Trichoderma asperellum Induces Maize Seedling Growth by Activating the Plasma Membrane H⁺-ATPase

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Although *Trichoderma* spp. have beneficial effects on numerous plants, there is not enough knowledge about the mechanism by which they improves plant growth. In this study, we evaluated the participation of plasma membrane (PM) H⁺-ATPase, a key enzyme involved in promoting cell growth, in the elongation induced by *T. asperellum* and compared it with the effect of 10 μ M indol acetic acid (IAA) because IAA promotes elongation and PM H⁺-ATPase activation. Two seed treatments were tested: biopriming and noncontact. In neither were the tissues colonized by *T. asperellum*; however, the seedlings were longer than the control seedlings, which also accumulated IAA and increased root acidification. An auxin transport inhibitor (2,3,5 triiodobenzoic acid) reduced the plant elongation induced by *Trichoderma* spp. *T. asperellum* seed treatment increased the PM H⁺-ATPase activity in plant roots and shoots. Additionally, the *T. asperellum* extracellular extract (TE) activated the PM H⁺-ATPase activity of microsomal fractions of control plants, although it contained 0.3 μ M IAA. Furthermore, the mechanism of activation of PM H⁺-ATPase was different for IAA and TE; in the latter, the activation depends on the phosphorylation state of the enzyme, suggesting that, in addition to IAA, *T. asperellum* excretes other molecules that stimulate PM H⁺-ATPase to induce plant growth.

Trichoderma spp. are beneficial fungi that colonize the root epidermis and the outer cortical layers (Harman 2006). Some *Trichoderma* strains promote plant growth, prevent plant infection, and increase root growth to cope with adverse nutritional conditions (Harman et al. 2004b; Hermosa et al. 2012; Keswani et al. 2014). Numerous studies have demonstrated the detrimental effects caused by *Trichoderma* spp. on soilborne plant pathogens; some *Trichoderma* strains, including *Trichoderma asperellum*, can be used for the biocontrol of *Phytophthora capsici*, *Rhizoctonia solani*, *Fusarium oxysporum*, and other important plant diseases (El_Komy et al. 2015; Ferrigo et al. 2014; Harman et al. 2004a,b). Although recent research has focused on biocontrol activity, the *Trichoderma* genus is extensively used around the world to promote plant growth; for example, *T. harzianum* strain T22 increases shoot and root development in several plants, including maize (Harman 2006).

Different mechanisms have been proposed to explain *Trichoderma* plant growth promotion; one involves auxins such as

indole acetic acid (IAA) (a major auxin in plants) and indole-acetaldehyde, which are synthesized by *T. virens* and *T. atroviride*, respectively, both of which induce plant root growth (Contreras-Cornejo et al. 2009). Furthermore, *Arabidopsis* auxin-transport mutants (*aux1-7*, *eir1*, and *doc1*) showed a reduced growth response when interacting with *Trichoderma* spp., suggesting that auxin is involved in *Trichoderma* plant growth promotion (Contreras-Cornejo et al. 2009). However, some *Trichoderma* strains induce plant growth independently of auxin biosynthesis (Hoyos-Carvajal et al. 2009).

It is well established that auxin promotes the elongation of epidermal cells, which leads to root hair (RH) formation (Overvoorde et al. 2010). In general, cell elongation induced by auxins has been explained by the acid growth theory, in which auxins stimulate proton extrusion as a requisite to activation of cell-wall-degrading enzymes to produce cell wall loosening and then allow the elongation of aerial and underground tissues (Enders and Strader 2015; Moloney et al. 1981; Rober-Kleber et al. 2003). The acidification of the apoplast is produced by ion imbalance, and the activity of the plasma membrane proton (PM H⁺) ATPase can contribute to acidification (Hager et al. 1991; Moloney et al. 1981).

PM H⁺-ATPase is the major plant PM pump; it belongs to the P-type ATPase family (Janicka-Russak 2011). This enzyme couples two reactions: ATP hydrolysis and proton transport to the apoplast space. PM H⁺-ATPase is tightly regulated because it is involved in many physiological processes, such as pH homeostasis, cell nutrition, stomata aperture, and cell growth (Morsomme and Boutry 2000; Morth et al. 2011). PM H⁺-ATPase activity is controlled at different levels: a multigene family of PM H⁺-ATPases that has been identified in many plants and isozymes with several differences in the catalytic and regulatory properties, in addition to the distinct abundance of each isozyme in each tissue (Falhof et al. 2016; Frías et al. 1996; Janicka-Russak 2011; Morsomme and Boutry 2000).

In addition to PM H⁺-ATPase abundance, its enzyme activity it is also modulated by its regulatory domain (R domain), located at the end of the carboxy-terminal region. Increased H⁺-ATPase activity is found when 110 amino acids of the PM H⁺-ATPase are removed. The R domain can change its conformation depending on its phosphorylation status or binding the regulatory 14-3-3 protein, or its interaction with specific lipids in their surroundings; therefore, the posttranslational modifications allow the enzyme to alternate in two activity states: upregulated or downregulated (Falhof et al. 2016; Haruta et al. 2015; Morales-Cedillo et al. 2015).

The 14-3-3 proteins bind to a variety of phosphorylated proteins; H⁺-ATPase binds at the penultimate phosphorylated Thr (Thr947 in AHA2), altering the conformation of the R domain, which increases ATP hydrolysis and proton extrusion (Falhof

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et al. 2016). Other amino acids at the carboxy terminus of the H⁺-ATPase are phosphorylated and are involved in changing the activity of the protein; Thr881 in AHA2 activates the enzyme, and Thr-924 or Ser-931 inhibits it (Falhof et al. 2016). Metabolites and peptides from pathogens change the PM H⁺-ATPase activity; for example fusicoccin, a fungal toxin, activates the enzyme by stabilizing the binding between the 14-3-3 protein and the R domain (Elmore and Coaker 2011; Falhof et al. 2016; Johansson et al. 1993; Korthout and de Boer 1994). In contrast to fusicoccin, FB1, a phytotoxin produced by *F. verticillioides*, inhibits PM H⁺-ATPase (Gutiérrez-Nájera et al. 2005). Lyso-phospholipids and free fatty acids also alter the ATPase activity by a mechanism involving the conformational change of the R domain (Morales-Cedillo et al. 2015).

