Inoculation with Azorhizobium caulinodans ORS571 enhances plant growth and salt tolerance of switchgrass (Panicum virgatum L.) seedlings

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Abstract

Background

Switchgrass (*Panicum virgatum* L.) is an important biofuel crop, making it possible to replace petroleum fuels. However, the slow-growing seedlings and soil salinization affected the growth and development of switchgrass. Beneficial microorganisms could enhance the salt tolerance of plants. Our previous study showed that *Azorhizobium caulinodans* ORS571 could colonize in wheat (*Triticum aestivum* L.) to promote its growth and development as well as regulated microRNAs (miRNAs). While the feasibility of inoculating *A. caulinodans* ORS571 with switchgrass to enhance the growth and salt tolerance of seedlings is unknown.

Results

In this study, we systematically studied the impact of *A. caulinodans* ORS571 on switchgrass growth and development as well as response to salinity stress; we also studied the undergoing mechanism during these biological processes. Inoculation with *A. caulinodans* ORS571 significantly alleviated the stress of salt on seedling growth. Under normal condition, *A. caulinodans* ORS571 significantly improved fresh weight, chlorophyll a content, protein content and peroxidase (POD) activity in switchgrass seedlings. Under salt stress, the fresh weight, dry weight, the length of shoots and roots as well as chlorophyll content were all significantly enhanced and even recovering to normal levels after inoculation with *A. caulinodans* ORS571. In addition, the contents of soluble sugar and protein as well as POD and superoxide dismutase (SOD) activities were also significantly increased, which was contrast with proline. It manifested that *A. caulinodans* ORS571 could enhance the salt tolerance of switchgrass seedlings by increasing the water content, photosynthetic efficiency, and scavenging ability of reactive oxygen species (ROS). Meanwhile, *A. caulinodans* ORS571 may alleviate salt stress by regulating miRNAs. Twelve miRNAs of switchgrass seedlings were all up-regulated to different degrees under salt stress. miR169, miR393, miR535 and miR844 were all decreased significantly after inoculation with *A. caulinodans* ORS571 under salt stress, which were in contrast with the expression level of miR399.

Conclusion

This study revealed that *A. caulinodans* ORS571 enhanced the salt tolerance of switchgrass seedlings by increasing biomass, photosynthetic efficiency, reactive oxygen species scavenging ability and regulating the expression of miRNAs. This provides a new and creative idea for improving the salt tolerance of switchgrass seedlings.

Background

Switchgrass, a perennial lignocellulose biofuel crop, has been developed as an important bioenergy feedstock due to its high production and viability [1, 2]. Based on average biomass yield of 15,000 kg/ha, switchgrass can produce theoretically ethanol of 5000 to 6000 L/ha [3], which makes switchgrass a great alternative candidate for replacing petroleum fossil fuels [4, 5]. However, the slow-growing rate of seedlings makes switchgrass less competitive with weeds, which significantly affected the utilization of switchgrass [4]. Improving the survival competitiveness of seedlings and optimizing the growth are of great significance to increase the yield of switchgrass and reduce the cost of planting [6, 7].

Soil salinization is one of the major abiotic stresses affecting global agriculture production. About 20% of the world's farmland is severely affected by salt stress [8, 9]. High Na^+ concentrations in soil leads to hyperosmotic and hyperionic conditions, which further inhibit plant nutrient intake [10]. Sodium toxicity causes osmotic stress and ionic toxicity in plants, especially oxidative stress. Stress induces plants to produce ROS, mainly including singlet oxygen ($^1O_2$), superoxide anion (O_2·-) and hydrogen peroxide (H_2O_2). These ROS attacks lipids and proteins, which causes cell damage and death [11, 12]. ROS also acts as a signal in response to external environmental stress. To eliminate excess ROS, plant cells have developed a series of enzymatic components to balance ROS production and scavenging. Both POD and SOD are involved in the cleanup of ROS [13]. In addition, various soluble osmotic substances are greatly increased under salt stress, which are extremely important for regulating the osmotic pressure in plants [9]. Salt stress also severely affects the biosynthesis of chlorophyll, which in turn affects photosynthesis [14, 15]. Thus, salt stress affects various aspects of plant physiology and anabolic metabolism. MicroRNAs (miRNAs) are a class of endogenous small RNAs, which are involved in regulating plant development and stress response to biotic or abiotic stresses by negatively regulating the expression of target genes at the mRNA level [16–18]. The abiotic stresses that plants need to deal with mainly include salt, drought, cold, and heat stress. A series of miRNAs responding to salt stress were identified, and the discovery of these miRNAs greatly enriched the salt stress regulatory network in plants [19].

