1a* gene, in comparison to individual applications of both BCAs. It could be hypothesized that this effect is mainly attributed to *C. rosea*. However, the effect of this combined treatment cannot be considered as additive, since the expression of *PR-1a* gene is even higher, as compared to the *C. rosea* IK726 treatment. There is no information in the literature that could help in explaining such a result and this effect should be studied further. We have previously reported that a combination of *C. rosea* IK726 and *P. chlororaphis* ToZa7 leads to successful biocontrol of Forl in tomato (Kamou et al. 2016). The results of the present study demonstrate that the biocontrol effect in this pathosystem by the combination of these two BCAs can at least partly be attributed to the induction of SA-related systemic resistance. Similar responses were documented

by other BCAs, such as *T. harzianum*, which elicited genes related to SA and JA/ETH pathways in tomato (Tucci et al. 2011; Harel et al. 2014).

Aims in this study included elucidation of the colonization ability and colonization pattern of *P. chlororaphis* ToZa7 on tomato roots. The results obtained confirm the colonizing ability of *P. chlororaphis* ToZa7. Microcolonies were located along the junctions of epidermal cells, where root exudates are reported to be accumulated (Jones et al. 2009), and this result corroborates previous studies regarding *P. chlororaphis* colonization patterns (Bolwerk et al. 2003). However, endophytic behavior observed using confocal microscopy was corroborated by colony counts from surface-disinfected tomato roots. A plethora of beneficial rhizobacteria is able to colonize the root system internally, without causing any negative effect to the host (Rosenblueth and Martínez-Romero 2006). Regarding the genus *Pseudomonas*, a wide number of non-pathogenic endophytes have been isolated and identified, from different geographical regions and hosts (Mercado-Blanco and Bakker 2007). Endophytic behavior is positively affecting both parties, since the bacterium can be protected by abiotic stresses and the host could benefit from all direct and indirect mechanisms of action of the BCAs (Sturz et al. 2000; Compant et al. 2005a, b; Rosenblueth and Martínez-Romero 2006; Mercado-Blanco and Prieto 2013).

Resuming, the present study provides valuable information regarding two effective BCAs, *P. chlororaphis* ToZa7 and *C. rosea* IK726. Their previously reported successful combination was now re-confirmed by the induction of defense-related responses in tomato plants challenged with Forl. Especially for *P. chlororaphis* ToZa7, its ability to trigger SA-related responses in tomato is underlined. Demonstration of this strain's endophytic colonization gives new viewpoints of its interaction with tomato.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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