Poly-γ-glutamic acid enhanced drought resistance of maize by improving photosynthesis and affecting rhizosphere microbial community

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Research Article

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Abstract

Background

Compared with other abiotic stresses, drought stress is a serious causal factor leading to crop yield reduction. Poly-γ-glutamic acid (γ-PGA), as an environmentally friendly biomacromolecule, plays an important role in plant growth and regulation.

Results

In this project, the effect of exogenous application of γ-PGA on drought tolerance of maize (Zea mays. L) and its mechanism were studied. Drought dramatically inhibited the growth and development of maize, but the exogenous application of γ-PGA significantly increased the dry weight of maize and the contents of ABA, soluble sugar, proline, chlorophyll and the photosynthetic rate under severe drought stress. RNAseq data showed that γ-PGA may enhance drought resistance of maize by affecting the expression of ABA biosynthesis and signal transduction related genes, photosynthesis-related genes and other stress-responsive genes, which were also confirmed by RT-PCR and promoter motif analysis. In addition, diversity and structure analysis of rhizosphere soil bacterial community demonstrated that γ-PGA enriched the plant growth promoting bacteria such as Actinobacteria, Chloroflexi, Firmicutes, Alphaproteobacteria and Deltaproteobacteria. Meanwhile, γ-PGA significantly improved roots development, urease activity and ABA contents of maize rhizospheric soil under drought stress. This study emphasized the possibility of using γ-PGA to improve crop drought resistance and soil environment under drought condition and revealed its preliminary mechanism.

Conclusions

Exogenous application of poly-γ-glutamic acid could significantly enhance the drought resistance of maize by improving photosynthesis, root development and affecting rhizosphere microbial community.

Background

As one of the major adverse environmental stresses hamper the crop productivity worldwide, drought stress threat is increasing due to the global climate change [1–4]. The growth and development of plants require sufficient water, water shortage can be fatal to crops and leads to the yield losses. Meanwhile, drought stress also cause a series of other problems, such as soil erosion, land desertification, ecosystem destruction and so on [5–7]. The shortage of water resources has been considered to be an urgent global environmental issue. Water scarcity had aroused great concern, and the effect of drought stress on plants is receiving more and more attention. In order to improve the drought resistance of crops, the morphological, physiological, metabolic, molecular and genetic mechanisms of drought resistance of various plants have been systematically studied [7–12]. Conventional breeding, molecular marker-
assisted selection, plant transgenic technology, exogenous application of hormones (such as ABA) or osmoprotectants (such as glycine betaine and proline) and drip irrigation are all used as the technical strategies to cope with drought stress [13–21].

Poly-γ-glutamic acid (γ-PGA) is a non-toxic, water-soluble, biodegradable and environment-friendly biopolymer, which is composed of D/L-glutamic acid monomers and fermented by Bacillus subtilis [22, 23]. In line with its different molecular weights, γ-PGA could be used in many fields, such as food, medicine, cosmetics and agriculture [24]. γ-PGA has been paid more and more attention as an environmentally friendly fertilizer synergist because of its strong water solubility and retention, biodegradability and innocuity [25]. Recent studies have found that γ-PGA plays an important role in plant growth and regulation, and can be used as a water retaining agent and soil conditioner to improve crop productivity [26–28]. It has been reported that exogenous application of γ-PGA could significantly enhance the stress resistance of plants [26, 29–31]. Most of the previous studies focused on cold and salt stress of vegetables such as Brassica napus and cucumber. For example, it was found that γ-PGA could increase salt and cold tolerance of Brassica napus by activating the crosstalk between H₂O₂ and Ca²⁺ signals [32] and enhance drought resistance of Brassica napus by promoting ABA accumulation. However, only few studies assessed the effect of γ-PGA on drought resistance of plants, especially crops. And the regulation mechanism of γ-PGA on drought resistance of maize remains unclear. Maize is an important crop integrating grain, feed, energy and industrial raw materials, and plays an extremely important role in world food security and economic development [33]. Its yield is always severely affected by drought stress [34]. In this study, the effect of γ-PGA on the growth of maize seedlings under drought stress was assessed by adding γ-PGA to soil. In addition, RNAseq was performed to study the gene expression of maize leaves after drought stress, and the changes of rhizosphere microbial community after exogenous application of γ-PGA were also studied, so as to understand the mechanism of exogenous application of γ-PGA to change drought resistance of maize. So as to understand the mechanism of exogenous application of PGA to change drought resistance of maize.

