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Trichoderma spp. Improve Growth of *Arabidopsis* Seedlings Under Salt Stress Through Enhanced Root Development, Osmolite Production, and Na^+ Elimination Through Root Exudates

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Salt stress is an important constraint to world agriculture. Here, we report on the potential of *Trichoderma virens* and *T. atroviride* to induce tolerance to salt in *Arabidopsis* seedlings. We first characterized the effect of several salt concentrations on shoot biomass production and root architecture of *Arabidopsis* seedlings. We found that salt repressed plant growth and root development in a dose-dependent manner by blocking auxin signaling. Analysis of the wild type and *eir1*, *aux1-7*, *arf7arf19*, and *tir1abf2abf19* auxin-related mutants revealed a key role for indole-3-acetic acid (IAA) signaling in mediating salt tolerance. We also found that *T. virens* (Tv29.8) and *T. atroviride* (IMI 206040) promoted plant growth in both normal and saline conditions, which was related to the induction of lateral roots and root hairs through auxin signaling. *Arabidopsis* seedlings grown under saline conditions inoculated with *Trichoderma* spp. showed increased levels of abscissic acid, L-proline, and ascorbic acid, and enhanced elimination of Na^+ through root exudates. Our data show the critical role of auxin signaling and root architecture to salt tolerance in *Arabidopsis* and suggest that these fungi may enhance the plant IAA level as well as the antioxidant and osmoprotective status of plants under salt stress.

Salinity is a major environmental factor affecting crop production. Up to 7% of the total land surface is saline and approximately one-third of the world's irrigated land is subjected to secondary-induced salinization (Hariadi et al. 2011; Shabala and Cuin 2008). Under salt stress, plants experience dehydration, nutrient deficiencies, membrane dysfunction, and oxidative stress, which lead to tissue damage or early senescence (Essah et al. 2003; Katori et al. 2010).

To avoid accumulation of toxic sodium (Na^+) levels in shoots, plants must take up no more than 3% of the Na^+ present in the rhizosphere, and many adaptive mechanisms are then activated (Zhang et al. 2008a). Salt tolerance is a complex trait that involves multiple physiological and biochemical mechanisms: for example, the salt-overly sensitive pathway, which regulates ionic homeostasis; the inducer of the CBF (C-repeat/dehydration-responsive element binding factor) expression and dehydration-responsive element–binding pathway that controls the expression of dehydration response element and C repeat-containing genes; and the mitogen-activated protein kinase cascade that regulates the generation of osmolytes and antioxidants with protective functions (Mehlmer et al. 2010; Seki et al. 2003; Xiong et al. 2002a and b; Zhu 2003).

The root system performs indispensable plant functions such as uptake of nutrients and water, anchorage in the soil, and interaction with symbiotic microorganisms (López-Bucio et al. 2003, 2005). Consequently, root system development is central for the plant to reach optimal growth and directly contributes to the levels of yield obtained in crops. The impact of the root on plant growth has become apparent not only in model plants such as *Arabidopsis thaliana*, *Medicago truncatula*, and *Lotus japonicus* but also in important crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), and maize (*Zea mays*) (Coudert et al. 2010; Hochholdinger and Tuberosa 2009). One way to minimize the negative impact of biotic and abiotic factors on yield is to manipulate root system architecture (RSA). The basic aspects of root architecture involve primary root growth and lateral and adventitious root formation. Branching structures are covered by root hairs (RH), a class of differentiated epidermal cells that further increase the exploratory potential. The primary root originates in the embryo and produces many lateral roots (LR) during vegetative growth, and each of these will produce more LR (Casimiro et al. 2003; López-Bucio et al. 2005; Malamy and Benfey 1997).

RSA is modified by the endogenous auxin level or in response to environmental factors that increase the auxin pool in the plant or affect auxin sensitivity (Himanen et al. 2002; López-Bucio et al. 2003; Pérez-Torres et al. 2008). In *Arabidopsis*, salt stress induces nitrilase genes *NIT1* and *NIT2,* which are involved in indole-3-acetic acid (IAA) biosynthesis (Bao and Li 2002). In addition, Wang and associates (2009) reported that high salt exposure suppresses LR initiation and organogenesis, which correlated with the concomitant reduction of expression of the auxin-inducible reporter *DR5::GUS* in primary root tips. These results suggest that auxin homeostasis is important for adaptive root system development under salt stress; however, it still remains to be determined whether auxin biosynthesis, transport, or sensitivity is the key target of salt stress. Other-

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wise, the ability of plants to adapt to stress conditions often appears to depend on their association with microbes. Recent reports have shown that auxin-like signals produced from rhizosphere microorganisms could improve root branching in *Arabidopsis*, with a dramatic impact in plant biomass production (Ortiz-Castro et al. 2011). The potential of plant-associated microorganisms to produce IAA, auxin precursors, or auxin signal mimics represents a means to influence the endogenous auxin pool of the host (Contreras-Cornejo et al. 2009; Felten et al. 2012; Hilbert et al. 2012). However, little is known about the implication of this hormone in symbiosis or plant tolerance to stress.

Trichoderma spp. are free-living fungi that are common in soil and root ecosystems. They have been widely studied for their capacity to produce antibiotics, parasitize other fungi, and compete with deleterious plant microorganisms (Harman et al. 2004). Until recently, these traits were considered to be the basis for how *Trichoderma* spp. exert beneficial effects on plant growth and development. However, it is clear that certain species also have substantial direct influence on plant health and development by producing phytohormones, activating defense responses, or conferring stress tolerance in plants (Brotman et al. 2013; Contreras-Cornejo et al. 2009, 2011, 2013; Velázquez-Robledo et al. 2011). *Trichoderma* spp. allowed the accumulation of proline in wheat plants under salt stress condition (Rawat et al. 2011), and microarray analysis of *Arabidopsis* and cucumber roots exposed to salt stress and inoculated with *Trichoderma* spp. revealed an increased expression of genes related to salt-tolerance, osmoprotection processes, and ascorbic acid (AA) production (Brotman et al. 2013).

Our previous research showed that *Trichoderma virens* and *T. atroviride* produce IAA and the indolic compounds indole-3-ethanol, indole-3-acetaldehyde, and indole-3-carboxaldehyde as part of their metabolism (Contreras-Cornejo et al. 2009, 2011). It was found that mutations in genes involved in auxin transport or signaling (*AUX1*, *BIG*, *EIR1*, and *AXR1*) reduced the growth-promoting and root developmental effects of *Trichoderma* spp. inoculation. Colonization of plant roots by fungal hyphae activated *DR5:uidA* expression, which correlated with an increased cell proliferation in LR tips. Application of all three indolic compounds produced by the fungus to

