The Root-Colonizing Endophyte *Piriformospora indica* Confers Drought Tolerance in *Arabidopsis* by Stimulating the Expression of Drought Stress–Related Genes in Leaves

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Piriformospora indica is an endophytic fungus that colonizes the roots of many plant species, including *Arabidopsis*. We exposed 18-day-old *Arabidopsis* seedlings, which were either cocultivated with the fungus or mock-treated for the last 9 days, to mild drought stress for 84 h. During the first 36 to 48 h, seedlings cocultivated with the fungus continued to grow, while the uncolonized controls did not. This results in a threefold difference in the fresh weight and a more than twofold difference in the chlorophyll content. The photosynthetic efficiency was only slightly reduced in the colonized (F variable/F maximum [Fv/Fm] at $t_{0h} = 0.82$ and $t_{36h} = 0.79$) and was severely impaired in the uncolonized (Fv/Fm at $t_{0h} = 0.81$ and $t_{36h} = 0.49$) seedlings, which also showed symptoms of withering. When seedlings exposed to drought stress for 72 or 84 h were transferred to soil, 10% (72 h) and none (84 h) of uncolonized seedlings reached the flowering stage and produced seeds, while 59% (72 h) and 47% (84 h) of the colonized seedlings flowered and produced seeds. After exposure to drought stress for 3 h, the message levels for RESPONSE TO DEHYDRATION 29A, EARLY RESPONSE TO DEHYDRATION1, ANAC072, DEHYDRATION-RESPONSE ELEMENT BINDING PROTEIN2A, SALT-, AND DROUGHT-INDUCED RING FINGER1, phospholipase D δ , CALCINEURIN B-LIKE PROTEIN (CBL)1, CBL-INTERACTING PROTEIN KINASE3, and the histone acetyltransferase (HAT) were upregulated in the leaves of *P. indica*-colonized seedlings. Uncolonized seedlings responded 3 to 6 h later, and the message levels increased much less. We identified an *Arabidopsis* ethylmethane-sulfonate mutant that is less resistant to drought stress and in which the stress-related genes were not upregulated in the presence of *P. indica*. Thus, *P. indica* confers drought-stress tolerance to *Arabidopsis*, and this is associated with the priming of the expression of a quite diverse set of stress-related genes in the leaves. Transfer to soil was again associated with a faster and stronger upregulation of the message levels for phospholipase D δ , CBL1, and HAT in *P. indica*-colonized seedlings, indicating that this response might also contribute to better survival on soil.

Chaves and Oliveira 2004; Denby and Gehring 2005; Seki et al. 2007; Shinozaki and Yamaguchi-Shinozaki 2007; Xiong et al. 2002; Yamaguchi-Shinozaki and Shinozaki 2006). Drought stress induces a range of physiological and biochemical responses in plants, such as stomatal closure (Roelfsema and Hedrich 2005; Schroeder et al. 2001), repression of growth and photosynthesis (Flexas et al. 2004), and activation of respiration (Rennenberg et al. 2006). Many drought-inducible genes have been identified (Seki et al. 2002) that can be classified into two major groups: i) proteins that function directly in abiotic stress tolerance and ii) regulatory proteins, which are involved in signal transduction or expression of stress-responsive genes (Shinozaki et al. 2003). The first group includes chaperones, late embryogenesis-abundant proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. The second group includes transcription factors, protein kinases and phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules such as calmodulin-binding proteins (Shinozaki and Yamaguchi-Shinozaki 2007). Many genes for drought stress–signaling components themselves are upregulated under drought stress. Absciscic acid (ABA)-dependent and -independent signaling pathways have been shown to convert stress-signal information into the alteration of the expression of responding genes (Yamaguchi-Shinozaki and Shinozaki 2005), and the drought stress–induced signaling pathways are embedded into complex regulatory networks, which are activated in response to different abiotic and biotic stressors (Chinnusamy et al. 2004; Christmann et al. 2006; Seki et al. 2003; Shinozaki and Yamaguchi-Shinozaki 2000; Vinocur and Altman 2005; Wang et al. 2003).

Piriformospora indica is an endophytic fungus of the Sebacinaceae family, which colonizes the roots of many plant species, including *Arabidopsis* (Peškan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Shahollari et al. 2005, 2007; Sherameti et al. 2005; Varma et al. 1999, 2001; Verma et al. 1998; Waller et al. 2005). Hyphae can be detected on the root surface, in the outer cell layers of the roots, and within the root cells, but unlike ectomycorrhizal fungi, they do not grow from arbuscular structures in the plant cells (Peškan-Berghöfer et al. 2004). Root colonization and association of fungal hyphae with roots resulted in promotion of plant growth and higher seed yield, especially under conditions that are not optimal for the plants. *P. indica* was isolated from the roots of plants grown in the Indian desert Thar (Verma et al. 1998),

Plant growth is greatly affected by drought stress, and plants must adapt to this stress to survive (Bartels and Sunkar 2005;

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suggesting that the fungus may confer fitness benefits under drought-stress conditions. Since the fungus is associated with different plant species, it is likely that mechanisms leading to drought tolerance are based on general and not plant-specific mechanisms. Using *Arabidopsis* as a model system, we demonstrate that *P. indica* confers drought tolerance by priming the aerial parts of the plants for the expression of a set of quite diverse (drought) stress-related genes.