Results

Exogenous application of γ-PGA enhanced drought resistance of maize

In order to investigate the effect of exogenous application of γ-PGA on maize under drought stress, the drought-resistant phenotype of maize with different concentrations of γ-PGA (0, 50, 70, 100mg/L) were examined (Additional file 1: Fig. S1). The results showed that the addition of γ-PGA could significantly enhance the drought resistance of maize, even at a lower concentration (50mg/L), and could regenerate maize rapidly after rewatering, while most of the control maize showed severe wilting and could not grow again after rewatering. 50mg/L γ-PGA treatment was used for the subsequent experiments.
Maize treated with 50mg/L γ-PGA exhibited a better phenotype after 7 days of drought stress (Fig. 1A). The dry weight, content of ABA, soluble sugar, proline, chlorophyll and the photosynthetic parameters of maize seedlings after 5 days drought treatment were determined. As shown in Fig. 1B, under drought condition, the dry weight of maize treated with γ-PGA (0.96g) was significantly higher than that of control maize (0.39g), indicating that γ-PGA could alleviate the inhibition of drought stress on the growth of maize seedlings. In addition, compared with the control group, the contents of ABA, soluble sugar, proline and chlorophyll in γ-PGA treatment group were 27.46%, 43.61%, 108% and 51.51% higher, respectively (Fig. 1B). This indicated that γ-PGA could promote the accumulation of ABA, soluble sugar, proline and chlorophyll in maize under drought stress. The photosynthetic parameters of the maize under drought for 5d were also measured, the results showed that both the net photosynthetic rate and stomatal conductance of the maize added γ-PGA were significantly higher than the control maize under drought stress (Fig. 1B).

In order to observe the effect of γ-PGA on maize growth under drought stress more directly, the simulated drought experiment with 18% PEG6000 solution was performed. It was found that the fresh weight of leaves and roots in γ-PGA treatment group was higher than that of the control group (Fig. 2), indicating that the drought resistance of leaves and roots in γ-PGA + PEG group was significantly higher than that of the control group.

**γ-PGA significantly improved roots development, urease activity and ABA contents of maize rhizospheric soil under drought stress**

It was found that γ-PGA significantly improved the roots development both under the normal condition and drought stress (Fig. 2A). Under normal growing conditions, the maize treated with γ-PGA had a better developed root system, and the fresh weight of roots was significantly increased than that of the control group. Under PEG simulate drought stress, the roots growth of the control group was significantly inhibited, however, the roots of the maize treated with γ-PGA were little affected by drought stress and the roots fresh weight was significantly higher than that of control group. Since maize rhizospheric soil was closely contacted with the roots, the urease activity (closely related to soil nitrogen transformation) and ABA contents (closely related to the drought resistance) of the maize rhizospheric soil under the severe drought stress were also detected. It was observed that the urease activity of rhizospheric soil of γ-PGA treatments was increased by 27.74%, while the ABA contents of γ-PGA treatments soil was also increased by 21.70% (Table 1).
Table 1

| Drought       | ABA (µg/g DW) | Urease activity (µg NH3-N/g/24h) |
|---------------|--------------|----------------------------------|
| 0mg/L γ-PGA   | 1.479 ± 0.011 | 767.583 ± 124.714                |
| 50mg/L γ-PGA  | 1.800 ± 0.002** | 980.524 ± 46.475**               |

Values are means ± sd (n ≥ 3 repeats). Significant differences are indicated by asterisks (**, P ≤ 0.01).

Differentially expressed genes (DEGs) between maize with γ-PGA addition and control under drought stress