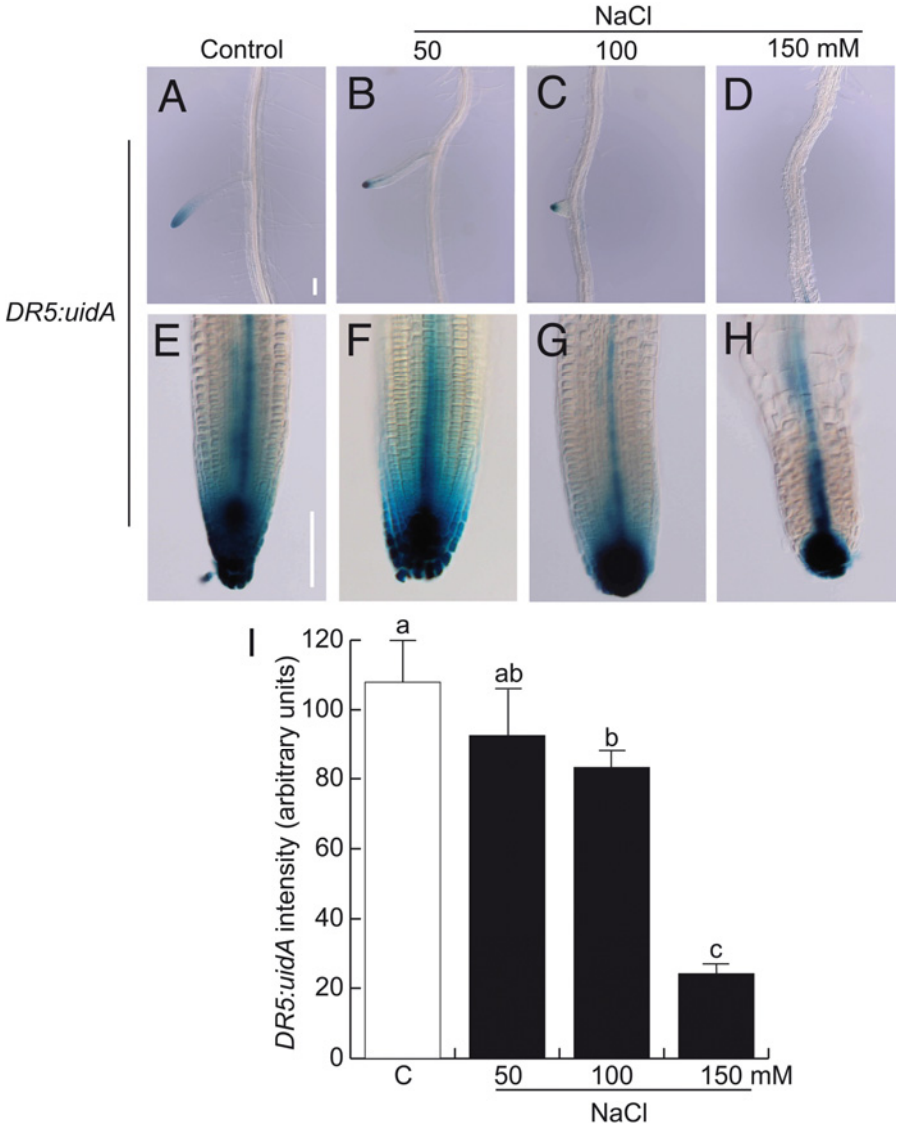

Fig. 1. Effect of NaCl on *DR5:uidA* expression. Representative photographs of **A** to **D,** lateral roots or **E** to **H,** primary root tips from transgenic *Arabidopsis* seedlings expressing the *DR5:uidA* auxin-inducible gene marker, which were grown on media with increasing concentrations of NaCl. **I,** Quantitative analysis of β-glucuronidase expression in primary root tips using the image J program. Photographs are representative individuals of at least 15 plants stained. Scale bar = 1 mm. The experiment was repeated two times with similar results.

Arabidopsis seedlings showed a dose-dependent effect on biomass production and in activation of *DR5:uidA* expression.

In this report, we show that salt affects plant biomass production and reduces root growth, LR formation, and RH development by decreasing auxin responsiveness. Cocultivation of plant roots with *T. virens* or *T. atroviride* normalized root development, likely because these fungi provide IAA to plants. Moreover, *Trichoderma* spp. improved the antioxidative and osmoprotective capacity and increased growth. Our results reveal that, by enhancing LR and RH development through sustained auxin production, *Trichoderma* spp. may affect plant performance and yield under saline conditions.

RESULTS

Salinity affects root architecture and decreases auxin responsiveness in *Arabidopsis* seedlings.

A common negative effect of salinity is reduced root growth and decreased plant biomass (Achard et al. 2006). Little is known about specific root architectural responses to salt and the contribution of auxin signaling in the different traits responsible for root adaptation to this stress. To evaluate the effect of salt on *Arabidopsis* growth and development, wild-type (WT) (Col-0) seedlings were germinated and grown for 9 days on vertically oriented agar plates containing 0.2× Murashige and Skoog (MS) medium supplied with increasing concentrations of salt (50 to 200 mM NaCl). It was found that increasing salt concentration in the medium affected total fresh weight, primary root length, LR number and density, and RH length in a dose-dependent manner (Supplementary Fig. S1A to E). These effects were more evident in seedlings grown in 100 to 150 mM or higher salt concentrations, which drastically decreased plant growth and biomass production. At 150 mM NaCl, LR and RH formation was totally blocked (Supplementary Fig. S2). These results show that NaCl affects fundamental cellular processes responsible for the configuration of RSA.

To test whether the repression of root branching by NaCl could be associated with changes in auxin accumulation or

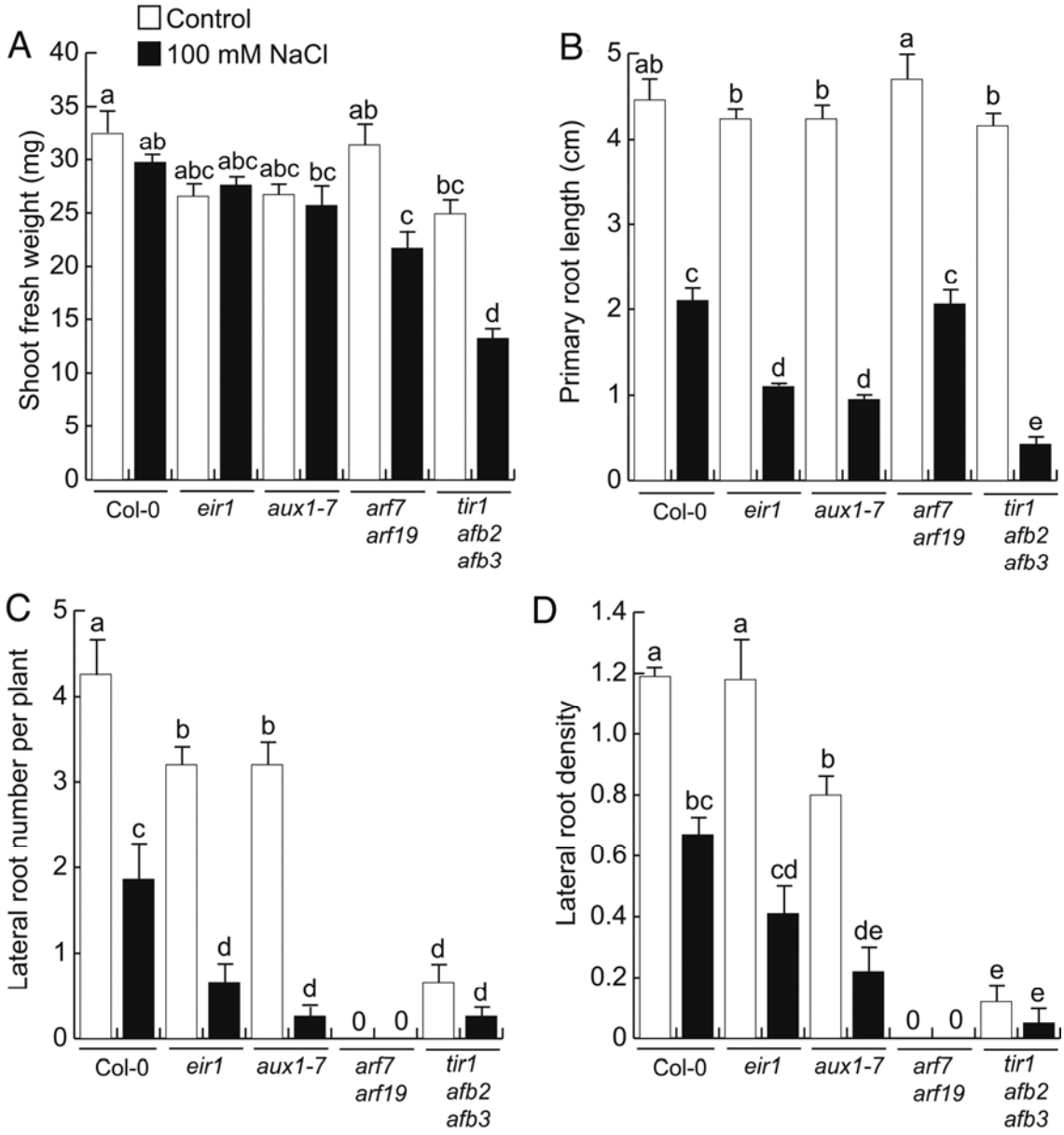

Fig. 2. Effect of NaCl on *Arabidopsis* wild type and mutants with defects in auxin transport or signaling. **A,** Shoot fresh weight. **B,** Primary root length (n = 15). **C,** Lateral root number per plant (n = 15). **D,** Lateral root density (n = 15). Error bars represent the standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

response, expression of the β-glucuronidase (GUS) reporter gene driven by the auxin-sensitive *DR5* promoter (Dubrovsky et al. 2008; Ulmasov et al. 1997) was examined in primary roots and LR. It was found that 50 to 150 mM NaCl affected the expression of *DR5:uidA* in primary roots and LR (Fig. 1A to H), which correlated with decreased root branching. The intensity of *DR5:uidA* gene expression was determined from images of primary root tips, which were processed using the Image J software and the data expressed as arbitrary units (Fig. 1I).These data show that salt inhibits auxin-inducible gene expression in a dose-dependent manner in primary root tips.

