The constitutive endopolygalacturonase TvPG2 regulates the induction of plant systemic resistance by *Trichoderma virens*

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ABSTRACT

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Trichoderma spp. are opportunistic fungi some of which are commonly present in the rhizosphere. Several species, such as *Trichoderma virens*, are also efficient biocontrol agents against phytopathogenic fungi and exert beneficial effects on plants. These effects are the consequence of interactions between *Trichoderma* and plant roots, which trigger enhanced plant growth and induce plant resistance. We have previously shown that *T. virens* I10 expresses two endopolygalacturonase genes, *tvpg1* and *tvpg2*, during the interaction with plant roots; *tvpg1* is inducible while *tvpg2* is constitutively transcribed. Using the same system, the tomato polygalacturonase-inhibitor gene *Lepgip1* was induced at the same time as *tvpg1*. Here we show by gene disruption that TvPG2 performs a regulatory role on the inducible *tvpg1* gene and in triggering the plant immune response. A *tvpg2*-knockout strain fails to transcribe the inducible *tvpg1* gene neither *in vitro* in inducing media containing pectin or plant cell walls, nor during the *in vivo* interaction with tomato roots. Likewise, the *in vivo* induction of *Lepgip1* does not occur, and its defence against the pathogen *Botrytis cinerea* is significantly reduced. Our data prove the importance of a *T. virens* constitutively produced endopolygalacturonase in eliciting plant Induced Systemic Resistance (ISR) against pathogenic fungi.

Keywords Endopolygalacturonase genes_ Gene knockout _ *Trichoderma virens*/tomato interaction _ polygalacturonase inhibiting proteins _ Induced Systemic Resistance

INTRODUCTION

Trichoderma is a fungal genus originating from mycoparasites that evolved into opportunistic fungi. It is able to colonize diverse habitats that harbor living organisms including plants (Druzhinina et al. 2011; Kubicek et al. 2011). Due to their mycoparasitic abilities, many species have been reported as suitable for the biocontrol of fungal plant pathogens (Benitez et al. 2004; Hermosa et al. 2012; Kubicek et al. 2001). In addition, the ability of some isolates to colonize the plant rhizosphere, and thereby to trigger plant resistance and enhanced plant growth, has fostered practical applications (De Meyer et al. 1998; Salas-Marina et al. 2011; Velázquez-Robledo et al. 2011). In the interaction with plants they can colonize the first or second layers of the root cell walls but – unlike plant pathogens – usually do not penetrate further (Chacón et al. 2007; Harman et al. 2004). They thus induce metabolic changes in the plants (Fiorini et al. 2015), which can cause systemic resistance to a wide range of pathogens (Shoresh et al. 2010), and sometimes also lead to growth promotion (Salas-Marina et al. 2011).

Examples are *Trichoderma asperellum* and *Trichoderma harzianum* which induce systemic resistance and plant defense responses in cucumber (Elsharkawy et al. 2013; Yedidia et al. 1999 respectively). *T. virens* can enhance biomass and root growth in *Arabidopsis* (Contreras-Cornejo et al. 2009), and induces phytoalexin production and resistance to *Rhizoctonia solani* in cotton roots (Howell et al. 2000), and to *Botrytis cinerea* in *Arabidopsis* (Velásquez-Robledo et al. 2011). Such plant responses are triggered by fungal proteins that come into contact with the plant cell wall (Kubicek et al. 2011; Kubicek et al. 2014; Mukherjee et al. 2012).

Plant cell wall degrading enzymes (CWDEs), such as xylanases, were the first fungal proteins that were found to induce such a reaction (Lotan and Fluhr 1990). In plant pathogenic fungi, pectin degrading enzymes, notably endopolygalacturonases (EPGs), act as virulence factors because they are produced in the early stages of contact with plant tissues (Annis and Goodwin 1997; Di Pietro and Roncero 1998). EPGs, together with pectin lyases, can disrupt the middle lamella of the plant

and consequently macerate the plant tissue (Herron et al. 2000), which is a prerequisite for penetration into the host and for using it as a carbon source. The importance of this step for plant pathogenic fungi is also reflected in the presence in plants of polygalacturonase inhibiting proteins (PGIPs) (Federici et al. 2001), and by the fact that oligogalacturonides - formed by the polygalacturonases - elicit the plant defense reactions (Cervone et al. 1989).

Although polygalacturonases are much more abundant in the genomes of plant pathogenic fungi, saprotrophic fungi also contain several polygalacturonase genes to feed on decaying plant material (Kubicek et al. 2014; Zhao et al. 2013). The genomes of *Trichoderma* spp. contain up to four polygalacturonase genes (Kubicek et al. 2011), but their potential role as plant elicitors has not been extensively investigated. One canonical EPG gene from the *T. harzianum* strain T34, which is induced upon contact with the plant, and is present in all *Trichoderma* spp. (C.P. Kubicek, unpublished findings), has been reported to be involved in beneficial associations with plants (Morán-Diez et al. 2009).

The *Trichoderma virens* strain I10 is an efficient biocontrol agent (Catalano et al. 2011; Sarrocco et al. 2009; Sarrocco et al. 2006; Vannacci and Pecchia 2000). We have previously shown that the transcripts of two EPG genes (*tvpg1* and *tvpg2*) were highly abundant in this strain during the *in vivo* association with tomato roots. Data also suggested an EPG-PGIP recognition in that system (Baroncelli et al. 2016). Interestingly, the expression of *tvpg1* was inducible, whereas *tvpg2* was constitutive. Constitutively expressed genes usually represent the core of an organism's physiological inventory because they encode essential proteins involved in basal pathways or processes. Constitutive EPGs may release pectin degradation products, which can induce other EPG encoding genes (Wubben et al. 1999).

In this study, we further investigated the role of *tvpg2*, and then of *tvpg1* during the interaction of *T. virens* with tomato. This entailed exploiting reverse genetics to investigate whether TvPG2

would elicit a defense response against plant pathogens in their host, such as an Induced Systemic Resistance (ISR), using *Botrytis cinerea* and tomato plants as a model.

MATERIALS AND METHODS

Fungal strains, culture conditions and plant material

The *T. virens* I10 isolate (CBS 116947) was used as the wild-type strain. The strain B14 (*T. virens* I10*Δtku70*, Catalano et al. 2011) was used to delete the *tvpg2* gene, since the disruption of *tku70* strongly increases the homologous recombination efficiency. *Botrytis cinerea* (SAS 56) was kindly provided by Prof. F. Faretra (Dept. Soil Science, Plant and Food, Bari University, Italy). All fungi were maintained on PDA (Potato Dextrose Agar, Difco, NJ, USA), under mineral oil, at 4°C, in the fungal collection of the Plant Pathology & Mycology Lab of Dept. Agriculture, Food and Environment (Pisa University). The fungi were grown on PDA plates at 24°C, with a daily cycle of 12 h light and 12 h dark. When needed, fungal liquid cultures in a basal mineral Fries medium containing glucose or apple pectin (Sigma Aldrich, Milan, Italy) or tomato cell walls, all at 0.5% (w/v), were set up as described in Baroncelli et al. (2016).

