Running head: The role of *Trichoderma*-induced MAPK in plant resistance.

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**Research area:** Plants interacting with other organisms.
Characterization of a MAPK gene from cucumber required for Trichoderma-conferred plant resistance

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ABSTRACT

The fungal biocontrol agent *Trichoderma asperellum* has been recently shown to induce systemic resistance in plants through a mechanism that employs jasmonic acid and ethylene signal-transduction pathways. MAPK (mitogen-activated protein kinase) proteins have been implicated in the signal transduction of a wide variety of plant stress responses. Here we report the identification and characterization of a *Trichoderma*-Induced MAPK (*TIPK*) gene function in cucumber (*Cucumis sativus*). Similar to its homologues, WIPK, MPK3 and MPK3a, TIPK is also induced by wounding. Normally, pre-inoculation of roots with *Trichoderma* activates plant defense mechanisms, which result in resistance to the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*. We used a unique attenuated virus vector, *Zucchini yellow mosaic virus* (ZYMV-AGII), to overexpress TIPK protein and antisense RNA. Plants overexpressing *TIPK* were more resistant to pathogenic bacterial attack than control plants, even in the absence of *Trichoderma* pre-inoculation. On the other hand, plants expressing *TIPK*-antisense revealed increased sensitivity to pathogen attack. Moreover, *Trichoderma* pre-inoculation could not protect these antisense plants against subsequent pathogen attack. We, therefore, demonstrate that *Trichoderma* exerts its protective effect on plants through activation of the *TIPK* gene, a MAPK that is involved in signal-transduction pathways of defense responses.
INTRODUCTION

*Trichoderma* spp. are effective biocontrol agents for a number of soilborne pathogens and are also known for their ability to enhance the plant-growth response. *Trichoderma* antagonizes plant pathogens by producing antibiotics, competing for nutrients in the rhizosphere and exhibiting mycoparasitism (Harman *et al.* 2004). *Trichoderma asperellum* (*T. harzianum* 203) can penetrate the roots of cucumber seedlings and colonize the epidermis and outer root cortex (Yedidia *et al.* 1999). Recently, it has been shown that these interactions induce systemic resistance (ISR) mechanisms in plants (Yedidia *et al.* 2003, Shoresh *et al.* 2005). Studies have shown that ISR induced by certain rhizospheric bacteria is not a SA-dependent phenomenon but rather requires components of the jasmonic acid (JA) signaling pathway followed by the ethylene signaling pathway (Pieterse *et al.* 2003). Analysis of signal molecules involved in defense mechanisms and application of specific inhibitors of plant hormones indicated the involvement of jasmonic acid (JA) and ethylene in the protective effect conferred by *Trichoderma* spp. against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans* (*Psl*). Moreover, examination of local and systemic gene expression in cucumber plants revealed that *Trichoderma asperellum* (T203) modulates the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (Shoresh *et al.* 2005).

Plants are exposed to a wide variety of environmental stresses and they have developed a broad range of responses to resist these stresses. Mitogen-activated protein kinase (MAPK) pathways have been implicated in signal transduction for a wide variety of stress responses and some may be involved in JA signaling pathways (Meskiene and Hirt 2000, Zhang and Klessig 2001). Exposure of plants to mechanical stress, such as touch or wounding, resulted in transcript accumulation of *AtMPK3* or *LeMPK3*, respectively (Mizoguchi *et al.* 1996, Mayrose *et al.* 2004). Wounding of tobacco leaves induced transcript accumulation and activity of WIPK (wound-induced protein kinase), both locally and systemically. In transgenic plants in which WIPK has been silenced, wounding did not induce activation of MAPK or accumulation of the wound- and JA-inducible genes PI-II and basic PR1 (Seo *et al.* 1995). On the other hand, WIPK-overproducing plants showed constitutive PI-II transcript accumulation and WIPK activity, and JA and methyl jasmonate (MeJA) levels were three- to fourfold higher than in the wild type (Seo *et al.* 1999). These observations demonstrated a role for WIPK in the production of jasmonate.
There are numerous examples for the involvement of MAPKs in signaling pathways of plant responses to pathogens. Direct proof of this involvement came from studies of parsley cells treated with Pep13, a 13 amino-acid oligopeptide fragment derived from an extracellular glycoprotein of Phytophthora sojae. By binding to a specific plasma-membrane receptor, Pep13 activated plant defense responses, including activation of the MAPK MPK3a, at both the transcriptional and post-translational levels (Ligterink et al. 1997, Kroj et al. 2003). WIPK was also activated by a fungal cell wall elicitor (Zhang et al. 1998) and by the avr9 protein from the fungal pathogen Cladosporium fulvum (Romeis et al. 1999). AtMPK3 was activated by flg22, a 22-amino-acid peptide corresponding to the most conserved domain of eubacterial flagellin (Asai et al. 2002). LeMPK3 was found to be transcriptionally upregulated by both pathogenic bacteria and fungal elicitor (Mayrose et al. 2004).

Plant RNA viruses have been shown to be an efficient tool for over expression and knock down expression of endogenous genes. This technology provides important new insights into the roles of specific genes in plant development and plant defense responses. Virus-induced gene silencing (VIGS) is a quick and efficient technique involving recombinant viruses for reverse genetics by down-regulation of target genes (Burch-Smith et al. 2004, Robertson 2004).

Zucchini yellow mosaic virus (ZYMV) is a member of the potyviridae family. ZYMV-AGII is a potyvirus based vector system which has been successfully used for over-expression of various foreign genes in cucurbits (Arazi et al. 2001, Aly et al. 2005). In contrast to other known viral vectors, which cause severe disease to host plants, the AGII vector was created from an attenuated engineered ZYMV potyvirus (Arazi et al. 2001) and does not elicit the severe phenotype or developmental impairment caused by wild type virus and no symptoms are developed in cucumbers (Gal-On and Raccah 2000,). Therefore, it is attractive for over or down regulation of endogenous gene expression.