Auxins also enhance the PM H⁺-ATPase activity by a variety of mechanisms. A rapid mobilization of membranes containing the enzyme from the endoplasmic reticulum to the PM is induced by auxin (Frías et al. 1996; Hager et al. 1991). Auxin binding to auxin binding protein 1 (ABP1) starts a phosphorylation cascade that activates the PM H⁺-ATPase by altering its phosphorylated status (Tromas et al. 2010). Etiolated coleoptiles lacking endogenous IAA are sensitive to the exogenous IAA addition and activate the PM H⁺-ATPase through the phosphorylation of its penultimate residue (Takahashi et al. 2012). PM H⁺-ATPase is rapidly induced by auxin through the activation of small auxin up-RNA (SAUR) genes; SAUR proteins specifically bind to a family of type 2C protein phosphatases, and this inhibition increases PM H⁺-ATPase activity (Spartz et al. 2014).

Auxin contribution to plant elongation induced by *Trichoderma* spp. has been demonstrated (Contreras-Cornejo et al. 2009, 2014); however, the *Trichoderma*-induced activation of PM H⁺-ATPase as an important ion pump in the plant cell membrane has not yet been determined. The approach to finding specific plant targets of *Trichoderma* metabolites may open the possibility of developing better strains or selecting specific metabolites to enhance plant performance because there is strong physiological evidence that PM H⁺-ATPase is a driver of plant growth (Enders and Strader 2015; Moloney et al. 1981; Rober-Kleber et al. 2003). In this study, PM H⁺-ATPase was studied in microsomal fractions in roots and shoots from seedlings treated with the fungus and compared with the enzyme from IAA-treated plants. Two models of *T. asperellum* seed treatment were applied to analyze the effect of the fungus on plant growth and H⁺-ATPase activity: biopriming, in which the seed were soaked in *T. asperellum* conidia, and noncontact treatment, in which the seed were grown in the same petri dish on agar medium with *T. asperellum* but without contacting the fungus. The rationale of the use of both treatments is to demonstrate that only fungus metabolite exudates could affect plant growth. Seed without treatment were used as a control. All of the seed were grown in agar, and the untreated control (C), biopriming (B), noncontact (NC), and IAA (A) seedlings were examined for plant growth, peroxide and auxin contents, and PM H⁺-ATPase activity.

RESULTS

T. asperellum promotes the growth of shoot and RH of maize seedlings without colonization.

It is known that *Trichoderma* fungi invade the roots of a variety of plants (Leroux et al. 2011; Yedidia et al. 1999); to identify hyphae from *T. asperellum* in B seedling roots, they were immersed in a solution of black ink Sheaffer (Vierheilig et al. 1998). Interestingly, at 72 h of germination, the fungus was located on the seed surface but not in the primary root or in the seed (Fig. 1A to F). Although *T. asperellum* does not invade the tissue at 72 h of germination, it modifies the seedling morphology (Fig. 2 and 3). However, at 10 days of germination

with *T. asperellum*, the hyphae were detected inside of the root epidermis (Fig. 1G to I).

Biopriming increases the shoot elongation, particularly of the mesocotyl, which represents 53% of the total shoot length (Fig. 2A and B). *Trichoderma* spp. growth-promotion activity has been linked to the hormone auxin (Contreras-Cornejo et al. 2009); therefore, to compare with the other treatments, a group of seed was imbibed in 10 μM IAA for 30 min and grown in agar medium for 72 h, and A seedlings were examined. A seedling shoots were as long as the B seedlings. For the NC treatment, the seed were germinated in potato dextrose agar (PDA) to allow *T. asperellum* to grow; then, as a control for this treatment, the C seed were grown on PDA plates. NC seedlings had shoot lengths similar to that of the C seedlings grown in either PDA or 1% agar (Fig. 2C). The observed shoot length increase in B, NC, and C seedlings did not correlate with an increase in fresh or dry weight (Supplementary Table S1).

Seedling root architecture was affected by *T. asperellum* and IAA treatment; the primary root lengths in B and A seedlings were shorter by 4 and 23%, respectively, compared with that of the C seedlings (Fig. 3A). In contrast, the NC seedlings had a longer primary root compared with that of the C seedlings germinated in PDA (Figs. 2C and 3A). However, the fresh and dry weights of the roots were not altered. In both *Trichoderma* treatments, the RH were affected; the length and density of the RH were measured in two of the three zones of the roots, and the root apex was not examined because of a lack of RH (Supplementary Fig. S1). The fungus and the auxin treatments promoted a long RH in the middle zone of the root (from 0.8 to 2.5 cm to the apex) but not in the basal zone (from 2.5 cm to the root basis) (Fig. 3B). Moreover, the RH density in the middle and basal zones of the root of B, NC, and A seedlings was higher than that in C seedling roots (Fig. 3C). It has been suggested that *Trichoderma* and auxin treatments improve root nutrient acquisition (Brown et al. 2013; Ma et al. 2001; Yedidia et al. 1999). To evaluate the increase in the root surface, we multiplied the primary root length by the RH density. The *T. asperellum* and IAA treatments gave five and two times higher values in the middle and basal zones, respectively, compared with the C seedlings (Fig. 3D). Denser and longer RH could indicate that *T. asperellum* and IAA treatments changed the root architecture to improve root nutrient acquisition (Ma et al. 2001; Miguel et al. 2015; Singh et al. 2013). The signal molecules H₂O₂ and IAA are produced when *T. asperellum* interacts with maize seedlings.