RESULTS

Arabidopsis seedlings cocultivated with *P. indica* are more drought tolerant than the uncolonized control.

We noticed that *Arabidopsis* plants cocultivated with *P. indica* were more resistant to drought stress in the greenhouse (data not shown). In order to obtain reproducible and quantitative data for this observation, we established standardized growth conditions, in which 9-day-old *Arabidopsis* seedlings were cocultivated with the fungus or were mock-treated for an additional 9 days before they were exposed to drought stress. Drought stress was applied by removing the lid from the petri dishes for up to 84 h. During this period, the development of the seedlings was monitored. At the 48-, 72-, and 84-h time-points after removal of the lids, the seedlings were also trans-

ferred to soil and the percentage of plants that had reached the flowering stage and produced seeds was determined.

By 24 h after exposure to drought stress, none of the seedlings showed visible effects of drought stress or withering (Fig. 1). However, *P. indica*-colonized seedlings continued to grow after the removal of the lids and weighed approximately 1.5 times as much as the uncolonized controls (Fig. 2). The chlorophyll level increased by 21%, while the chlorophyll level in the uncolonized control plants remained constant (Fig. 2). Furthermore, drought had a strong effect on photosynthesis (Flexas et al. 2004; Ramachandra-Reddy et al. 2004). The F variable/F maximum (Fv/Fm) values decreased in the uncolonized, dark-adapted controls (from $t_{0h} = 0.81$ to $t_{24h} = 0.61$), while no significant change was observed for the dark-adapted colonized seedlings (from $t_{0h} = 0.82$ to $t_{24h} = 0.81$; Fig. 2). Fv/Fm values around 0.83, measured after dark adaptation of plants, reflected the potential fluorescence quantum efficiency of photosystem II and are sensitive indicators of plant photosynthesis performance (Björkman and Demming 1983). Values lower than 0.83 indicated that plants are exposed to stress (Maxwell and Johnson 2000). This demonstrates that root-colonized seedlings suffered less from drought stress than the uncolonized controls.

Visually, the maximum difference between colonized and uncolonized seedlings was detectable approximately 36 h after

Fig. 1. *Arabidopsis* seedlings in petri dishes, which were cocultivated with *Piriformospora indica* (right) or mock-treated (left) before removal of the lid for 24 to 72 h. After 48 or 72 h, the seedlings were transferred to soil for 2 weeks (right, with *P. indica*; left, without *P. indica*). **Bottom,** Close-up of seedlings 48 h after the removal of the lid.

the removal of the lids. Colonized plants weighed more than twice as much as the uncolonized controls (Fig. 2), and the difference in the Fv/Fm values clearly demonstrated the beneficial effect of the fungus on photosynthetic efficiency under drought stress (Fig. 2).

By 48 h after exposure to drought stress, the agar in the petri dishes was dry. Uncolonized seedlings showed strong symptoms of drought stress; they withered and their weights decreased, although the chlorophyll levels remained constant (Figs. 1 and 2). The Fv/Fm value of $t_{48h} = 0.49$ indicated that photosynthetic efficiency was dramatically impaired in the uncolonized seedlings. *P. indica*-colonized seedlings also began to suffer from drought stress; no additional growth could be detected, and the Fv/Fm value decreased to $t_{48h} = 0.72$.

By 72 h after exposure to drought stress, the aerial parts of most uncolonized seedlings lay on the bottom and the chlorophyll content was approximately half the amount present before drought stress. We could not determine meaningful Fv/Fm values, indicating that photosynthetic machinery was badly damaged. In contrast, the aerial parts of the *P. indica*-colonized seedlings were still upright, although the leaves showed severe withering symptoms. The Fv/Fm value of $t_{72h} = 0.59$ indicated that photosynthesis performance in the colonized seedlings was now also severely impaired. Approximately 20% of the colonized but none of the uncolonized seedlings bolted (data not shown).

By 84 h after exposure to drought stress, $52 \pm 7\%$ of the colonized seedlings were still standing, while uncolonized seedlings were dried out (data not shown).

Transfer of drought stressed *Arabidopsis* seedlings to soil.