Arabidopsis mutants defective on auxin receptors are oversensitive to salt treatments.

Previous work showed that *Arabidopsis* mutants with defects in auxin influx (*aux1-7*) or efflux (*pin2*) were slightly oversensitive to salt stress, indicating that auxin transport might play a role in root system remodeling under salt stress (Wang et al. 2009). To further characterize the contribution of auxin transport and response in plant salt tolerance and its relationship with root adaptive traits, we compared the growth and development of WT and *Arabidopsis* mutants, including *eir1-1* and *aux1-7*, defective on auxin transporters, and *arf7arf19* and *tir1afb2afb3*, defective in transcription factors or receptors involved in auxin response, respectively. When grown under 100 mM NaCl, it was found that the *eir1-1* and *aux1-7* mutants showed a slight decrease of shoot fresh weight under salt treatment in a manner similar to WT seedlings. Interestingly, the *arf7arf19* and *tir1afb2afb3* mutants were clearly oversensitive to this salt treatment, with a 35 and 50% inhibition in shoot fresh weight, respectively (Fig. 2A). Salt treatment repressed approximately 55 to 60% of primary root growth, LR number per plant, and LR density in WT seedlings; whereas, in *eir1*, *aux1-7*, and *tir1afb2afb3*, these root architectural traits were more severely affected (Fig. 2B to D). The fact that the *tir1afb2afb3* triple mutant, which is defective in three putative auxin receptors of the TIR1 family, shows salt oversensitivity in biomass production and primary root growth suggests that auxin signaling (and not only auxin transport) is an important target of salinity.

T. virens and *T. atroviride* show differential growth and produce auxin under salt stress.

High salinity may affect plants, their microbial partners, and the outcome of the plant-microbe interaction. We next determined the effect of salinity on growth of *T. virens* and *T. atroviride*. In all, 10^6 conidia from each species were germinated and grown for 5 days on agar plates containing 0.2× MS medium supplied with increased concentrations of salt (50 to 300 mM NaCl). It was found that salt affected the growth of both *Trichoderma* spp. in a dose-dependent manner (Fig. 3A). However, both fungal strains tolerated 150 mM NaCl well (Fig. 3B and C) and differential effects were observed with the 300 mM salt treatment, in which the growth of *T. virens* and *T. atroviride* was repressed 27 and 74%, respectively. These results show that *T. virens* tolerates higher NaCl levels than *T. atroviride* and may represent a very promising agent to test salt responses in plant-fungus interactions.

Trichoderma spp. release auxin and auxin precursors as part of their metabolism (Contreras-Cornejo et al. 2009); thus, it was important to know whether salinity could affect auxin production in these fungi. The production of IAA by *T. virens* and *T. atroviride* was determined in liquid medium with or without 100 mM NaCl, a salt concentration that drastically affects LR formation in *Arabidopsis*. It was found that salt slightly increased IAA production in *T. virens* from 4.21 to 5.88 ng/ml, whereas in *T. atroviride*, a higher and sustained auxin production from 7.65 to 7.64 ng/ml was registered under both normal and saline growth conditions. These data show that *Trichoderma* spp. are able to produce auxin when subjected to salt treatment, which could benefit plant growth under salt stress.

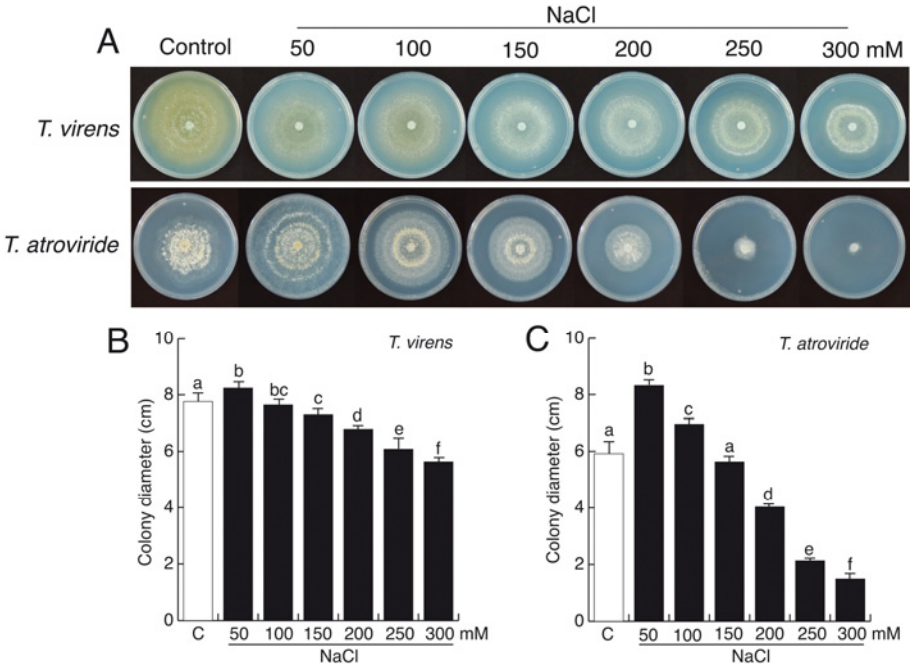

Fig. 3. Effect of NaCl on *Trichoderma* spp. growth. To determine the effect of NaCl on fungi, 1×10^6 spores from *Trichoderma* spp. were used to inoculate 0.2× Murashige and Skoog medium with or without NaCl. **A,** Representative photographs showing the aspect of the colonies of the strains grown at 24°C and photographed after 5 days. **B,** Kinetic of *Trichoderma virens* growth determined by measuring colony diameter. **C,** Kinetic of *T. atroviride* growth. Bars represent the means ± standard deviation, based on two independent experiments with three petri dishes each. Different letters represent means statistically different at the 0.05 level.

Trichoderma spp. promote growth and confer salt tolerance in *Arabidopsis*.

To investigate whether *Trichoderma* spp. could confer salt tolerance to plants, *Arabidopsis* (Col-0) seedlings were germinated and grown on petri plates containing agar-solidified 0.2× MS medium with or without 100 mM NaCl. At 4 days after germination, the seedlings were treated with sterilized water (control treatment) or with 10^6 conidia of *T. virens* or *T. atroviride*. Fungal spores were placed at a 5-cm distance from the primary root tip to test the possibility that auxins released by the fungal colony could reach the root system and affect growth and development.

Plants inoculated with *T. virens* or *T. atroviride* showed enhanced shoot growth when compared with control seedlings when grown in medium with or without 100 mM salt. The dif-

