Piriformospora indica confers drought tolerance on Zea mays L. through enhancement of antioxidant activity and expression of drought-related genes

Le Xu¹, Aiai Wang¹, Jun Wang, Qiao Wei, Wenying Zhang※

Hubei Collaborative Innovation Center for Grain Industry/Research Center of Crop Stresses Resistance Technologies, Yangtze University, Jingzhou 434025, China

ARTICLE INFO

Article history:
Received 20 May 2016
Received in revised form 13 September 2016
Accepted 2 November 2016
Available online 12 November 2016

Keywords:
Antioxidants
Drought-related genes
Drought tolerance
Piriformospora indica
Maize

ABSTRACT

Drought stress is one of the most severe environmental constraints to plant growth and crop productivity. Plant growth is greatly affected by drought stress, and plants, to survive, adapt to this stress by invoking different pathways. Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales, promotes plant growth and confers resistance to biotic and abiotic stresses, including drought stress, by affecting the physiological properties of the host plant. The fungus strongly colonizes the roots of maize (Zea mays L.) and promotes shoot and root growth under both normal growth conditions and drought stress. We used polyethylene glycol (PEG-6000) to mimic drought stress and found that root fresh and dry weight, leaf area, SPAD value, and leaf number were increased in P. indica-colonized plants. The antioxidative activities of catalases and superoxide dismutases were upregulated within 24 h in the leaves of P. indica-colonized plants. Drought-related genes DREB2A, CBL1, ANAC072, and RD29A were upregulated in drought-stressed leaves of P. indica-colonized plants. Furthermore, after drought treatment, proline content increased, whereas accumulation of malondialdehyde (MDA), an indicator of membrane damage, decreased in P. indica-colonized maize. We conclude that P. indica-mediated plant protection against the detrimental effects of drought may result from enhanced antioxidant enzyme activity, proline accumulation, and expression of drought-related genes and lower membrane damage in maize plants.

© 2016 Crop Science Society of China and Institute of Crop Science, CAAS. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Drought is one of the most severe abiotic stresses constraining and destabilizing maize grain production [1], and the sensitivity of this crop to water stress severely reduces its yield [2]. Piriformospora indica, a basidiomycete of the Sebacinaeae family, was first isolated from bush rhizosphere zones of the Thar Desert in India [3]. P. indica, which is easily cultivated in axenic culture, is an endophytic fungus that colonizes the roots of a broad range of hosts, promoting host plant growth and

* Corresponding author.
E-mail address: wyzhang@yangtzeu.edu.cn (W. Zhang).
Peer review under responsibility of Crop Science Society of China and Institute of Crop Science, CAAS.
1 These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.cj.2016.10.002
2214-5141/© 2016 Crop Science Society of China and Institute of Crop Science, CAAS. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

increasing plant tolerance to biotic and abiotic stresses by affecting physiological properties [3–5]. Drought can induce the production of reactive oxygen species (ROS), which cause damage to lipids, carbohydrates, proteins, and DNA [6–9]. Plants possess a collection of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD) that alleviate oxidative stress [10].

P. indica confers drought tolerance on Chinese cabbage leaves by stimulating antioxidant enzymes and expression of drought-related genes [11]. The fungus also confers drought tolerance on Arabidopsis in a manner that is associated with priming of the expression of a set of stress-related genes in leaves [12]. However, it remains unclear whether P. indica confers drought tolerance on maize and whether the mechanisms are similar to those in Chinese cabbage and Arabidopsis.

The goal of this study was to determine the effect of P. indica inoculation on maize tolerance to drought induced by PEG-6000 treatment. Maize inoculated with P. indica showed increases in fresh and dry weight, irrespective of the application of drought stress. It is concluded that the mechanisms by which P. indica protects maize plants from the detrimental effects of drought may enhance antioxidant enzyme activity and proline accumulation, lower membrane damage, and stronger expression of drought-related genes.