P. indica-colonized and control seedlings exposed to 48, 72, or 84 h of drought stress were transferred to soil, and their growth was monitored over a period of 4 months. Since the recovery from drought stress was quite different for individual plants (Fig. 1), we determined the percentage of plants that set flowers and produced seeds. After 48 h of drought stress, $31.2 \pm 3.6\%$ of the uncolonized and $96.2 \pm 1.6\%$ of the colonized plants produced seeds. After 72 h of drought stress, $9.8 \pm 0.8\%$ of the uncolonized plant and $59.0 \pm 7.1\%$ of the colonized seedlings produced seeds. After 84 h of drought stress, none of the uncolonized plants recovered and survived the seedling stage, while $46.5 \pm 4.0\%$ of the *P. indica*-colonized plants produced seeds. This confirmed that *P. indica* conferred drought tolerance to *Arabidopsis*.

Drought stress-related genes were upregulated earlier after exposure of *P. indica*-colonized *Arabidopsis* seedlings to drought stress.

We performed a differential display with mRNAs from leaves of colonized and uncolonized *Arabidopsis* seedlings 6 h after the removal of the lids. Of the 109 candidate genes, which were isolated in the screen and were identified by sequence analyses, nine were chosen for further analysis. Eight of them represented well-characterized markers for drought stress, and the gene products were involved in quite diverse processes of drought and other stress responses, i.e., *RESPONSE TO DEHYDRATION (RD)29A*, *EARLY RESPONSE TO DEHYDRATION (ERD)1*, *phospholipase D δ (PLD)*, the transcription factor gene *ANAC072*, *DEHYDRATION-RESPONSE ELEMENT BINDING PROTEIN (DREB)2A*, *SALT-AND DROUGHT-INDUCED RING FINGER (SDIR)1*, *CALCINEURIN B-LIKE PROTEIN (CBL)1*, *CBL-INTERACTING PROTEIN KINASE (CIPK)3*. Furthermore, the histone acetyltransferase (*HAT*) gene was selected as the ninth gene. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis demonstrated that all nine genes were

upregulated in the leaves of *P. indica*-colonized *Arabidopsis* seedlings 3 h after exposure to drought stress (Fig. 3). After 9 h, some of the message levels (*PLD*, *DREB2A*, *SDIR1*) started to decline, although a drought stress phenotype was not yet visible. In the uncolonized controls, a significant increase in these message levels could only be observed 6 or 9 h after exposure to drought stress, and for almost all these genes, the response was much weaker than in the colonized seedlings (Fig. 3). The drought-stress response of many genes in the uncolonized seedlings was slower as compared with previously published data, presumably because the seedlings were kept on wet agar after removal of the lids. This indicates that *P. indica* primes aerial parts of plants to respond to drought stress.

Fig. 2. Fresh weight, chlorophyll content, and F variable/F maximum values of *Arabidopsis* seedlings, which were either cocultivated with *Piriformospora indica* (rhombi) or mock-treated (squares) before opening the lids ($t = 0$). The fresh weight and chlorophyll content of *P. indica*-colonized seedlings at $t = 0$ was set as 100%, and all other values are expressed relative to it. Bars represent standard errors, based on five independent experiments with 60 seedlings each.

Before exposure to drought, colonized seedlings weighed $25 \pm 3\%$ more than the mock-treated seedlings. To exclude the possibility that this size difference is responsible for the observed phenomena, we compared *Arabidopsis* seedlings that were mock-treated for 9 days (weight = 26.6 ± 0.5 mg) with those that were cocultivated with *P. indica* for 7 days (weight = 26.4 ± 0.5 mg). After removal of the lids for 48 h, the importance of *P. indica* for drought tolerance could easily be seen. While the weight of the colonized seedlings increased to $212 \pm 21\%$ and the chlorophyll content to $121 \pm 9\%$ within 48 h, the weight of the uncolonized control decreased to $66 \pm 5\%$ and the chlorophyll content to $71 \pm 5\%$ within 48 h (data are based

on five groups of 60 seedlings, plus or minus standard error [SE]). The Fv/Fm values at t_{48h} were identical to those determined for seedlings described above (Fig. 2). Furthermore, the earlier response of the investigated drought-related mRNA levels can also be observed in colonized seedlings that were two days younger than the uncolonized controls (Fig. 3, bottom). Thus, the fungal effect on drought tolerance is not caused by differences in the size of the seedlings at the beginning of the drought-stress period.

The message levels for *PLD*, *CBL1*, and *HAT* were also upregulated after transfer of *P. indica*-colonized seedlings to soil.