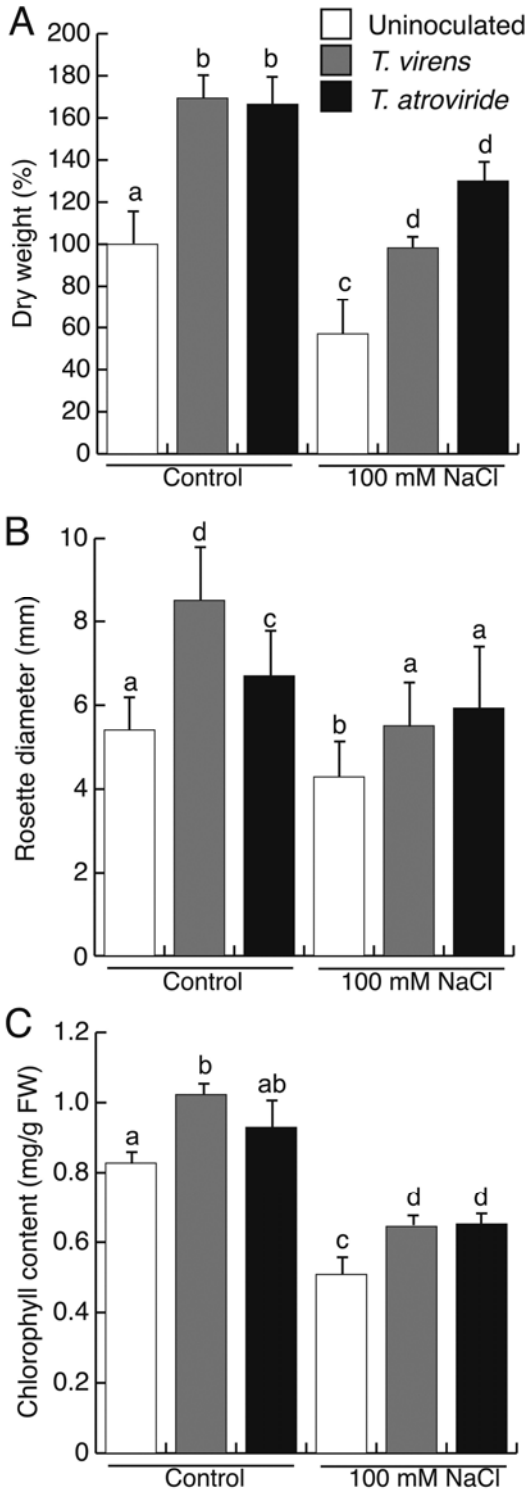

Fig. 4. *Trichoderma* spp. confer salt tolerance in *Arabidopsis*. **A,** Effect of *Trichoderma* spp. on total biomass accumulation represented as dry weight. **B,** Shoot diameter ($n = 60$). **C,** Total chlorophyll content. Rosettes were excised after 5 days of inoculation and chlorophyll content was determined. Values shown represent means of six groups of 20 seedlings ± standard error. Asterisks are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated three times with similar results.

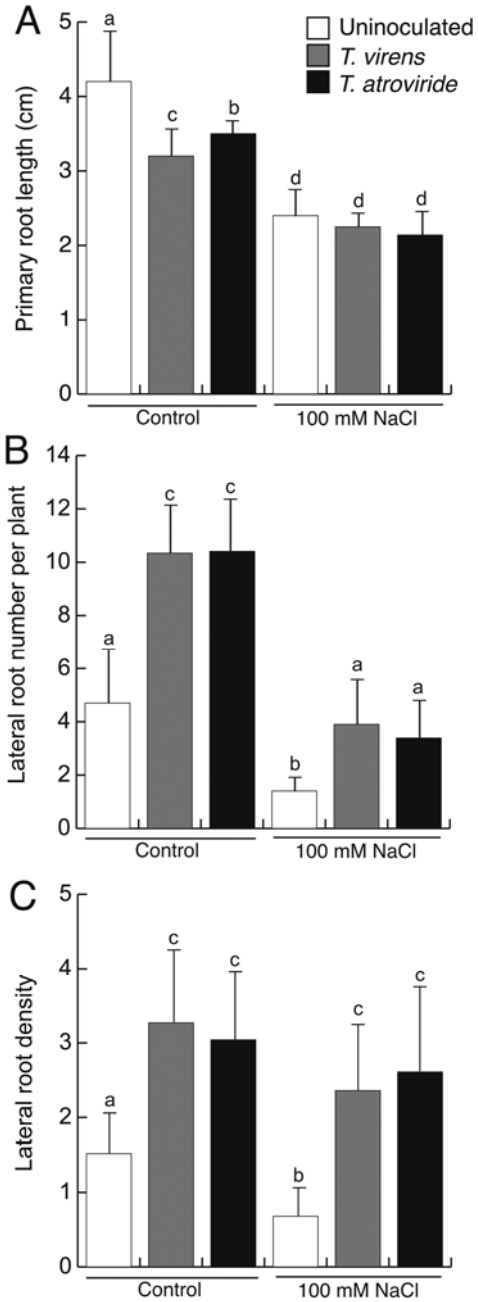

Fig. 5. Effects of *Trichoderma* spp. on root architecture of *Arabidopsis* seedlings grown under salinity. Seedlings were germinated and grown for 5 days on the surface of agar plates containing 0.2× Murashige and Skoog medium. Plates were inoculated with *Trichoderma virens* or *T. atroviride* at a distance of 5 cm from the primary root tip and grown for an additional 5-day period. **A,** Primary root length. **B,** Lateral root number per plant. **C,** Lateral root density. Bars represent the means ± standard deviation, based on three independent experiments with 60 seedlings each. Different letters represent means statistically different at the 0.05 level.

ference in dry weight and rosette diameter clearly demonstrated the beneficial effects of these fungi under salt stress (Fig. 4A and B). Saline stress also had a negative effect on chlorophyll content. The content of chlorophyll in plants grown with 100 mM NaCl decreased by 38.56% when compared with the control treatment. However, the chlorophyll level increased by 15.66% in plants inoculated with *T. virens* and 16.87% by *T. atroviride* (Fig. 4C). These results show the beneficial effects of *Trichoderma* spp. to confer salt tolerance.

Trichoderma spp. improve root-system architecture of *Arabidopsis* seedlings grown under saline conditions.

The mechanisms by which plants incorporate microbial signals into root-system development under saline stress are poorly understood. We performed bioassays to determine whether *Trichoderma* spp. could affect root plasticity in *Arabidopsis* grown under salt stress. We found that seedlings grown in normal conditions or with 100 mM NaCl co-cultivated with *T. virens* or *T. atroviride* developed a more branched root system. There were slight differences in primary root growth in *Trichoderma* spp.-inoculated seedlings when compared with axenically grown seedlings. Salt stress repressed by 50% primary root growth in control or inoculated seedlings (Fig. 5A). As expected, the LR number and density (LR number per centimeter) were reduced by 100 mM salt treatment. Interestingly, *Trichoderma* spp. normalized LR formation, increasing both LR number and density to the levels shown in uninoculated seedlings grown without salt (Fig. 5B and C). These results show that *Trichoderma* spp. can normalize root branching in *Arabidopsis* under salt stress.

RH are specialized tubular structures formed from differentiated epidermal cells of roots called trichoblasts. To investigate the effect of *Trichoderma* spp. on RH formation under elevated salinity, *Arabidopsis* seedlings were germinated and grown for 4 days on 0.2× MS with or without salt; after this period, seedlings were treated with water as a control or inoculated with *Trichoderma* spp. and grown for 5 additional days. Both RH length and density decreased significantly with 100 mM NaCl (Fig. 6G and H). Importantly, *Trichoderma* spp. increased the RH length and density in *Arabidopsis* grown in normal or saline conditions when compared with their respective controls (Fig. 6A to H). These data show that *Trichoderma* spp. can promote RH length and density.

Trichoderma spp. increase auxin-inducible gene expression in *Arabidopsis* under salt stress.

The results described above strongly suggest that *Trichoderma* spp. induce LR formation under salt stress, likely providing auxin to plants. Because auxin triggers various developmental effects through the activation of auxin-responsive