Tomato seeds, *Solanum lycopersicum* cv. Micro-Tom, were provided by Nicky's Nursery Ltd, Kent, UK (www.nickys-nursery.co.uk).

Deletion of *tvpg2* and transformation

In order to disrupt *tvpg2* (ID 192705 in the *T. virens* Gv29-8 genome sequence database v2.0, http://genome.jgi.doe.gov/TriviGv29_8_2/TriviGv29_8_2.home.html), 1.32 kb of its 5'non-coding and 1.45 kb of its 3'non-coding regions were amplified by PCR. The 5' upstream region was amplified with the primer pair A/B (the forward A containing the restriction site *Spe*I, the

reverse B with a *Bam*HI site); the 3' downstream region was amplified with the primer pair C/D (the forward C with a *Hind*III site, the reverse D with a *Xho*I site) (Table 1). Both regions were cloned into a pGEM-T easy vector (Promega, Madison, WI, USA). The hygromycin phosphotransferase B gene, *hphB*, conferring resistance to hygromycin B, was excised as a *Bam*HI/*Hind*III fragment from pLHhph (Hartl et al. 2007), whereas *Pdm*I/*Xmn*I were used to linearize the vector. First the PCR upstream (*Spe*I/*Bam*HI), then the downstream (*Hind*III/*Xho*I), fragment and finally the *hphB* resistance marker (2.4 kb between up- and downstream regions *Bam*HI/*Hind*III), were ligated into the vector pBluescript SK, resulting in the *tvpg2* knockout vector pSGEPG (8.1 kb). The right orientation of fragments inside the deletion cassette (5.2 kb) was checked by PCR with the primer pair A/D (Table 1), using the Expand Long Template PCR System (Roche, Indianapolis, IN, USA). The 5.2 kb cassette was used to delete the *tvpg2* gene in *T. virens* I10*Δtku70* (B14) strain. The transformation was carried out with B14 protoplasts, as previously described by Catalano et al. (2011).

PCR and Southern blot analysis of transformants

Fungal genomic DNA was isolated from transformants and control strains I10 and B14 as described in Hartl and Seiboth (2005). Southern blot analysis was carried out following standard protocols (Sambrook and Russell 2001). Probe labelling and hybridization were performed with the DIG nonradioactive system according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). A specific probe, including both flanking regions of *tvpg2* and the *hphB* gene, was amplified from the pSGEPG DNA with the primer pair A/D (Table 1) and used on genomic DNA digested by *Eco*RV.

A PCR analysis was performed with the Expand Long Template PCR System (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Genomic DNA was

amplified with the primer pair A/D (Table 1). All primers were provided by MWG-BIOTECH (Ebersberg, Germany).

Physiological analysis of transformants

Fungal growth rates were quantified in 96-well microplates (PbI International, Milan, Italy) in PDB (Potato Dextrose Broth, Difco, NJ, USA), using a spectrophotometric approach (Catalano et al. 2011). OD_{595} values were plotted against time to create growth curves, which were then subjected to ANOVA for regression, in order to compare the slope of each curve, assuming $P \leq 0.05$ as a significant level. The experiment was independently replicated three times.

To quantify conidial germination, three spore suspensions (10^3 conidia·ml^{-1}) were prepared for each strain grown on PDA and used to evaluate the germination ability, as described by Catalano et al. (2011). Data were submitted to one-way ANOVA, assuming $P \leq 0.05$ as a significant level. Three independent biological replicates were made, each consisting of three slides for each strain. Forty conidia were counted for each slide.

For *in vitro* confrontation tests the antagonistic and mycoparasitic abilities of transformants were tested in dual cultures against *Botrytis cinerea* as described by Catalano et al. (2011). In order to compare the growth of the pathogen in the presence/absence of the antagonist (wt or transformants), growth curves on PDA were subjected to ANOVA for regression using Systat (SPSS Inc., Chicago, IL) $P \leq 0.05$ was assumed as a significant level. Three independent replicates were made for each antagonist/pathogen combination.

On the same plates, after 21 days, overgrowth and sporulation of the antagonists on *B. cinerea* colonies were assessed. Interaction zones and overlapping regions for each antagonist/pathogen combination were then analysed on water agar (agar 15 g·l^{-1} of distilled water) by microscopic investigations in order to detect possibly occurring coilings of *T. virens* strains around the host hyphae.

RNA isolation and expression analysis by one step RT-PCR

Samples obtained from fungal liquid cultures containing glucose or pectin or tomato cell walls were collected after 48, 72 and 96 h. Total RNAs were then isolated following a guanidinium isothiocyanate/phenol–chloroform method (Baroncelli et al. 2016).

A one step Reverse Transcription PCR (RT-PCR) analysis was performed using 1 µg of RNA from each sample, according to the manufacturer's instructions (AccessQuick™ RT-PCR System, Promega, Madison, WI, USA). The primers used are listed in Table 1.

Quantitative RT-PCR

Tomato roots were inoculated by *T. virens* strains in order to monitor the expression of fungal EPG genes *tvpg1*, *tvpg2* (accession numbers KF963272 and KF963273 respectively) and the tomato endopolygalacturonase inhibitor gene *Lepgip1* (Accession Number L26529). Experimental conditions for *in vivo* root colonization, total RNA isolation, primers (Table 1) and protocols for quantitative RT-PCR (qRT-PCR) were those described by Baroncelli et al. (2016). Two biological replicates were carried out for each experimental condition. Specific primers used in this experiment were: for *T. virens tvpg1* E/F, for *tvpg2* G/H, and for *S. lycopersicum Lepgip1* M/N. The following primers targeting housekeeping genes were used as a reference: for *T. virens* both *β-tubulin* I/J, and the translation elongation factor 1-alpha *tef1* K/L; for tomato the translation elongation factor *LeEF1A* O/P. All primers, supplied by MWG-BIOTECH (Ebersberg, Germany), are listed in Table 1. Three internal technical replicates were made for each of the two biological replicates.

***In vivo* biocontrol assay**

Tomato seeds were sterilized for 5 min with 50% NaClO (1% active chlorine) in a solution (v/v) of 50% ethanol, and then rinsed for 5 min each three times with distilled water. A pre-germination three-day step was performed in a humid chamber in the dark at 4°C. The germinated seeds were then transferred at room temperature to pots containing a commercial plant growing substrate (Universal soil Esselunga®, supplied by Berco Srl, Calcinate, Bergamo, Italy). Four days later, the soil around the plantlets was pre- inoculated by 0.5 ml of *T. virens* spore suspensions (10^6 conidia·ml^{-1}) or by water alone (control). After additional seven days, a *B. cinerea* suspension containing 10^6 conidia·ml^{-1} was sprayed on all leaves (0.5 ml/plant) of pre-inoculated and untreated plants (*B. cinerea* control). Both control and inoculated plants were evaluated for the presence of foliar symptoms two weeks after inoculation. The disease was quantified by counting the number of necrotic lesions developed on each inoculated true leaf. The experiment consisted of two pots with three plants each per treatment, and was performed three times. ANOVA was carried out using Systat (SPSS Inc., Chicago, IL).