Studies have reported that reactive oxygen species (ROS) are produced during germination and are required for RH elongation and development (Carol and Dolan 2006). The levels of H₂O₂ were determined in the apex, middle, and basal root zones in the C, B, NC, and A seedlings (Fig. 4A). The level of H₂O₂ was similar in the root apex between C, B, and NC seedlings but a slow reduction in H₂O₂ was detected in A seedlings. In the middle zone of the roots of all the seedlings in which the RH were longer and abundant, no change in H₂O₂ was detected (not significantly different, *P* < 0.05, Tukey's test). In contrast, in the basal zone of the root, *T. asperellum* treatment increased the amount of H₂O₂ by two times, coinciding with the increase in the RH density measured in the B and NC seedlings.

IAA has been proposed as a growth promotor molecule produced by the genus *Trichoderma* (Contreras-Cornejo et al. 2009). IAA was identified by high-performance liquid chromatography (HPLC) from a total extract of *T. asperellum* HK703 grown in liquid potato dextrose for 3 days. Fungal culture produces IAA at 72.52 ± 15.14 μg/g of dry weight, which might explain the improved seedling growth. Furthermore, the treatments with *T. asperellum* increased the IAA content in the aerial and root tissues; the IAA content was higher in B seedlings by fivefold in shoots and by twofold in

roots. The NC seedlings had 3.5- and 2.5-fold more IAA in the shoots and roots, respectively. A significant amount of IAA was found in A seedlings, being 21 times greater in the shoots and 57 times greater in the roots (Fig. 4B).

Despite the significant increase of IAA in A seedlings, these seedlings grew similarly to the B and NC seedlings (Figs. 2 and 4C). IAA is stored in different organs and released during germination and growth (Hohm et al. 2014; Locascio et al. 2014); therefore, to explore the effect of reduced IAA transport during germination, seed were imbibed in 2,3,5 triiodobenzoic acid (TIBA), an auxin transport inhibitor (Choi et al. 2001), before undergoing biopriming, NC, or auxin imbibition. All of the seed that were treated with TIBA appeared stunted, with the TIBA + C (T+C) seedlings being the shortest, indicating an absolute requirement of IAA transport for seedling growth (Fig. 4C).

***Trichoderma* prompts the extracellular acidification of root maize seedlings.**

According to the acid growth theory, decreased pH in the extracellular zone is a prerequisite for tissue elongation (Takahashi et al. 2012). Because treatments with *Trichoderma* and IAA induce RH growth, the roots of these seedlings were immersed in water to monitor the pH for 4 h. A decrease in one unit of pH at 20 min was found in the solution in which the B, NC, and A seedlings roots were immersed, whereas the roots of the C seedlings produced a decrease of 0.5 units at this time. At the end of 4 h, the pH was lower in the water in which the roots of B, NC, and A seedlings were immersed (Fig. 5). The same result, a progressive acidification of media, was obtained when C, B, NC, and A seedlings were transplanted in tubes containing agar supplemented with 0.003% bromocresol purple, with a higher acidification of the medium by the roots from seedlings treated with fungus and IAA (Supplementary Fig. S2).

High PM H⁺-ATPase activity in the root and shoots in *T. asperellum*-treated seedlings.

Extracellular root acidification and growth induced by *T. asperellum* could be explained by a higher proton cell extrusion mediated by the activation of PM H⁺-ATPase, an essential enzyme that participates in cellular expansion, nutrition, and intracellular pH regulation (Haruta et al. 2015). High ATPase activity was found in microsomal fractions (MF) from the shoots and primary roots of B and NC seedlings compared with C seedlings (Fig. 6). It is known that IAA induces the activity of PM H⁺-ATPase (Haruta et al. 2015; Hohm et al. 2014; Takahashi et al. 2012); as expected, the MF of A seedlings showed high PM H⁺-ATPase activity. Therefore, the increased ATPase activity in the roots and shoots of B and NC seedlings could be due to the presence of auxin because *T. asperellum* enhanced IAA accumulation in these seedlings (Fig. 4B).

An elevated enzyme level might explain the higher PM H⁺-ATPase activity (Hager et al. 1991; Frías et al. 1996); however, no change in the enzyme was detected on the Western blot of the MF of the roots and shoots of all seedlings using an antibody against the PM H⁺-ATPase (Fig. 6A). A larger amount of enzyme was found on the roots compared with the shoots in all of the treated seedlings, which could account for the higher ATPase activity detected in the roots compared with the shoots (Fig. 6). Then, the PM H⁺-ATPase activity and not the protein abundance was enhanced in the seedlings treated with the fungus and auxin.

PM H⁺-ATPase activity is stimulated by auxin and by another molecule secreted by *T. asperellum*.

Posttranslational changes can increase the PM H⁺-ATPase activity. The C-terminal domain constitutes the autoinhibitory region of PM H⁺-ATPase; to activate the enzyme, it is required to remove the region or modify its conformation. Phosphorylation at serine and threonine and the interaction with the 14-3-3 protein

Fig. 1. *Trichoderma asperellum* detection in biopriming treated seedling. Staining of hyphae was made as described by Vierheilig et al. (1998). Tissues were longitudinally hand cut, then clarified in boiling 5% KOH for 5 min, submerged in acetic acid solution with black ink Sheaffer (Sheaffer Slovakia s.r.o., Sered, Slovak Republic), and washed. This method stains the hyphae in black and the plant tissue sometimes acquires a brownish color. **A to C**, Untreated control (C) seedling; **D to F**, biopriming (B) seedling from 72-h germinated seed; **G to I**, B seedling from 10-day germinated seed. **A, D, and G**, Seedling close-up; **B, E, and H**, primary root; **C, F, and I**, cortex cells of primary root. In B seedlings from 72-h germinated seed, the mycelium adhered to the root hairs (rh) (D) but it was not present inside of the cortex cells of the primary root (F). Abundant mycelium adhered to the epidermis was detected on B seedling at 10 days of germination (H) inside of the cortex of the primary root (I). Photographs are representative of five different tissues. Arrows indicate *T. asperellum* hypha.