Azorhizobium caulinodans ORS571 is originally isolated from stem nodule of the tropical leguminous plant *Sesbania rostratak* and it could nodulate on the roots and stems of the host [20, 21]. Our previous study showed that *A. caulinodans* ORS571 could colonize in wheat to promote its growth and development [20, 22]. In recent years, researchers demonstrated that certain microorganisms, including *Enterobacter sp.* SA187 and *Piriformospora indica*, also improved plant salt tolerance [23–25]. However, there is no any report on whether *A. caulinodans* ORS571 affects biofuel crop and whether it affects plant response to salt tolerance.

This study provided evidence for inoculation with *A. caulinodans* ORS571 as well as its effect on switchgrass seedling. Our study shows that *A. caulinodans* ORS571 not only inoculated with switchgrass but also enhanced switchgrass growth and development as well as tolerance to salinity stress, which will significantly enhance switchgrass production and biofuel development.

Methods And Materials

Plant growth and inoculation

The switchgrass Alamo seeds were kindly provided by Dr. Neal Stewart at The University of Tennessee at Knoxville. Switchgrass seeds were planted in the Plant Growth Room, which were 16 h of light at a constant temperature of 28 ℃ and 8 h of dark at 18 ℃. The *gfp-A. caulinodans* ORS571 was kindly provided by the Jing lab of the Institute of Botany, Chinese Academy of Sciences. *A. caulinodans* ORS571 was cultured in Trypton Yeast (TY) liquid medium at 28 ℃, 140 rpm until OD_{600} was 0.6, and then suspended in sterilized water to OD_{600} of 0.05.

Switchgrass seeds used in this experiment were soaked in the 70% ethanol and 5% sodium hypochlorite (NaClO) successively for 90 s and 10 min, respectively. The residual ethanol and NaClO were washed 10 times by using sterilized water [20]. Sterilized seeds were placed in 24 cm diameter pots filled with vermiculite for germination. Three pots were used every single treatment as well as each pots contained 50 seeds. The pots were irrigated with 1/4 strength Hoagland solution (pH 6.5) before planting. Three days after switchgrass germination, 0.5% NaCl was applied for 5 days followed by 1% NaCl was instead for 10 days. *A. caulinodans* ORS571 was inoculated to the roots zone of switchgrass seedlings for 50 mL at 1 and 6 day after first salt treatment as well as the sterilized water was applied for the controls.

Observation of *A. caulinodans* ORS571 in root tissue

Two weeks after treatment, roots of switchgrass seedlings were observed by laser confocal microscope (Leica, Bensheim, Germany) with a wavelength of 488 nm to monitor *A. caulinodans* ORS571 colonization.

Measurement of growth parameters

The total biomass of switchgrass seedlings was determined after 15 days of treatment. The fresh weight and dry weight of the seedling were measured, respectively. The dry weight was measured by placing the washed seedlings in an oven at 80 °C until the weight was constant [26]. At the same time, the length of the shoots and roots were also measured.

Chlorophyll and proline measurement

After 15 days of treatment, the chlorophyll and proline contents were determined. Chlorophyll was extracted by using ethanol as extraction solvent. Approximate 0.5 g of switchgrass leaves were washed with distilled water and cut into pieces, added with an appropriate amount of quartz sand and calcium carbonate, and grinded with 95% ethanol. The above grinding liquid was diluted to 25 mL with 95% ethanol after filtering and then measured using spectrophotometer at 665 nm and 649 nm respectively.