Root and rhizosphere colonization test

The same experimental scheme used for the biocontrol assay was employed for the root and rhizosphere colonization test. Seven days after the *T. virens* pre-inoculation (in the biocontrol assay, *B. cinerea* was also inoculated at the same time), treated and control (untreated) tomato roots were collected. The roots were gently shaken to detach loosely adherent plant growing substrate and were then rinsed with 20 ml of sterile distilled water, and the washing water was collected. This represented the rhizosphere suspension. The roots were then washed again under tap water and sterilized as described in the previous section for seeds, except for the sterilization time which was reduced to 1 min. Each root was drained on sterile filter paper and divided into four-five pieces which were laid on *Trichoderma* semi-selective agar plates (TSM: PDA amended with 50 ppm streptomycin sulphate, 7500 u.i·l^{-1} bacitracin sulphate, and 0.3 g·l^{-1} hymexazol). At

the same time, 200 µl of the rhizosphere suspension from each root were spread on the surface of a TSM plate. All plates were incubated for up to two weeks at room temperature in order to detect the presence of *Trichoderma* colonies emerging from root tissues or from the rhizosphere. Whenever *T. virens* developed out of at least one piece of each root, that root was considered as colonized. As an additional control, particles of plant growing substrate used for potting plants were scattered over the surface of TSM plates (about 0.1 g per plate, 18 plates) and incubated for up to two weeks at room temperature.

To verify the presence/absence of *T. virens*, *Trichoderma* colonies arising from roots, rhizosphere and plant growing substrate (as a control) were isolated on TSM plates. They were then compared with *T. virens* I10 reference colonies of the same age grown on TSM.

RESULTS

Generation and verification of *T. virens tvpg2* knockout mutants

The gene to be deleted was the *T. virens* constitutive endopolygalacturonase *tvpg2* (Baroncelli et al., 2016). A 5.2 kb deletion cassette, including a 5' *tvpg2* upstream region (1.32 kb), the *hphB* marker (2.4 kb) and a 3' *tvpg2* downstream region (1.45 kb), was inserted into the final pSGEPG vector and used for transformation. In order to facilitate the integration of the vector pSGEPG into the native *tvpg2* locus, we made use of a *T. virens* mutant (B14) derived from the I10 isolate which bears a knocked out *tku70* gene responsible for ectopic integration (Catalano et al. 2011). The transformation and the selection for mitotically stable, hygromycin resistant transformants resulted in three strains (2, 8 and 12) with the *tvpg2* gene potentially replaced.

To prove the knockout in these transformants, they were analyzed by PCR with primers including the *tvpg2* 5' and 3' flanking regions (A/D in Table 1) comparing them to wild-type I10 and I10Δ*tku70* B14 strains. The expected molecular amplicon weights, 4.0 kb in the wild-type and

B14, and 5.2 kb (displaced *tvpg2* + cassette) in mutants were observed (Fig. 1A), confirming the deletion event in all transformants. To verify the integration of the vector into only a single site of the mutants' genomes, an *Eco*RV Southern blot analysis was performed, since this enzyme cuts at 5' and 3' *tvpg2* flanking regions, which were both inserted in the deletion cassette. The probe was obtained by PCR on the plasmid pSGEPG with A/D primers (including the deletion cassette). The results were consistent with expectations: a 2.8 kb fragment (unaltered *tvpg2*, 2829 bp) in I10 wild type and in B14, and a 4.1 kb fragment (deleted *tvpg2*, 4061 bp) in knockout strains were found, indicating a single site insertion in the transformants' genomes (Fig. 1B).

Phenotype comparison of the knockout strains

Since the presence of the knocked out *tku70* locus in the transformants impaired all attempts to complement the deleted *tvpg2* strains, we used three independent transformants (2, 8 and 12) in order to assess their basal physiological properties.

Fungal growth rates. After regression analysis of growth curves (all highly significant, $R^2 \geq 0.95$ and $P<0.0001$) no differences between the parent strains I10 or B14 and the three mutant strains were detected ($P_{slope} \geq 0.108$) (Fig. 2A).

Conidial germination. Spore germination percentages were submitted to ANOVA, after angular transformation, to compare I10 and B14 with the three transformants. Efficiency of germination was not statistically altered in 2 and 12 compared to strain I10, but 8 showed a significant reduction in the percentage of germination. No differences were recorded between B14 and the three transformants (Fig. 2B).

In vitro confrontation tests. The ability of the three mutants to antagonize and mycoparasitize *Botrytis cinerea* was evaluated and compared with I10 and B14, previously reported as antagonist against this pathogen (Catalano et al. 2011). When growth curves of the pathogen in dual cultures

were analysed, all mutant strains significantly inhibited the growth of *B. cinerea* ($P_{slope} \leq 0.002$) (Fig. 2C). After 21 days, all transformants sporulated on the pathogen, thus indicating mycoparasitism. No differences were apparent among the three knockout strains and I10 or B14 (Fig. 2D). No coilings around *B. cinerea* hyphae were observed, neither by the wt I10 and B14, in agreement with Catalano et al. (2011), nor by any of the transformants.

Because of the consistency in the analyzed phenotypes, a single mutant (number 2) was selected for further analyses, hereafter Δ*tvpg2*.

In vitro expression analysis in response to pectin or plant cell walls

In order to check the expression of *tvpg1* and *tvpg2* genes on different carbon sources, the strains I10, B14 and Δ*tvpg2* were grown in liquid cultures amended with glucose as a control and two potentially inducing substrates, pectin and tomato cell walls. Samples were collected at 48, 72, and 96 h after inoculation and analysed by Reverse Transcription PCR. No differences were seen in the expression shown by the parent strains I10 and B14 (Fig. 3A and B respectively), which is in accordance with the pattern reported earlier in strain I10 (Baroncelli et al. 2016). Instead, none of the transcripts were present in the Δ*tvpg2* knockout mutant (Fig. 3C) suggesting that *tvpg2* plays a regulatory role in *tvpg1* expression.

The fungal growth on different media was also evaluated. The biomasses obtained from strains I10, B14, and Δ*tvpg2* in each liquid inducing medium were measured, and no significant differences were detected among the three fungi in any condition (data not shown).

TvPG2 plays an essential role in the expression of *T. virens tvpg1* and tomato *Lepgpi1* during the interaction with tomato roots

To investigate the consequences of *tvpg2* deletion on the interaction between *T. virens*-and tomato roots, we tested the expression of *tvpg1*, encoding the inducible endopolygalacturonase (Baroncelli et al. 2016), and of tomato *Lepgpi1*, which encodes an endopolygalacturonase inhibitory protein (PGIP). To this end, tomato roots were *in vivo* inoculated with I10wt, B14 or Δ*tvpg2* for 24, 48 and 72 h. *T. virens* strains (I10, B14, Δ*tvpg2*) in the soil without plants, and non-inoculated roots were grown as controls. Consistent with earlier findings (Catalano et al. 2011), the I10 isolate constitutively expressed *tvpg2* whereas *tvpg1* was induced with a peak at 48 h (Fig. 4 A), and the *Lepgip1* expression paralleled that of *tvpg1* with highest levels at 48 h (Fig. 4 B). As expected, the B14 expression pattern overlapped the pattern shown by I10 (Fig. 4 A/B). On the other hand, during the interaction with tomato roots the expression of all Δ*tvpg2* genes exhibited a different pattern compared to I10 and B14 genes. The expected lack of *tvpg2* transcripts was evident, however *tvpg1* and *Lepgip1* were not expressed either (Fig. 4 A/B). These results suggest that TvPG2 plays a regulatory role in the induction of *tvpg1*, and as a consequence is also crucial for the expression of *Lepgip1*.