or the addition of molecules such as L- α -lysophosphatidylcholine (LPC) and some phytotoxins induces PM H⁺-ATPase activity through the C-terminal domain (Axelsen et al. 1999; Elmore and Coaker 2011; Fuglsang et al. 2007; Takahashi et al. 2012). With the purpose of identifying the mechanism by which *T. asperellum* induces PM H⁺-ATPase activity and to determine whether the *T. asperellum* exudate (TE) can induce the enzyme, the MF from the roots and shoots of C seedlings were incubated with phosphatase alkaline-agarose to dephosphorylate the enzyme. A control with MF incubated with a denatured phosphatase alkaline-agarose was included. After treatment, the PM H⁺-ATPase activity was determined in an ATP hydrolysis reaction assay supplemented with the activators 100 μ M LPC and 10 μ M

IAA (final medium concentration) (Morales-Cedillo et al. 2015; Takahashi et al. 2012) or TE obtained from *T. asperellum* potato dextrose broth (PDB) liquid culture.

The PM H⁺-ATPase activity in the MF from C seedling shoots was stimulated by the addition of IAA, LPC, and TE, with TE inducing the higher activity (Table 1). Similarly high PM H⁺-ATPase activity was obtained in root MF when IAA and TE were added. As an exception, LPC did not stimulate the activity; thus, it is possible that the treatment with the denatured phosphatase alkaline-agarose modified the ATPase conformation in the membrane.

Dephosphorylation treatment of the MF decreased the PM H⁺-ATPase activity by one-half in roots and shoots; it is well established that the dephosphorylation of the carboxy-terminus domain inhibits ATPase activity (Spartz et al. 2014; Takahashi et al. 2012). The addition of TE, IAA, and LPC to dephosphorylated MF also stimulated the PM H⁺-ATPase activity but reached different levels of activity depending on the molecule added; the largest increase was attained with LPC. IAA enhanced the ATPase activity independently of the dephosphorylation treatment, reaching the same level of activity in dephosphorylated and nondephosphorylated membranes. In contrast, TE stimulated the ATPase activity at the maximum only when the MF were not dephosphorylated. Because the activation by IAA and TE was different depending on the phosphorylation of PM H⁺-ATPase, the results suggest that the TE not only has IAA but also other molecules that stimulate PM H⁺-ATPase, because a low amount of IAA was present in 8 μ l of TE (0.31 μ M IAA).

DISCUSSION

Trichoderma spp. are known plant growth promoters; they induce short- and long-term plant improvement, which affects plant productivity, such as increasing the biomass accumulation, the number of leaves, and the photosynthetic rate (Akladios and Abbas 2014; Doni et al. 2014; Harman 2000). In this study, *T. asperellum* biopriming stimulated maize shoot enlargement, conferring an important advantage over noncolonized plants. Plant morphology is genetically determined but is affected not only by environmental factors such as light, temperature, and nutrient supply but also by internal hormonal changes (Kazan 2013). For example, light stops mesocotyl elongation, and the addition of auxin and gibberellin reverse light inhibition (Vanhaelewyn et al. 2016). A similar and significant increase in mesocotyl length in B and A seedlings may indicate an auxin effect. Zhao and Wang (2010) analyzed five maize inbred lines and, in one, found mesocotyl elongation linked to a high auxin content in that tissue. The elongation process was related to cell enlargement and not to cell division. The lack of shoot length stimulation by the NC treatment could be because the aerial tissue did not contact the PDA in which some of the *T. asperellum* exudated molecules were diffused.

However, the two *T. asperellum* seed treatments produced strong changes in root architecture, although the fungus did not internally colonize the tissues at 72 h of seed germination, suggesting that the effect on plant growth was caused by *T. asperellum* exudates. A contrasting root length was achieved by B seedlings and NC seedlings, with shorter and longer primary roots, respectively, but both *T. asperellum* treatments induced longer and denser RH. Similar changes in RH development were observed in *Arabidopsis* seedlings when they interacted with *T. virens* and *T. atroviride* in a noncontact treatment (Contreras-Cornejo et al. 2014).

RH are cytoplasmic extensions from specialized root epidermal cells that extend the contact surface between the root and the soil by 70% and can facilitate water and nutrient absorption (Richardson et al. 2009). *T. asperellum* induces root

Fig. 2. Morphological changes induced by *Trichoderma asperellum* and the auxin (indol acetic acid [IAA]). **A**, Representative images of maize seedlings treated with *T. asperellum* and 10 μ M IAA. C = control, B = biopriming, NC = noncontact, and A = auxin seedlings. Bar = 5 cm. **B**, Seedling shoot length. **C**, Comparison of C and NC seedlings grown in potato dextrose agar (PDA). Seed of the noncontact treatment were germinated in PDA medium to allow *T. asperellum* to grow. Data represent means and standard deviation (error bars) from 30 seedlings in triplicates. Different letters statistically represent different values of the total shoot length at the $P < 0.05$ level according to Dunn's test.

architecture changes, suggesting that the fungus provokes adjustments to improve the root surface contact with the soil and nutrient acquisition capacity (Brown et al. 2013; Ma et al. 2001; Thomas and Hedden 2006). In *Arabidopsis*, phosphate deficiency induced longer and denser RH, and mutants in RH development under phosphate deficiency accumulate less phosphate and biomass (Bates and Lynch 2000). These results suggest that root architecture is important for nutrient uptake under poor-nutrient soils. Changes in root architecture have been observed in *Arabidopsis* seedlings when they interacted with *T. virens* and *T. atroviride*; longer and denser RH are induced, especially when *Arabidopsis* is grown under salt stress (Contreras-Cornejo et al. 2014).

