Involvement of Jasmonic Acid/Ethylene Signaling Pathway in the Systemic Resistance Induced in Cucumber by *Trichoderma asperellum* T203

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ABSTRACT

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Trichoderma spp. are effective biocontrol agents for a number of soilborne plant pathogens, and some are also known for their ability to enhance plant growth. It was recently suggested that *Trichoderma* also affects induced systemic resistance (ISR) mechanism in plants. Analysis of signal molecules involved in defense mechanisms and application of specific inhibitors indicated the involvement of jasmonic acid and ethylene in the protective effect conferred by *Trichoderma* spp. against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*. Moreover, examination of local and systemic gene expression by real-time reverse

transcription-polymerase chain reaction analysis revealed that *T. asperellum* (T203) modulates the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (*Lox1*, *Pal1*, *ETR1*, and *CTR1*) in cucumber plants. We further showed that a subsequent challenge of *Trichoderma*-preinoculated plants with the leaf pathogen *P. syringae* pv. *lachrymans* resulted in higher systemic expression of the pathogenesis-related genes encoding for chitinase 1, β -1,3-glucanase, and peroxidase relative to noninoculated, challenged plants. This indicates that *Trichoderma* induced a potentiated state in the plant enabling it to be more resistant to subsequent pathogen infection.

Additional keyword: salicylic acid.

Growing awareness of the environmental damage caused by the use of chemical substances for plant disease control in agriculture has raised the need to study biological alternatives, such as activating the defense response of plant crops by inducers that are not toxic to the environment. *Trichoderma* spp. are effective biocontrol agents for a number of soilborne pathogens and are also known for their ability to enhance plant growth (32,40). However, most studies on reducing disease incidence in soil treated with *Trichoderma* spp. have focused on microbial interactions rather than host plant responses (12).

Recently, it has been shown that *T. asperellum* (formerly *T. harzianum* T203) (19) can penetrate the roots of cucumber seedlings and colonize the epidermis and outer root cortex (38). These interactions also induce host plant resistance to pathogens even in the upper part of the plant (38–40). *Trichoderma* root inoculation has been shown to be effective against different types of pathogens in a wide variety of plants, including cucumber (9).

Contact with pathogenic and nonpathogenic microorganisms triggers a wide range of defense mechanisms in plants that protect them against invasion. One such response is known as systemic acquired resistance (SAR). SAR, commonly triggered by local infection, can provide long-term resistance throughout the plant to subsequent infection by different pathogens. The SAR response correlates with the activation of pathogenesis-related (PR) genes. This process generally requires the involvement of the signal molecule salicylic acid (SA), which has been shown to increase in both infected and uninfected tissues (34). Colonization of roots by certain rhizospheric bacteria confers another form of dis-

ease resistance termed induced systemic resistance (ISR). Several studies have shown that ISR is not an SA-dependent phenomenon but rather requires components of the jasmonic acid (JA) signaling pathway followed by the ethylene signaling pathway (26,27,36).

The first enzyme in the biosynthesis pathway of JA is lipoxygenase (LOX). LOX utilizes linoleic and linolenic acids for the production of JA and controls a feed-forward loop in jasmonate synthesis (21,28).

Several components of the ethylene signal transduction pathway have been identified and include ETR1 and CTR1 (5,13). The *ETR1* gene is related to the super-family of catalytic receptors in prokaryotes referred to as two-compound regulators (4). The ETR1 protein is an ethylene receptor with histidine protein kinase activity. The *CTR1* gene acts downstream of the ethylene receptor and encodes a protein most similar to the Raf family of serine/threonine protein kinases that initiate the mitogen-activated protein kinases cascades. Both ETR1 and CTR1 are negative regulators of ethylene signal (14).

The gene encoding for phenylalanine ammonia-lyase (PAL) is believed to be activated by the JA/ethylene signaling pathway in context of induced plant defenses (7,17,23). PAL1 is the first enzyme in the phenylpropanoid biosynthesis pathway, which provides precursors for lignin and phenols, as well as for SA (34). Other enzymes of the phenylpropanoid pathway, including peroxidases, are also induced in resistant reactions (6,39). Peroxidases are also known for their role in the production of phytoalexins, reactive oxygen species, and formation of structural barriers (10).

PR proteins such as β -1,3-glucanase (PR2) and chitinase (PR3), known to disrupt the fungal mycelial wall, can be induced by SA as well as by pathogenic attack (34). However, studies indicate that some chitinase classes, such as class III chitinases (PR8), are induced by JA and not SA (30,31).

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In order to study the mechanisms by which *T. asperellum* induces plant resistance to pathogen infection, we examined the involvement of the plant signal molecules (JA, ethylene, and SA) by measuring hormone levels and the effect of specific inhibitors. We also analyzed the local and systemic expression of defense-related genes in cucumber seedlings inoculated with *T. asperellum* T203 and challenged with the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*. Our study indicates that unlike pathogen-induced SAR, the JA/ethylene signaling pathway is involved in the *Trichoderma*-mediated plant protection process.