In this study, we demonstrate that in cucumber, a MAPK is activated by inoculation of the roots with the biocontrol agent Trichoderma asperellum. Moreover, activation of this gene is necessary for the plant's Trichoderma-conferred defense against bacterial pathogens: silencing of this MAPK completely eliminates this protection. We present evidence suggesting that this MAPK is also involved in other plant stress responses, including that to wounding.

RESULTS

A MAPK is induced by Trichoderma inoculation of plants.
We recently demonstrated the involvement of the plant signal molecules JA and ethylene in the *Trichoderma*-induced plant defense response. We were therefore interested in analyzing the expression of genes involved in signal transduction during the plant-*Trichoderma* interaction. Since many MAPKs have been found to be involved in the plant defense response and their regulatory effects on the control of plant defense responses have been described, we decided to focus on these kinases. PCR with degenerate primers designed according to resistance-associated MAPK plant proteins allowed us to isolate a putative resistance-associated cucumber MAPK clone. We used real-time PCR analysis to examine the expression profile in reaction to *Trichoderma* inoculation of plant roots. The specificity of the primers was confirmed by having one clear peak of melting curve indicating only one fragment is produced by this primer pair and by sequencing the PCR product. Six hours after *Trichoderma* inoculation, the MAPK mRNA levels in the roots increased, peaking at four- to fivefold the level in untreated plants (Fig. 1A). This was followed by another, higher peak 24 h post-inoculation (hpi). In leaves, the expression levels of the gene also began to rise 6 hpi and reached a peak at 24 hpi, which was about two and half times higher than in non-inoculated plants (Fig. 1B). Expression of this MAPK in leaves exemplifies a systemic gene response to root inoculation. Our results suggest that this *Trichoderma*-induced MAPK (TIPK) participates in the *Trichoderma*-induced defense response.

*TIPK* is a homologue of wound-induced MAPKs.

Using RACE analysis, we obtained the full-length cDNA of *TIPK*. Further PCR and sequence analysis enabled us to deduce the genomic organization of the gene encoding TIPK in the cucumber genome (Fig. 2A). When genomic DNA was digested with the appropriate restriction endonucleases and hybridized at high-stringent conditions with a *TIPK* cDNA probe, we observed unique hybridization signals, which were expected according to the deduced restriction map (Fig. 2B). This indicated that the gene encoding *TIPK* is apparently present as a single-copy gene in the cucumber genome. This MAPK is 84% identical and 93% similar to WIPK (*Nicotiana tabacum*), 82% identical and 92% similar to MPK3 (*Arabidopsis thaliana*), and 81% identical and 91% similar to MPK3a (*Petroselium crispum*) (Fig. 3). The expressions of *wipk* and *mpk3* have been shown to be wound-induced. We therefore examined whether *TIPK* also responds to wounding. Wounding leaves with carborundum resulted in a rise in *TIPK* expression levels, starting from 10 min and peaking 1 h post-wounding, with or without pre-inoculation with *Trichoderma* (Fig. 4A). *TIPK* was also upregulated in systemic (non-wounded)
leaves 24 h post-wounding (Fig. 4B). These results demonstrate the close functional relationship of TIPK with other wound-induced MAPKs.

**Effect of plant hormones and their inhibitors on TIPK expression.**

Since both *Trichoderma* inoculation and wounding exert their effects via the JA and ethylene signal molecules, we examined whether plant hormones or their inhibitors might affect TIPK expression levels. Treatment of cucumber roots with JA or salicylic acid (SA) did not seem to affect the TIPK expression in roots (Fig. 5A). The effect of JA and SA treatments was validated by examining the JA- and SA-inducible genes, lipoxygenase and chitinase, respectively (Fig. 5A). Since we identified a peak of TIPK expression at 24 h post *Trichoderma* inoculation we decided to focus on this time point for hormone treatment experiments, however, it may be that an activation of the gene occurred at a different time point under these treatments. We also examined whether inhibitors of JA production (DIECA) or ethylene action (STS) would reduce the induction of TIPK expression in roots by *Trichoderma* inoculation. We noticed that treatments with JA, SA, and DIECA could repress TIPK expression when compared to control. However, using both inhibitors, induction of TIPK gene expression was similar to that in the control experiment (Fig. 5B). This suggests that TIPK may act prior to the hormones' actions.

**Expression of TIPK after Psl infection is potentiated by prior Trichoderma inoculation.**

The expression of TIPK in roots of *Trichoderma*-inoculated and Psl-challenged plants was higher than in plants subjected to only one of those treatments (Fig. 6). Moreover, whereas leaves of *Trichoderma*-inoculated plants did not differ in TIPK expression level from the controls, leaves of plants inoculated with *Trichoderma* and challenged with Psl expressed three- to fourfold higher TIPK mRNA levels than plants challenged only with Psl (Fig. 6). It should also be noted that while systemic expression of TIPK decreased in *Trichoderma*-inoculated plants 72 hpi (Fig. 1B) and remained low at 96 hpi (Fig. 6, +T-P treatment), the high levels of TIPK transcript in leaves of the +T+P treatment 96 hpi (Fig. 6) suggest that *Trichoderma* inoculation prior to pathogen challenge also results in prolonged induction of the gene. This indicates that the plant's interaction with *Trichoderma* enables a stronger defense reaction to subsequent pathogen attack.