Induction of systemic resistance in tomato

The results described so far prompted us to investigate whether TvPG2 is necessary for triggering an ISR in plants. Tomato plants were thus *in vivo* pre-inoculated with the *T. virens* strains I10, B14 or Δ*tvpg2* and after one week plants were inoculated by *B. cinerea*. Specific *B. cinerea* necrotic symptoms occurring on plant leaves were evaluated 15 days after infection (dpi). In order to quantify the plant response, the number of lesions per leaf was recorded for all inoculated leaves present on plants. The resulting data (Table 2) revealed a significant difference between I10/B14 and Δ*tvpg2*, and all three were in turn significantly dissimilar to the *B. cinerea* control (in all cases $P<0.001$). Evidently, the induction of the resistance mechanism prompted by I10 and B14 strains in the plant is strongly reduced (about 100-fold) using the mutant Δ*tvpg2*. Pre-treating roots

with the strain Δ*tvpg2* still resulted in some protection of the tomato against *B. cinerea* (about five times less than the *B. cinerea* control) revealing, however, that TvPG2 is not the only signal of *T. virens* that acts as an elicitor of ISR.

Root and rhizosphere colonization

The ability of I10, B14 and Δ*tvpg2* to grow in the tomato roots or to colonize the rhizosphere was evaluated on two-week-old plants, seven days after inoculation of the roots. Observations were made two weeks after plating roots and rhizosphere suspensions on TSM. The plant growing substrate itself was also plated on TSM and no *T. virens* colonies were observed. The few *Trichoderma* colonies present had a clearly different morphology compared to *T. virens* I10 when grown on the same medium (data not shown). Moreover, no *Trichoderma* colonies were observed also in plates seeded by roots or rhizosphere suspensions derived from control plants, suggesting that the isolates naturally present in the plant growing substrate were unable to grow in the roots and unable to colonize the rhizosphere (H_2O ctrl, Table 3).

From this semi-quantitative analysis, no differences in the percentage of plant roots colonized by each strain was detected. The morphology of all the developed colonies was similar to that of a pure colony of *T. virens* I10 grown for the same time on TSM. The ability of *T. virens* I10 and its mutants to grow in plant roots is, therefore, very limited (11%) and is not affected by the mutations investigated in this paper. Instead *T. virens* was recorded on all plates (100%) derived from the rhizosphere of plants inoculated with I10, B14 and Δ*tvpg2* (Table 3). These results confirm that I10 is a good rhizosphere competent isolate and not an endophyte. This suggests that mutations in B14 and in Δ*tvpg2* do not modify the relationships established between I10 and roots/rhizosphere of tomato plants.

DISCUSSION

Some *Trichoderma* spp. colonize the rhizosphere and establish interactions with plant roots, although the physical association is usually limited to the outermost external layers (Harman et al. 2004). This association triggers a molecular cross-talk between the two organisms which leads to reciprocal beneficial events, such as nutrients supply for *Trichoderma* and growth promotion, or immunity against pathogens in plant. Pectinases, and especially endopolygalacturonases (EPGs), are required by plant pathogenic fungi to gain access to the host. In turn, plants recognize the presence of pectinases and activate appropriate plant defense responses (Cervone et al. 1989).

In contrast to other plant cell wall degrading enzymes (Enkerli et al. 1999), the activity of endopolygalacturonases is essential for this stimulation, since plant cell wall-derived oligogalacturonides within a limited range of polymerization, function as elicitors of plant responses (Cervone et al. 1989). The release of these oligomers initiates a PGIP (PolyGalacturonase Inhibiting Protein) mediated signaling mechanism and elicits defence reactions (Federici et al. 2006). PGIPs thereby reduce the pectinase activity, which would otherwise continue to degrade the oligomers and produce molecules that are too short to function as elicitors (D'Ovidio et al. 2004).

Previous *in vivo* investigations on tomato roots inoculated by *T. virens* showed the expression of two EPGs (an inducible gene, *tvpg1*, and a constitutive gene *tvpg2*), and the induction of a plant PGIP encoding gene, *Lepgip1*, which paralleled the expression of *tvpg1* (Baroncelli et al. 2016). These data suggested the activity of an EPG-PGIP recognition system. In addition, the availability of both constitutive and inducible EPGs in *T. virens* suggests the potential for different functionalities during its interaction with plants, as reported elsewhere (Kemp et al. 2004). In fungi, the presence of constitutively expressed EPGs has been linked to aggressiveness and pathogenic potential against plants (Shieh et al. 1997; Wubben et al. 1999). It may shorten the time of contact with the plant and allow the pectin methyl esterase to degrade the esterified pectin

and thus decrease the pH thereby triggering the expression of the inducible genes (Kemp et al. 2004). In this regard it is notable that, with the exception of *T. atroviride,* TvPG2 has no ortholog in other *Trichoderma* spp. Proteins with the highest similarity were found in *Fusarium* spp. (C.P. Kubicek, unpublished data) suggesting its possible origin as a result of horizontal gene transfer. This would explain the "pathogen-like" behaviour of the constitutive TvPG2 towards plants. Furthermore the alkaline isoelectric point of TvPG2, theoretical 9.6, fits models where a constitutive enzymatic activity opens the plant cell wall and thus provides access for other degrading enzymes working at a lower local pH, such as TvPG1 which has a theoretical isoelectric point of 6.3 (Baroncelli et al. 2016; Kemp et al. 2004).

Our study showed, both *in vitro* and *in vivo*, that the inducible *tvpg1* is also not expressed in the absence of TvPG2, thus indicating a regulative action by the constitutive *tvpg2* on *tvpg1*. In addition, the deletion of *tvpg2* leads to a simultaneous loss of *tvpg1* and *Lepgip1* transcription during the interaction between *T. virens* and tomato. This outcome confirms the coordinated action of the two fungal genes and the plant gene under analysis, implying that TvPG2 is needed in order to induce the expression of both *tvpg1* and *Lepgip1*.

These findings may be explained by hypothesizing that the constitutive TvPG2 works at the first contact with plant roots and consequently triggers the expression of *tvpg1* in *T. virens* and of *Lepgip1* in tomato. For the moment it is unclear whether *tvpg2* induces *Lepgip1* directly (by releasing oligogalacturonides) or indirectly (by activating the expression of *tvpg1* which in turn releases oligogalacturonides).

Structural properties of EPGs have been widely studied and the target amino acids crucial for the PGIP recognition, the formation of an EPG-PGIP complex and therefore EPG inhibition by PGIP have been identified (Raiola et al. 2008; Stotz et al. 2000). Note that both *T. virens* TvPG1 and TvPG2 (Baroncelli et al, 2016) contain, at fixed positions in relation to the active site, the three amino acid residues that are strictly required for interacting with PGIPs in *Fusarium moniliforme*

pectinases, i.e. H188, R267 and K269, (Federici et al. 2001). This *in silico* result is consistent with the potential ability of TvPG1 and TvPG2 to recognize a plant PGIP.