MATERIALS AND METHODS

Plant material. Seeds of cucumber (*Cucumis sativus* L. cv. Delila) from Gedera Seeds Co. (Gedera, Israel) were used in this experiment. Plant growth medium (PGM) was prepared according to Yedidia et al. (38).

Fungal material. *T. asperellum* (*T. harzianum* strain T203) was grown on potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD). Synthetic medium (SM) for *T. asperellum* was prepared according to Yedidia et al. (38). The inoculum consisted of 1 ml (10^9 spores, counted by hemacytometer) of 10-day-old *T. asperellum* cultured on PDA added to a 250-ml flask containing 100 ml of SM. The flask was shaken at 150 rpm for 16 to 18 h at 30°C to allow spore germination. The inoculum was then separated from the growth medium by centrifugation at 5,000 rpm at 4°C followed by two washes with 100 ml of distilled water.

Axenic growth system. Seeds were surface-sterilized in 2.0% NaOCl for 2 min and thoroughly washed with sterile distilled water. Seeds (25 per box) were placed on a sterile gauze sheet, which was then placed in an axenic hydroponics growth system (38). Plants were grown in a controlled environment: 26°C, 80% relative humidity, light 300 $\mu\text{E}/\text{m}^2 \text{ s}$, and a circadian cycle of 14 h light and 10 h darkness. The growth chambers enabled the development of cucumber seedlings for up to 3 weeks.

***Trichoderma* plant inoculation.** Mycelial inoculum was added under aseptic conditions to the PGM of 7-day-old seedlings to a final concentration of $\pm 10^5$ germinated spores per ml (38). Control plants were treated with sterile distilled water.

Plants were harvested 1, 3, 6, 9, 24, 48, and 72 h postinoculation (hpi). The induced expression of defense-related genes was examined in roots and leaves. These experiments were repeated twice and each time point represents approximately 20 plants per experiment.

Bacterial inoculum. *P. syringae* pv. *lachrymans* was grown in tryptic soy broth overnight at 30°C. Bacterial cells were pelleted at 5,000 rpm and resuspended in sterile saline-phosphate buffer (5 mM, pH 7.2). Challenge was performed 48 h after inoculating *Trichoderma* to the PGM. *P. syringae* pv. *lachrymans* bacterial suspension (20 μl ; OD = 0.5) containing 0.01% (vol/vol) surfactant (Tween 20) was applied to the surface of the cotyledons and gently smeared with a sterile tip. Bacterial inoculation was performed under aseptic conditions. Multiplication of *P. syringae* pv. *lachrymans* in the cotyledon was assessed 48 h after bacterial inoculation. For each treatment, 10 randomly selected leaves were rinsed thoroughly in sterile water and homogenized in a sterile solution of 10 mM phosphate-saline buffer. Dilutions were plated onto *Pseudomonas*-selective King's B agar supplemented with a 1 ml/liter solution containing basic fuchsin at 9 mg/ml, cycloheximide at 200 mg/ml, nitrofurantoin at 10 mg/ml, and nalidixic acid at 23 mg/ml. After incubation at 28°C for 2 days, the number of *P. syringae* pv. *lachrymans* colony forming units per gram of infected tissue was determined.

For controls, we used noninoculated, nonchallenged plants (–T–P); noninoculated, challenged plants (–T+P); and inoculated, nonchallenged plants (+T–P). Plant roots and leaves were sampled 48 h post-*P. syringae* pv. *lachrymans* infection to exam-

ine expression levels of the studied genes. These experiments were repeated three times with approximately 15 plants per treatment in each experiment.

For measurement of the number of necrotic lesions, *P. syringae* pv. *lachrymans* bacterial suspension (optical density at 600 nm [OD₆₀₀] = 0.1) was applied to the surface of the first and second leaves of cucumber seedlings. Six droplets of 10 μl were applied on each leaf. Necrotic lesions were examined after 5 days.

Treatment with plant hormone inhibitors. Diethylthiocarbamic acid (DIECA; Sigma, St. Louis), a potent inhibitor of jasmonate biosynthesis (22), was added to the root compartment at final concentrations of 50 and 100 μM , 1 h after *Trichoderma* inoculation.

Silver thiosulfate (STS), an inhibitor of ethylene action (1), was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M of silver nitrate in a 4:1 ratio. STS was added to the root compartment at final concentrations of 0.25 and 0.5 mM, 3 h after *Trichoderma* inoculation.