**Construction of AGII-TIPK and AGII-TIPK antisense.**
The TIPK cDNA and an antisense (AS) fragment of TIPK were inserted between the NIb and coat protein (CP) genes of the AGII virus vector using a polylinker-cloning site next to the Nla proteinase cleavage site in the NIb gene at the 3’ end of AGII (Fig. 7A). Inserted genes were designed to create an in-frame translational fusion with both flanking Nla processing sites. Proteolysis of the nascent AGII-TIPK polyprotein by Nla protease in trans was predicted to yield recombinant TIPK protein lacking the first methionine and having an additional seven amino-acid residues (VDTVMLQ) at the C-terminus. AGII-AS was designed to produce a polyprotein that would be processed to produce all the viral proteins necessary for its reproduction in the plant, while the AS fragment embedded in the viral RNA genome was significant for the induction of TIPK silencing, via virus-induced gene silencing (VIGS). The presence of the intact TIPK and AS sequences was verified by RT-PCR of the viral progeny 14 and 17 days post inoculation (dpi) (Fig. 7B), as well as by direct sequencing of the amplified products. As a control treatment, we used the AGII-GFP construct.

Expression of TIPK is modified by infection with the different AGII constructs.

The AGII-GFP construct has been reported to be stable in cucumber plants for at least 60 dpi. Nevertheless, we verified the viral accumulation in these plants in our hydroponic system over the time interval needed for our experiments. The second leaves of systemic AGII-GFP-infected cucumbers were analyzed for GFP by visualization under UV light. Green fluorescence was observed in many regions of the leaves, but not in those that were mock-inoculated (Fig. 8). Real-time PCR analysis using primers designed according to the CP gene demonstrated a consistent rise in virus quantity from 8 to 17 dpi, resulting in a 40-fold higher quantity on day 17 than on day 8 (data not shown). We then measured the endogenous RNA levels of TIPK in the AGII-GFP-, AGII-TIPK- and AGII-AS-infected plants. The RNA levels of TIPK in the AGII-TIPK plants were three- and sixfold of controls, 14 and 17 dpi, respectively (Fig. 9), whereas TIPK mRNA levels in the AGII-AS were 50 and 60% of controls, 14 and 17 dpi, respectively (Fig. 9). Therefore, infection with the AGII constructs allowed us to modify endogenous TIPK RNA levels and produce TIPK-overexpressing and underexpressing plants.

To verify the specificity of the antisense construct we cloned a fragment of another cucumber MAPK which had 97% and 96% identity to the MSK7 (Medicago sativa; gi:298019) and MPK6 (Arabidopsis thaliana; gi:15224359) proteins, respectively, but only 87% identity to the TIPK protein. The RNA levels of this MAPK gene in the AGII-GFP and AGII-AS-infected
plants had no significant difference at 14dpi (Means of relative RNA levels ± SE and the number of repeats were as follows: AGII-GFP 14dpi- 1.00±0.21, n=5; AGII-AS 14dpi- 0.93±0.19, n=6). At 17dpi we also observed no significant difference between AGII-GFP and AGII-AS plants (AGII-GFP 17dpi- 0.59±0.19, n=6; AGII-AS 17dpi- 0.70±0.21, n=5).

Examination of TIPK expression in AGII-GFP plants treated with Trichoderma (Fig. 9, plants marked GFP+T) revealed the expected up-regulation of the gene at 14dpi (which is 24h post Trichoderma inoculation) and reduction of the expression to basal level at 17dpi (which is 96h post Trichoderma inoculation). These results are similar to TIPK expression observed in cucumber plants treated with Trichoderma (Fig. 1).

In addition we also compared the expression of TIPK in AGII-AS plants after Trichoderma inoculation (Fig. 9, plants marked AS+T) to AGII-GFP plants treated with Trichoderma (Fig. 9, plants marked GFP+T). While in the AGII-GFP plants treated with Trichoderma, TIPK expression increased 24h post Trichoderma inoculation (14dpi) at this time point not only the TIPK expression was not elevated in AGII-AS plants post Trichoderma inoculation, but it was decreased (Fig. 9).

Sensitivity to pathogen challenge is modified by changes in TIPK RNA levels.

To determine whether TIPK plays a role in the Trichoderma-induced defense response, we challenged AGII-TIPK- and AGII-AS-infected plants with Psl and determined the levels of Psl proliferation in the plants’ leaves. As a control, we used AGII-GFP-infected plants. Some samples varied greatly from the average of their group with respect to pathogen multiplication. Since the distribution of AGII virus in leaves was not uniform (Fig. 8), we speculate that when the pathogenic bacteria penetrate the leaf in a region where the AGII virus exists, the latter can affect the bacterial propagation based on its transgenic construct. On the other hand, when the bacteria and the virus do not co-localize, the pathogen will behave as in control leaves. For example, in the group of AGII-AS transformants, one plant had 150 fold less bacteria than the average, another transformant in this group had 5 fold less bacteria. We assume that in these transformant the virus and the Psl did not co-localize, or the viral infection was not efficient. In the AGII-TIPK transformants we had four plants with levels of bacteria that were more similar to the AGII-GFP control plants. This could also result from non co-localization of the virus and the Psl, or inefficient viral infection. Nevertheless the AGII-TIPK transformants group was significantly different from the AGII-GFP plants (Fig. 10). In the AGII-AS plants treated with
*Trichoderma* we had five plants with 5-10 fold higher Psl counts than all other plants. In these plants the virus either co-localized with the bacteria perfectly or the efficiency of the antisense inhibition was greater. In either case it fits well our hypothesis that inhibition of *TIPK* expression decrease plant defense even in the presence of *Trichoderma*.