In order to verify whether the PGIP activation by *T. virens* in tomato is able to also elicit a plant immune response, a three-way plant-pathogen-antagonist interaction system has been developed. *Trichoderma*-plant-pathogen interactions have already been used for monitoring the induction of plant resistance (Howell et al. 2000), for the analysis of the *Trichoderma atroviride* proteome (Marra et al. 2006), and to study the function of selected *Trichoderma* genes during biological control (Mukherjee et al. 2012; Vinale et al. 2008). With regards to the latter, a gene encoding an inducible EPG, *Thpg1*, was silenced in *T. harzianum* and shown to be involved in tomato root colonization. However, and unlike the present study, *in vivo* biocontrol assays on plants infected with *B. cinerea* did not show significant differences between the wild-type and the *Thpg1* silenced strain (Morán-Diez et al. 2009). In our approach, tomato roots had been *in vivo* inoculated by *T. virens* and leaves were then sprayed by *B. cinerea*. The effective systemic response of the tomato plants to *B. cinerea* infection, which was promoted by root pre-treatment with the *T. virens* wild type strain, was strongly impaired when the *T. virens* mutant with a deleted *tvpg2* was used. These data again underline the importance of constitutively expressed EPGs and confirm the crucial role of the *tvpg2* gene in triggering the PGIP signaling pathway, which in turn activates an ISR response in plants.

Data regarding the root and the rhizosphere colonization showed no difference in behavior between the mutant and the two control strains. This observation, although within the limits of a semi-quantitative technique, suggests that there is no interference of a possible impaired colonization with the reduced induction of the plant response by Δ*tvpg2*. Furthermore the poor ability to penetrate into plant roots and the high rhizosphere competence shown by I10 isolate is consistent with other reports on rhizosphere competent fungi inducing systemic resistance without needing to be endophytic (Druzhinina et al, 2011; Harman et al, 2004).

In conclusion we believe that our investigation contributes to understanding the interaction of a biocontrol agent with a plant and how it modulates the plant reaction to a pathogen attack. The data set reported shows a coordinated regulation between the two *T. virens* EPGs and a tomato PGIP, which results in the activation of ISR against *B. cinerea*. All these events are dependent on the presence of the constitutive *tvpg2*, which appears to regulate the cross-talk between *T. virens* and tomato, mimicking the behavior of a fungal plant pathogen. Given the importance of eliciting systemic plant defense responses as an antagonistic mechanism versus pathogens, the beneficial effects of this *T. virens* isolate and the role of its TvPG2 offer strategies for further improvements towards successful biological control.

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LITERATURE CITED

Annis, S. E., and Goodwin, P. H. 1997. Recent advances in the molecular genetics of plant cell wall-degrading enzymes produced by plant pathogenic fungi. Eur. J. Plant Pathol. 103:1-14.

Baroncelli, R., Matarese, F., Moncini, L., Vannacci, G., and Vergara, M. 2016. Two endopolygalacturonase genes in *Trichoderma virens*: *in silico* characterisation and expression during interaction with plant. J. Phytopathol. 164:18-28. doi: 10.1111/jph.12414

Benitez, T., Rincòn, A. M., Limòn, M. C., and Codòn, A. C. 2004. Biocontrol mechanisms of *Trichoderma* strains. Int. Microbiol. 7:249-260.

Catalano, V., Vergara, M., Hauzenberger, J. R., Seiboth, B., Sarrocco, S., Vannacci, G., Kubicek, C. P., and Seidl-Seiboth, V. 2011. Use of a non-homologous end-joining-deficient strain

(delta-ku70) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene *lcc1* in sclerotia degradation. Curr. Genet. 57:13-23.

Cervone, F., Hahn, M. G., De Lorenzo, G., Darvill, A., and Albersheim, P. 1989. Host-pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. 90:542-548.

Contreras-Cornejo, H. A., Macias-Rodríguez, L., Cortés-Penagos, C., and López-Bucio, J. 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin dependent mechanism in Arabidopsis. Plant Physiol. 149:1579-1592.

De Meyer, G., Bigirimana, J., Elad, Y., and Hofte, M. 1998. Induced systemic resistance in *Trichoderma harzianum* T39 biocontrol of *Botrytis cinerea*. Eur. J. Plant Pathol. 104:279-286.

Di Pietro, A., and Roncero, M. I. G. 1998. Cloning, expression, and role in pathogenicity of *pg1* encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol. Plant Microbe Interact. 11:91-98.

D'Ovidio, R., Mattei, B., Roberti, S., and Bellincampi, D. 2004. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant-pathogen interactions. Biochem. Biophys. Acta 1696:237-244.

Druzhinina, I. S., Seidl-Seiboth, V., Herrera-Estrella, A. et al. 2011. *Trichoderma*: the genomics of opportunistic success. Nat. Rev. Microbiol. 9:749-759. doi: 10.1038/nrmicro2637

Elsharkawy, M. M., Shimizu, M., Takahashi, H., Ozaki, K., and Hyakumachi, M. 2013. Induction of Systemic Resistance against Cucumber mosaic virus in Arabidopsis thaliana by *Trichoderma asperellum* SKT-1. Plant Pathol. J. 29:193-200.

Enkerli J, Felix G, and Boller T. 1999. The enzymatic activity of fungal xylanase is not necessary for its elicitor activity. Plant Physiol. 121:391-397.

Federici. L., Caprari, C., Mattei, F., Savino, C., Di Matteo, A., De Lorenzo, G., Cervone, F., and Tsernoglou, D. 2001. Structural requirements of *endo*-polygalacturonase for the interaction with PGIP (polygalacturonase-inhibiting protein). Proc. Natl. Acad. Sci. USA 98:13425-13430.

Federici, L., Di Matteo, A., Fernandez-Recio, J., Tsernoglou, D., and Cervone, F. 2006. Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends in Plant Sci. 11:65-70.

Fiorini, L., Guglielminetti, L., Mariotti, L., Curadi, M., Picciarelli, P., Scartazza, A., Sarrocco, S., and Vannacci, G. 2016. *Trichoderma harzianum* T6776 modulates a complex metabolic network to stimulate tomato cv. Micro-Tom growth. Plant Soil. 400:351-366. doi: 10.1007/s11104-015-2736-6

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2:43-56.

Hartl, L., Kubicek, C. P., and Seiboth, B. 2007. Induction of the *gal* pathway and cellulase genes involves no transcriptional inducer function of the galactokinase in *Hypocrea jecorina.* J. Biol. Chem. 282:18654–18659.

Hartl, L., and Seiboth B. (2005) Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette. Curr. Genet. 48:204–211.

Hermosa R., Viterbo A., Chet I., and Monte E. 2012. Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology 158:17-25.

Herron, S. R., Benen, J. A. E., Kester, H. C. M., and Visser, J. 2000. Structure and function of pectic enzymes: virulence factors of pathogens. Proc. Natl. Acad. Sci. USA, 97:8762-8769.

Howell, C. R., Hanson, L. E., Stipanovic, R. D., and Puckhaber, L. S. 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. Phytopathology 90:248-252.