Root colonization efficiency. *Trichoderma* ability to colonize roots with or without STS or DIECA was tested in cucumber roots 2 days after *Trichoderma* inoculation and treatment with the inhibitors. Five plants per replicate were harvested, and their root systems washed, weighed, and homogenized using Ultra-Turrax apparatus (Janke & Kunkel, Staufen, Germany) with water for 1 min. Serial dilutions from both treated and untreated plants were assayed for colony forming units on *Trichoderma*-selective plates. Each treatment was repeated six times. Data were subjected to one-way ANOVA at $P = 0.05$.

Trichoderma-selective plates contained $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g/liter); K_2HPO_4 (0.9 g/liter); KCl (0.15 g/liter); NH_4NO_3 (1 g/liter); glucose (3 g/liter); chloramphenicol (0.25 g/liter); p-dimethylaminobenzenediazo sodium sulfonate (Dexon 70% wettable powder [w.p.], Farbenfabrik Bayer AG, Germany) (0.3 g/liter); pentachloronitrobenzene (Terraclor 75% w.p., Olin Chemicals, Charlestown, TN) (0.2 g/liter); Rose-bengal (0.15 g/liter); and Bacto agar (Difco Laboratories) (20 g/liter).

Ethylene measurement. Two-week-old plants with or without prior *Trichoderma* inoculation were weighed and placed in 30-ml gas-tight tubes. After 1 h, 1-ml gas samples were withdrawn through the rubber seal. The concentration of ethylene was detected by gas chromatography as described by Thompson et al. (35). Wounding of leaves was done to verify our ability to detect ethylene production in plants (data not shown).

SA measurement. Total SA was extracted from cucumber roots and leaves and quantified by high-performance liquid chromatography analysis with spectrofluorescence monitoring as described by Enyedi et al. (8). Treatment with isonicotinic acid, a functional SA analog, was performed to verify our ability to detect SA production in plants (data not shown).

RNA isolation. For RNA analysis, roots, cotyledons, and leaves were harvested at a set of time points after the inoculation and stored at –70°C until required. Total RNA was extracted using the EZ-RNA Total RNA Isolation Kit (Biological Industries Co., Beit-Haemek, Israel). RNA was treated with RNase-free DNase I in 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM MgCl_2 , and 10 mM CaCl_2 for 30 min at 37°C (Roche, Mannheim, Germany). This was followed by a phenol/chloroform and chloroform extraction and a subsequent ethanolic precipitation.

Reverse transcription. After treatment with DNase, 1 μg of total RNA was used for a reverse transcription (RT) reaction using Expand Reverse Transcriptase (Roche) according to the manufacturer's instructions.

Cloning and sequencing. Degenerate primers designed according to several known *Pal1* and *CTR1* plant genes were used to isolate these genes with the Expand High Fidelity PCR System (Roche). Primer sequences are as follows: *Pal1*, forward 5'-TGGAACA(T/C)(A/C)TTTTGGATGGAA-3' and reverse 5'-GCTGTTTT(T/C)CTTG(C/A)TGAGATTA-3'; and *CTR1*, for-

ward 5'-(C/T)ACCGTG(G/C)TGA(T/G)TGG(C/A)ATGG-3' and reverse 5'-CCCACAA(A/G)ATGAC(A/C)CC(A/G)AAG-3'. Polymerase chain reaction (PCR) fragments were cloned in pGEM-T Easy Vector (Promega, Madison, WI) and both strands were sequenced. Sequences are deposited as GenBank accession nos. AF529240 and AF529239 for *Pal1* and *CTR1*, respectively.