In order to explain the mechanism of γ-PGA in improving the drought resistance of maize, the leaves of γ-PGA treatment and the control maize under drought condition for 5 days were used for RNA sequencing to identify the DEGs and pathways in response to drought stress. The total raw reads, clean reads, genome mapping ratio, and uniquely mapping ratio were listed in Additional file 10: Table S1. 16126 DEGs were identified and the distribution of the DEGs was illustrated in Fig. 3A. These DEGs were subjected to enrichment analysis of KEGG pathways and Gene Ontology (GO) functions. Based on KEGG pathway analysis, all DEGs were significantly enriched into 6 pathways (Q value ≤ 0.05), namely photosynthesis-antenna proteins (31 DEGs), photosynthesis (105 DEGs), glyoxylate dicarboxylate metabolism (102 DEGs), oxidative phosphorylation (156 DEGs), alanine, aspartate and glutamate metabolism (73 DEGs) and carotenoid biosynthesis pathway (65 DEGs) (Fig. 3B). The results of GO annotation functions enrichment analysis also showed that GO terms such as photosynthesis and photosystem, response to abiotic stimulus, chlorophyll metabolic process, response to biotic stimulus, electron transport chain and so on were significantly enriched (Additional file 2: Fig. S2B). A more detailed classification of the terms of response to abiotic stimulus showed that these DEGs were mainly related to the response to stress (osmotic stress, salt, heat, cold, reactive oxygen species, and hydrogen peroxide), the response to hormone (ABA, JA, and SA), ABA biosynthetic process, chlorophyll metabolic process, proline biosynthetic process, protein folding, and so on (Additional file 2: Fig. S2B).

γ-PGA improved drought resistance of maize by affecting the expression of photosynthesis-related genes

As known, drought could significantly reduce the photosynthesis capability of plants. However, KEGG analysis showed that under drought stress, compared with the control maize, the photosynthesis related genes of maize treated with γ-PGA were significantly enriched (Fig. 3B), with most of related genes were dramatically upregulated. As shown in Fig. 4 and Additional file 10: Table S1, most genes in DEGs of photosystem II complex were upregulated, except PsbA, PsbB, PsbC, PsbE, PsbF and 1 for PsbP, which were downregulated. In photosystem I complex, all of the DEGs were upregulated. In cytochrome b6/f complex, 7 genes encoding PetA, 2 genes encoding PetC and 1 genes encoding PetG were upregulated, while only 1 gene encoding PetD and 1 gene encoding PetA were downregulated. In photosynthetic
electron transport, other 16 genes encoding PetE, PetF, PetH and PetJ were all up-regulated except 3 genes encoding PetF and 2 for PetH. In F-type ATPase complex, except 1 gene encoding beta, 1 for gamma and 1 b which were downregulated, the other 14 genes encoding alpha, beta, gamma, delta, epsilon, a, b and c subunits respectively were upregulated. Additionally, all DEGs (67 genes) encoding antenna proteins were also up-regulated (Additional file 3: Fig. S3). To confirm the results, 14 genes with different transcript abundances were validated by real-time RT-PCR (Additional file 4: Fig. S4). The expression of these genes showed good consistency between the two detection methods. Meanwhile, the motifs in the promoter region of these genes were analyzed, higher percentage of drought, low-temperature, salicylic stress and ABA response elements were found (Additional file 5: Fig. S5, Additional file 6: Fig. S6).

**γ-PGA promoted ABA accumulation and affected ABA signaling to improve drought resistance of maize**

ABA, as an important drought response hormone, plays an important role in the response of maize to abiotic stress. Based on KEGG pathway analysis, it was found that DEGs related with carotenoid biosynthesis pathway which contains ABA biosynthesis pathway were significantly enriched (Fig. 3B), γ-PGA could promote ABA accumulation under drought condition (Fig. 1B). CHY2, ABA1, NCED, ABA2 and AAO3 were reported to be involved in ABA biosynthesis [35–38], 8'-hydroxyase was reported to play an important role in the catabolism of ABA [39]. RNAseq results showed that 2 genes encoding CHY2, 7 genes encoding ABA1, 3 genes encoding NCED, 2 genes encoding ABA2, and 1 genes encoding AAO3 were significantly upregulated, while 2 genes encoding 8'-hydroxyase were downregulated. In addition, ABA signaling pathway related genes, including ABA receptor (PYR/PYL), PP2C, SnRK2 and ABFs were also differentially expressed. Among these DEGs, 3 for PYL, 4 for SnRK2, and 4 for ABF were upregulated, 10 for PP2C were downregulated (Additional file 7: Fig. S7).