We noticed that—in addition to drought—transfer of *Arabidopsis* seedlings to soil is associated with the upregulation of stress-related genes (data not shown). To investigate the role of *P. indica* in this scenario, we analyzed the message level of the above-mentioned genes in the leaves of 18-day-old seedlings that were either cocultivated with *P. indica* or mock-treated for the last 9 days, during the first 9 h on soil. The *PLD*, *CBL1*, and *HAT* mRNA levels but not those of the other genes were upregulated in the leaves of the colonized seedlings, and this response was weak if at all detectable in the uncolonized controls (Fig. 4). Thus, the higher message levels of these genes might also contribute to the better adaptation to soil.

A *P. indica*-insensitive mutant was more sensitive to drought stress.

An ethylmethane sulfonate-induced *P. indica*-insensitive (*pii*) mutant was originally isolated because it lacked several responses normally induced by the fungus (Oelmüller et al. 2005). The mutant did not show an obvious phenotype under normal growth conditions in petri dishes or the greenhouse, except that it did not grow taller in the presence of *P. indica*. Furthermore, the growth of fungal hyphae associated with *pii* roots was not different from that found in wild-type roots. This can be measured by fluorescence emission at 595 nm, which originates from

Fig. 3. Top, Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis for various message levels in *Piriformospora indica*-colonized (+*P. indica*) and control seedlings (-*P. indica*) after exposure to mild drought stress. The seedlings were cocultivated with the fungus for 9 days before removal of the lids. The numbers across the top refer to hours after removal of the lids. *RD29A* = *RESPONSE TO DEHYDRATION29A*, *ERD1* = *EARLY RESPONSE TO DEHYDRATION1*, *PLD* = *phospholipase Dδ*, the transcription factor gene *ANAC072*, *DREB2A* = *DEHYDRATION-RESPONSE ELEMENT BINDING PROTEIN2A*, *SDIR1* = *SALT- AND DROUGHT-INDUCED RING FINGER1*, *CBL1* = *CALCINEURIN B-LIKE PROTEIN1*, *CIPK3* = *CBL-INTERACTING PROTEIN KINASE3*, *HAT* = *histone acetyltransferase*. Representative data from five independent RT-PCR experiments are shown. Numbers under the PCR products represent relative mRNA levels based on real-time PCR analysis in which the level for the 0 h -*P. indica* timepoint was taken as 1 and the other values are expressed relative to it. Average of three real-time PCR reactions; standard errors (SE) are between 2 and 27%. **Bottom,** Comparative analysis of *DREB2A* mRNA accumulation for seedlings that were cocultivated (squares) or mock-treated (rhombi) with *P. indica* for either 7 (gray) or 9 (black) days. All values are expressed relative to $t = 0$ for those seedlings that were cocultivated with the fungus for 9 days before removal of the lids. Average of three real-time PCR reactions; bars represent SE.

Fig. 4. Semiquantitative reverse transcriptase-polymerase chain reaction analysis (RT-PCR) analysis for various message levels in *Piriformospora indica*-colonized (+*P. indica*) and control seedlings (-*P. indica*) after transfer of the seedlings to soil. The numbers across the top refer to hours on soil. *PLD* = *phospholipase Dδ*, *CBL1* = *CALCINEURIN B-LIKE PROTEIN1*, *HAT* = *histone acetyltransferase*, *RD29A* = *RESPONSE TO DEHYDRATION29A*, *ACTIN* = *Actin* gene. Representative data from five independent RT-PCR experiments are shown. Numbers under the PCR products represent relative mRNA levels based on real-time PCR analysis in which the level for the 0 h -*P. indica* timepoint was taken as 1 and the other values are expressed relative to it. Average of three real-time PCR reactions; standard errors are between 5 and 33%.

a not-yet-identified metabolite synthesized by the fungus (Peškan-Berghöfer et al. 2004). The fluorescence is detectable in *pii* roots but not in the uncolonized control. We also performed RT-PCR for the translation elongation factor 1 α gene (*Pitefa*) from *P. indica* (Bütehörn et al. 2000) and compared levels of *Pitefa* to levels of *ACTIN* gene in *Arabidopsis* roots. No difference can be detected between the fungal signals in wild-type and *pii* roots (Fig. 5, top), confirming again that *pii* roots are colonized by *P. indica*. In the drought stress experiment described above, *pii* showed, in the presence of the fungus, very little drought-stress tolerance and behaved more like the uncolonized control plants. The Fv/Fm values 48 and 60 h after removal of the lids were not significantly different from those determined for the uncolonized wild-type seedlings (data not shown and Fig. 2). The mutant phenotype became easily visible by comparing *pii* and wild-type plants, which had been exposed to drought stress for 60 h before transfer to soil for 4 weeks. Only $15 \pm 1\%$ of the *P. indica*-colonized *pii* seedlings and $17 \pm 4\%$ of the uncolonized *pii* seedlings recovered on soil. This is comparable to the results obtained for the uncolonized wild-type seedlings ($17 \pm 2\%$), while $61 \pm 8\%$ of the colonized wild-type seedlings recovered and produced seeds. Those *P. indica*-colonized *pii* plants that recovered were much smaller than the wild-type controls (Fig. 5, bottom). Furthermore, the stress-related mRNA levels were not or were only a little upregulated in 18-day-old *P. indica*-colonized *pii* seedlings that were exposed to drought stress for 6 h or that were transferred to soil for 6 h (Fig. 6). This suggests that *pii* is affected in at least three *P. indica*-induced processes, growth promotion, drought stress tolerance, and upregulation of stress-related genes upon transfer of the seedlings to soil.