Quantitative PCR. According to these sequences and known sequences of other *C. sativus* genes, primers were designed for the quantitative PCR experiments. The size of amplified fragment for all genes was 200 bp, and the annealing temperature of all primers was 60°C. The sequences of the primers used were as follows: *Chit1*, forward 5'-TGGTCACTGCAACCCTGACA-3' and reverse 5'-AGTGGCCTGGAATCCGACT-3' (gi|167514); *ETR1*, forward 5'-GCCATTGTTGCAAAAGCAGA-3' and reverse 5'-GCCAAAGACCACTGCCACA-3' (gi|3641253); β -1,3-glucanase gene, forward 5'-TCAATTATCAAACTTGTTTC-GATGC-3' and reverse 5'-AACCAGGTCTCGGATACAACAAC-3' (gi|3273193); *Lox1*, forward 5'-AAGGTTTGCTGTCCCAAG-3' and reverse 5'-TGAGTACTGGATTAATCCAGCCAA-3' (gi|3668062); *Pal1*, forward 5'-ATGGAGGCAACTTCCAAGGA-3' and reverse 5'-CCATGGCAATCTCAGCACCT-3'; peroxidase gene, forward 5'-AGAGCAACAAGGTCGGTTTCA-3' and reverse 5'-GTGCCGACATCCTAGCTCAAG-3' (gi|553047); *CTR1*, forward 5'-AAACACGTCGGATAAATATGGCTT-3' and reverse 5'-CATCCATTCAAGCGTTCCAG-3'; and 18s, forward 5'-GTGTGCTTTAAGGACTCCGCCA-3' and reverse 5'-AGGGGTACCTCCGCATAGCTAG-3' (gi|7595414). PCR reactions were carried out in 96-well plates (25 μ l per well) in a reaction buffer containing 1 $\times$ SYBR Green PCR Master Mix (PE Applied Biosystems, Foster, CA), 120 nM primers (for each forward and reverse primers), and 1/20 of the RT reaction. Quantitative analysis was performed using the GeneAmp 5700 Sequence Detection System (PE Applied Biosystems) with PCR conditions of 95°C for 15 s and 60°C for 1 min for 40 cycles. The absence of primer-dimer formation was checked in no-template controls. Specificity of primers to cucumber genes was examined by using *Trichoderma* DNA and reverse-transcribed RNA as templates. The 18s ribosomal cDNA was used as a control reference. Each sample was examined in duplicate. We first normalized the expression of the specific gene versus the control reference with the formula $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_T$ specific gene - C_T reference gene, and $\Delta\Delta C_T = \Delta C_T$ - arbitrary constant (the highest ΔC_T). More details can be found in the PE Applied Biosystems Sequence Detector User Bulletin 2. The C_T (threshold cycle) value is defined as the PCR cycle number that crosses an arbitrarily placed threshold line.

RESULTS

Involvement of plant signal molecules in the defense responses induced by *Trichoderma* inoculation. An aseptic hydroponics growth system (38) was used to control growth conditions and medium composition and to eliminate unwanted microorganisms as well as to enable spatial separation of the inducing agent from the pathogen.

First, we tested the protective effect of *Trichoderma*-induced resistance on pathogen challenge. We assessed *P. syringae* pv. *lachrymans* proliferation in infected cucumber cotyledons with or without *Trichoderma* preinoculation. Leaves and roots of the same plants were also used for the gene expression study. Multiplication of *P. syringae* pv. *lachrymans* was significantly lower in the +T+P plants compared with that of the -T+P plants, indicating that inhibition of bacterial multiplication had occurred (Fig. 1A). In addition, we infected leaves and the proportion of necrotic lesions per leaf was assessed. A 50% reduction in the proportion of necrotic lesions was observed in the +T+P plants compared with the -T+P plants (Fig. 1B), 5 days postchallenge.

Ethylene production was measured in plants at several time points post-*Trichoderma* inoculation. No significant difference was observed between *Trichoderma*-inoculated and noninoculated plants (Fig. 2). Similar results were obtained with detached roots (data not shown). Nevertheless, when plants were treated with STS (an inhibitor of ethylene action), the ability of *Trichoderma*

Fig. 2. Time course of ethylene production by plants with or without *Trichoderma* inoculation to the root compartment. The presented values are means ($\pm$ SE) for seven repeats.

Fig. 1. **A**, Quantification of *Trichoderma* protective effect against multiplication of *Pseudomonas syringae* pv. *lachrymans* (in challenged cotyledons, 96 h after infection). Control plants (+T-P and -T-P) were treated with distilled water. Bars are means ± 1 standard deviation of three independent experiments from which 10 cotyledons were randomly selected. Different letters indicate statistically significant differences between treatments by one-way analysis of variance (ANOVA) and multiple range test for comparing means ($\alpha = 0.05$, $n = 10$). **B**, Disease severity is expressed as proportion of necrotic lesions, viewed after 5 days, relative to the number of droplets applied to each leaf. Different letters indicate statistically significant differences between treatments by one-way ANOVA and multiple range test for comparing means ($\alpha = 0.05$, $n = 15$).

to protect plants against challenge infection with *P. syringae* pv. *lachrymans* was significantly reduced (Fig. 3A). Similarly, treating plants with DIECA (inhibitor of JA biosynthesis) could abolish the protective effect of *Trichoderma* on plants against challenge infection with *P. syringae* pv. *lachrymans* in a quantitative dependent manner (Fig. 3B). Both root treatments with STS or DIECA did not inhibit root inoculation by *Trichoderma* (data not shown), nor did they affect the ability of *P. syringae* pv. *lachrymans* to multiply in the leaves of control plants (Fig. 3).

We also examined the levels of total SA produced by treated plants. No difference in both roots and leaves of *Trichoderma*-inoculated and noninoculated plants was observed (Fig. 4). Further, challenging the plants with the pathogen *P. syringae* pv. *lachrymans* did not produce any differences in SA levels between roots of *Trichoderma*-preinoculated and noninoculated plants. In the leaves, an elevation of SA level was observed in *P. syringae* pv. *lachrymans*-infected plants 96 h post-*P. syringae* pv. *lachrymans* infection but not in the *Trichoderma*-preinoculated plants.