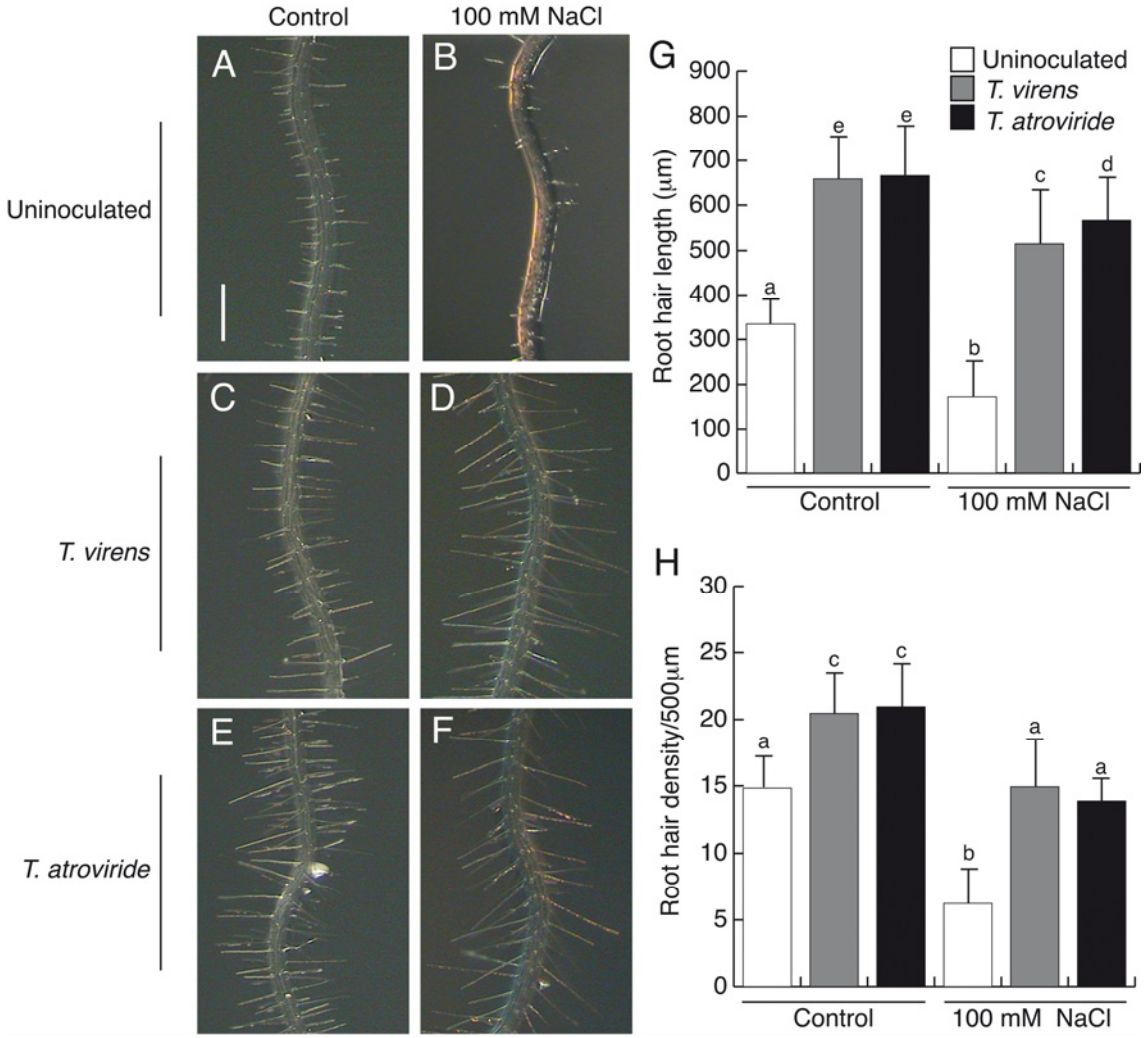

Fig. 6. Effect of *Trichoderma* spp. on root-hair development in *Arabidopsis* seedlings grown under salt stress. **A** to **F,** Representative photographs of root hairs from seedlings grown in normal or saline conditions and inoculated with *Trichoderma* spp. Bar = 500 µm. **G,** Length of root hairs formed in the primary root and **H,** root-hair density. Values shown represent the mean of 100 root hairs ± standard deviation. Different letters are used to indicate means that differ significantly ($P < 0.05$).

genes, we evaluated the expression of the auxin-responsive marker gene *DR5:uidA*. *T. virens* and *T. atroviride* increased the expression of *DR5:uidA* in LR and primary root tips in nonsaline conditions (Fig. 7A to F). Although expression of *DR5:uidA* was reduced in plants treated with 100 mM NaCl, both *Trichoderma* spp. clearly induced the expression of *DR5:uidA* in LR and primary root tips under elevated salinity (Fig. 7G to L), which correlated with an improved growth of LR. An analysis of the *DR5:uidA* expression domain in primary root tips processed using the Image J software showed that *T. virens* and *T. atroviride* increased auxin-responsive gene expression by 40% (Fig. 7M). These results show that *Trichoderma* spp. increase auxin-responsive gene expression under saline stress, which positively affect LR development.

***Trichoderma* spp. increase abscissic acid, L-proline, AA content, and salt exudation.**

To determine the biochemical and metabolic events that occur in *Arabidopsis* during the interaction with *Trichoderma* spp. under saline or normal conditions, we quantified the amounts of metabolites related to salt stress. Abscissic acid (ABA) is considered the universal plant stress hormone (Verslues and Zhu 2005; Wasilewska et al. 2008). *Arabidopsis* seedlings inoculated with *T. virens* or *T. atroviride* showed a twofold increased ABA concentration in shoots when compared with axenically grown seedlings. ABA levels in uninoculated plants were sixfold increased in response to saline stress. Interestingly, plants subjected to 100 mM NaCl treatment and inoculated with *Trichoderma* spp. displayed similar levels of ABA compared with nonstressed plants (Fig. 8A). L-proline (L-Pro) is accumulated in response to salinity, and its accumulation frequently correlates with tolerance to drought or salt stress in plants (Ben et al. 2008; Parida et al. 2008). Saline stress induced twofold the amount of L-Pro in uninoculated seedlings when compared with nonstressed seedlings, and a further increase in L-Pro was evident in *Arabidopsis* seedlings inoculated with *Trichoderma* spp. (Fig. 8B). Several reports have suggested that AA has a crucial function as antioxidant. *Arabidopsis* seedlings inoculated with *T. virens* grown under nonsaline conditions showed increased AA levels when compared with uninoculated seedlings. No changes were found in seedlings inoculated with *T. atroviride*. In contrast, the AA amounts in *Arabidopsis* under salt-stress inoculated with *Trichoderma* spp. increased further when compared with the saline control (Fig. 8C).

Excessive Na^+ accumulated in plant cells is the primary cause of inhibition of plant growth (Ghoulam et al. 2002; Ungar 1996). Because detoxification mechanisms would prevent salt toxicity, we determined whether *Trichoderma* spp. inoculation could affect the levels of Na^+ in root exudates. Seedlings were grown in normal or saline conditions and inoculated with *Trichoderma* spp. by 5 days. Then, seedlings were transferred to falcon tubes containing tridistilled water. Root exudates were collected 2 days later, samples were filtered and Na^+ was determined by atomic absorption spectrophotometry. In plants grown in medium without salt, no significant differences in Na^+ exudation were found among treatments. However, plants grown under salinity increased exudation of Na^+ by roots, with a clearly enhanced amount of Na^+ being exuded in *Trichoderma* spp.-inoculated plants (Fig. 8D). These data show that *Arabidopsis* seedlings inoculated with *Trichoderma* spp. are better adapted to cope with salt stress, likely by increasing plant vigor, osmolite and antioxidant production, and salt exudation.

DISCUSSION

Plants synthesize and require a variety of signals to adjust growth and development throughout their life cycle. Auxins,