Psl multiplication was significantly lower at 72 dpi in the AGII-TIPK (29 x 10^6 cfu/g FW) plants than in AGII-GFP control plants (181 x 10^6 cfu/g FW)(Fig. 10). In fact, Psl multiplication in AGII-TIPK plants was similar to that observed in AGII-GFP plants which had been inoculated with *Trichoderma* prior to pathogen challenge (37 x 10^6 cfu/g FW). This demonstrates that overexpression of the *TIPK* gene can confer the same level of protection as *Trichoderma* inoculation. Although Psl multiplication was higher in AGII-AS plants (303 x 10^6 cfu/g FW) than in AGII-GFP control plants (181 x 10^6 cfu/g FW) these differences were not statistically significant (Fig 10). More importantly, the significantly higher Psl multiplication in plants infected with AGII-AS and pre-inoculated with *Trichoderma* (940 x 10^6 cfu/g FW) than in AGII-GFP control plants (Fig. 10) demonstrates that reduced *TIPK* expression abolishes the protective effect normally conferred by *Trichoderma*.

**DISCUSSION**

In recent years it has become clear that MAPK signaling pathways are involved in plant resistance. We isolated and characterized a MAPK gene that is activated by root inoculation with the biocontrol fungus *T. asperellum* (Fig. 1). Sequence analysis demonstrated that the *Trichoderma*-induced MAPK (*TIPK*) is homologous to *MPK3a, WIPK* and *MPK3* (Fig. 3), genes which have been shown to be upregulated by pathogenic bacterial and fungal elicitors (Lijten et al. 1997, Zhang et al. 2000, Schenk et al. 2003, Mayrose et al. 2004). Moreover, similar to these genes, *TIPK* is also induced by wounding. The transcriptional response is fast and transient, with transcript levels starting to accumulate 10 min after wounding and reaching a maximum at 1 h (Fig. 4A). This fast and transient accumulation is very similar to the reaction of *AtMPK3, LeMPK3* and *WIPK* to mechanical stress and wounding (Seo et al. 1995, Mizoguchi et al. 1996, Mayrose et al. 2004). The kinetics of the accumulation of *TIPK* mRNA in response to *Trichoderma* root inoculation was slower than that in response to wounding, reaching a maximum at 24 hpi (Fig. 1A). This demonstrates that *TIPK* is induced with distinct activation kinetics by different stimuli, and suggests that *TIPK* may be a convergence point for different
stimuli and that different induction kinetics may play a role in the types of downstream processes it mediates.

However, we also observed systemic expression of the gene in leaves post-Trichoderma inoculation (Fig. 1B) and post-wounding (Fig. 4B). Moreover, the systemic expression post-Trichoderma inoculation was much higher and more prolonged when plants were inoculated with Trichoderma prior to pathogen challenge (Fig. 6). The potentiation effect of Trichoderma on plant-defense-related gene expression has been recently demonstrated (Yedidia et al. 2003, Shoresh et al. 2005). It has also been shown that plants pre-inoculated with Trichoderma are more resistant to subsequent Psl challenge (Shoresh et al. 2005). Together with the results presented here, the plant’s interaction with Trichoderma prior to pathogen challenge appears to enable a stronger defense response.

The expression of TIPK in wounded plant leaves post-Trichoderma root inoculation did not differ from that in wounded leaves from non-inoculated plants (Fig. 4A), and no potentiation effect was observed post-wounding in plants pre-inoculated with Trichoderma (data not shown). This demonstrates that TIPK potentiation post-Trichoderma inoculation is specific to the plant's response to pathogen challenge.

We have recently demonstrated the involvement of the JA signaling pathway in the Trichoderma-induced plant response (Shoresh et al. 2005). WIPK-overproducing plants show three- to fourfold higher JA and MeJA levels than wild-type plants (Seo et al. 1999). Similarly, rice plants transformed with MK1, a homologue of WIPK from pepper, showed a threefold higher level of JA than the wild type (Lee et al. 2004). On the other hand, wipk-silenced plants produced much less JA and MeJA after wounding than wild-type plants (Seo et al. 1995). We found that JA or SA could not activate transcription of TIPK, even at high concentrations (Fig. 5A). In fact, the hormones seem to inhibit the basal expression of TIPK suggesting that there may be some modulation of the gene activity by those hormones. But this modulation of gene expression is different from what we observe with the Trichoderma inoculation. JA is also unable to induce expression of LeMPK3 (Mayrose et al. 2004), or activate WIPK or its alfalfa homologue, SAMK (Bögre et al. 1997, Kumar and Klessig 2000). We previously demonstrated that root treatments with either STS or DIECA do not affect root inoculation by Trichoderma (Shoresh et al. 2005). While, a JA-production inhibitor (DIECA) can decrease Trichoderma's protective effect on plants against the pathogen (Shoresh et al. 2005) it did not affect TIPK-
induced expression post- *Trichoderma* root inoculation (Fig. 5B). We also found that while an ethylene-action inhibitor (STS) can decrease *Trichoderma*'s protective effect on plants against the pathogen (Shoresh *et al.* 2005) it does not inhibit TIPK induction by *Trichoderma* (Fig. 5B). This is consistent with the observation that WIPK is not activated by ethylene (Kumar and Klessig 2000). These results together with previous studies of TIPK homologues suggest a role for TIPK upstream of these signaling molecules during plant reaction to *Trichoderma* inoculation.

If signaling through the MAPK cascade is the primary or only route by which *Trichoderma*-interaction information can be transmitted to trigger a defense response, then interfering with the cascade by reducing TIPK expression should reduce the plant's resistance to subsequent pathogen challenge, even in the presence of *Trichoderma*. Therefore, we modified the expression levels of TIPK by employing an attenuated potyvirus-vector, ZYMV-AGII (Arazi *et al.* 2001). This vector represents a unique system for gene expression in cucurbits cytosol. Because it was engineered from an attenuated strain, it is non-pathogenic and does not impair growth of the host plant (Arazi *et al.* 2001). The AGII-GFP construct has been reported to be stable in cucumber plants for at least 60 dpi (Arazi *et al.* 2001) and we also validated the propagation of the virus during our experimental period. We determined the stability of the viral constructs throughout the experiments (Fig. 7B). The distribution of AGII virus in leaves was not uniform (Fig. 8). Similar observation of non-uniform expression via the AGII vector was observed in other studies while using this vector (Arazi *et al.* 2001, Aly *et al.* 2005). Nevertheless, the AGII vector provides a good systemic expression system for a gene of interest.