Expression of signal molecules-related genes post-*Trichoderma* root inoculation. *CTR1*, an ethylene signal-associated gene, and *Pall1*, a gene coding for an enzyme activated by the JA/ethylene signaling pathway and involved in the production of antimicrobial compounds, were cloned from *C. sativus* by PCR. Our partial protein sequences were found to strongly resemble the homologous proteins from *Arabidopsis* spp. (88 and 93% identity, respectively, and 94 and 96% similarity, respectively, Fig. 5A and B).

In addition to these genes, we also analyzed the expression of two other known cucumber genes: *Lox1*, involved in JA synthesis, and *ETR1*, coding for the ethylene receptor.

To assess the expression of these genes in response to root inoculation with *Trichoderma* spp., 1-week-old cucumber seedlings were inoculated with *Trichoderma* and harvested 1, 3, 6, 9, 24, 48, and 72 hpi. Expression levels of these genes were analyzed both locally (roots) and systemically (leaves).

Lox1 mRNA expression level was already induced in the roots 1 hpi with *Trichoderma* spp. and a second increase was observed within 9 h. Maximal levels of *Lox1* mRNA were obtained at 24 hpi. The first peak was fourfold and the second peak was two- to threefold larger than the control (Fig. 6A). *Lox1* was hardly expressed in leaves (data not shown), regardless of whether the plants had been inoculated with *Trichoderma* spp. or not. Other studies also demonstrate that *Lox1* is hardly expressed in seedling leaves (37).

Expression levels of *Pall1* increased in roots as early as 1 hpi with *Trichoderma* spp. *Pall1* expression peaked again at 24 hpi. In leaves, however, *Pall1* expression levels began to increase between 9 and 24 hpi, reaching a maximum at 48 hpi (Fig. 6B).

Expression levels of *ETR1* and *CTR1* were studied in order to determine the involvement of ethylene signaling in plant response to *Trichoderma* inoculation. Expression levels of both *ETR1* and *CTR1* were elevated in roots starting from 9 hpi and peaking at 24 hpi. In leaves, a similar time course of expression was observed for *ETR1*. In contrast, *CTR1* expression level increased in noninoculated plants approximately three times more than in *Trichoderma*-inoculated plants (Fig. 7A and B). Since *ETR1* and *CTR1* are negative regulators of ethylene response, down-regulation of these genes is an indication of ethylene involvement in the defense response.

In general, we demonstrated that *Trichoderma* interaction with plants affected expression levels of plant genes related to the JA/ethylene signaling pathway.

Effects of *P. syringae* pv. *lachrymans* infection on *Trichoderma*-induced gene expression. One-week-old cucumber seedlings were inoculated with *Trichoderma* spp. and 48 h later the cotyledons were challenged with the leaf pathogen *P. syringae* pv. *lachrymans* (+T+P plants). Expression of defense-related genes was examined in leaves and roots of these plants (+T+P) and

control plants, i.e., nontreated plants (-T-P), *Trichoderma*-inoculated and *P. syringae* pv. *lachrymans*-noninfected plants (+T-P), or *Trichoderma*-noninoculated and *P. syringae* pv. *lachrymans*-infected (-T+P) plants.

There was no significant difference between the *ETR1* expression levels in roots and leaves of plants treated either with *Trichoderma* spp., *P. syringae* pv. *lachrymans*, or *Trichoderma* spp. and *P. syringae* pv. *lachrymans*. However, in both tissue types, *ETR1* expression level tended to be lower in plants preinoculated with *Trichoderma* spp. and challenged with *P. syringae* pv. *lachrymans* (+T+P) than in *P. syringae* pv. *lachrymans*-challenged plants (-T+P; Fig. 8A).

Whereas *ETR1* revealed only minor reduction of expression, *CTR1* expression in roots of plants inoculated with *Trichoderma* spp. and challenged with *P. syringae* pv. *lachrymans* (+T+P) was significantly lower than in plants treated only with either *Trichoderma* spp. (+T-P) or *P. syringae* pv. *lachrymans* (-T+P). In leaves, however, we observed no significant difference between plants challenged with *P. syringae* pv. *lachrymans* with or without prior *Trichoderma* inoculation (Fig. 8B).

Three genes encoding for proteins with known antipathogenic activity (chitinase 1, β -1,3-glucanase, and peroxidase) were examined in order to monitor the defense status of the plant.

Fig. 3. Effect of **A**, silver thiosulfate (STS) and **B**, diethyldithiocarbamic acid (DIECA) treatments on the protective effect of *Trichoderma* inoculation against *Pseudomonas syringae* pv. *lachrymans* multiplication in cotyledons. The presented values are means ($\pm$ SE, $n = 5$). Different letters indicate statistically significant differences between treatments (one-way analysis of variance and multiple range test for comparing means, $\alpha = 0.05$). The experiments were repeated with similar results.