2. Materials and methods

2.1. Plant cultivation

Maize seeds (jixiang 1) were surface-sterilized with 75% ethanol for 2 min and 0.75% NaClO for 10 min and then washed approximately 5 times with sterilized water, as described by Varma et al. [5]. The seeds were germinated on double-layer filter paper in the dark at 25 °C for 3 days. Young seedlings were inoculated or not with P. indica and transplanted to sterilized sand. Twelve plants (used mainly for antioxidant measurement or gene expression analysis) were grown in a single plastic pot (18.5 cm diameter; 13.5 cm height; 3 pots used for one treatment). Four plants (mainly for phenotypic analysis) were grown in a square plastic pot (18.5 cm diameter; 13.5 cm height; 4 pots for one main experiment). The plants were grown in a greenhouse under 700 μmol m−2 s−1 light intensity and at temperatures between 25 °C and 28 °C, as described by Ghanem [13].

2.2. Inoculation and root colonization with P. indica

Samples of P. indica were provided by Dr. Ralf Oelmüller (University of Jena, Germany). The fungus was cultivated in a 250 mL flask filled with Aspergillus (ASP) medium. This flask was then placed on a shaker (150 r min−1) and grown in the dark at a constant temperature of 25 °C. After 14 days, the liquid was filtered and the excess culture medium was carefully removed from the mycelia. The maize seedlings germinated on filter paper were inoculated with 10 mL of a 0.1% P. indica suspension after being transferred to sand (1 g fresh mycelium per liter of water, injection of the suspension into the sand), and dead mycelia were added into the sand as a control. Root colonization by P. indica was examined one week after inoculation. Root systems were harvested from each plant per replication plot and stained with trypan blue. Colonization of roots was assessed using a Leica microscope (DM5000 B; Germany).

2.3. Drought and PEG-6000 treatment

Only one genotype (jixiang 1) was used for all experiments. For natural drought treatment, 12 plants (per treatment) were grown in a plastic pot containing sterilized sand, and water was withheld. For PEG-6000 treatment, seedlings were carefully removed from sand, washed, and transplanted into a pot with 1/2 strength Hoagland solution containing 20% PEG-6000 (with or without P. indica inoculation). Control plants were grown in 1/2 strength Hoagland solution for the duration of the experiment. Samples were harvested within 24 h. All plants were grown in a greenhouse (28 °C/16 h light, 25 °C/8 h dark, light intensity 700 μmol m−2 s−1, relative humidity 60%). The experiments were replicated 4 times.

2.4. Vegetative characteristics

After PEG-6000 treatment, root fresh weight, root dry weight, length of the longest root, leaf number, and leaf area of 12 plants per treatment were measured using a balance or a ruler. The SPAD index with or without P. indica inoculation was measured using a SPAD-502 Plus (Japan).

2.5. Antioxidative enzyme activity

Antioxidative enzyme activity was determined as described in a previous study [14]. In brief, 0.2 g leaves (fresh weight) was ground and the powder was transferred to tubes containing pre-chilled 50 mmol L−1 phosphate buffer (pH 7.8, containing 1% PVP) and centrifuged at 4 °C, 10,000 r min−1 for 20 min. Then, 5 mL of the supernatant was transferred to new tubes for SOD, CAT, and POD activity assays according to Giannopolitis and Ries [15], Kraus and Fletcher [16], and Cakmak and Marschner [17], respectively. Five replicates were used for each independent experiment.

2.6. MDA and proline measurements

MDA levels were determined according to the thiobarbituric acid (TBA) method of Hodges [18]. The components in the supernatant of the extract were precipitated with 0.5% TBA. The suspension was then boiled for 10 min and immediately cooled on ice. After centrifugation at 8000×g for 10 min, the MDA concentrations of the samples were quantified by measuring absorbance at 532, 600, and 450 nm using a Spectrostar Nano Spectrometer (UV-5800PC, Yuanxi, Shanghai, China).

Proline measurements were performed as previously described by Ashraf and Foolad [19]. Maize leaves were harvested, weighed (approximately 0.2 g fresh weight), and ground to powder in liquid nitrogen; 1 mL 75% ethanol was added, and the sample was shaken overnight. The mixture was centrifuged at 20,000×g and aliquots of the extract were used for measurements. For proline measurement, 100 μL of the aliquot was incubated with 900 μL ninhydrin reagent (1% ninhydrin (w/v), 60% glacial acetic acid (v/v), and 40% H2O) at 100 °C for 1 h. Then, 3 mL toluene was added, followed by
vortexing and incubation at 23 °C for 24 h. Absorbance was measured at 520 nm.