**γ-PGA affected the bacterial community diversity and structure of rhizospheric soil**

In order to study the influence of γ-PGA on bacterial community diversity under drought stress, the relative abundance and diversity of maize rhizospheric soil bacteria were analyzed by high-throughput sequencing of 16S rRNA. The species curve showed that the samples were representative enough to obtain a true bacterial community (Additional file 8: Fig. S8). NMDS (stress = 0.00422) of the weighted UniFrac distance ordinations were conducted (Fig. 5A), the results indicated that the bacterial community composition of the soil with γ-PGA application brought shifts compared with that of the soil without γ-PGA under the drought stress, the communities in maize rhizospheric soil with γ-PGA were grouped together and significantly separated from those in soil without γ-PGA under the drought stress. The obtained high-quality sequences were belonged to 36 phylum, among which the main phylum was Proteobacteria, followed by Actinobacteria, Chloroflexi, Bacteroidetes, and Acidobacteria. Although the diversity of bacterial community changed after the addition of γ-PGA under drought stress, the predominant phylum were similar. There was no difference in species composition among these samples, but the relative abundances of some species changed (Fig. 5B). Compared to the control, the relative abundance of Actinobacteria and Chloroflexi were higher in soil added γ-PGA under drought stress. LEfSe
analysis (LDA ≥ 3) showed the species with the most significant variation (Fig. 5C). Under drought stress, the application of γ-PGA could significantly enrich **Actinobacteria**, **Chloroflexi** and **Cyanobacteria** at phylum level, while **Alphaproteobacteria** and **Deltaproteobacteria** were enriched at class level. At the genus level, bacteria such as **Rhodobacter**, **Sphingobium**, **Sphingomonas**, **Sphingopyxis**, **Haliangium**, **Methyllobium**, **Lysobacter**, **Azoarcus** and **Arenimonas** of **Proteobacteria**, **Aeromicrobium**, **Lechevalieria** and **Streptomyces** of **Actinobacteria**, **Subgroup_10** of **Acidobacteria**, **Clostridium** and **Pelotomaculum** of **Firmicutes**, **Chloronema**, **A4b** and **KD4-96** of **Chloroflexi** were dominant in γ-PGA added rhizosphere soil under the persistent severe drought condition. The abundances of these genera in maize rhizospheric soil with γ-PGA addition were all higher than that of control (Additional file 9: Fig. S9), while **Bacillus** of **Proteobacteria** was dominated in control (Fig. 5C).

**Discussion**

Among all abiotic stresses, drought has the greatest impact on soil organisms and plants [40]. Drought could adversely affect the important physiological and biochemical processes of plants, resulting in serious loss of crop yield worldwide [41]. It is critical to improve the plant tolerance to drought stress. As a natural and environment friendly biopolymer, γ-PGA has been widely used in agricultural production [42]. However, there are few reports about the effect and mechanism of γ-PGA on drought resistance of plants, especially crops. In this study, the effect of γ-PGA on maize drought resistance and its comprehensive mechanism by RNAseq and rhizosphere soil bacterial community diversity analysis were firstly reported.

The effects of exogenous application of γ-PGA on dry weight, the contents of ABA, soluble sugar, proline and chlorophyll of maize leaf under severe drought stress were characterized. These physiological indexes have been often used to evaluate the drought resistance of plants. As the osmoprotectants, proline and soluble sugar could provide osmotic adjustments in plants under drought stress [43]. Proline has strong hydration ability, which can protect cell structure and enzymes, reduce cell acidity and regulate redox potential under stress. ABA is considered to be the most critical hormone regulating tolerance to drought stress. Drought stress could trigger a huge increase in ABA biosynthesis. As a key chemical messenger of drought signal, ABA could activate a series of signal transduction reactions to regulate stomatal closure, calcium signal and the expression of some ABA-responsive genes to resist the drought stress. Drought stress can significantly decline the chlorophyll content of leaves [44, 45]. Plants with higher chlorophyll contents under drought stress could use light energy more efficiently and have better drought resistance. In this study, we found that under drought stress, γ-PGA could promote the accumulation of ABA, soluble sugar, proline and chlorophyll, the drought resistance of maize was significantly enhanced by adding γ-PGA. In addition, γ-PGA could increase the dry weight of maize under drought stress, indicating that maize added with γ-PGA could still maintain a certain growth compared with that of control. In order to observe the root morphology under drought stress more directly, PEG6000 was used to simulate the drought treatment in the solution culture process. The results showed that, under PEG treatment, maize added with γ-PGA had more developed roots than that of control, which could make maize absorb deeper and more water of soil during drought stress.
To explore the molecular mechanism of enhanced drought resistance by exogenous application of γ-PGA, the differentially expressed genes (DEGs) of the leaves were evaluated by RNAseq analysis. KEGG analysis showed that photosynthesis related genes were significantly enriched which was consistent with the increase of photosynthetic rate in the maize treatment with γ-PGA under the drought stress. Most of the photosynthesis related genes, including 20 genes in photosystem I, 28 genes involved in photosystem II, 16 genes in photosynthetic electron transport, 10 genes in cytochrome b6/f complex, 14 genes in ATPase complex, and 31 genes encoding antenna proteins (9 genes encoding LHCI complex, 22 genes encoding LHClI complex), were dramatically upregulated in γ-PGA treatment maize compared with that of control. Photosynthesis is one of the main processes affected by drought [46]. However, under severe drought stress, the photosynthesis related genes in maize added with γ-PGA still maintained a high expression level than that of control, which may be the main reason for the higher drought resistance of maize treated with γ-PGA, while the reduced chlorophyll contents under drought in control leaded to the inactivation of photosynthesis.