Fig. 4. Time course of the total salicylic acid (SA) content in roots and leaves of plants with or without *Trichoderma* root inoculation (**A** and **B**), and after *Pseudomonas syringae* pv. *lachrymans* (Psl) infection, with or without prior *Trichoderma* inoculation (**C** and **D**). The presented values are means ($\pm$ SE, $n = 5$).

Fig. 5. Protein sequence alignment of the partial sequences of cucumber **A**, CTR1 (serine/threonine protein kinase-CTR1) and **B**, PAL1 (phenylalanine ammonia-lyase) with their homologues from *Arabidopsis thaliana*.

These genes were upregulated locally within 24 h post-*Trichoderma* inoculation (data not shown). In leaves, however, elevation of expression levels of these genes appeared within 48 h post-*Trichoderma* inoculation and was small and temporary (*Chit1*, peroxidase gene) or hardly expressed at all (β -1,3-glucanase gene; data not shown).

In leaves of plants inoculated with *Trichoderma* spp. and infected with *P. syringae* pv. *lachrymans*, *Chit1* expression levels were twofold higher than in plants infected only with *P. syringae* pv. *lachrymans*, whereas leaves of plants inoculated only with *Trichoderma* spp. did not express *Chit1* at this time point at all (Fig. 9A). Leaves of plants inoculated with *Trichoderma* spp. and infected with *P. syringae* pv. *lachrymans* also manifested a significant elevation in β -1,3-glucanase gene mRNA levels compared with plants infected only with *P. syringae* pv. *lachrymans* (Fig. 9B). Plants inoculated with *Trichoderma* spp. and challenged with *P. syringae* pv. *lachrymans* expressed elevated levels of expression of peroxidase gene in leaves relative to plants treated only with *Trichoderma* spp. or *P. syringae* pv. *lachrymans* (Fig. 9C).

DISCUSSION

While most studies focus on direct antagonistic mechanisms between *Trichoderma* spp. and plant pathogens, we examined the ability of *Trichoderma* spp. to induce defense response in the plant. The protective effect of *Trichoderma*-mediated plant resistance to pathogen challenge was demonstrated by both the reduction of bacterial multiplication in the leaves and the number of necrotic lesions per leaf in the *Trichoderma*-preinoculated plants (+T+P) relative to noninoculated controls (-T+P, Fig. 1A and B). Application of autoclaved *T. asperellum* mycelial inoculum to the roots did not appear to have any effect on disease reduction (data not shown), indicating that only live *Trichoderma* interacting with plant roots could exert this effect.

It is important to note that *Trichoderma* was not detected on shoots of inoculated plants. Moreover, *Trichoderma* could not antagonize *P. syringae* pv. *lachrymans* growth in dual cultures (data not shown). These observations indicate that the protective effect conferred by *Trichoderma* to the plant against the pathogen infection was not due to direct antagonism but rather a plant-mediated phenomenon.

The present study was aimed at identifying plant signals involved in the induction of systemic defense responses by *T. asperellum*.

Three major signal molecules are known to be involved in systemic defense responses of plants: SA, commonly shown to be involved in SAR, and JA and ethylene, which were shown to be involved in ISR (1,27,34). Our results demonstrated that no significant difference occurred in ethylene levels in any of the treatments (Fig. 2). However, treatment with an inhibitor of ethylene action (STS) strongly inhibited the protective effect of *Trichoderma* on plants (Fig. 3A), thus indicating that ethylene signal is required for this effect. Moreover, application of JA-production inhibitor (DIECA) completely abolished the protective effect of *Trichoderma* on plants (Fig. 3B). These results strongly suggest the involvement of JA/ethylene signal transduction pathway in the plant-induced resistance mediated by *Trichoderma*. This coincides with other studies, which have shown that JA and ethylene are the signal molecules involved in ISR mediated by biocontrol rhizobacteria (reviewed in literature citation 27). Such studies also demonstrated that rhizobacteria-mediated ISR is not accompanied by a detectable increase in JA or ethylene levels although it requires sensitivity to these hormones (18,26).

On the other hand, SA content did not differ between *Trichoderma*-inoculated and noninoculated plants even post-*P. syringae* pv. *lachrymans* challenge infection (Fig. 4). The levels of SA rose only in leaves of *P. syringae* pv. *lachrymans*-infected plants with-

out *Trichoderma* pretreatment (-T+P), indicating the development of SAR in these plants.