Biopriming and noncontact treatments modified the RH similarly, indicating that, in both seed treatments, similar molecules were secreted by the fungus, which changed the RH morphology. The opposite primary root growth in both treatments indicates the presence of different types or concentrations of molecules secreted by the fungus depending on the growing medium. Regardless of the different root primary elongation responses that have been reported, the increase in primary root length produced by *T. harzianum*-colonized root was explained by Björkman (2004). It is neither by change in auxin sensitivity nor by antiauxin molecule secreted by the fungus but by faster growth response. The inhibition on primary root elongation in *Arabidopsis* during the interaction with *T. virens* and *T. atroviridae* was found to be dependent of the auxin transporters and signaling (Contreras-Cornejo et al. 2009). In our case, TIBA reduced the effect of *T. asperellum* on maize seedling growth and, thus, it is possible that auxin transport is implicated in the morphological changes induced by *T. asperellum*.

The *T. asperellum* exudate was only analyzed for its IAA content but it has been reported that *Trichoderma* spp. secrete a wide variety of molecules involved in different aspects of disease development such as attaching to plant tissues, eliciting the defense response, degrading plant and fungi cell walls, metabolites having antimicrobial properties, and another set of molecules having growth properties (Adav and Sze 2014; Contreras-Cornejo et al. 2009; Keswani et al. 2014; Pazzagli et al. 2014). Some of the growth regulators secreted by various *Trichoderma* strains are trichocaranes, trichosetin, cyclo-nerodiol, trichokonin, viridiol, harzianolide, koniginins, and 6-pentyl-2H-pyran-2-one, most of which have plant growth inhibitory or promoting properties, depending on the concentration used (Keswani et al. 2014). Therefore, it is possible that more than one of the molecules secreted by *T. asperellum* can induce seedling physiological modifications and, in the case of the noncontact treatment, the PD medium could affect its metabolism, which influences the seed development differently (here, an observed increase in primary root growth). The TE effect demonstrates that a low level or absence of IAA is not necessary to activate H^+ -ATPase and promote plant cell growth. Recently, it was found that 6-pentyl-2H-pyran-2-one, a volatile molecule excreted by *Trichoderma* spp., affects auxin transport and the ethylene-response modulator EIN2 (Garnica-Vergara et al. 2016).

Different mechanisms have been proposed to promote plant growth when *Trichoderma* spp. interact with plants. One of these mechanisms involves auxin signaling (Harman et al. 2004a). *T. virens* induces *Arabidopsis* growth and secretes IAA, indole-3 acetaldehyde, and indole-3 ethanol. *Arabidopsis* mutants in auxin transport genes (AUX1, BIG, EIR1, and AXR1) failed to grow

Fig. 3. Primary root architecture in seedling treated with *Trichoderma asperellum* and indol acetic acid (IAA). **A**, Percentage of primary root elongation in C = control, B = biopriming, C-NC = control growing in potato dextrose agar, NC = noncontact, and A = auxin seedlings. **B**, Root hair (RH) length; **C**, density expressed in RH per square millimeter; and **D**, values from multiplied RH length by RH density. In each treatment, 100 RH were measured. Data represent means and standard deviations (error bars) from three experiments in duplicates. Different lowercase letters indicate significant differences at $P < 0.05$ level (Tukey's test).

when they interacted with *T. virens* (Contreras-Cornejo et al. 2009). The high expression of DR5:uidA, an auxin-responsive marker gene, occurred in *Arabidopsis* infected with *Trichoderma* spp. (Contreras-Cornejo et al. 2014). This evidence strongly favors the hypothesis that auxin is responsible for growth promotion by *Trichoderma* spp.

Furthermore, when tissues of B and NC seedlings were analyzed, a significant amount of IAA was found, and incubation with TIBA, an auxin transport inhibitor, produced a severe growth reduction, indicating that auxin induces the morphological changes observed in the seedlings treated with *Trichoderma* spp. Nevertheless, strains of *Trichoderma* unable to produce IAA also stimulate plant growth (Hoyos-Carvajal et al. 2009).

H₂O₂ is one ROS molecule that plays an important role in many physiological processes, including senescence, development, and adaptation (Tognetti et al. 2012). In addition, ROS

affects auxin biosynthesis, transport, metabolism, and signaling (Tognetti et al. 2012). In maize and *Arabidopsis*, ROS accumulates at the root apex and regulates the activity of calcium channels required for polar growth (Carol and Dolan 2006; Liskay et al. 2004). H₂O₂ accumulation in the middle zone of the roots resulting from *T. asperellum* seed treatment could be a signal to maize seedlings to carry out adaptive changes in root morphology, an adaptation to stress and not only a growth response (Voothuluru and Sharp 2013). Other ROS might participate in growth promotion; for example, superoxide anion produced by the PM NADPH oxidase and the hydroxyl radical produced by the cell wall peroxidase activity participate in the growth process, inducing cell wall loosening and RH growth

Fig. 4. Signaling molecules and 2,3,5 triiodobenzoic acid (TIBA) effect in shoot and root of treated seedlings. **A**, Hydrogen peroxide content in the apex, middle, and basal zone of primary root of C = control, B = biopriming, NC = noncontact, and A = auxin seedlings. Data represent means and standard deviations (error bars) from three experiments in triplicate. Different letters indicate significant differences at the $P < 0.05$ level according to Tukey's test. **B**, Indol acetic acid (IAA) content in shoot and primary root tissues determined by high-performance liquid chromatography; the IAA content is expressed in micrograms of IAA per gram of dry weight tissue. Data represent means and standard deviation (error bars) from two experiments in triplicate. Different letters indicate significant differences at the $P < 0.05$ level according to Tukey's test. **C**, Effect of TIBA on seedling elongation. Morphological characteristics of the seedling are affected when the seed were previously incubated in 10 μM TIBA (T) for 30 min before each treatment: T, T + B, T + NC, and T + A seedlings. Representative photographs of 10 seedlings and five independent tissue samples. Bar = 5 cm.