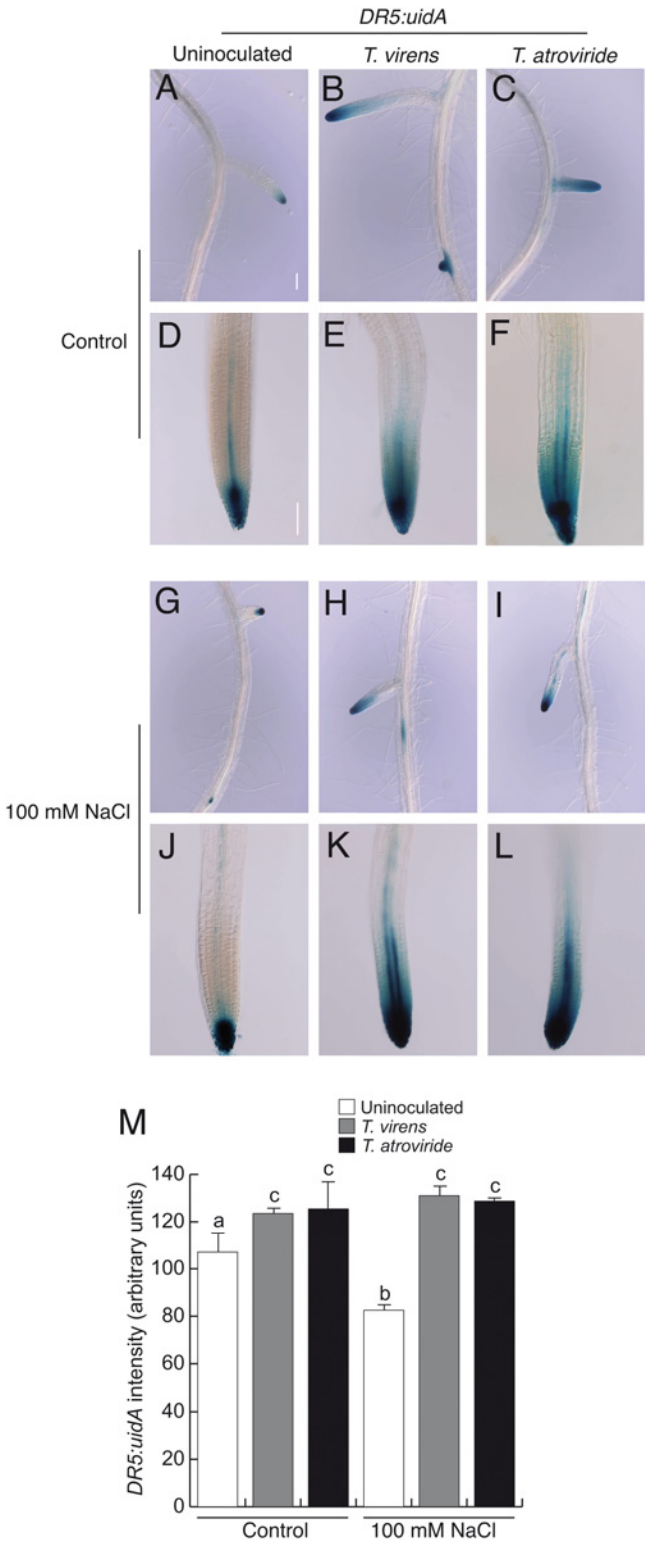

Fig. 7. Effect of *Trichoderma* spp. on the expression of *DR5:uidA* in normal or saline growth conditions. *Arabidopsis* transgenic seedlings were grown by 4 days on agar plates containing 0.2× Murashige and Skoog medium with or without 100 mM NaCl and inoculated with *Trichoderma* spp. by 5 days. **A to F,** Representative images from *DR5.uidA* seedlings uninoculated or inoculated with *Trichoderma* spp. in normal conditions. **G** to **L,** Photographs from seedlings uninoculated or inoculated with *Trichoderma* spp. under elevated salinity. **M,** Quantitative analysis of β-glucuronidase expression in primary root tips using the image J program. Photographs are representative individuals of at least 15 plants stained. Scale bar = 100 μm. The experiment was repeated three times with similar results.

including IAA, comprise a group of tryptophan-derived signals which are involved in most aspects of plant development (Woodward and Bartel 2005) These compounds exert a strong biological activity at very low concentrations in both in vivo and in vitro systems. Optimal plant growth requires tight control of IAA activity, which is accomplished by diverse mechanisms that include IAA biosynthesis, its transport among tissues, cycling between active and inactive forms, and signal perception through a family of auxin transporters, transcription factors, and IAA receptors (Leyser 2006; Ljung et al. 2002; Mockaitis and Estelle 2008). Auxins have also been implicated in an abiotic stress response (Bao and Li 2002). Recent breeding improvements in terms of salt resistance of maize have led to a genotype with improved growth under saline conditions. By comparing this salt-resistant hybrid with a sensitive hybrid, it was possible to show differences in hormone concentrations in expanding leaves and roots. In response to salinity, the salt-resistant maize significantly increased indole-butyric acid concentrations in leaves and maintained IAA concentration in roots (Zörb et al. 2013). These hormonal adaptations were suggested to activate β-expansin gene expression to maintain growth of resistant maize hybrids under salt stress. Moreover, ABA concentrations significantly increased in resistant maize leaves under salt stress, which may contribute to acidifying the apoplast which, in turn, is a prerequisite for growth (Zörb et al. 2013). In consonance with this information, it was reported that auxin transport is required for remodeling RSA under salt stress because *aux1-7* and *pin2 Arabidopsis* mutants showed slight oversensitivity to salt exposure and because salt treatments repress expression of the auxin efflux carrier *PIN2*, and led to a stable reduction in PIN2 protein abundance (Galvan-Ampudia and Testerink 2011; Wang et al. 2009).

Despite this available information, little is known about the relationship between salinity stress and auxin levels in plants and the role of auxin in alleviating salt stress. Our data further extend these previous observations by showing that auxin responsiveness is an important target of salinity, because NaCl treatments decrease biomass production, root growth, and LR formation in *Arabidopsis* seedlings, which correlates with a dose-dependent reduction of auxin-inducible gene expression (Fig. 1). The genetic analysis of growth and root architectural responses of the WT and *eir1*, *aux1-7*, *arf7arf19*, and *tir1abf2abf19* auxin-related mutants further supports the hypothesis that an intact auxin signaling pathway and not only auxin transport is required for salt tolerance, because *arf7arf19* and *tir1abf2abf19* were the most sensitive of the mutants tested regarding salt repression of growth (Fig. 2).

The use of plant symbionts is a promising strategy for agriculture sustainability because they improve plant health under different conditions. For instance, the root endophytic basidiomycete *Piriformospora indica* increased resistance against biotic stress and tolerance to abiotic stress in many plants. Root

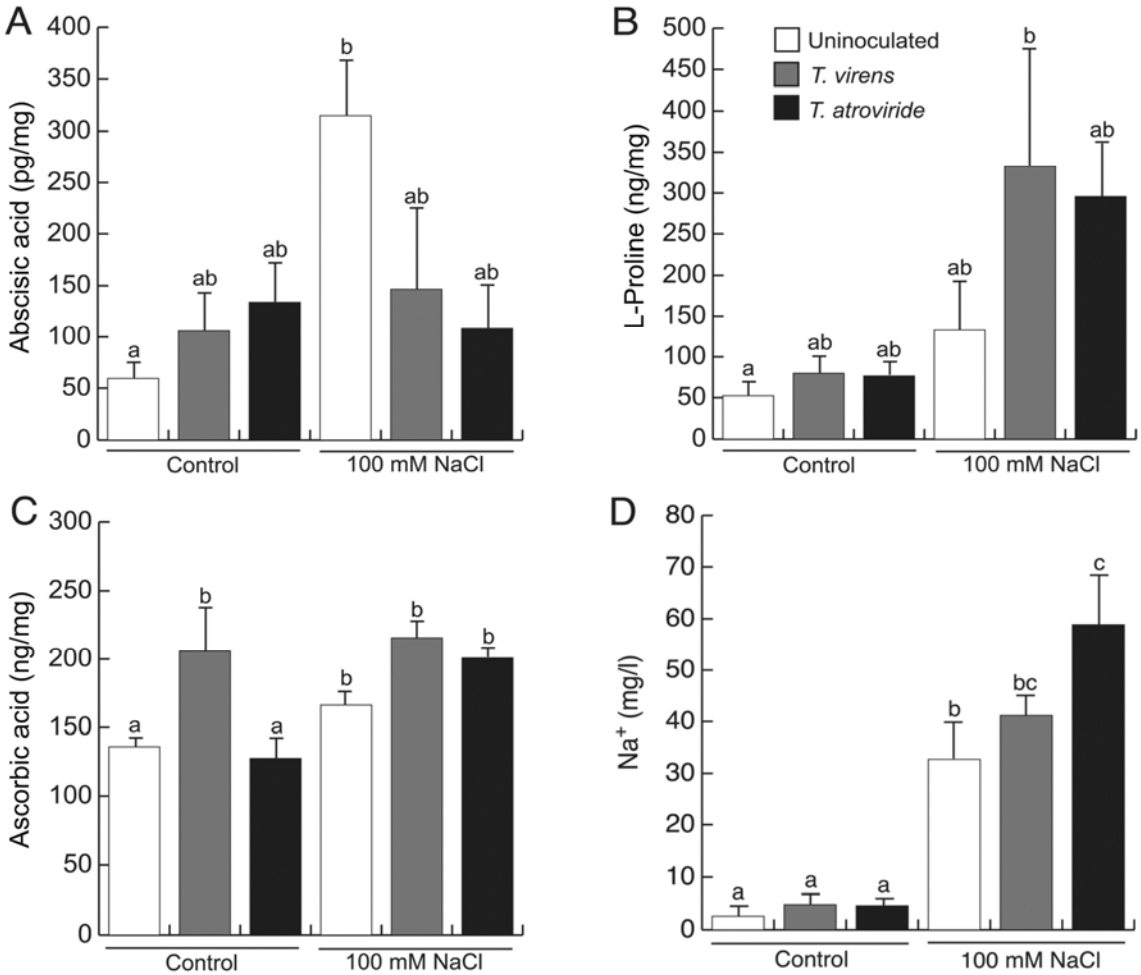

Fig. 8. Effects of *Trichoderma* spp. on biochemical changes and Na^+ elimination through root exudates**.** Metabolite determination was performed after 5 days of fungal inoculation. **A,** Endogenous content of *cis*, *trans*-abscisic acid (ABA). **B,** Content of free L-proline. **C,** Content of ascorbic acid. **D,** Root exudates were collected by 2 days from 9-day-old *Arabidopsis* maintained hydroponically. Aqueous Na^+ was determined by atomic absorption spectrophotometry ($n = 3$). Error bars represent the standard error. Different letters are used to indicate means that differ significantly ($P < 0.05$). The experiment was repeated four times with similar results.