DISCUSSION

P. indica confers drought stress tolerance in *Arabidopsis*.

P. indica, originally isolated in a desert, confers drought tolerance to *Arabidopsis* by priming the aerial parts of the plant for an early and high expression of (drought) stress-related genes (Fig. 3). Some stress-related genes were also upregulated after transfer of the seedlings from tissue culture to soil (Fig. 4), suggesting that similar mechanisms also contributed to the hardening of the plants. The fungus is unique, in that hyphae positioned in the vicinity of the growing seedling sufficed to colonize the roots and induced stress tolerance. In the colonized plants, genes involved in different stress-related processes were upregulated, including genes for proteins directly involved in stress responses and those that were involved in ABA-dependent and -independent signaling, transcription and chromatin remodeling (Fig. 3). The availability of a mutant that did not confer stress tolerance to *Arabidopsis* (Figs. 5 and 6) and that was also affected in other *P. indica*-induced responses, such as stimulation of root and shoot growth (Oelmüller et al. 2005), suggests that the mutation affects more general processes in this beneficial interaction between the two symbiotic partners. Since root colonization and association of fungal hyphae with the roots, either separately or together, were not affected in the mutant (Fig. 5, top), it is tempting to speculate that either recognition of the two symbiotic partners or early signaling events in the root cells was affected by the mutation.

The described drought-tolerance assay is easy to perform. Drought tolerance could be visualized within 24 h and quantified with a pulse amplitude modulation fluorometer, by measuring the Fv/Fm values representing photosynthetic efficiency. We also applied this assay to other plant species (such as mustard, different cabbages, cress, tobacco), which can be germinated on tissue-culture medium, and found that they were also

more drought tolerant in the presence of *P. indica* (data not shown). Many endophytic bacteria and fungi (Schardl et al. 2004) and mycorrhizal fungi (Ruiz-Lozano 2003) have been reported to confer drought tolerance to various plant species. The use of *Arabidopsis* for these studies and the availability of markers that were regulated by the fungus in *Arabidopsis* roots or shoots (Peškan-Berghöfer et al. 2004; Sherameti et al. 2005; Shahollari et al. 2005, 2007) provided us with a model system to study microbe-induced drought tolerance.

The information flow from the roots to the shoots is still enigmatic. The fast response in the aerial parts of the root-colonized seedlings to drought stress suggests that the information transfer from the roots to the shoots is either fast or had already occurred before the seedlings were exposed to the stress. It would be interesting to see if components already known to be involved in long distance signaling are also involved in this process and whether the information flow is specific for drought tolerance or also affects other responses in the leaves. For instance, *P. indica* has been implicated to confer resistance against leaf pathogens (Waller et al. 2005). It is also unknown whether the *P. indica*-derived signal targets the individual genes in the leaves separately or acts on a major target from which other processes are initiated in the leaves. The established model system will allow us to study these effects at the molecular level and to identify appropriate genes and mutants.

P. indica-responsive genes involved in stress responses.

Substantial progress has been made in the last years to understand drought tolerance (Alpert 2006; Seki et al. 2007). Plants activate different signaling pathways when exposed to drought (Boudsocq and Laurie 2005; Seki et al. 2003), and these pathways cross-talk with those involved in other stress responses (Chinnusamy et al. 2004; Fujita et al. 2006; Kolukisaoglu et al. 2004; Shinozaki and Yamaguchi-Shinozaki 2000). Overexpression of crucial regulators of drought stress-regulated genes in different plant species had conferred drought-stress tolerance to many plant species, and this is important for their biotechnological applications (Denby and Gehring 2005; Umezawa et al. 2006; Vinocur and Altman 2005; Wang et al. 2003; Zhang et al. 2004). However, several overexpressors were severely affected in growth or development or exhibit other undesired traits that prevent their direct application in agriculture (Denby and Gehring 2005). *P. indica* has clear advantages over the manipulation of individual components in (drought) stress tolerance, since it targets several drought-related genes simultaneously and has no obvious side effects for biotechnological applications.