ABA is considered to be the most critical hormone involved in the adaptive responses of plants to drought stress. DEGs related with carotenoid biosynthesis pathway which contains ABA biosynthesis pathway were also found to be significantly enriched in this study. In ABA biosynthesis, β-carotene is converted to zeaxanthin by CHY2 enzyme firstly, the epoxidation of zeaxanthin and antheraxanthin to violaxanthin was catalyzed by zeaxanthin epoxidase (ZEP/ABA1) afterwards [35]. Violaxanthin is converted to 9-cis-violaxanthin after a series of structural modifications. The next step is also a rate-limiting step, that is, 9-cis-violaxanthin is converted to xanthoxin under the catalysis of 9-cis-epoxycarotenoid dioxygenase (NCED) [36]. Subsequently, xanthoxin is converted to abscisic aldehyde, and then ABA is produced by two-step reaction via ABA-aldehyde. The enzyme (alcohol dehydrogenase/reductase) encoded by ABA2 catalyzes the first step of this reaction and generates ABA aldehyde[37], and abscisic aldehyde oxidase encoded by AAO3 catalyzes the last step of ABA synthesis [38]. In this study, it was found that the ABA biosynthesis related genes including 2 genes encoding CHY2, 7 genes encoding ABA1, 3 genes encoding NCED, 2 genes encoding ABA2, and 1 genes encoding AAO3 were significantly upregulated, while 2 genes encoding 8’-hydroxyase which played important role in the catabolism of ABA were downregulated in maize with the application of γ-PGA. The expression level of these DEGs led to the increase of ABA level in γ-PGA treated maize under drought stress. The results indicated that γ-PGA could promote ABA accumulation under drought condition, and the accumulation of ABA can activate the core ABA signaling pathway including PYR/PYL/RCAR receptor, PP2C proteins, SnRK2 family members, AREB/ABF transcription factors and downstream regulatory genes, as well as ABA-activated signaling pathway to resist drought stress [47]. In addition, many reports have shown that among the promoters of the stress-responsive genes, there was a major cis-acting element (ABRE) which was regarded to be necessary for ABA response [48]. We found that ABRE element were present in the promoters of these upregulated photosynthesis related genes, suggesting that these genes may also be regulated by ABA. In addition, it was also found that many stress-responsive genes, including the DEGs response to abiotic stimulus, were significantly enriched (Fig. 6).
Many reports have shown that drought stress has a great impact on soil microbial communities which play an important role in regulating plant response to drought stress [49]. Drought stress could lead to a significant reduction of microbial biomass [50–52] and change the composition of plant rhizosphere microbial. The drought tolerance of plants is related to the change of relative abundance of specific bacterial groups [29–32, 40]. Although our understanding of the interaction between plants and soil microbial in drought responses is advancing, most of the knowledge comes from non-crop plants. The results in this study showed that the application of γ-PGA under the drought stress did not affect the species of dominant bacteria, but change the bacterial community diversity. Under drought stress, Actinobacteria and Chloroflexi were significantly enriched in soil supplemented with γ-PGA (Fig. 5C). Actinobacteria and Chloroflexi were reported to be the most prominent phylum of drought enrichment [53]. Actinobacteria was previously found to promote the decomposition or formation of humus, making it easier to be absorbed [54, 55], and it was also reported to have the important role in plant defense and growth promotion [56–58]. In this study, it was also found that Alphaproteobacteria and Deltaproteobacteria were enriched at class level after addition of γ-PGA. Most members of Proteobacteria were reported to play important roles in nitrogen fixation [59, 60]. Exogenous application of γ-PGA under drought stress could also enrich Sphingobium, Sphingomonas, Sphingopyxis, Haliangium of Proteobacteria. In addition, Subgroup_10 of Acidobacteria, Clostridium and Pelotomaculum of Firmicutes, which was previously reported to promote plant growth through nitrogen fixation, phosphate solubilization and production of plant hormone [54], were also found to be significantly enriched in the γ-PGA added soil in this study.