Kemp, G, Stanton, L., Bergmann, C.W., Clay, R.P., Albersheim, P., and Darvill, A. 2004. Polygalacturonase-Inhibiting Proteins Can Function as Activators of Polygalacturonase. Mol. Plant Microbe Interact. 17:888-894.

Kubicek, C. P., Herrera-Estrella, A., Seidl-Seiboth, V., et al. 2011. Comparative genome sequence analysis underscores mycoparasitism as the ancestral life style of *Trichoderma.* Genome Biol. 12:R40. doi: 10.1186/gb-2011-12-4-r40

Kubicek, C. P., Mach, R. L., Peterbauer, C. K., and Lorito, M. 2001. *Trichoderma*: from genes to biocontrol. J. Plant Pathol. (Special Issue) 83:11-23.

Kubicek, C.P., Starr, T.L., and Glass, N.L. 2014. Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. Annu. Rev. Phytopathol. 52:427-451.

Lotan, T., and Fluhr, R. 1990. Xylanase, a novel elicitor of pathogenesis-related proteins in tobacco, uses a non-ethylene pathway for induction. Plant Physiol. 93:811-817.

Marra, R., Ambrosino P.,·Carbone, V., Vinale, F., Woo S., L., Ruocco., M., et al. 2006. Study of the three-way interaction between *Trichoderma atroviride*, plant and fungal pathogens by using a proteomic approach. Curr. Genet. 50:307-321.

Morán-Diez, E., Hermosa, R., Ambrosino, P., Cardoza, R. E., Gutiérrez, S., Lorito, M., and Monte, E. 2009. The *thpg1* endopolygalacturonase is required for the *Trichoderma harzianum*-plant beneficial interaction. Mol. Plant Microbe Interact. 22:1021-31.

Mukherjee, M., Mukherjee, P. K., Horwitz, B. A., Zachow, C., Berg G., and Zeilinger, S. 2012. *Trichoderma*–Plant–Pathogen Interactions: Advances in Genetics of Biological Control. Indian J. Microbiol. 52:522–529.

Raiola, A., Sella, L., Castiglioni, C., Balmas, V., and Favaron, F. 2008. A single amino acid substitution in highly similar endo-PGs from *Fusarium verticillioides* and related *Fusarium* species affects PGIP inhibition. Fungal Genet. Biol. 45:776-789.

Salas-Marina, M. A., Silva-Flores, M. A., Uresti-Rivera, E. E., Castro-Longoria, E., Herrera-Estrella, A., and Casas-Flores, S. 2011. Colonization of *Arabidopsis* roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonic acid/ethylene and salicylic acid pathways. Eur. J. Plant Pathol. 131:15-26.

Sambrook, J., and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Lab. Press, Painview, NY.

Sarrocco, S., Guidi, L., Fambrini, S., Degl'Innocenti, E. and Vannacci, G. 2009. Competition for cellulose exploitation between *Rhizoctonia solani* and two *Trichoderma* isolates in the decomposition of wheat straw. J. Plant Pathol. 91:331-338.

Sarrocco, S., Mikkelsen, L., Vergara, M., Jensen, D. F., Lübeck, M., and Vannacci, G. 2006. Histopathological studies of sclerotia of phytopathogenic fungi parasitized by a GFP transformed *Trichoderma virens* antagonistic strain. Mycol. Res. 110:179-187.

Shieh, M-T., Brown, R. L., Whitehead, M. P., Cary, J. W., Cotty, P. J., Cleveland, T. E., and Dean, R. A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63:3548–3552.

Shoresh M, Harman GE, and Mastoury F. 2010. Induced systemic resistance and plant responses to fungal biocontrol agents. Annu. Rev. Phytopathol. 48:21-43.

Stotz, H. U., Bishop, J. G., Bergmann, C. W., Koch, M., Albersheim, P., Darvill, A. G., and Labavitch, J. M. 2000. Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. Physiol. Mol. Plant Pathol. 56:117-130.

Vannacci, G., and Pecchia, S. 2000. Discovery of *Trichoderma* I252 and *Gliocladium* I10, components of the biofungicide GT10/252. European Cost 830 Workshop 'Selection Strategies for Plant Beneficial Microorganisms', Nancy, 3-5 April 2000.

Velázquez-Robledo, R., Contreras-Cornejo, H.A., Macías-Rodríguez, L., Hernández-Morales, A. Aguirre, J., Casas-Flores, S., López-Bucio, J., and Herrera-Estrella A. 2011. Role of the 4-Phosphopantetheinyl transferase of *Trichoderma virens* in secondary metabolism and induction of plant defense responses. Mol. Plant Microbe Interact. 24:1459-1471.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., Marra, R., Woo, S. L., and Lorito, M. 2008. *Trichoderma*–plant–pathogen interactions. Soil Biol. Biochem. 40:1-10.

Wubben, J. P., Mulder, W., Ten Have, A., Van Kan, J. A. L., and Visser, J. 1999. Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. Appl. Environ. Microb. 65:1596-1602.

Yedidia, I., Benhamou, N., and Chet, I. 1999. Induction of defense responses in cucumber plants (*Cucumis Sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Appl. Environ. Microb. 65:1061-1070.

Zhao, Z., Liu, H., Wang, C., and Xu, J.R. 2013. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. BMC Genomics 14:274. doi: 10.1186/1471-2164-14-274

CAPTIONS FOR FIGURES

Fig. 1. Molecular analyses of transformants

I10: *T. virens* wild type strain; B14: I10Δ*Ku70* strain; 2, 8 and 12: *tvpg2* knockout transformants derived from B14. **A.** PCR with A/D primers external to the *tvpg2* coding region. **B.** Southern blot with genomic DNAs restricted by *Eco*RV and a probe containing the deletion cassette.

Fig. 2. Phenotypic analyses of transformants

The samples are the same as in Fig. 1. **A:** Growth curves of I10, B14 and transformants 2, 8, 12 grown in PDB. Each value represents the average of three independent replicates. Curves were used for the ANOVA for regression. **B:** Conidial germination (expressed as percentage) of I10, B14 and transformants. Different letters correspond to significantly different values according to ANOVA analysis ($P \leq 0.05$). Data represent the average of three biological replicates, each consisting of three slides per strain; forty conidia were counted for each slide. **C:** Growth curves of *B. cinerea* in the presence/absence of each of the three transformants. For each antagonist/pathogen combination three independent replicates were made. Curves were used for the ANOVA for regression. **D:** Overgrowth and sporulation of I10, B14 and transformants on *B. cinerea*.

Fig. 3. RT-PCR patterns of I10, B14 and Δ*tvpg2* in response to pectin or plant cell walls.

G48, G72, G96: glucose cultures after 48, 72, 96 h; P48, P72, P96: pectin cultures at the same times; W48, W72, W96 plant cell wall cultures at the same times. A: I10; B: B14; C: Δ*tvpg2*. For each isolate, the first line corresponds to E/F primers for *tvpg1* (171 bp), the second line to G/H primers for *tvpg2* (194 bp), and the third line to K/L primers for *tef1* (258 bp) as a reference gene.