Nine genes have been investigated in this study, which were earlier upregulated in response to drought stress in *P. indica*-colonized seedlings compared with the controls (Fig. 3). The gene products are involved in quite different cellular processes, starting from phospholipid metabolism at the plasma membrane (PLD δ), via cytoplasmic signaling through CBL1/CIPK3, control of gene expression in the nucleus (HAT, DREB2A, ANAC072) to cytoplasmic functions associated with RD29A and protein degradation at the endomembrane system (SDIR1) as well as in the plastids (ERD1). It is likely that a more comprehensive analysis will uncover more *P. indica*-responsive genes and proteins involved in (drought) stress tolerance. RD29A (Yamaguchi-Shinozaki and Shinozaki 1993) and ERD1 (Nakashima et al. 1997) are widely used reporter genes for drought-stress responses. ERD1 codes for a plastid-localized caseinolytic protease, which is induced by dehydration, independent of ABA or cold stress (Nakashima et al. 1997). Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of ABA in *Arabidopsis* cell culture (Takahashi et al. 2001). Downstream of this

Fig. 5. Top, Semiquantitative reverse transcriptase-polymerase chain reaction analysis for *Pitef1* and *ACTIN* for 18-day-old wild-type (WT) and *Piriformospora indica*-insensitive (*pii*) seedlings, which were either cocultivated with *Piriformospora indica* for the last 9 days (+) or mock-treated (-). Representative data from five independent experiments are shown. Real-time PCR analysis suggests that the *Pitef1* signal in colonized wild-type seedlings corresponds to $1.22 \pm 0.23\%$ and, in the colonized *pii* seedlings, to $4 \pm 0.19\%$ of the *ACTIN* signals, which were set as 100% (data not shown). **Bottom**, Wild-type (WT) and *pii* plants exposed to drought stress for 60 h and transferred to soil for 4 weeks. The picture shows representative plants from those that survived on soil.

response, PLD (in particular the α and δ forms) and their products, phosphatidic acid, are involved in different signaling networks and are activated by drought, salinity stress, and cold (Bargmann and Munnick 2006). Katagiri and associates (2001) have demonstrated that PLD δ is specifically induced by dehydration. The transcription factor ANAC072 binds to the promoter of *ERD1*, and DREB2A interacts with the *cis*-acting dehydration-responsive element involved in drought stress-responsive gene expression. The ANAC072 and DREB2A message levels themselves are drought-inducible (Fujita et al. 2004; Liu et al. 1998; Nakashima et al. 2000; Sakuma et al. 2002; Tran et al. 2004; Yamaguchi-Shinozaki et al. 1992) and earlier upregulated in *P. indica*-colonized drought-stressed plants than in the uncolonized control (Fig. 3). Overexpressors of ANAC072 (Tran et al. 2004) and of a constitutive active form of DREB2A (Sakuma et al. 2006) showed improved drought-stress tolerance in *Arabidopsis*. SDIR1 is an E3 ubiquitin ligase located in intracellular membranes of all tissues of *Arabidopsis*. SDIR1 is a positive regulator of ABA signaling and its message level is upregulated by drought and salt stress but not by ABA. Overexpression of *SDIR1* also leads to enhanced drought resistance, in addition to many other ABA-associated phenotypes (Zhang et al. 2007). Thus, unlike DREB2A (Shinozaki and Yamaguchi-Shinozaki 2007), SDIR1 is involved in an ABA-dependent pathway leading to stress tolerance. CBL1 is an important player in Ca^{2+} signaling and integrates plant responses to abiotic stresses, including drought stress, through one or more ABA-independent pathways. CBL1 also contributes to the regulation of early stress-related transcription factors of the C-repeat-binding factor/DREB type (Albrecht et al. 2003; Cheong et al. 2003). CIPK3, a calcium sensor-associated kinase, has multiple functions in stress responses and might be a cross-talk node in stress and ABA signal transduction (Batistic and Kudla 2004; Kim et al. 2003). Although CIPK3 modulates primarily cold- and salt-induced but not drought-induced gene expression, its message level was earlier upregulated in *P. indica*-colonized seedlings exposed to drought stress. The same was observed for the *HAT*

Fig. 6. Semiquantitative reverse transcriptase-polymerase chain reaction analysis (PCR) for various message levels. **A**, *Piriformospora indica*-colonized (+), **B**, control (-) wild-type (WT), and **C**, *Piriformospora indica*-insensitive (*pii*) seedlings 6 h after the removal of lids and **D** to **F**, 6 h after transfer to soil. **G**, *ACTIN* control. Representative data from five independent experiments are shown. Numbers under the PCR products represent relative mRNA levels based on real-time PCR analysis in which the level for the -WT level was taken as 1 and the other values are expressed relative to it. Average of three real-time PCR reactions; standard errors are between 6 and 26%.