To further investigate the involvement of these hormones in plant reaction to *Trichoderma* inoculation, we have studied the expression pattern of several defense-related genes. Several genes were shown to be involved in signal transduction, one such gene is *Lox*. LOX catalyzes the addition of molecular oxygen at either the C9 or C13 residue of linoleic and linolenic acids. The 13-hydroperoxylinolenic acid, formed by 13-LOX, is the substrate for allene oxide synthase, giving rise after reduction and a series of β -oxidations to JA.

The amino acid sequence of LOX1 was found to be identical to that of the cucumber lipid body lox (X92890), except for one conserved amino acid substitution outside the active site. The lipid body lox has been reported to have 13-LOX activity (11), indicating that cucumber LOX1 has the same activity leading eventually to the formation of JA.

Fig. 6. Time course of the induction of **A**, *Lox1* and **B**, *Pal1* gene expression. Expression was measured in roots and leaves of cucumber plants after *Trichoderma* inoculation to the root compartment (time zero). Two experiments were conducted, each including approximately 20 plants. Open symbols signify experiment 1; closed symbols signify experiment 2. The internal standard deviation values for each experiment were smaller than the size of the symbols. ■ and □ indicate inoculation with *Trichoderma*; ● and ○ indicate controls.

We observed two peaks of *Lox1* expression in the roots (Fig. 6A) in response to *Trichoderma* inoculation. The first peak was observed as early as 1 hpi, while the second peak appeared at 24 hpi. Using *Trichoderma* cellulase, Martinez et al. (20) demonstrated that LOX activity peaked a few hours after infiltration of melon cotyledons followed by a JA peak at 12 h. Other studies have shown that methyl jasmonate (MeJA) can induce several of the enzymes in the LOX pathway, as well as their corresponding genes (2,3). Correspondingly, our results indicated local induction of *Lox1* by *Trichoderma* followed by a second peak possibly resulting from the initiation of the octadecanoic pathway and the synthesis of JA. Another indication for the activation of octadecanoic pathway comes from the analysis of hydroperoxide lyase gene, which is also upregulated by the interaction of *Trichoderma* with plant roots (41). Taken together, our results indicate that *Trichoderma* activates the octadecanoic pathway in plant roots with *Lox1* being activated immediately. This activation apparently leads to the production of JA signal.

Ethylene response is considered to act downstream to JA response in nonpathogenic rhizobacteria-mediated ISR. In order to better understand the ethylene response during *Trichoderma*-mediated ISR, expression of two genes from the ethylene signaling pathway, *ETR1* and *CTR1*, was analyzed. *ETR1* and *CTR1* gene products work together to negatively regulate ethylene response pathway in the absence of ethylene (13,14,29). Ethylene binding to the receptor would down-regulate the activity of the *ETR1/CTR1* complex and result in de-repression of the response pathway.

In roots, both *ETR1* and *CTR1* expression peaked at 24 hpi (Fig. 7A and B), suggesting that ethylene response in the roots is inhibited. Such phenomenon was well documented in symbiotic systems where the plant–fungus interaction results in local silencing of the plant defense response to enable symbiotic interaction to develop.

In the leaves, an increased expression of *ETR1* was recorded in response to *Trichoderma* inoculation. However, this increase was followed by reduction to below control level (Fig. 7A at 72 hpi).

Fig. 7. Time course of the induction of **A**, *ETR1* (ethylene receptor 1 gene) and **B**, *CTR1* (constitutive triple response 1 gene) gene expression, respectively. Expression was measured in roots and leaves of cucumber plants after *Trichoderma* inoculation to the root compartment (time zero). Two experiments were conducted, each including approximately 20 plants. Open symbols signify experiment 1; closed symbols signify experiment 2. The internal standard deviation values for each experiment were smaller than the size of the symbols. ■ and □ indicate inoculation with *Trichoderma*; ● and ○ indicate controls.

Fig. 8. Relative expression levels of **A**, *ETR1* (ethylene receptor 1 gene) and **B**, *CTR1* (constitutive triple response 1 gene). Expression was measured in roots (hatched boxes) and leaves (black boxes) of cucumber plants after the following treatments: -T-P, control; +T-P, *Trichoderma* inoculation in the root compartment at time zero; -T+P, *Pseudomonas syringae* pv. *lachrymans* infection of cotyledons at 48 h; and +T+P, *Trichoderma* inoculation at time zero and *P. syringae* pv. *lachrymans* infection at 48 h. Roots and leaves for expression measurements were harvested 48 h after time of *P. syringae* pv. *lachrymans* infection.

Similarly, the peak of *CTR1* expression detected in the leaves of noninoculated plants was almost abolished in the *Trichoderma*-inoculated plants, indicating down-regulation of the gene (Fig. 7B). This down-regulation of both genes suggests that the negative regulation of the ethylene response was withdrawn in the leaves. This may enhance ethylene sensitivity of the leaves, leading to a higher response in subsequent pathogen challenge. In plants preinoculated with *Trichoderma* and challenged with *P. syringae* pv. *lachrymans*, expression of *ETR1* and *CTR1* tend to be reduced suggesting that the negative regulation of *ETR1/CTR1* on the ethylene defense response was withdrawn in those plants (Fig. 8).