Approximate 0.5 g of switchgrass leave extract were added into 5 mL of 3% sulfosalicylic acid, and placed in a water bath at 100 °C for 10 min. Equal volumes of glacial acetic acid and acidic ninhydrin solution were added to 2 mL extract and then placed in boiling water for 30 min until the solution turn red.

Then 4 mL toluene was added and shaken for 30 s, centrifuged at 3000 rpm for 5 minutes, the upper layer liquid was taken and measured at a wavelength of 520 nm.

Determination of soluble sugar and soluble protein contents

The contents of soluble sugar and soluble protein were measured after 15 days of treatment. Soluble sugar content was measured by anthrone colorimetric method. Approximate 0.5 g of fresh leaves were put into 10 mL distilled water, placed in boiling water for 30 minutes and filtered into a 25 mL volumetric flask after cooling. The mixture was prepared including1.5 mL distilled water, 0.5 mL anthrone ethyl acetate, 0.5 mL sample and 5 mL concentrated sulfuric acid, which were shaken vigorously for 30 s, bathed in boiling water for 10 minutes, and measured at a wavelength of 620 nm after cooling down.

Coomassie brilliant blue method was used for the determination of soluble protein. Approximate 0.5 g of fresh leaves were grinded to powder by distilled water, centrifuged to take the supernatant and then diluted to 10 mL. Then 5 mL of Coomassie brilliant blue was mixed with 0.1 mL the sample, shaken vigorously for 2 minutes and measured at 595 nm wavelength.

Measurement of antioxidant enzyme activities

After 15 days of treatment, approximate 0.2 g leaves of switchgrass were powdered in 5 mL potassium phosphate buffer (50 mM, pH 7.8) containing 1% PVP and then centrifuged at 12,000 g for 10 min at 4 °C, followed by supernatant was for subsequent measurements. The enzymatic activity of SOD was determined according to its ability to inhibit O_2^--induced NBT reduction [27]. The activity of POD was detected according to the rate at which guaiacol was oxidized by hydrogen peroxide. Each 0.01 increase in the absorbance at 470 nm was regarded as one unit of enzyme activity [28].

RNA extraction and quantitative real-time PCR

After 15 days of treatment, three switchgrass seedlings from different pots were selected, washed, chopped and mixed, and then the samples were weighed. Total RNAs were extracted by using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of extracted total RNAs was checked by the Nanodrop ND-1000 and gel electrophoresis. Reverse transcription (RT) reaction was implemented by One Step PrimeScript® miRNA cDNA synthesis kit (TaKaRa, Dalian, China). qRT-PCR was run on BIO-RAD CFX96TM and each miRNA had three biological replicates. The results of the qRT-PCR were analyzed by using the $2^{-\Delta\Delta CT}$ method [29].

Statistical analysis

One-way ANOVA was employed to analyze the significance ($P < 0.05$) among different treatments.

Results

A. caulinodans ORS571 relieves salt stress on switchgrass seedlings

Inoculating *A. caulinodans* ORS571 significantly improved switchgrass growth under salt stress (Fig. 1a). Two weeks after inoculation, *A. caulinodans* ORS571 was observed in the roots of switchgrass seedlings (Fig. 1b). Under salt stress treatments, colonization of *A. caulinodans* ORS571 resulted in significantly increased the total fresh weight, dry weight, shoots and roots length of switchgrass seedlings (Fig. 2). The biomass of seedlings inoculating with *A. caulinodans* ORS571 were increased by 8.7% and 67.2% under normal conditions and salt stress respectively (Fig. 2a). Salt stress inhibited both shoot and root development, while *A. caulinodans* ORS571 inoculation significantly increased shoot and root development by 19.8% and 18.3%, respectively (Fig. 2d, e). Under salt stress, *A. caulinodans* ORS571 promoted the fresh weight, dry weight and shoots length of switchgrass seedlings to recover to normal levels.