2.7. RNA extraction and real-time quantitative RT-PCR (qRT-PCR)

At indicated time points (0, 3, 6, 12, and 24 h), 1 g (fresh weight) of plants was harvested and used immediately for qRT-PCR analysis. Total root RNA was isolated by grinding the tissue to fine powder in liquid nitrogen and following the RNAiso Plus reagent manufacturer’s instructions (Code No. 9109). Total RNA (2 μg) was reverse-transcribed using a reverse transcription kit (Takara, M-MLV Version). qRT-PCR reactions were performed with an Applied Biosystem 7300 with SYBR premix (TaKaRa Biotechnology, Dalian, China) according to the manufacturer’s instructions. The experiment was performed in three biological replicates, always with similar results. The primers used can be found in Table 1.

3. Results

3.1. *P. indica* colonizes root cells and enhances biomass production and drought tolerance in maize

Microscopic inspection of *P. indica*-inoculated maize plants showed that the fungus enters roots and grows intracellularly in the root cortex (Fig. 1A). Only colonized plants were used in each experiment. Enhanced growth was verified in *P. indica*-inoculated plants under PEG-6000 induced drought stress or normal conditions (Figs. 2A and 1B). The rate of colonization was not significantly affected by exposure to drought stress (data not shown). Maize plants exposed to PEG-6000-induced or natural drought stress exhibited stunted growth and underwent chlorosis and subsequent necrosis, whereas reduced damage was observed in *P. indica*-colonized plants (Figs. 2A and 1C). Compared with uncolonized plants, root fresh and dry weight, leaf area, and SPDA values of *P. indica*-colonized maize were significantly increased under drought conditions (Fig. 2B–F), although the length of the longest root showed the opposite trend (Fig. 2G).

Table 1 – Primer sequences for drought stress-responsive genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sequence (5’–3’)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANAC072</td>
<td>F: 5’-ACTAGTGCAGAAGTCTCCTC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-AAGCCATCTCAGGCAGGCCT-3’</td>
</tr>
<tr>
<td>SDIR1</td>
<td>F: 5’-GACAGATCTGAGAAGCAGCC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACGCATCCTGTCAGTG-3’</td>
</tr>
<tr>
<td>CIPK3</td>
<td>F: 5’-GAGGGCATCTGACGAGCC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GAGGGCATCTGACGAGCC-3’</td>
</tr>
<tr>
<td>PLDα</td>
<td>F: 5’-GAGGCTACGGTCGATAG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GTGAGTCTGCTGCTGCTG-3’</td>
</tr>
<tr>
<td>DREB2A</td>
<td>F: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td>ERD1</td>
<td>F: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td>CBL1</td>
<td>F: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td>HAT</td>
<td>F: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td>Tubulin</td>
<td>F: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GACAGCGACGAGGAGGACG-3’</td>
</tr>
</tbody>
</table>

F: Forward primer; R: reverse primer.

Given that accumulation of proline in plants enhances stress tolerance [19], we compared the proline contents of *P. indica*-inoculated plants with uninoculated plants after PEG-6000 treatment. The proline levels in PEG-6000-treated plants were significantly higher than those in the control (Fig. 3). Compared to uncolonized plants, *P. indica*-colonized plants accumulated more proline, starting at 12 h. This finding suggests that accumulation of proline may play a role in drought stress tolerance.
role in enhancing drought tolerance in *P. indica*-colonized maize PEG-6000-induced drought stress caused accumulation of MDA, an indicator of the peroxidation of membrane lipids, at 3 h and 24 h. The MDA content in *P. indica*-colonized plants decreased compared with that in uninoculated plants, indicating that cell membrane damage caused by PEG-6000 treatment was reduced in the former (Fig. 4).