It is worth noting that γ-PGA increased the urease activity of rhizosphere soils of maize under the severe drought stress (Table 2). The activities of soil urease play an important role in soil nitrogen transformation, which produces NH₃, NH₄⁺ and CO₃²⁻ in the process of urea hydrolysis and provides nutrition for plants. The results implied that exogenous application of γ-PGA could contribute to improve the soil biochemical reaction and plant growth under the drought stress condition. In addition, interestingly, we also detected a significant increase in ABA content in the rhizosphere soil after γ-PGA application, which will also play an important role in drought resistance of maize. The mechanism of the increase of urease activity and ABA content in soil by exogenous application of γ-PGA needs further study. Our results showed that exogenous application of PGA not only affected the physiological and biochemical indexes and gene expression related to drought resistance of plants, but also profoundly affected the microbial community and physiological and biochemical properties of rhizosphere soil.

Conclusions

Our study demonstrated that exogenous application of γ-PGA could significantly enhance the drought resistance of maize under severe drought stress. γ-PGA can regulate the expression of ABA biosynthesis, ABA signal transduction related genes, photosynthesis-related genes and other stress-responsive genes. At the same time, γ-PGA could enrich the plant-promoting bacteria such as Actinobacteria, Chloroflexi,
Firmicutes, Alphaproteobacteria and Deltaproteobacteria. This study highlighted the possibility of using γ-PGA to improve crop drought resistance and soil environment under drought condition.

Materials And Methods

Plant materials and drought treatments

Maize (inbred line KN5585) seeds (provided by Weimi Biotechnology (Jiangsu) Co., Ltd (Changzhou, China)) were sown in a soil box (10cm*10cm*10cm). When seeds germinated, the seedlings were watered with different concentrations (0, 50, 70, 100 mg/L) of γ-PGA (10KD) solution and grown in greenhouse at 28±2°C under nature light and 25±2°C at night. All seedlings at three-leaf stage were exposed to a drought stress treatment by stopping watering to select the most suitable treatment concentration of γ-PGA. After drought for 7 days (the soil water content decreased to 4.9 %, and the control plants wilted seriously), the seedlings were rewatered. After rewatering for 1 day, the recovered maize added with γ-PGA were recorded and compared with the control maize. 50 mg/L γ-PGA was selected for the subsequent experiment according to the results of drought lethal test. The physiological parameters including photosynthetic parameters (net photosynthetic CO₂ assimilation rate, stomatal conductance), soluble sugar, proline, chlorophyll and ABA contents of the maize added with γ-PGA and the control maize were measured after 5 days treatment (soil water content decreased to 9.8 %). Soil water content was monitored by using Soil Moisture Content Meter (TZS, TOP instrument, China). Each experiment had at least three biological repetitions, and the determination of photosynthetic parameters was repeated at least five times. The leaves were taken for RNA sequencing. Finally, the dry weights of plants under drought conditions were measured.

In the experiment of using PEG to simulate drought stress, maize (inbred line KN5585) seeds were surface sterilized using 75% alcohol and germinated on moist filter paper in sterile petri dishes (diameter:12.5 cm) in the dark at 28°C. After 4 days, the germinated seeds were transferred to the culture flasks (height: 15cm, diameter: 7cm) with Hoagland Solution, and grown at 28°C/25°C (16h light/8h dark) until maize reached to two-leaf stage. Then the maize seedlings were divided into four groups and cultured as follows: group1, cultured with Hoagland Solution only; group2, cultured with Hoagland Solution supplemented with 18% (m/v) PEG6000 (~0.77 MPa) solution; group3, cultured with Hoagland Solution supplemented with γ-PGA (10 kDa, 50 mg L⁻¹); group 4, cultured with Hoagland Solution supplemented with γ-PGA (10 kDa, 50 mg L⁻¹) + 18% (m/v) PEG6000. The nutrient solution was renewed every 2 days, aerated with a mini air pump and supplemented with fresh solution. The phenotypes of plants were examined, the leaf and root fresh weight were measured.