colonization by *P. indica* increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux, and fatty acid desaturation in leaves of the salt-sensitive barley 'Ingrid'. In addition, *P. indica* significantly elevated the amount of AA and increased the activities of antioxidant enzymes in barley roots under salt stress conditions. Likewise, a sustained upregulation of the antioxidative system was demonstrated in NaCl-treated roots of the salt-tolerant barley 'California Mariout' (Waller et al. 2005). These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

T. virens and *T. atroviride* were found to produce IAA and auxin-related substances, which may normalize root growth under salinity stress. *Arabidopsis* and cucumber (*Cucumis sativus* L.) plants treated with *Trichoderma* spp. prior to salt stress show significantly improved seed germination (Brotman et al. 2013). In addition, *Trichoderma* spp. modulated the expression of several genes related to osmo-protection and general oxidative stress in roots of both plant species. The MDAR gene coding for monodehydroascorbate reductase was significantly upregulated and, accordingly, the pool of reduced AA was increased in *Trichoderma* spp.-treated plants. Therefore, it was interesting to examine whether *T. virens* or *T. atroviride* could contribute to salt tolerance in *Arabidopsis* seedlings via auxin production or through other mechanisms. We found that both *Trichoderma* spp. tested were able to sustain prolific growth at 150 mM NaCl. However, a differential salt response was found because, at greater salt concentrations, *T. virens* was more halotolerant than *T. atroviride* (Fig. 3). Importantly, *T. virens* and *T. atroviride* were able to produce IAA when grown in medium supplied with 100 mM NaCl or even greater IAA levels than those quantified in medium without salt, suggesting their great potential as plant growth-promoting fungi to cope with salinity in crops.

In *Arabidopsis* seedlings cocultivated with *T. virens* or *T. atroviride*, there were an increased number of LR, RH, and biomass accumulation (Figs. 4 to 6). RH development is a useful marker of differentiation processes that take place in the root (López-Bucio et al. 2005). It has been reported that NaCl treatment inhibits RH growth (Halperin et al. 2003). In contrast, *Trichoderma* spp. increased both RH number and length (Fig. 6). These data suggest that root plasticity could be part of a mechanism by which *Trichoderma* spp. can confer salt tolerance to plants. In *Arabidopsis* and other plant species, exogenous auxin application can induce both LR and RH formation (Laskowski et al. 1995); therefore, we speculated that, by providing auxins, *Trichoderma* spp. could restore auxin homeostasis and, consequently, growth and development could be normalized when grown under stressing salt levels. To verify this, the effect of cocultivation with *Trichoderma* spp. on *DR5:uidA* expression was tested under salinity. It was found that *T. virens* or *T. atroviride* could increase the expression of *DR5:uidA* in LR and that more of these structures were formed in response to fungal interaction (Fig. 7). Therefore, it is tempting to speculate that auxins derived from *Trichoderma* spp. may be important to sustain root developmental programs under saline stress. A key role for IAA in plant salt tolerance induced by fungi has started to be revealed. For example, Redman and associates (2011) reported that *Fusarium culmorum* and *Curvularia protuberata* enhanced growth of rice plants under salinity. Similarly, the endophytic fungi *Phoma glomerata* and *Penicillium* sp. significantly increased plant biomass of cucumber under saline stress (Waqas et al. 2012). A *Streptomyces* sp. isolate increased the growth and development of wheat plants in normal and saline conditions. This isolate also produced IAA in axenic conditions, which increased when salt was added (Sadeghi et al. 2012). Thus, plant-growth-promoting fungi may be advantageous to plants grown under salt stress by producing auxins.

Salt tolerance is a complex trait involving the coordinated action of many gene families that perform a variety of functions such as control of water loss through stomata, ion sequestration, metabolic and osmotic adjustments, and antioxidative protection (Abogadallah 2010). For example, under severe saline stress, reactive oxygen species (ROS) production can damage cell components (Mittler 2002). The osmoprotectant L-Pro is accumulated in many plant species in response to drought and salinity, and its accumulation frequently correlates with stress tolerance. This amino acid functions as a scavenger of hydroxyl radicals, controlling redox homeostasis (Ben et al. 2008; Fabro et al. 2004). Another potent antioxidant molecule is AA, which detoxifies ROS, particularly hydrogen peroxide (Smirnoff 2000). Here, we show that *Trichoderma* spp.-induced plant salt tolerance in *Arabidopsis* is correlated with increases in L-Pro and AA (Fig. 8). Roots secrete many substances which include ions, free oxygen and water, enzymes, mucilage, and a diverse array of carbon-containing primary and secondary metabolites (Bais et al. 2006). Root exudates are transported across the cellular membrane and secreted into the surrounding rhizosphere. Na^+ toxicity is considered one of the most important factors limiting root growth (Ghoulam et al. 2002; Ungar 1996) and, therefore, it is expected that exclusion or detoxification mechanisms might be integral to plant adaptation to salinity. We tested whether *Trichoderma* spp. induced the Na^+ elimination through root exudates as part of a plant detoxification mechanism. The content of Na^+ exudated from roots grown in normal conditions was similar among treatments (Fig. 8D). In contrast, uninoculated seedlings grown under salinity exuded an enhanced amount of Na^+ when compared with nonstressed plants. Importantly, the Na^+ exuded by roots was increased by 25.76 and 79.65% when cocultivated with *T. virens* or *T. atroviride,* respectively. These effects may allow plants to better tolerate excess Na^+ levels. One possibility to explain the increased production of osmolite, antioxidants, and Na^+ exclusion is that the beneficial effects of *Trichoderma* spp. in roots through increasing the auxin pool improves plant health and activates metabolic or transport processes, which lead to an improved capacity to react or adapt to salt stress.

In conclusion, our research demonstrates the beneficial role of *Trichoderma* spp. to improve saline stress tolerance in *Arabidopsis*. Our data reveal a novel facet of auxins produced by fungi in promoting plant health, which may lead to potential applications in agriculture.

MATERIALS AND METHODS

Plant material and growth conditions.

A. thaliana was used in this work. The transgenic and mutant lines were derived from the parental *Arabidopsis* ecotype Columbia-0 (Col-0). The lines *aux1-7* (Picket et al. 1990), *eir1-1* (Roman et al. 1995), *arf7arf19* (Okushima et al. 2007), and *tir1afb2afb3* (Dharmasiri et al. 2005) are auxin-related mutants defective on auxin transporters AUX1 or PIN2 or affected in the transcription factors ARF7 and ARF19 or auxin receptors TIR1, AFB2, and AFB3, respectively. *DR5:uidA* is an auxin responsive marker (Ulmasov et al. 1997). Seed were surface sterilized with ethanol for 5 min and 20% (vol/vol) bleach for 7 min. After five washes in distilled water, seed were germinated and grown on agar plates containing 0.2× MS medium (Murashige and Skoog basal salts mixture, catalog number M5524; Sigma-Aldrich, St. Louis). Plates were placed vertically at a 65° angle to allow root growth along the agar surface and unimpeded aerial growth of the hypocotyls. Plants

were grown at 24°C in a chamber with a photoperiod of 16 h of light (200 μmol m^2 s^{-1}) and 8 h of darkness.