Fig. 5. Physiological test of extracellular root acidification. Roots of 72-h treated seedlings were immersed in 4 ml of water, and the pH was recorded during 4 h. C = control, B = biopriming, NC = noncontact, and A = auxin seedlings. Data represent means and standard deviations (error bars) from three experiments.

Fig. 6. Plasma membrane (PM) H⁺-ATPase content and activity. **A**, Immunodetection of PM H⁺-ATPase in microsomal fractions of shoot and primary root using an antibody against the H⁺-ATPase of *Arabidopsis thaliana* from C = control, B = biopriming, NC = noncontact, and A = auxin seedlings. Representative image of three independent immunoblots is presented. **B**, PM H⁺-ATPase activity was induced in seedlings treated with *Trichoderma asperellum* or indol acetic acid. Data represent means and standard deviations (error bars) from three experiments in six replicates. Values with the same letter do not differ significantly at the $P < 0.05$ level according to Tukey's test.

(Carol and Dolan 2006). In addition, high superoxide production and pH acidification induced by auxin facilitate cell wall loosening, which is required for cell elongation (Hayashi et al. 2010; Tognetti et al. 2012).

T. asperellum and IAA induce root extracellular acidification, suggesting the activation of proton pumping or organic acid cell exudation (Hohm et al. 2014; Jones et al. 2009). Apoplastic acidification, close to pH 4.5, increases the activity of cell-wall-remodeling enzymes such as expansins and swollenins and leads to cell wall loosening (Cosgrove 2015). PM H⁺-ATPase is involved in pH homeostasis, plant nutrition, and apoplast acidification. Barley leaves incubated in vanadate, an inhibitor of PM H⁺-ATPase, maintain the pH of the medium, and the addition of fusicoccin, a PM H⁺-ATPase activator, produces a sudden pH decrease (Visnovitz et al. 2011). Higher extracellular acidification and PM H⁺-ATPase activity have been observed in the *Arabidopsis* open stomata 2 mutant, which constitutively expresses an active PM H⁺-ATPase allele (Spartz et al. 2014).

According to the acid growth theory, auxin induces acidification and cell wall loosening by increasing the PM H⁺-ATPase activity (Hager 2003; Hohm et al. 2014; Kazan 2013). *Trichoderma* spp. promote RH elongation, increase extracellular acidification, and, at the molecular level, induce IAA accumulation and PM H⁺-ATPase activity. Despite the differences in root enlargement between the B and NC seedlings and PM H⁺-ATPase activation, *Trichoderma* spp. are able to secrete other molecules in addition to auxins that possibly affect the activity of the PM H⁺-ATPase; however, as far as we know, there are no reports about how *Trichoderma* spp. affect ATPase activity.

In addition to enzyme activation, auxin may influence PM H⁺-ATPase isoform transcription. MHA2 is the ATPase isoform 2 from maize and was enriched in nonvascular tissues from coleoptiles incubated in 20 µM IAA (Frías et al. 1996). In this study, increased PM H⁺-ATPase activity was due to the activation of the preformed enzyme, suggesting posttranscriptional regulation by *Trichoderma* exudates.

During plant–microorganism interaction, some microorganisms are able to modulate PM H⁺-ATPase activity through different mechanisms. Fusicoccin is a mycotoxin secreted by *Fusicoccum amygdali* that stimulates apoplast acidification by the activation of PM H⁺-ATPase (Elmore and Coaker 2011; Johansson et al. 1993). Fusicoccin stabilizes the binding between the 14-3-3 protein to the carboxy terminus of the enzyme, maintaining its active form (Elmore and Coaker 2011). *Rhynchosporium secalis* secretes necrosis inductor protein 1 (NIP1), which is used to invade barley; PM H⁺-ATPase is activated after NIP1 binds to its receptor (Elmore and Coaker 2011; van't Slot et al. 2007). In contrast, reduced activity is

caused by FB1, a mycotoxin produced by *Fusarium*, proposed as an uncompetitive inhibitor (Gutiérrez-Najera et al. 2005) and by beticolins produced by *Cercospora beticola* (Simon-Plas et al. 1996). Beticolins need Mg²⁺ to form a pore-like structure in the membrane (Goudet et al. 2000); the ion channel could change the electrochemical gradient induced by ATPase.

The carboxy-terminal regulatory domain of PM H⁺-ATPase is 100 amino acids long (Falhof et al. 2016; Morsomme and Boutry 2000; Okumura et al. 2012; Pedersen et al. 2007; Piette et al. 2011). The phosphorylation of the penultimate residue leads to an increase in activity once the 14-3-3 protein binds to this phosphothreonine residue (Hayashi et al. 2010), although there are other phosphorylation sites that induce or inhibit enzyme activity (Falhof et al. 2016; Spartz et al. 2014; Takahashi et al. 2012). It was not possible to determine, using antibodies, the change in the phosphorylation state of the enzyme or the presence of protein 14-3-3. Instead, the dephosphorylation protocol (Desbrosses et al. 1998) was used to determinate the mechanism by which *Trichoderma* spp. induced ATPase activity. As expected, dephosphorylation treatment decreased the activity in the native MF.

Some interesting responses were noted. (i) The increased activity due to LPC was higher in dephosphorylated ATPase caused by conformational changes due to alkaline phosphatase treatment. It is known that LPC induces ATPase activity through the displacement of the carboxyl region due to the binding of LPC to the carboxyl domain or by modifications in enzyme conformations induced by LPC (Morales-Cedillo et al. 2015). (ii) The PM H⁺-ATPase activity induced by LPC was also different in aerial and root tissues. This may be explained if, in each tissue, different enzyme isoforms are expressed, as occurs in *Nicotiana tabacum*, in which LPC decreases the K_M for ATP in the roots but not in the leaves because the tissues have different isoforms (Olsson et al. 1995). (iii) The increase in PM H⁺-ATPase activity in the microsomal fraction of control seedlings was different between auxin and the extracellular extract of *T. asperellum*. Although auxin induces a similar activation independently of the microsomal phosphorylation state, the activity induced by TE was higher in the native microsomal fraction (nondephosphorylated). This may indicate the presence of diverse molecules in TE besides IAA and explain why *Trichoderma* strains unable to produce IAA also induce plant growth.