Introducing the coding region of TIPK into the AGII viral genome allowed us to create TIPK-overproducing plants. In these plants, 14 days after AGII-TIPK infection, the level of TIPK RNA was threefold higher than in control AGII-GFP plants and 17 dpi, the levels of TIPK were six fold that in controls (Fig. 9). When AGII-GFP plants were inoculated with *Trichoderma* we observed a threefold elevation in TIPK mRNA levels (Fig. 9), which is on the same order of magnitude as in TIPK-overproducing plants. Challenging the latter with a bacterial pathogen resulted in pathogen resistance which was at least as effective as with the *Trichoderma* root treatment of AGII-GFP control plants (Fig. 10). In a recent study, transformation of rice plants with the gene MK1, the pepper homologue of WIPK, resulted in expression of the transgene at both the RNA and protein levels, as well as increased resistance to rice blast disease (Lee *et al.*
Together, this demonstrates that plant resistance to subsequent pathogens is conferred by overexpression of *TIPK*.

Southern analysis of DNA digested by several restriction enzymes revealed that *TIPK* is present as a single-copy gene (Fig. 2), thus simplifying the interpretation of the antisense experiments. Similarly, the TIPK homologues WIPK and MPK3a have been shown to be single-copy genes (Seo *et al.* 1995, Ligterink *et al.* 1997).

When we infected cucumber plants with AGII virus harboring an antisense fragment of *TIPK*, a reduction of ca. 50% in *TIPK* mRNA levels was obtained (Fig. 9). It is important to note that the primers used to detect these mRNA levels did not overlap with the antisense fragment cloned into the AGII-AS construct, thus enabling us to detect endogenous mRNA levels of *TIPK* gene. Virus-derived siRNA has been shown to accumulate in plants infected with *Potyviruses* (Xie *et al.* 2004 and Gal-On unpublished results in ZYMV infected plants). This indicates that the silencing process is initiated in the cells of the plant despite the presence of the potyviral suppressor HC-Pro. We assume that in plants infected with AGII-AS, ZYMV siRNAs are produced and include the *TIPK* antisense sequence. The *TIPK* siRNAs may target, and cause cleavage of the *TIPK* mRNA through the RISC complex. Additionally the *TIPK* siRNA could serve as a primer for plant RdRp mediated *TIPK* dsRNA amplification, which would then be degraded by DICER-like proteins as part of the plant silencing mechanism. The mRNA levels of a homologous MAPK were not decreased in the AGII-AS plants as compared to AGII-GFP plants indicating the specificity of the antisense. *TIPK* expression was also silenced in AGII-AS plants inoculated with *Trichoderma* (Fig. 9). More importantly, Psl multiplication in TIPK-silenced plants pre-inoculated with *Trichoderma* was higher than in AGII-GFP control plants, while in AGII-GFP plants pre-inoculated with *Trichoderma*, Psl multiplication was largely reduced. Therefore, in our TIPK-silenced plants, *Trichoderma* treatment failed to protect the plants from subsequent pathogenic challenge (Fig. 10). Altogether, this clearly demonstrates that *TIPK* is a crucial component in the pathway of signals being transferred from the interaction site of *Trichoderma* with the plant. Moreover, it is shown clearly that *Trichoderma* exerts its protective effect on plants through activation of the *TIPK* gene. So it appears that without the ability to activate this gene, the plant cannot be protected by *Trichoderma*.

**MATERIALS AND METHODS**
**Plant material.** Seeds of cucumber (*Cucumis sativus* L. cv. Kfir) from Gedera Seeds Co. (Gedera, Israel) were used in this experiment. Plant growth medium (PGM) was prepared according to Yedidia *et al.* (1999).

**Axenic growth system.** Seeds were surface-sterilized in 2.0% (v/v) 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 hydroponic growth system (Yedidia *et al.* 1999). Plants were grown in a controlled environment: 26°C, 80% relative humidity, light 300 µE/m²/s and a circadian cycle of 16 h light and eight h darkness.

**Fungal material.** *Trichoderma asperellum* (*Trichoderma harzianum* strain T203) was grown on potato dextrose agar (PDA) (Difco). Synthetic medium (SM) for *T. asperellum* was prepared according to Yedidia *et al.* (1999). The inoculum consisted of 1 ml (10⁹ spores, as counted by hemocytometer) of 10-day-old *T. asperellum* cultured on PDA added to a 250-ml flask containing 100 ml 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 5000 rpm at 4°C, followed by two washes with 100 ml distilled water.

**Trichoderma plant inoculation.** Inoculum was added under aseptic conditions to the PGM of 7-day-old seedlings to a final concentration of ±10⁵ germinated spores/ml (Yedidia *et al.* 1999). Control plants were treated with sterile distilled water.

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

**Treatment with plant hormones and plant-hormone inhibitors.** Plants were grown in a hydroponic growth system for 12 days and then transferred to 50-ml tubes in closed chambers, five plants per tube. Each tube contained hydroponic medium +/- the hormone. Hormone concentrations (both JA and SA) were: 0 (control), 0.5, 1 and 2 mM. Plants were harvested at 24 h post-exposure to the hormones (five plants per treatment at each time point).

Diethyldithiocarbamic acid (DIECA; Sigma), a potent inhibitor of jasmonate biosynthesis (Menke *et al.* 1999), was added to the root compartment at a final concentration of 100 µM, 1 h after *Trichoderma* inoculation.