Fungal growth and plant inoculation experiments.

The following fungal strains were used in this work: *T. virens* Gv29.8 and *T. atroviride* (formerly *T. harzianum*) IMI 206040. Fungal strains were grown in 0.2× MS medium with (50 to 300 mM NaCl) or without salt (catalog number 11830-031; Gibco BRL, Bethesda, MD, U.S.A.). *T. virens* and *T. atroviride* were evaluated in vitro for their ability to promote salt tolerance in *Arabidopsis*. Fungal spore densities of 10^6 spores were inoculated on 0.2× MS medium with 100 mM NaCl or without salt by placing the spores at 5 cm in the opposite ends of agar plates containing 4-day-old germinated *Arabidopsis* seedlings (10 seedlings/plate). Plates were arranged in a completely randomized design. The seedlings were cultivated in a Percival AR95L growth chamber.

Quantification of shoot and root growth.

For shoot diameter quantification, seedlings were photographed 9 days after the treatments using a stereoscopic microscope (Leica MZ6; Leica Microsystems, Ryswyk, The Netherlands). Measurements were determined by using the Image J software. Growth of primary roots was registered using a ruler. LR numbers were determined by counting the LR present in the primary root. LR densities were determined by dividing the LR number by the primary root length. RH were measured in a 500-μm region at a 1-cm distance from the primary root tip. The average length of RH was determined by measuring 100 hairs for each root, taking as a reference the root protoxylematic plane to locate the radical hair base in the epidermal cell.

Chlorophyll content measurement.

Chlorophyll content was determined from excised *Arabidopsis* shoots 5 days after fungal inoculation. Shoots were homogenized in 1 ml of 80% aqueous acetone. Chlorophyll from different samples was extracted by 48 h in darkness at –20°C. Supernatant readings were taken at 647 and 663 nm. Total chlorophyll content was calculated as (7.15 × A663) + (18.71 × A647) divided by 1,000 × shoot fresh weight and was reported as milligrams of chlorophyll per gram of fresh weight, as described by Zhang and associates (2008b).

Root exudate collection and Na^+ content measurement.

Arabidopsis WT (Col-0) plants were grown as described above and root exudates from 40 seedlings per sample were collected. Five days after fungal inoculation, seedlings were transferred to falcon tubes containing tri-distilled water supplemented with 0.2× sucrose and placed at 24°C in a chamber with a photoperiod of 16 h of light (200 μmol m^2 s^{-1}) and 8 h of darkness. Root exudates were collected 2 days later. Each treatment consisted of three replicates and each replicate consisted of a total volume of 4 ml of exudate. The collected root exudates were filtered using nylon filters (Econofilter 25/0.45 μm; Agilent Technologies Netherlands BV, Amsterdam) to remove root-border cells. After filtration, the exudates were stored at –72°C for further analyses. Next, aqueous Na^+ was determined by atomic absorption spectrophotometry (Model AAnalyst 200; Perkin Elmer, Norwalk, CT, U.S.A.).

L-Pro determination.

L-Pro extraction and determination were performed in *Arabidopsis* (ecotype Col-0) seedlings at 5 days after inoculation. For sample preparation, plant tissues were frozen and ground in liquid N_2. Approximately 250 mg of ground tissue was placed in an Eppendorf tube. Tissue was homogenized with 1 ml of 0.7% (vol/vol) concentrated HCl in methanol and shaken for 5 min. Samples were centrifuged at 11,500 rpm for 3 min, and supernatants were collected and evaporated under a stream of gaseous nitrogen. L-Pro was derivatized with acetyl chloride in methanol (500 μl per 2 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the methylated sample was evaporated and added to acetic anhydride (1.5 ml) and dichloromethane (1 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and redissolved in 50 μl of methanol for gas chromatography mass spectrometry (GC-MS) analysis. GC-selected ion-monitoring mass spectrometry (GC-SIM-MS) and retention time were established for N-acetyl-proline methyl ester (*m/z* 70, 112 and 171 M^+, 4.62 min), respectively. L-Pro (Merck, Amsterdam) was derived and used as pure standard. To estimate the amount of L-pro in *Arabidopsis* seedlings, we constructed a standard curve.

ABA and AA determinations.

ABA and AA were extracted and determined in *Arabidopsis* (Col-0) 5 days after *Trichoderma* spp. inoculation. Plant tissues were frozen and ground in liquid N_2. Approximately 200 mg of ground tissue was placed in an Eppendorf tube, homogenized with 500 μl of isopropanol/H_2O/concentrated HCl (2:1:0.002, vol/vol), and shaken for 30 s. Samples were centrifuged at 11,500 rpm for 3 min, and supernatants were collected and subjected to ABA or AA extraction with 300 μl of dichloromethane. ABA was derivatized with acetyl chloride in methanol (500 μl per 2 ml), sonicated for 15 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and resuspended in 25 μl of ethyl acetate for GC-MS analysis. GC-SIM-MS and retention time were established for *cis*, *trans*-ABA methyl ester (ABA-ME; *m/z* 134, 190, and 278 M^+, 15.51 min); *cis*, *trans*-ABA was purchased from Sigma-Aldrich and used as standard.

AA was derivatized with acetic anhydride and dichloromethane (1.5 ml per 1.0 ml), sonicated for 25 min, and heated for 1 h at 75°C. After cooling, the derivatized sample was evaporated and redissolved in 25 μl of ethyl acetate for GC-MS analysis. GC-SIM-MS and retention time were established for AA and 2, 3, 5, 6-tetra-acetyl ester (*m/z* 200 and 344 M^+, 14.79 min) respectively. L(+) AA was obtained from Merck, derivatized, and used as standard.

The identity of each compound was further confirmed by comparison with the pure standard and, to estimate the amount, we constructed an independent standard curve.

IAA determination and GC-MS analysis.

For the production of IAA, an active inoculum of 1 × 10^6 spores of *T. virens* or *T. atroviride* was added to 200 ml of potato dextrose broth (Fluka Analytical; Sigma-Aldrich) and grown for 3 days at 24°C, with shaking at 180 rpm. To evaluate the effect of salt supply on IAA production, the medium was supplemented or not with NaCl at a concentration of 100 mM. The fungal cultures were filtered and the supernatant was adjusted to pH 3 using 1 N HCl. IAA in supernatant solution was extracted two times with 500 ml of ethyl acetate. The extracts were combined and evaporated to dryness under a stream of nitrogen and then diluted in 1 ml of ethyl acetate. IAA was methyl esterified with 500 ml of acetyl chloride in 2 ml of dry methanol, sonicated for 15 min, and heated at 75°C for 1 h. IAA content was determined as described previously (Contreras-Cornejo et al. 2009).

Samples were injected in an Agilent 6850 Series II gas chromatograph equipped with an Agilent MS detector model 5973 and 30-m by 0.2-μm by 0.25-mm, 5% phenyl methyl silicone capillary column (HP-5 MS). The operating conditions used

were helium at 1 ml/min^{-1} as carrier gas, 300°C detector temperature, and 250°C injector temperature. The column was held for 5 min at 150°C and programmed at 5°C min^{-1} to a 278°C final temperature for 5 min.

Histochemical analysis and GUS expression measurements.

Transgenic plants that expressed the *uidA* reporter gene (Jefferson et al. 1987) were incubated 10 h at 37°C in GUS reaction buffer (5-bromo-4-chloro-3-indolyl-β-D-glucuronide at 0.5 mg/ml in 100 mM sodium phosphate, pH 7). The stained seedlings were cleared using the method of Malamy and Benfey (1997). For each treatment, at least 15 transgenic plants were analyzed. The processed seedlings were included in glass slips and sealed with commercial nail varnish. For each treatment, a representative plant was chosen and photographed using a Leica MZ6 stereomicroscope.

GUS expression was determined in primary root tips of 9-day-old *Arabidopsis* seedlings grown, stained, and cleared as described above. Fifteen obtained images were processed using the Image J software and the data expressed as arbitrary units.

Data analysis.

Experiments were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Multivariate analyzes with a Tukey's post hoc test was used for testing differences in the fresh and dry weight, shoot length, chlorophyll content, ABA, L-Pro, AA, aqueous Na^+, and RSA. Different letters are used to indicate means that differ significantly ($P \leq 0.05$).

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