message. Chromatin histone acetylation is crucial for transcriptional regulation. Highly acetylated chromatin is normally associated with active transcription, whereas chromatin regions with low acetylation are normally found in repressed regions

of the genome (Eberharther and Becker 2004; Eberharther et al. 2005). HAT do not directly interact with their target promoters, they are directed to specific DNA sites by recruiting factors. DREB1B, a transcription factor involved in dehydration and cold responses, is one of the recruiting factors interacting with HAT (Stockinger et al. 1997). Thus, *P. indica* might control gene expression more generally by regulating crucial factors involved in histone acetylation. Finally, the earlier upregulation of genes involved in stress responses in *P. indica*-colonized plants is consistent with the idea that the fungus primes the aerial parts of the plant to become more resistant to water stress. Although more studied in plant defense responses (Conrath et al. 2002, 2006), chemical priming for drought-stress tolerance has also been reported for *Arabidopsis* (Jakab et al. 2005).

Besides an early response to drought stress, we also observed that the *PLD*, *CBL1*, and *HAT* message levels in *P. indica*-colonized seedlings were upregulated after transfer of the seedlings to soil. A crucial role of *P. indica* in promoting the survival rate of various plant species after their transfer to soil has previously been reported (Pham et al. 2004). In agriculture, this common procedure is often associated with a severe loss of plants. It appears that the presence of the fungus also strengthens the seedlings to cope with such a stress situation by stimulating the expression of stress-related genes. *PLD* and *CBL1*, for example, are early mediators in several stress-related signaling pathways. HAT recruits transcription factors, including those involved in stress responses (Benhamed et al. 2006; Gao et al. 2007; Mao et al. 2006). Expression profiling and the analysis of knock-out lines will help to identify genes and proteins that are targeted by *P. indica* in *Arabidopsis* and that might participate in plant hardening.

MATERIALS AND METHODS

Growth conditions of plant and fungus.

Arabidopsis wild type and *pii* mutant seeds (ecotype Columbia) were surface-sterilized and were placed on petri dishes containing MS nutrient medium (Murashige and Skoog 1962). After cold treatment at 4°C for 48 h, plates were incubated for 7 days at 22°C under continuous illumination (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$). *P. indica* was cultured as described previously (Peřkan-Berghöfer et al. 2004; Verma et al. 1998) in petri dishes on a modified KM medium (NaNO_3 , 7.0 mM; KCl, 7.0 mM; MgSO_4 , 2.1 mM; KH_2PO_4 , 9.2 mM; ZnSO_4 , 0.77 mM; H_3BO_3 , 0.18 mM; MnSO_4 , 0.02 mM; CoCl_2 , 0.007 mM; CuSO_4 , 0.0065 mM; FeSO_4 , 0.02 mM; EDTA, 0.02 mM; ammonium molybdate, 0.001 mM; thiamine, 0.003 mM; glycine, 0.005 mM; nicotinic acid, 0.002 mM; pyridoxine, 0.0004 mM; glucose, 110 mM; 2 g of peptone per liter; 1 g of yeast extract per liter; 1 g of casein hydrolysate per liter, pH 6.5) with 1% (wt/vol) agar. The plates were inoculated with the fungus and were kept at room temperature in the dark for 1 to 2 weeks.

Cocultivation experiments and estimation of plant growth.

At 9 days after the beginning of the experiments, *A. thaliana* seedlings were transferred to nylon disks (mesh size 70 μm) and were placed on top of a modified PNM culture medium (5 mM KNO_3 , 2 mM MgSO_4 , 2 mM $\text{Ca}(\text{NO}_3)_2$, 0.01 μM FeSO_4 , 70 μM H_3BO_3 , 14 μM MnCl_2 , 0.5 μM CuSO_4 , 1 μM ZnSO_4 , 0.2 μM Na_2MoO_4 , 0.01 μM CoCl_2 , 10.5 g of agar per liter, pH 5.6) in petri dishes. One seedling was used per petri dish. A plug (5 mm in diameter) with fungal hyphae growing on a KM plate was removed and placed at a distance of 3 cm from the roots. Control seedlings were mock-treated as follows. The plug was taken from a fresh KM plate without fungus. The

PNM plates were incubated at 22°C under continuous illumination from the side (80 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 9 days. Then, the lids were removed and the development of the seedlings was followed within the next 84 h.

Fresh weight, chlorophyll content, and Fv/Fm values.

The fresh weights were determined directly after seedlings were removed from the plates. Chlorophyll was determined according to Oelmüller and Briggs (1990). The Fv/Fm data were determined as described by Maxwell and Johnson (2000) after dark adaptation of the seedlings.

RNA analysis.