T. asperellum, similar to other *Trichoderma* spp., promotes shoot and root growth. It has been reported that the observed plant changes are attributable mostly to auxin signaling; in the present work, it is clear that the fungus modulates PM H⁺-ATPase by enhancing ATP hydrolysis and not protein abundance in roots and shoots by either auxin or other fungus-exudated

Table 1. Plasma membrane H⁺-ATPase activity is modulated by the extracellular extract of *Trichoderma asperellum*^z

Alkaline phosphatase	Modulator	ATPase activity (U/µg of protein)	
		Shoot (%)	Primary root (%)
–	...	12.54 ± 2.34 c (100)	17.15 ± 0.7 d (100)
–	TE	26.62 ± 4.43 b (212.28)	61.37 ± 5.62 a (357.84)
–	10 µM IAA	20.24 ± 3.02 b (161.4)	33.17 ± 0.46 c (193.41)
–	LPC	24.71 ± 3.44 b (197.05)	16.17 ± 0.87 d (94.29)
+	...	7.21 ± 1.23 d (57.50)	9.18 ± 1.95 d (53.53)
+	TE	13.38 ± 2.06 c (106.70)	13.8 ± 2.06 d (80.47)
+	10 µM IAA	24.06 ± 3.22 b (191.87)	29.73 ± 7.62 c (173.35)
+	LPC	39.80 ± 5.46 a (317.38)	47.97 ± 6.22 b (279.71)

^z ATPase activity was determined in a microsomal fraction of shoot and primary root previously incubated with alkaline phosphatase (+) or denatured alkaline phosphatase (–). Modulators were added to the ATP hydrolysis medium: 100 µM lysophosphatidylcholine (LPC), 10 µM indol acetic acid (IAA), or *T. asperellum* extracellular extract (TE). Data represent means and standard deviations (error bars) from two experiments in six replicates. Different lowercase letters indicate significant differences at the *P* < 0.05 level according to Tukey's test. Values in parentheses are the percentages of the activity related to the control, considering that ATPase activity in the nondephosphorylated microsomal fractions without any modulator in each tissue represents 100%.

metabolites. These results also suggest that the activation of PM H⁺-ATPase is one key element that improves plant growth because *T. asperellum* synthesizes more than one molecule that activates the enzyme.

MATERIALS AND METHODS

Biological material and growth conditions.

T. asperellum inoculum. *T. asperellum* HK703 (NRRL50191) conidia were obtained from 2-week-old PDA cultures by washing the plates with 5 ml of sterile distilled for 30 min in orbital agitation. Liquid was recovered and centrifuged for 15 min at 13,000 rpm and 4°C in a benchtop centrifuge. The pellet was washed once in 1.5 ml of sterile distilled water. The pellet was resuspended in 1.0 ml of sterile distilled water and stored at 4°C until used.

Plant material. Seed of *Zea mays* 'Chalqueño' were surface sterilized in 2% (vol/vol) Clorox regular bleach (final concentration: 0.12% NaOCl) for 2 min and rinsed at least five times with sterile water. For internal sterilization, the seed were subjected to a heat-shock treatment of 60°C for 5 min (Glenn et al. 2008). The maize-*T. asperellum* interaction occurred in two forms. The first was the biopriming, in which the seed were shaken for 1.5 h under constant agitation in a solution containing *T. asperellum* at 1,000 conidia ml⁻¹ and then placed in 20-by-20-cm plastic containers with 1% agar. The second form was the noncontact treatment, in which the seed were placed in petri dishes with PDA medium (39 g/liter) and, simultaneously, 1 × 10⁶ conidia per seed were inoculated at the opposite end of the 1% agar plates. To compare the effect of seed *Trichoderma* treatment with auxins, another group of seed was imbibed in 10 μM IAA for 30 min with constant agitation and then placed in 20-by-20-cm plastic containers with 1% agar. Seed without treatment were considered controls. All of the seed were germinated in agar in the dark at 29°C for 72 h, after which the B, NC, A, and C seedlings were examined.

Measurement of signaling molecules.

Peroxide. The H₂O₂ content in the primary roots was determined as previously described (Gay et al. 1999), with a few modifications. The root was cut into three different sections (apex, middle, and base) and fresh tissue sections (1 g each) were homogenized in liquid nitrogen. The fine powder was mixed with 1 ml of 25 mM Tris-HCl, pH 7.5, and then centrifuged at 4,000 rpm for 40 s and allowed to stand for 1 min. This was added to 100 μl of the supernatant and 1 ml of xylenol orange solution prepared at the moment of use with 10 ml of solution A (125 μM xylenol orange and 100 μM sorbitol) mixed with 100 μl of solution B (0.5 ml of 5 M H₂SO₄, 0.1 ml of 250 mM [NH₄]₂SO₄, and 0.4 ml of 62.5 mM FeSO₄). Immediately, the sample was mixed in a vortex and incubated for 30 min at room temperature. H₂O₂ was quantified by monitoring the absorbance at 560 nm and compared with a standard curve made with 0.5 to 6 nM H₂O₂.

T. asperellum extracellular extract. *T. asperellum* was grown on 200 ml of PDB culture inoculated with 10,000 conidia and incubated for 3 days in the dark at 29°C under constant agitation (100 rpm). After that, the culture was filtered through a 0.22-μm polyvinylidene difluoride (PVDF) membrane and centrifuged at 2,000 × g for 15 min at 4°C. This TE supernatant was stored at -70°C until used.