Silver thiosulfate (STS), an inhibitor of ethylene action (Abeles *et al.* 1992), was prepared by mixing solutions of 0.1 M sodium thiosulfate with 0.1 M silver nitrate in a 4:1 (v/v)
ratio. STS was added to the root compartment at a final concentration of 0.25 mM, 3 h after *Trichoderma* inoculation. Plants roots were harvested 24 h post-*Trichoderma* inoculation.

The treatments and the controls were: -T-inhibitor; -T+inhibitor; +T-inhibitor; +T+inhibitor.

**Wound treatments.** Plants were grown in a hydroponic growth system for 11 days. The roots were inoculated with germinated *Trichoderma* (T203) spores (as already described). After 96 h, leaves were wounded by rubbing with carborundum, and then harvested from each treatment at the following time points: 0 min, 10 min, 30 min, 1 h, 2 h, 6 h, and 24 h post-wounding, with 10 plants per time point.

**RNA isolation.** For RNA analysis, roots and leaves were harvested and placed immediately in liquid nitrogen and then stored at -70°C until use (1-2 weeks). 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 MgCl2, 1 mM CaCl2 for 30 min at 37°C (Roche). This was followed by a phenol/chloroform and chloroform extraction and a subsequent ethanolic precipitation.

**Cloning and sequencing.** Degenerate primers designed according to several known MAPK plant genes were used to isolate the MAPK gene using the Expand High Fidelity PCR System (Roche). Primer sequences used to clone TIPK were:

Forward 5'-GG(C/T)GCTTA(T/C)GG(T/A/C)AT(T/G)GT(C/T)TGT-3',
Reverse 5'-ACC(A/G)AC(A/T)GACCA(A/T)ATATCAA-3'.

Primer sequences used to clone MPK6 were:

Forward 5'- C(A/C/T)TT(T/C)AA(T/C)GATGT(G/T)TA(C/T)AT(T/C)GC(A/G)TA-3'
Reverse 5'- TCTGA(A/T)GG(T/A)GT(G /T/A)CC(A/T)AT(C/G)A(G/A)CTCCA-3'.

PCR fragments were cloned in pGEM-T Easy Vector (Promega) and both strands were sequenced.

**RACE and genomic walk analysis.** A 5'/3' RACE Kit (Roche) was used to isolate 5’ and 3’ sequences according to the manufacturer's instructions. The gene-specific primers used were: reverse 5’-CAAGGCAGCCTACAATTCGAAGATGCGGT-3’, forward 5’-TGTGACAAGATGGTACAGAGCACCTGA-3’. The universal Genome Walker Kit (Clontech) was used to isolate 5’ upstream sequences to the gene. The gene-specific primer used was: 5’-AGGCATAATCGGAGGACGATATTGGA-3’.
Comparison of TIPK cDNA to genomic sequences of homologous genes was performed to design primers surrounding the postulated introns, and the corresponding introns were isolated. The Genbank accession no. of the TIPK sequence is: DQ118734. Accession number of the second cucumber MAPK is: DQ841553.

**Southern analysis.** Genomic DNA of C. sativus was digested with EcoRI, SacI, PstI, and XhoI, separated on an agarose gel and blotted onto a nylon membrane. A fragment of 1.2 kb from the cDNA was labeled with $^{32}$P and used as a probe. ULTRAhyb (Ambion) was used as a hybridization solution at 42°C. Membrane was washed according to manufacture’s instructions with one modification: stringent washing was done at 45°C.

**Reverse transcription.** After treatment with DNaseI, 1 µg of total RNA was used for a RT reaction using Superscript II (Invitrogen) according to the manufacturer’s instructions.

**Quantitative PCR.** The size of all amplified fragments was 200 bp and the annealing temperature of all primers was 60°C. The sequences of the primers used were

- **TIPK:** forward 5'- CCGTCATGCATTTCATTTTCAGAA-3',
  reverse 5'-TCCGCTCCAACCAAGTATTATC-3';
- **18S:** forward 5'-GTTGCTTTAAGGACTCCGCCA-3',
  reverse 5'-AGGGGTACCTCCGCATAGCT-3' (gi|7595414).
- **MPK6:** forward 5'- CCAGATACTTCGTGGATTGAAG 3',
  reverse 5'- AGACATCAATAAGCTGCAGTG 3'.

The specificity of the primers to the genes they were designed for was tested by using melting curve analysis of the PCR reaction (standard protocol of the real-time PCR machine), as well as sequence analysis of the PCR product amplified. The primers described above are those who passed these tests. PCR was carried out in 96-well plates (20 µl per well) in a reaction buffer containing 1X SYBR Green PCR Master Mix (PE Applied Biosystems), 350 nM primers (for each forward and reverse primer) and 1/40 of the RT reaction for TIPK detection or 1/1000 for 18S detection. Quantitative analysis was performed using the GeneAmp®7000 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 examined 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 triplicate, using relative quantification analysis. This
method normalizes the expression of the specific gene versus the control reference with the formula $2^{-\Delta\Delta C_T}$ where $\Delta C_T = C_T_{\text{specific gene}} - C_T_{\text{reference gene}}$; $\Delta\Delta C_T = \Delta C_T - \text{arbitrary constant}$ (the highest $\Delta C_T$) (for further elaboration, see 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.

For gel visualization of quantitative PCR, we used the same conditions and primers but with a standard PCR instrument for 20 cycles (for TIPK) or 18 cycles (for 18S) and ran 10 µl on the gel. Gels were then blotted and hybridized with a probe of TIPK cDNA or 18S DNA, respectively, using standard protocols (Sambrook et al. 1989). Images were quantified using ImageJ 1.36. For verification of hormone treatments, we performed PCR for 25 cycles using primers designed to amplify chitinase and lipoxygenase genes as described in Shoresh et al. (2005).