Studies of rhizobacteria-induced ISR demonstrated that there was no change in ethylene production or in expression of ethylene biosynthetic genes in roots or leaves during ethylene-dependent ISR development (18,26). These studies demonstrate that sensitivity to JA and ethylene is required for the rhizobacteria-mediated ISR. Thus, a JA signal induced in *Trichoderma*-inoculated plants may increase sensitivity to ethylene, perhaps by reducing the negative regulation on the ethylene response pathway.

During plant defense processes, *Pal* is considered to be activated by the JA/ethylene signaling pathway and was shown to be

a key regulatory enzyme in the synthesis of SA (7,17,23,34). We observed two peaks of *Pal1* expression in roots inoculated with *Trichoderma*, at 1 and 24 hpi (Fig. 3B). Using active *Trichoderma* cellulase, Martinez et al. (20) also revealed two local peaks of PAL activity after infiltration of melon cotyledons, the first of which appeared before the production of SA. Using a JA signaling inducer (inactive cellulase), they were able to show that the second peak was induced by JA signaling. Correspondingly, our results may suggest that the first peak is independent of SA and the second peak is induced by JA signaling. On the other hand, only the second peak of *Pal1* expression was observed in leaves of *Trichoderma*-inoculated plants. This suggests that the induction of resistance in systemic tissues by *Trichoderma* involves only JA signal and not SA signal. In agreement with this, we observed no difference in SA content in leaves of *Trichoderma*-inoculated and noninoculated plants over a range of several days (Fig. 4).

This idea is supported by additional testing of the expression level of several PR genes. Chitinase 1, a member of the class III chitinases, and β -1,3-glucanase code for hydrolytic enzymes that disrupt the mycelial cell wall. Chitinase activity is induced in plants by various stimuli, including ethylene and SA (15,24,30). However, β -1,3-glucanase has been found to be induced mainly by SAR and SAR inducers, such as SA (16,25,33,37). ISR-inducing rhizobacteria and MeJA failed to induce the β -1,3-glucanase gene in both roots and leaves (37). In *Trichoderma*-inoculated plants, temporary activation of the *Chit1* gene in roots preceded its temporary activation in leaves (data not shown), suggesting that *Chit1* activation in leaves relies on the development and transduction of a signal to the leaves. However, β -1,3-glucanase was induced only locally (data not shown), indicating that SAR is not involved in *Trichoderma*-mediated systemic gene expression.

The temporary nature of the increase in defense-related gene expression in response to *T. asperellum* inoculation raised a notable question as to what extent these genes may be activated following challenge infection with a pathogen. A significant elevation in the mRNA levels of *Chit1*, β -1,3-glucanase gene, and peroxidase gene was observed in leaves of *Trichoderma*-induced plants, 48 h postchallenge with *P. syringae* pv. *lachrymans*, relative to plants treated exclusively with *Trichoderma* or *P. syringae* pv. *lachrymans* (Fig. 9). The same phenomenon was described for the hydroperoxide lyase gene in the same biological system (41). Similar response was described with the *Atvsp* gene in rhizobacteria-induced ISR in *Arabidopsis* plants and termed potentiation (37). Our results demonstrate that potentiation of defense-related gene expression may have a wider spectrum, and thus, this mechanism may have a significant contribution to the plant's resistance to pathogens.

Taken together, the results implicate that the main signal transduction pathway through which *Trichoderma*-mediated ISR is activated uses JA and ethylene as signal molecules, as shown by the involvement of several JA/ethylene pathway-related genes. In addition, the results demonstrate that the *Trichoderma*-induced state sensitizes the plant to respond more efficiently to subsequent pathogenic attack. This sensitization is apparent from both the reduction in disease symptoms and the systemic potentiation of the PR genes *Chit1*, β -1,3-glucanase, and peroxidase. The wide spectrum of this potentiated gene expression suggests its crucial role in ISR mediated by *Trichoderma* spp.

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Fig. 9. Relative expression levels of **A**, *Chit1*, **B**, β -1,3-glucanase gene, and **C**, peroxidase gene. Expression was measured in leaves of cucumber plants after the following treatments: -T $\geq$ P, control; +T-P, *Trichoderma* inoculation in the root compartment at time zero; -T+P, *Pseudomonas syringae* pv. *lachrymans* infection of cotyledons at 48 h; and +T+P, *Trichoderma* inoculation at time zero and *P. syringae* pv. *lachrymans* infection at 48 h. Leaves were harvested for expression measurements 48 h post-*P. syringae* pv. *lachrymans* infection.

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