IAA content. IAA was extracted from 1 g of fresh roots or shoots homogenized in liquid nitrogen or from 5 ml of TE. To extract IAA, 6 ml of 1-propanol/H₂O/HCl (2:1:0.002 [vol/vol]) was added to each sample and mixed by inversion for 30 min at 4°C. Then, 10 ml of CH₂Cl₂ was added, and this procedure was repeated. The upper phase was discarded, and the remainder

was centrifuged at 13,000 rpm for 5 min at 4°C. The lower phase was dried in a rotary evaporator at 45°C and 80 rpm for 15 min (Pan et al. 2008). The samples were resuspended in 3 ml of 1-propanol, filtered through a 0.22-μm PVDF, and stored in amber vials at 70°C until IAA determination.

IAA was analyzed by HPLC (Waters, Milford, MA) with a C18 (ACE 5, 250 by 4.6 mm) column using solvent A (methanol) and solvent B (water with 0.05% acetic acid) (A/B, 60:40), previously degassed at a flow rate of 0.7 ml min⁻¹ (Castillo et al. 2005). IAA was detected with a model 474 (Waters) fluorescence detector using λ excitation of 280 nm and λ emission of 340 nm. The standard curve was made from 0.1 g of IAA (Sigma-Aldrich, St. Louis) dissolved in methanol, which was diluted in 1-propanol to obtain a series of concentrations (0.1, 0.3, 0.5, 0.7, and 1 μg ml⁻¹ for IAA). The standard solution was analyzed under the same conditions described above.

Physiological test for root proton extrusion.

Extracellular acidification was determined from all seedlings. Each root was immersed in 4 ml of sterile distilled H₂O, and the pH was recorded at different times over 4 h. Groups of five seedlings for each treatment were analyzed in duplicate.

PM H⁺-ATPase activity.

MF were obtained from 3 g of shoots or roots by differential centrifugation according to Sánchez-Nieto and associates (1997). The homogenization buffer contained 50 mM HEPES/BTP (pH 7.8), 250 mM sorbitol, 2 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 1.5 mM benzamidine, 1 mM KCl, 4 mM dithiothreitol, and complete EDTA-free protease inhibitor cocktail at 40 μg/ml (Roche, Basilea, Switzerland).

ATPase activity was determined as ATP hydrolysis, as described by Sánchez-Nieto and associates (1998). Total ATPase activity was obtained by adding 10 μg of MF protein to 150 μl of assay medium (250 mM sucrose, 50 mM MOPS/benzyltriphenylphosphonium chloride (BPH) [pH 6.5], 10 mM ATP, 10 mM MgCl₂, 7 μM carbonyl cyanide 3-chlorophenylhydrazon, and 0.015% Brij 58), followed by incubation for 20 min at 30°C. The PM H⁺-ATPase (EC 3.6.3.6) activity was obtained by two ways. In the first, a PM H⁺-ATPase inhibitor of 150 μM Na₃VO₄ (final concentration) was added to the ATP hydrolysis medium; therefore, the difference between total ATPase activity and the activity obtained with Na₃VO₄ was the PM H⁺-ATPase. In the second type of assay, the ATPase activity was evaluated adding a mixture of 2 mM Na₂MoO₄, 2 mM NaN₃, and 150 mM KNO₃, which are inhibitors of acid phosphatase (EC 3.1.3.2), tonoplast (EC 3.6.1.3), and mitochondrial (EC 3.6.1.34) ATPases, respectively; as a result, the residual ATPase activity comes from the PM H⁺-ATPase. The reaction was stopped by adding sodium dodecyl sulfate (SDS; 12% final concentration). Released Pi was determined with the method of (González-Romo et al. 1992).

PM H⁺-ATPase immunodetection.

The MF (30 μg of protein) was subjected to electrophoresis (8% SDS polyacrylamide gel electrophoresis) (Laemmli 1970), gels were stained on Coomassie blue or electroblotted onto PVDF membrane using a wet chamber (Towbin et al. 1979), and the proteins were transferred at 40 V during 2.5 h. The membrane was blocked for 1 h in 5% Svelty milk in 10 mM Trizma base, 10 mM NaCl, and 10 mM MgCl₂ (pH 9.5) solution (TBS₂) with constant agitation. The membrane was rinsed twice in TBS₂ plus 0.1% Tween 20 and once in TBS₂ with 5 min of orbital agitation each time. Then, the membrane was incubated overnight at 4°C with an antibody against the PM H⁺-ATPase from *Arabidopsis* (1:1000 in TBS₂) (Agrisera, Vännäs, Sweden). At the end of the incubation time, the membrane was

rinsed three times as described above. Then, it was incubated with the secondary antibody, goat antirabbit immunoglobulin G conjugated with alkaline phosphatase (1:4000 in TBS₂) (Millipore, Darmstadt, Germany). The detection of PM H⁺-ATPase was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate for 15 min, as described by Rosenberg (1996). The blots were scanned and a densitometric analysis was carried out with the Image J software (U.S. National Institutes of Health, Bethesda, MD).

Modulation of the PM H⁺-ATPase activity.

The H⁺-ATPase dephosphorylation was made using 5 µg of MF protein of C seedlings and incubated with 6 µl (100 U ml⁻¹) of phosphatase alkaline-agarose from calf intestine (Sigma-Aldrich), during 20 min at 37°C, according to Desbrosses and associates (1998). Then, it was centrifuged at 12,000 × g for 8 min at 4°C, and the supernatant was used for ATPase activity assays as described above. Modulators were then added: 100 µM LPC, 10 µM IAA (final concentration), and 8 µl of TE.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

College of Agricultural Science, 2014. Root hair density:
<http://plantscience.psu.edu/research/labs/roots/methods/computer/simroot/root-trait-demo/root-hair-density>