A. caulinodans **ORS571 significantly increased the content of chlorophyll, soluble sugar and protein in switchgrasss seedlings under salt stress**

A significant increase was found in the content of chlorophyll a in inoculated seedlings under both normal and salt stress conditions, which reached to 1.19 and 1.15 mg g^{-1}, respectively (Fig. 3a). Although the chlorophyll b content of inoculated seedlings was also significantly improved by 33.2% in contrast with non-inoculated seedlings under salt stress, it was almost unchanged under normal conditions (Fig. 3b). Total chlorophyll content were consistent with chlorophyll b content (Fig. 3c). Salt stress significantly increased the contents of soluble sugar and soluble protein to 10.9 and 24.6 mg g^{-1}, which were further significantly increased after inoculation with *A. caulinodans* ORS571 (Fig. 3d, e). In addition, inoculation with *A. caulinodans* ORS571 under normal condition resulted in a significant rise in the soluble protein content (Fig. 3e).

Proline accumulated abundantly under salt stress, but it is noteworthy that the content of proline significantly decreased to 29.9 μg g^{-1} after inoculation with *A. caulinodans* ORS571 (Fig. 3f).

A. caulinodans **ORS571 significantly increased POD and SOD activities in switchgrass seedlings under salt stress**

The activities of POD and SOD in *Zea mays*, *Arabidopsis* and *Trichoderma longibrachiatum* were remarkably enhanced under stress [30–32], which were consistent with switchgrass seedlings. At the same time, the activities of POD and SOD in *A. caulinodans* ORS571-colonized plants were significantly raised by 10.3% and 8.8% respectively under salt stress (Fig. 4a, b). There was a significant increase in the activity of POD after inoculation with *A. caulinodans* ORS571 under normal conditions (Fig. 4a).

A. caulinodans **ORS571 affected differential expression of miRNAs in switchgrass seedlings under salt stress**

To study the role of miRNAs in switchgrass response to *A. caulinodans* ORS571 and the potential mechanism of enhanced plant growth and tolerance of *A. caulinodans* ORS571-infected switchgrass to abiotic stresses, we detected the expression of miRNAs associated with plant growth and stress

response. Among the 12 miRNAs involved in this study (Table 1), 10 of them have been shown to respond to multiple abiotic stress, including miR156, miR159, miR160, miR162, miR169, miR171, miR319, miR393, miR396, and miR399 [33–41]. miR535 and miR844 were shown to be associated with plant disease resistance [42, 43]. In this study, we found that all miRNAs were up-regulated under salt stress, and miR399 were further enhanced expression after inoculation with *A. caulinodans* ORS571 (Fig. 5). However, the expressions of miR169, miR171, miR319, miR393, miR535 and miR844 were significantly decreased after incubating with *A. caulinodans* ORS571.

Table 1
Primers of twelve miRNAs for qRT-PCR.

Name of primers	Primer sequence (5’ – 3’)
miR156	UGACAGAAGAGAGUGAGCAC
miR159	UUUGGAUUGAAGGGAGCUCUA
miR160	UGCCUGGCUCCCUGUAUGCC
miR162	UCGAUAAACCUCUGCAUCCAG
miR169	CAGCCAAGGAUGACUUGCCGA
miR171	UUGAGCCGCGUCAAUAUCUCC
miR319	UUGGACUGAAGGGUGCUCCC
miR393	UCCAAAGGGAUCGCAUAUC
miR396	UCCACAGGCUUUCUUGAACUG
miR399	UGCCAAAGGAGAUUUGCCCUG
miR535	UGACAACGAGAGAGAGCACGC
miR844	UGGUAAGAUUGCUUAUAAGCU

Discussion

A. caulinodans **ORS571 significantly increased switchgrass seedling biomass under salt stress**

There is growing evidence that beneficial microorganisms enhance plant tolerance to stresses [23, 44, 45]. Salt stress adversely affected the growth and development of switchgrass seedlings, which was significantly alleviated by inoculation with *A. caulinodans* ORS571 (Fig. 1a). Our previous study manifested the colonization of *A. caulinodans* ORS571 in roots and leaves of wheat [20, 22], and we also observed its colonization in the roots of switchgrass seedlings (Fig. 1b). *A. caulinodans* ORS571 alleviated the stress of salt on seedlings and showed a significant increase in shoot and root length as well as dry and fresh weight in switchgrass (Fig. 2). Additionally, these parameters except root length were all almost recovering to normal levels. Contrary to the results of previous studies, the biomass of

switchgrass seedlings did not change after inoculation with *A. caulinodans* ORS571 under normal condition [22], which may be caused by species diversity. The significant increase in fresh weight implied that it was beneficial to increase the water content of seedlings (Fig. 2c). Moreover, the results indicated that *A. caulinodans* ORS571 improved the stress resistance of seedlings by directly promoting their fresh weight, dry weight, shoot length and root length and even recovering to normal levels.