Fig. 4. qRT-PCR during the *T. virens*/tomato interaction

A: *tvpg1* and *tvpg2* gene expression; **B:** *Lepgip1*gene expression. Tc: I10 control; TR: roots inoculated by I10. Bc: B14 control; BR: roots inoculated by B14. Δc: Δ*tvpg2* control; ΔR: roots inoculated by Δ*tvpg2*; Rc: tomato roots control. Times of sampling: 24, 48, 72 hours after fungal inoculation on roots in perlite. Transcript levels are indicated in relative units, assuming as unitary the sample with the lowest expression value. Each value is the mean +/- s.d. To normalize data *tef1* and *β-tubulin* were used as fungal reference genes for *tvpg1* and *tvpg2* expression, *LeEF1A* as a plant reference gene for *Lepgip1* expression. Data represent the average of three internal technical replications, carried out for each of the two biological replicates of the experiment.

Table 1 Primers used for transformation and RT-PCR

Name	Sequence 5'-3'	Gene	T annealing	Product size
A: Pga5*Spe*If	CGACTAGTGAAAGAAACCGGCTATACACG*	5' flanking *tvpg2*	56 °C	1321 bp
B: Pga5*Bam*HIr	GCGGATCCTAAGAGTTTACTGGCGGACAAG*			
C: Pga3*Hind*IIIf	GATAAGCTTCAGAGAATGCGAGTAGCCTC*	3' flanking *tvpg2*	56 °C	1449 bp
D: Pga3*Xho*Ir	GTCTCGAGGGATGAGTTTGTTGGATTTG*			
E: Tvpg1F	GTA TCA AGG CGA CTG CTG GT	*tvpg1*	57 °C	171 bp
F: T2NR	AGATGTTCTGGACGACGAGG			
G: Tvpg2F	TGG AGC GAC TGG TTC TGT TT	*tvpg2*	57 °C	194 bp
H: EPT1IR	ACG TAT TTG TGC CAC CCG AC			
I: BT2A	GGTAACCAAATCGGTGCTGCTTTC	*β-tubulin*	53 °C	258 bp
J: BT2B	ACCCTCAGTGTAGTGACCCTTGGC			
K: EF1728F	CATCGAGAAGTTCGAGAAGG	*tef1***	53 °C	258 bp
L: EF1986R	TACTTGAAGGAACCCTTACC			
M: PGIPtomF	CCG GAT AAA TGC TCT CAC C	*Lepgip1*	55 °C	203 bp
N: PGIPtomR	ATT CAG GGA TCG GAC CTG T			
O: LeEF1AF	GCTGCTGTAACAAGATGGATGC	Tomato *tef1***	60 °C	119 bp
P: LeEF1AR	GGGGATTTTGTCAGGGTTGTAA			

*Underlined nucleotides correspond to target sites of restriction enzymes

***tef1*: translation elongation factor 1-alpha

1
2

Table 2. *Botrytis cinerea* disease severity on tomato leaves

Treatment	Lesions*		Total leaves**
B. cinerea ctrl	5.12 ± 3.14	a***	107
B. cinerea + I10	0.01 ± 0.08	c	145
B. cinerea + B14	0.01 ± 0.11	c	141
B. cinerea + Δ*tvpg2*	0.96 ± 1.52	b	121

Plants infected by *B. cinerea* (15 dpi) without any pre-treatment (ctrl), or pre-inoculated by I10, B14 or Δ*tvpg2*. *Averaged numbers of lesions (± standard deviation) per leaf, resulting from three replicates each consisting of two pots, three plants per pot. **Number of leaves observed for each treatment, corresponding to the sum of inoculated leaves in all replicates. ***Different letters correspond to significantly different values ($P<0.001$), according to ANOVA analysis.

3
4

5

1

2

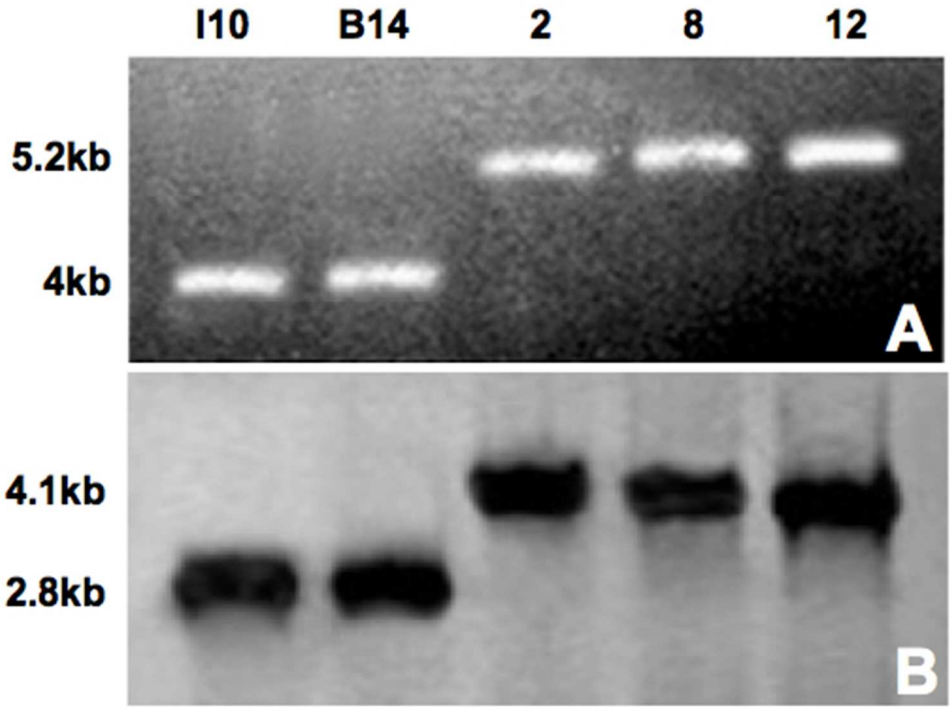
Table 3. Colonization of tomato roots or rhizosphere by *T. virens* strains

Treatment	Roots (%)	Rhizosphere (%)
H_2O ctrl	0	0
I10	11	100
B14	11	100
Δ*tvpg2*	11	100

Plant roots, one week old, were inoculated by I10, B14 or Δ*tvpg2* and by water (ctrl). Roots: samples rinsed, removed from the plant, sterilized, cut in pieces and laid on TSM plates. Rhizosphere: roots washing off spread on TSM plates. All samples were observed up to two weeks after plating for checking the presence of *T. virens*. Data represent the percentage of roots or rhizosphere samples giving rise to *T. virens* colonies. Three independent replicates, each consisting of two pots per treatment, each one containing three plants, were tested.