Determination of physiological parameters

The leaves and roots disk from the plants were excised, and the fresh weights (FWs) were recorded immediately. The dry weights (DWs) of leaves were obtained after drying in an oven at 80°C. The photosynthetic parameters (net photosynthetic CO₂ assimilation rate and stomatal conductance) were
measured at 28°C and PAR 1000 µmol/m²/s by a portable infrared gas analyser-based photosynthesis system (Yaxin-1105, China). Total soluble sugars of leaves (approximately 100 mg) were extracted in boiling water for 30 min and determined by anthrone reagent using glucose as the standard according to the methods described by Yemm and Willis [61]. Proline was detected using the protocol described by Bates et al [62]. Approximately 200 mg of the maize leaves was excised to measure chlorophyll content following the method described by Amon [63]. The ABA content was measured with ELISA kit (code JM-01148P2, Jingmei Bio Inc., Jiangsu, China) according to the manufacturer’s protocol. The urease activity was determined according to the described method by Guan [64]. In this study, at least three biological repeats were sampled for one treatment, each replicate contained tissues from four plants, and the determination of photosynthetic parameters was repeated at least five times.

RNA extraction and real-time RT-PCR

Total RNA was isolated from maize leaves as described by the manufacturer’s instructions using HiPure RNA Kit (Magen, Guangzhou, China). 2 µg of total RNA was reverse transcribed into cDNA using Reverse transcription kit (TAKARA). The cDNA was diluted to 200 µL by sterile DEPC water. Real-time RT-PCR of the candidate genes were performed by SYBR Green I Master Mix (Roche, Indianapolis, USA). Three biological and three technical replicates for each reaction were analyzed on LightCycler 480 (Roche, USA) with the first step of 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Melting curves were generated using the following program: 95 °C for 15 s, 60 °C for 15 s, and for 15 s. ZmTub was used as an internal control. Data analysis was calculated by 2-ΔΔCT method. Significant differences between different samples were tested with IBM SPSS Statistics 22.0 software. Real-time PCR of the candidate genes and data analysis was performed and primers used were list in Additional file 11: Table S2.

RNA sequencing and analysis

RNA sequencing and primary bioinformatics analysis were performed by BGI Tech Solutions Co., Ltd. (Shenzhen, China). Each treatment was made three biological replicates. Primary sequencing data (raw read) were produced by Illumina HiSeq™ 2000. After QC, raw reads were filtered into clean reads which will be aligned to the reference sequences. The alignment data was utilized to calculate distribution of reads on reference genes and mapping ratio. Gene expression was measured as fragments per kilobase of transcript per million fragments mapped (FPKM) using Cuinks. Differentially expressed genes (DEGs) were determined using DEseq2. The false discovery rate was used to adjust the P-values. Genes with significant differences in expression, |log2Fold Change| ≥ 1, and adjusted P-value <0.05 were considered as DEGs. GO analysis and pathway enrichment analysis of all DEGs (Q value ≤ 0.05) were performed by AgriGO (http://bioinfo.cau.edu.cn/agriGO/) and KEGG (http://www.genome.jp/kegg/). The promoter motif analysis was conducted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Bacterial Community Analysis of maize rhizosphere soil
The two-leaf stage maize seedlings watered with γ-PGA (0, 50mg/L) were treated under drought stress and kept the soil moisture content at 8.0% by replenishment. After 30 days, the tightly bound soils of roots (served as rhizosphere soils) were taken to analyze the microbial community, and three biological replicates were performed. Amplification and High-throughput sequencing of 16s rRNA from maize rhizosphere soil bacterial were performed as described by Wang et al.[65]. The primers of V4 region of bacterial 16S rRNA were 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). High-throughput sequencing was conducted by Illumina Hiseq 2000 (Illumina Inc., San Diego, USA). Nonmetric multidimensional scaling (NMDS) was performed on distance matrices and the coordinates were used to draw 2D graphical outputs. Taxa abundances at the phylum, class, order, family and genus levels were statistically compared among samples or groups by Metastats. The LEfSe analysis (LDA≥3) was carried out to obtain the important indicator taxa with significant changes in relative abundance.