**Insertion of TIPK gene and TIPK-antisense (AS) into the AGII genome.** ZYMV-AGII (AGII) is a potyvirus-based vector system that has recently been developed for the expression of foreign genes in cucurbits (Arazi et al. 2001). To construct AGII-TIPK and AGII-AS, the TIPK gene (1200 bp) and a 300-bp antisense fragment of TIPK were amplified from the TIPK cDNA clone by PCR. For AGII-TIPK, the primers used were: forward 5’- ATACTGCAGGCTGATGTTGGTCAGAACAAC-3’, reverse 5’- ATAGTCGACTGCAAATTCTGGATTGAGTGC-3’, with the added PstI and SalI sites, respectively (underlined). For AGII-AS, the primers used were: forward 5’- ATACTGCAGAGTAAGCTTAATCTCACGGAACG-3’, reverse 5’- ATAGTCGACGAAGGCCTTCTGTGACAGG-3’, with the added PstI and SalI sites, respectively (underlined). The amplified fragments were double-digested with the respective enzymes and cloned into the AGII genome between the CP and the NIb-coding regions. Sequence analysis was used to verify that no mutations were inserted by the PCR. We also used the AGII-GFP construct described in Arazi et al. (2001).

**Plant growth conditions and virus inoculation.** Potted squash (Cucurbita pepo L cv. Ma’ayan) was grown in a greenhouse. Particle bombardment was used to propel microprojectiles containing plasmid with the AGII-TIPK, AGII-AS, or AGII-GFP constructs into the fully expanded cotyledons of each plant as described in Gal-On et al. (1997). Infected squash leaves (10-14 dpi) were extracted with ice-cold water (1 g/5 ml) and centrifuged at 4000 rpm for 5 min.
Cotyledons of 10-day-old cucumbers grown in the hydroponic system were mechanically inoculated by rubbing sap extract on them with a sterile gauze sheet. *Trichoderma* inoculation of AGII-infected plants was performed 13 days post-AGII infection.

**RT-PCR analysis of recombinant virus progeny.** The second leaf from each plant was harvested. RNA extraction and reverse transcription were conducted as already described. The PCR was performed using AGII polylinker flanking primers: 5’-AAGGGAGCGGATACAAGTGA-3’ and 5’-TGATGAGACGCTCGTGTGTT-3’. PCR conditions were: 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min, for 40 cycles.

**Bacterial inoculum.** *Pseudomonas syringae* pv. *lachrymans* (Psl) was grown in Tryptic Soy Broth (Difco) overnight at 30°C. Bacterial cells were pelleted at 5000 rpm and resuspended in sterile saline-phosphate buffer (5 mM, pH 7.2). Challenge was performed 48 h post-*Trichoderma* inoculation to the PGM. Psl bacterial suspension (20 µl; OD 0.5) containing 0.01% (v/v) surfactant (Tween 20) was applied to the surface of the second leaf and gently smeared with a sterile tip. Bacterial inoculation was performed under aseptic conditions. Psl multiplication in the leaves was assessed 72 h post-bacterial challenge. These experiments were repeated three times. Leaves were weighed and each leaf was homogenized in a sterile solution of 10 mM phosphate-saline buffer (1 ml/leaf). Tenfold dilutions were plated onto *Pseudomonas*-selective King’s B agar supplemented with 1 ml/l of 9 mg/ml basic fuchsin, 200 mg/ml cycloheximide, 10 mg/ml nitrofurantoin and 23 mg/ml nalidixic acid. After incubation at 28°C for 2 days, the number of Psl colony-forming units per gram of infected tissue was determined. We also harvested the upper leaf for real-time analysis and to confirm the AGII virus’s presence via RT-PCR analysis (as already described).

**Statistical analysis.** Statistical analysis was performed using STATISTICA 7 software. Data were subjected to one-way ANOVA analysis and Tukey-Kramer HSD for comparison of means.
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FIGURE LEGENDS

FIGURE 1: Time course of TIPK gene expression. Expression was measured in roots (A) and leaves (B) of cucumber plants after Trichoderma inoculation of the root compartment (time zero) and normalized versus the control gene. Two experiments (empty and full symbols) were conducted, each including ca. 20 plants per time point. Relative mRNA levels were determined by real-time PCR (see Materials and Methods). The internal standard deviation values for each experiment were smaller than the size of the symbols. ■□—Inoculated with Trichoderma; ⋄〇—control, mock-inoculated plants.

FIGURE 2: Genomic organization of TIPK gene. (A) Schematic illustration of the TIPK gene as deduced from sequence and PCR analyses. (B) Southern analysis of total cucumber DNA digested by the indicated restriction enzyme using TIPK cDNA as a probe. EcoRI digested DNA was made in separate membrane in order to get a more clear data (Faint bands correspond to residuals of partial digestion).

FIGURE 3: Protein-sequence alignment of the TIPK sequence (CsTIPK) with its homologues from Arabidopsis thaliana (AtMPK3; gi21431794), Nicotiana tabacum (NtWIPK; gi18143321) and Petroselinum crispum (PcMPK3a; gi2231034). Fully conserved residues are indicated by black boxes; gray and white colors represent similar and different amino acids, respectively.

FIGURE 4: Time course of TIPK gene expression. (A) Expression was measured in wounded-leaves of cucumber plants after wounding (time zero), 96 h post-Trichoderma inoculation into the root compartment, and normalized versus the control gene. Relative levels of TIPK mRNA were determined by real-time PCR (see Materials and Methods). Symbol at each time point represents the average of 10 plants ± SE. ⋄—Non-Trichoderma inoculated and wounded; ■—inoculated with Trichoderma and wounded. (B) Expression was measured in the non-wounded upper leaves of wounded plants (hatched boxes) and control non-wounded plants (black boxes). The presented values are means (±SE, n = 4).