3

4



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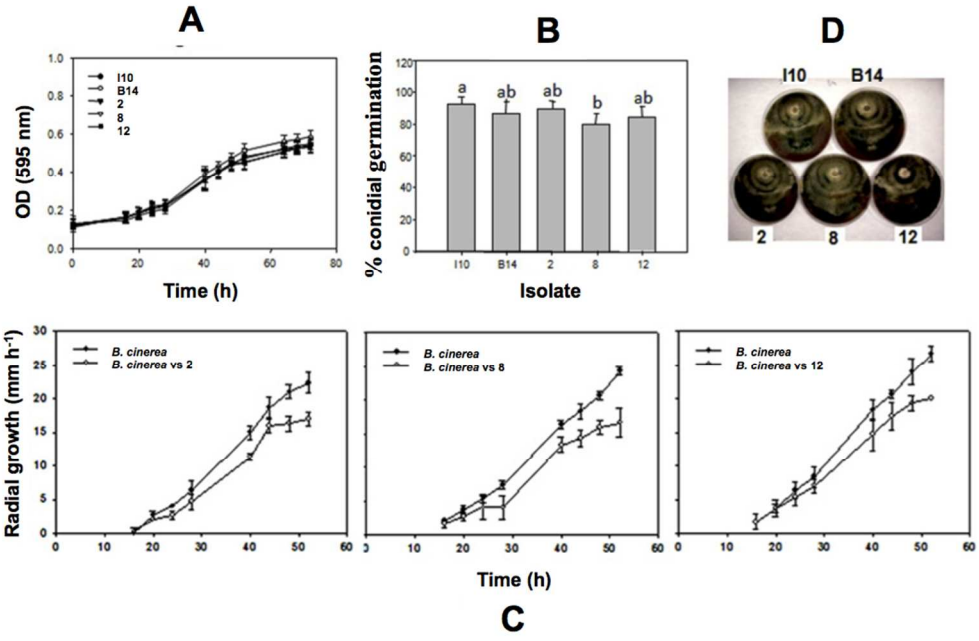

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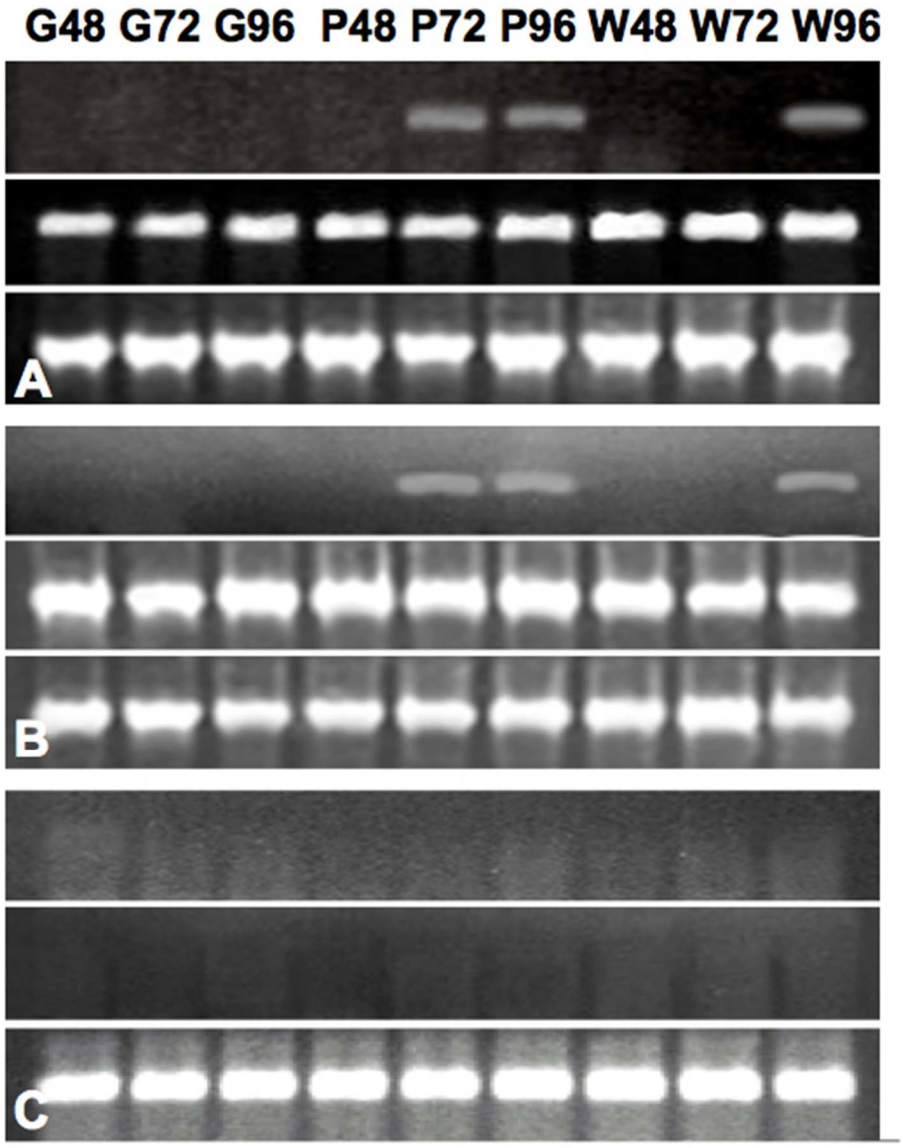
183x120mm (150 x 150 DPI)



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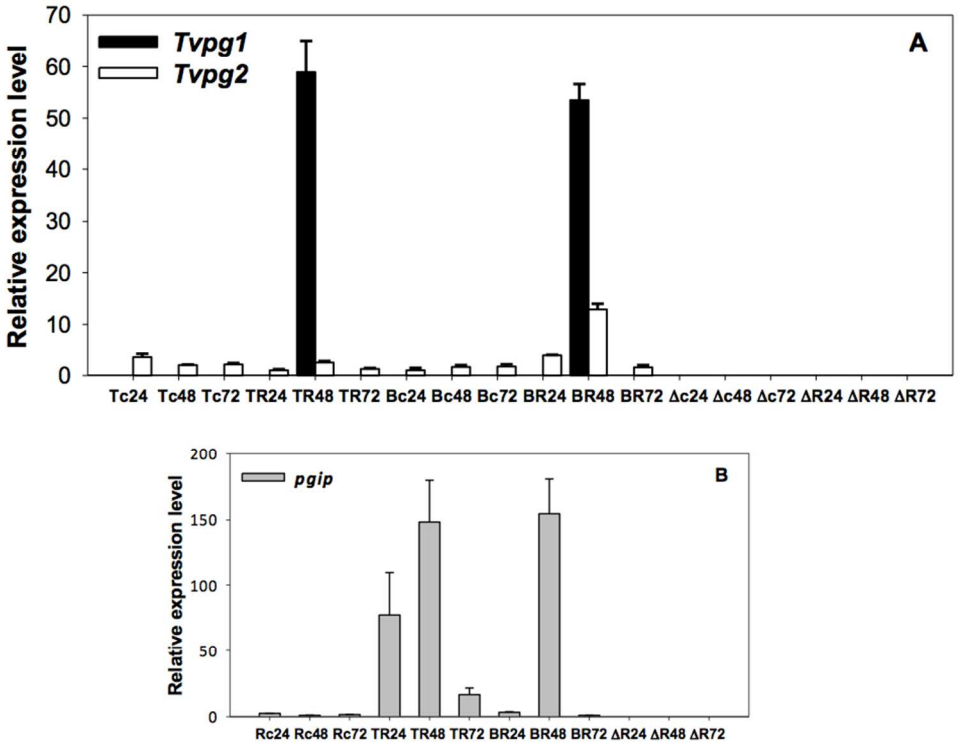

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168x132mm (150 x 150 DPI)