RNA extraction, reverse transcription, and semiquantitative RT-PCR were described in Shahollari and associates (2007). PCR products were separated on 1.5% agarose gels and were stained with ethidium bromide, and visualized bands were quantified with the ImageMaster Video system (Amersham Biosciences). The following primer pairs were used:

PLD δ (At4g35790): CGTGGTTAAAGTGTCAGGAAG AGCC and AATCGCCATGGGCGCATACCAACC; *RD29A* (At5g52310): CGTCGAGACCCCGATAACGTTGG and CC CGTCGGCACATCCTTGTCGAT; *ERD1* (At5g51070): GGC TCTCCTCCTGGTTATGTTGGC and TCCGGAGTGGTCGT GCACCGTA; *ANAC072* (=RD26) (At4g27410): ACCCACTC GAGCTGTACCCG and CTCGTAGCCATGGAAGCTCC; *DREB2A* (At5g05410): GGGGTAAATGGGTTGCTGAG and TTGGCAACACTGTTCCCG; *SDIR1* (At3g55530): CCTTG CTGTCTCAACCATGGATC and ACTTAGGCTCCAGCTGG CTCTT; *CBL1* (At4g17615): AGCTTGCTCTGAGACAGC and CGACCTCCGAATGGAAGAC; *CIPK3* (At2g26980): CGCGCTCCTCTCAATGTTATCC and ACGTGACATGTG GTCATGCGGTG; *HAT* (At5g56740): GCGTTTGACCACTC TTGGAGAC and GATGTCACTGATCCTGACTGGC.

Piriformospora indica was monitored with a primer pair for the *Pitefl* gene (Bütehörn et al. 2000): ACCGCTTGGGGTT GTATCC and TCGTCGCTGTCAACAAGATG. Roots were harvested and carefully dipped into distilled water (12 times) to remove loosely associated fungal hyphae. In pilot experiments, we have shown that six washing steps were sufficient to remove those fungal hyphae that were only loosely associated with the roots and that the intensity of the PCR signal for *Pitefl* was constant after six and more washing steps, irrespective of the amount of fungal hyphae in the root environment.

Differential display with RNA from the leaves of seedlings that were grown in the presence or absence of *P. indica*, 6 h after exposure to drought stress, and analysis of the identified clones was described by Krüger and associates (2004). The SMART PCR cDNA synthesis kit and the Clontech PCR-select cDNA subtraction kit (BD Biosciences, Franklin Lakes, NJ, U.S.A.) were used.

Real-time PCR.

Real-time quantitative RT-PCR was performed using an iCycler iQ real-time PCR detection system and iCycler software version 2.2 (Bio-Rad, Munich). Total RNA was isolated from three independent replicates of *Arabidopsis* leaves, which were grown in the absence or presence of *P. indica* after exposure to drought stress or after transfer to soil. SE values of the three replicates are given as percentages. For the amplification of the RT-PCR products, iQ SYBR green supermix was used according to the manufacturer's protocol in a final volume of 25 μl . The iCycler was programmed to 95°C for 2 min, 40 $\times$ cycle of 95°C for 30 s, 55°C for 40 s, and 72°C for 45 s, and then, 72°C for 10 min, followed by a melting curve program of 55 to 95°C in increasing steps of 0.5°C. All reactions were performed in triplicate. The mRNA levels for each

cDNA probe were normalized with respect to the actin mRNA level, which was taken as 1.0. The relative mRNA levels of the genes in the figures are expressed relative to the mRNA levels found in control seedlings (not cocultivated with *P. indica*) before removal of the lid or transfer to soil (Figs. 3, 4, and 6).

Analysis of the plants after transfer to soil.

At 48, 72, or 84 h after exposure to drought stress, seedlings were directly transferred to soil. Before being transferred to soil, the roots of the seedlings cocultivated with the fungus were examined under the microscope to ensure that hyphae and spores have been developed within the roots (discussed below). Cultivation occurred in multitrays with Aracon tubes in a temperature-controlled growth chamber at 22°C under long-day conditions (light intensity = 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Plant sizes were continuously monitored. Seed production was monitored by collecting seeds from individual plants grown under the standardized conditions described above. The data given in the above are based on five independent experiments with 60 colonized and uncolonized plants per experiment.

For the RNA analysis presented in Figures 4 and 6D to F, 18-day-old *P. indica*-colonized and control seedlings (wild type and *pii*) were transferred directly to soil.

Staining fungal hyphae and spores.

To monitor root colonization, small parts of the roots from seedlings that were cocultivated with *P. indica* were transferred to 10% potassium hydroxide and were boiled for 10 min. After washing with water for 1 min, the roots were put into a 0.01% acid fuchsin-lactic acid solution and were boiled again for 10 min. Excess dye was removed with water prior to microscopy.

Fluorescence measurements.

Autofluorescence in the developing root hairs as a result of coculture with *P. indica* was detected with the LSM 510 META microscope (Carl-Zeiss Jena GmbH, Jena, Germany). Relative values (595 nm) were obtained from the emission spectra (Peškan-Berghöfer et al. 2004).

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