3.3. Antioxidant enzyme activities are upregulated in leaves of colonized, drought-exposed plants

MDA is generated mainly via ROS-induced degradation of polyunsaturated lipids [20,21], and *P. indica* could prevent or retard degradation of these lipids by preventing excessive ROS formation under stress conditions. We determined...
enzyme activities rather than the expression levels of genes, given that most antioxidant enzymes are regulated posttranslationally in response to oxidative stress. No significant difference between *P. indica* inoculation or non-inoculation was found in the activities of antioxidant enzymes. However, after drought stress induced by 20% PEG-6000, enzyme activities were markedly increased. *P. indica* further increased the activities induced by drought

**Fig. 4** – MDA content in leaves of *P. indica*-colonized and uncolonized maize under PEG-6000 treatment. PD, *P. indica* + drought; CD, control + drought; PC, *P. indica* + water; CC, control + water. Maize plants were treated with either 200 mL of water or 200 mL of PEG-6000 solution for 24 h. Bars represent SEs. The experiments were repeated four times, with similar results, and representative data from one replicate are shown. Data are average values ± SD (n = 3) calculated from three technical replicates. Asterisks indicate significant differences between *P. indica* inoculation or non-inoculation under normal or drought conditions (*P* < 0.05; **P** < 0.01 by Student’s t-test).

**Fig. 5** – Antioxidative enzyme activities in leaves of *P. indica*-colonized and uncolonized maize under PEG-6000 treatment. PD, *P. indica* + drought; CD, control + drought; PC, *P. indica* + water; CC, control + water. Maize plants were treated with either 200 mL of water or 200 mL of PEG-6000 solution for 24 h, and SOD, CAT, and POD activities were assessed. Bars represent SEs. The experiments were repeated four times, with similar results, and representative data from one replicate are shown. Data are average values ± SD (n = 3) calculated from three technical replicates. Asterisks indicate significant differences between *P. indica* inoculation or non-inoculation under normal or drought conditions (*P* < 0.05; **P** < 0.01 by Student’s t-test).

treatment, peaking at 24 h. SOD activity increased 1.21-fold in *P. indica*-inoculated plants after 24 h of drought treatment, and CAT activity was increased after 3 h and 24 h of drought treatment compared to plants not inoculated with *P. indica* (Fig. 5). We speculate that such elevated antioxidant levels could be a reason for the observed better survival of maize after inoculation with *P. indica*. These findings are consistent with previous observations that activation of antioxidant enzyme systems is a major target of this fungus in leaves [12,22,23]. Further research to determine how the signal is transferred from roots to leaves in maize is of great importance for better understanding of the use of this fungus in agriculture.

3.4. *P. indica* promotes expression of drought-related genes after PEG-6000 treatment

Previously studies have demonstrated that many drought-induced genes are more quickly and strongly upregulated in drought-exposed *Arabidopsis* and Chinese cabbage leaves when the roots are colonized by *P. indica* [11,12]. Accordingly, a similar experiment was conducted using maize plants exposed to PEG-6000 treatment. RNA was isolated from the leaves of colonized and uncolonized maize plants after PEG-6000 treatment for 0, 3, 6, 12, and 24 h. The mRNA levels of *ZmANAC072*, *ZmDREB2A*, *ZmCIPK3*, and *ZmERD1* were expressed at much stronger levels in colonized plants than in uncolonized controls.

Fig. 6 – Drought-related gene expression in leaves of *P. indica*-colonized and uncolonized maize under PEG-6000 treatment. PD, *P. indica* + drought; CD, control + drought; PC, *P. indica* + water; CC, control + water. Bars represent SEs based on 4 independent experiments. The experiments were repeated three times, with similar results, and representative data from one replicate are shown. Data are average values ± SD (n = 3) calculated from three technical replicates. Asterisks indicate significant differences between *P. indica* inoculation or non-inoculation under normal or drought conditions (*P* < 0.05; **P** < 0.01 by Student’s *t*-test).
after 24 h of PEG-6000 treatment. ZmCBL1 and ZmHAT responded more strongly to PEG-6000 treatment in colonized plants than in uncolonized controls at 3 h and 12 h, and ZmPLDδ was upregulated in inoculated plants during the entire PEG-6000 treatment (Fig. 6). The products of these genes are involved in diverse cellular processes, including phospholipid metabolism at the plasma membrane (PLDδ), cytoplasmic signaling through CBL1/ CIPK3, control of gene expression in the nucleus (HAT, DREB2A, ANAC072), and cytoplasmic functions associated with protein degradation in the endomembrane system (SDIR1) as well as in plastids (ERD1). Plants displaying ANAC072, DREB2A, and SDIR1 overexpression showed improved tolerance to drought stress [24–26]. Our findings confirmed previous results obtained with Arabidopsis seedlings and Chinese cabbage, extending such effects to maize plants grown in soil. Thus, enhanced expression of drought-related genes may be responsible for the observed drought tolerance of P. indica-inoculated maize.