FIGURE 5: RT-PCR analysis of the TIPK gene and the control gene 18S (A) after JA or SA root treatments at concentrations of 0, 0.5, 1 and 2 mM each, and (B) after DIECA (inhibitor of jasmonate biosynthesis) or STS (inhibitor of ethylene action) root treatments at concentrations of 100 μM and 0.25 mM, respectively. PCR was conducted for 20 cycles for the TIPK gene, 25 cycles for lipoxygenase and chitinase genes, and 18 cycles for the 18S gene to keep the
amplification within the linear region of the reaction. For each gene we first normalized the intensity of the band versus its corresponding 18S band. We then calculated the ratio between each hormonal treatment to the control (no hormonal treatment). The Ratio therefore, indicates the expression of each gene as compared to control treatments. C – control; T – *Trichoderma* inoculation; D – DIECA treatment; TD – *Trichoderma* inoculation and DIECA treatment; S – STS treatment; TS – *Trichoderma* inoculation and STS treatment.

**FIGURE 6:** Relative expression levels of *TIPK* post-pathogen challenge. Expression was measured in roots (hatched boxes) and leaves (black boxes) of cucumber plants after the following treatments: -T-P – control (mock inoculations); +T-P – *Trichoderma* inoculation in the root compartment, at time zero; -T+P – *Pseudomonas syringae* pv. *lachrymans* (Psl) infection of cotyledons at time 48 h; +T+P – *Trichoderma* inoculation at time zero and Psl infection at time 48 h. Roots and leaves for expression measurements were harvested 48 h after time of Psl infection. These experiments were repeated three times with approximately 15 plants per treatment. The presented values are means of all plants in each treatment (±SE).

**FIGURE 7:** Expression of *TIPK* gene via ZYMV-AGII. (A) Schematic presentation of the AGII genome. AGII non-coding (stippled) and coding (open boxes) regions, and the inserted foreign sequences (*TIPK*, *AS* and *GFP*) are shown. Arrows indicate NIa protease, involved in proteolysis of the foreign gene products. NIa cleavage sites are indicated by /. Amino-acid sequences corresponding to the NIa protease recognition motif are indicated in bold. (B) Analysis by RT-PCR of AGII-GFP, AGII-AS, and AGII-TIPK viral RNA accumulation 14 and 17 days post-inoculation. Total RNA was extracted from infected and non-infected plants and subjected to RT-PCR with primers flanking the insertion point. The expected sizes of the PCR fragments are: AGII-GFP – 1180 bp; AGII-AS – 680 bp; AGII-TIPK – 1480 bp.

**FIGURE 8:** Virus localization in leaves. Visualization of GFP fluorescence in leaves of AGII-GFP-infected plants and mock-infected plants (bottom) compared with the leaves (top), 17 days post-infection.

**FIGURE 9:** Relative expression levels of *TIPK* in AGII-construct-infected plants. Expression was measured in leaves of cucumber plants 14 days (black boxes) and 17 days (hatched boxes) post AGII-construct infection. When ever plants were also treated with *Trichoderma* it was done 24 h prior to time point of 14dpi. The number of plants in each treatment were as follows: no treatment, 14 dpi – six plants; mock inoculation, 14 dpi – 10 plants; +T – *Trichoderma* inoculation, 14 dpi – 10 plants; -T+P – Psl infection, 14 dpi – 10 plants; +T+P – Psl infection, 14 dpi – 10 plants; +T+P – Psl infection, 17 dpi – 10 plants.
plants; AGII-GFP infected (GFP), 14 dpi – 12 plants and 17 dpi – 20 plants; AGII-GFP infected with *Trichoderma* inoculation (GFP+T) -14 dpi – six plants and 17 dpi – six plants; AGII-AS infected (AS), 14 dpi – 16 plants and 17 dpi – 22 plants; AGII-AS infected with *Trichoderma* inoculation (AS+T), 14 dpi – six plants and 17 dpi – six plants; AGII-TIPK infected (TIPK), 14 dpi – six plants and 17 dpi – six plants. The presented values are means (±SE). Different letters indicate statistically significant differences between treatments by one-way ANOVA data analysis and Tukey-Kramer for comparing means (α = 0.05).

**FIGURE 10:** Quantification of *Pseudomonas syringae* pv. *lachrymans* (Psl) multiplication in AGII-construct-treated plants. Cotyledons of 10-day-old cucumbers grown in the hydroponic system were mechanically inoculated with AGII-virus construct and after 13 days inoculated with *Trichoderma*. Challenge was performed 48 h post-*Trichoderma* inoculation. Leaves were harvested 72 h post-challenge with Psl. The treatments and the number of repeats in each treatment were as follows: AGII-AS challenged with Psl, n = 12; AGII-AS pre-inoculated with *Trichoderma* and challenged with Psl, n = 20; AGII-GFP challenged with Psl, n = 23; AGII-GFP pre-inoculated with *Trichoderma* and challenged with Psl, n = 26; AGII-TIPK challenged with Psl, n = 18. The presented values are means (±SE). Different letters indicate statistically significant differences between treatments by one-way ANOVA data analysis and Tukey-Kramer for comparing means (α = 0.05).
Figure 1
Figure 2
Figure 3
Figure 4

Relative mRNA levels vs. time post wounding (h)

- C
- T

Relative mRNA levels

Time post wounding (h)

0 5 10 15 20

0 2 4 6 8 10 12 14 16 18

B

Relative mRNA levels

control
wound

Time post wounding (h)

3 h 24 h 48 h
Figure 5
Figure 6
Figure 7
Mock AGII-GFP

Figure 8
Figure 9
Figure 10