Statistical Analysis

All data have at least three biological replicates. The data were presented as the mean ± standard deviation (SD). The statistical analysis between the maize with and without γ-PGA was performed using T-test and Duncan's tests of one-way ANOVAs in SPSS (version 22.0.0.0). Significant differences were indicated by asterisks, *p < 0.05; **p < 0.01.

Declarations

Ethics approval and consent to participate

There is no ethics approval and consent to participate in this manuscript.

Consent to publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

All datasets generated for this study are included in the article/Supplementary Materials.

The RNA-Seq raw data have been uploaded to a public database: https://doi.org/10.6084/m9.figshare.14495775.v1

The data of 16s rRNA from maize rhizosphere soil bacterial were deposited in the figshare database: https://doi.org/10.6084/m9.figshare.14496006.v1
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Authors’ contributions

Tao Xia and Haizhen Ma designed the research project. Haizhen Ma performed the experiments and analyzed the data. Xingwang Liu Panpan Li, Can Li, Shengkui Zhang and Xiaohan Wang assisted in the determination of some physiological indexes, drought stress treatment and bacterial community analysis. Haizhen Ma and Tao Xia wrote this manuscript.

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**Figures**
Figure 1

Phenotypes of maize added 50mg/L γ-PGA under drought stress, and the determination of related physiological indexes. (A) Phenotypes of maize added 50mg/L γ-PGA under drought stress. (B) The determination of related physiological indexes (dry weight, contents of ABA, soluble sugar, proline, chlorophyll, net photosynthetic rate, and stomatal conductance) under drought stress for 5 d. Values are means ± sd (n ≥3 repeats). Significant differences are indicated by asterisks (**, P ≤0.01).
Figure 2

Phenotypes of maize added γ-PGA under drought stress treatment with the 18% PEG6000 solution. (A) Phenotypes of maize added γ-PGA (10KD, 50mg/L) under drought stress treatment with the 18% PEG6000 solution. (B) Fresh weight of the leaf and root of maize added γ-PGA and control maize under drought stress treatment for 5d with the 18% PEG6000 solution. Values are means ± sd. Bars represent means ± sd (n≥3 repeats). Significant differences are indicated by asterisks (**, P ≤ 0.01). Bars=5cm.
Figure 3

The differentially expressed genes (DEGs) identified by RNA sequence analysis and KEGG enrichment analysis of DEGs. (A) The number of differentially expressed genes (DEGs) identified by RNA sequence analysis. (B) The KEGG enrichment analysis of DEGs, Q value ≤ 0.05.
Figure 4

The DEGs involved in photosynthesis. Leaves from maize added γ-PGA under drought stress was collected for RNA sequencing. The absolute values of log2 (CK+ γ-PGA/CK) ≥ 1 and FDR < 0.001 were used as the criteria for DEGs. The color of the box represented up (red) and down (green)-regulated (CK+ γ-PGA/CK) genes, the value in the box was the log2 (CK+ γ-PGA/CK) of the genes in the leaves (CK+ γ-PGA/CK) under drought stress. The pattern of photosynthesis came from KEGG (http://www.genome.jp/kegg/).
Figure 5

The NMDS, relative abundance and LEfSe analysis. (A) Non-metric multidimensional scaling (NMDS) showed the grouping patterns of the samples based on weighted UniFrac distance of all community. Each colored dot represented a sample. (B) The influence of γ-PGA on the relative abundances of bacterial communities at phylum level in the rhizosphere soil of maize. (C) LEfSe analysis (LDA ≥ 3) showed the species with the most significant variation in the rhizosphere soil of control and γ-PGA added maize under drought stress.
Figure 6

Proposed model for the role of γ-PGA on maize under long-term drought. γ-PGA can improve the drought resistance of maize by regulating the expression of ABA biosynthesis, ABA signal transduction related genes, photosynthesis-related genes and other stress-responsive genes (osmotic protection, stress response and protein folding genes) and enriching the plant-promoting bacteria such as Actinobacteria, Chloroflexi, Firmicutes, Alphaproteobacteria and Deltaproteobacteria. In addition, ABA contents and urease activity in maize rhizosphere soil were also increased.

Supplementary Files

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