4. Discussion

In this study, we characterized the phenotypic changes of maize seedlings when colonized with P. indica under drought stress. P. indica colonization resulted in apparent promotion of plant host growth and conferred drought tolerance on maize.

To adapt to osmotic stress, plants accumulate organic solutes such as proline and other amino acids [27]. Interestingly, P. indica-colonized plants have higher concentrations of proline than corresponding controls, which could partially explain their increased tolerance to osmotic stress [28]. Our study also showed that P. indica-colonized Z mays L. plants accumulated more proline, starting from 12 h, than uncolonized plants, suggesting that this accumulation of proline may play a role in enhancing drought tolerance.

It has been proposed that P. indica-conferring abiotic stress tolerance relies on enhanced synthesis of corresponding antioxidants, such as increased conversion of dehydroascorbate to ascorbate and higher levels of glutathione, which are the two main antioxidants [29–32]. Compared with non-inoculated plants, wheat inoculated with P. indica showed better vegetative growth, significantly lower levels of both hydrogen peroxide and lipid peroxidation, and increased levels of antioxidant enzymes such as CAT, APX, and POD and leaf chlorophyll at various moisture levels [33]. Other studies have also suggested that activation of antioxidant enzyme systems is a major target of the fungus in leaves [12,22,23]. We speculate that elevated antioxidant levels could be one reason for the observed better survival of maize after inoculation with P. indica.

Higher levels of expression of several genes putatively involved in stress responses is induced by drought in plants colonized by P. indica. Our findings showed that P. indica promotes expression of drought-related genes in maize after 24 h of PEG-6000 treatment, in agreement with a previous study in which a large number of drought-induced genes were more quickly and strongly upregulated in drought-exposed Arabidopsis and Chinese cabbage leaves when the roots were colonized by P. indica [11,12]. Taking these results together, P. indica may confer drought tolerance to maize by increased antioxidative enzyme activity and proline accumulation, reduced membrane damage and enhanced expression of drought-related genes.

The rapid response in the aerial parts of root-colonized seedlings suggests that information transfer from roots to shoots either occurs quickly or has already occurred before the seedlings are exposed to the stress [13]. It would be interesting to determine whether components already known to be involved in long-distance signaling are also involved in this process and whether the information flow is specific to drought tolerance or also affects other responses in leaves. For instance, P. indica has been implicated in conferring resistance to leaf pathogens [29]. It is also unknown whether the P. indica-derived signal separately targets individual genes in leaves or acts on a major target from which other processes are initiated.

Different phytohormones might act as signaling molecules for relaying information from roots to shoots and work in concert to promote drought tolerance after P. indica colonization. Synthesis of abscisic acid (ABA) is activated by water stress, triggering stomatal closure, growth arrest, and large-scale transcriptional changes, and also plays a significant role in drought tolerance [34–36]. Previous studies have shown that ABA might promote the plant–fungus interaction under moderate stress conditions by suppressing root innate immunity [37]. Cytokinin is a hormone that not only promotes cell division and differentiation but also mediates many other biological processes, including drought stress response [38]. Our study suggested the involvement of ABA and cytokinin in P. indica-mediated drought tolerance (unpublished data), but the exact mechanism requires further investigation.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31471496). We thank Prof. Lijuan Qiu of Institute of Crop Science, Chinese Academy of Agricultural Sciences for technology support. We thank Ntambo Mbuya Sylvain for manuscript revision.

References