Chlorophyll content directly determines the photosynthetic efficiency of plants, which is positively correlated with salt tolerance in rice [46]. In this study, salt stress significantly reduced the contents of chlorophyll a and chlorophyll b in switchgrass seedlings, which were significantly improved after inoculation with *A. caulinodans* ORS571(Fig. 3a-c). Therefore, our results demonstrated that *A. caulinodans* ORS571 may improve the salt tolerance of switchgrass seedlings by enhancing their photosynthetic efficiency and eventually recovering to normal levels.

A. caulinodans **ORS571 significantly increased soluble solids and decreased proline content in switchgrass seedlings under salt stress**

Soluble sugar as the main product of photosynthesis and the substrate of respiration, plays a critical role in the process of plant growth and metabolism [47–49]. Soluble sugar is also used to maintain the osmotic homeostasis of cells and improve the stress resistance of plants [9, 50]. Soluble proteins were usually accumulated under abiotic stresses. In addition to being an osmotic regulator in plants, it is also crucial for the precise transmission of stress signals [51]. The results showed that inoculation with *A. caulinodans* ORS571 significantly increased the content of soluble sugar and soluble protein under salt stress, thereby further enhanced the salt tolerance of switchgrass seedlings (Fig. 3d, e). Under normal conditions, the content of soluble protein was significantly increased (Fig. 3e), which may be related to inoculation with *A. caulinodans* ORS571 in switchgrass. Proline acts as a solute to maintain cellular osmotic balance and protect plant cells from various stresses [13, 52–54]. In this study, the content of proline was increased significantly under salt stress. Interestingly, inoculation with *A. caulinodans* ORS571 resulted in a significant decrease under both salt stress and normal condition (Fig. 3f), which was different from the soluble sugar and protein. However, under normal conditions, excessive free proline inhibited leaf development and even induced cell death in *Arabidopsis* [55, 56]. Therefore, *A. caulinodans* ORS571 may be beneficial to degrade excess proline in plant cells under normal conditions or when salt stress is alleviated.

A. caulinodans **ORS571 significantly induced the activity of antioxidant enzymes in switchgrass seedlings under salt stress**

Under salt stress, plants produce and accumulate ROS, causing cell damage or death [11]. The increase of POD and SOD activities were beneficial to scavenge excess ROS [57]. Our study determined the activities of POD and SOD to explore whether inoculation with *A. caulinodans* ORS571 improved the tolerance of switchgrass seedlings to salt stress. The results showed that *A. caulinodans* ORS571 resulted in significant increase in POD and SOD activities under salt stress condition, and POD activity was significantly increased even under normal conditions (Fig. 4a, b). These results indicated that the

enhanced ability to scavenge ROS benefits improving salt tolerance of switchgrass seedlings via inoculation with *A. caulinodans* ORS571.

The Differential Expression Of Mirnas In Switchgrass Seedlings Under Salt Stress

In previous studies, we identified a series of salt stress-related miRNAs in switchgrass by high-throughput deep sequencing [39]. In this study, we pretreated with 0.5% NaCl and then treated with 1% NaCl. The results showed that twelve miRNAs were all up-regulated to varying degrees under salt stress (Fig. 5). The changes of miR156, miR159, miR162, miR169, miR171, miR393 and miR396 were also consistent with our previous results in switchgrass [39, 41]. However, the expression of miR160 was up-regulated under 1% NaCl treatment, which was opposite to the previous results [39]. It is inferred that the concentration of salt used in this experiment was twice of that. In previous studies, miR399 was up-regulated under low-concentration salt stress, and slightly down-regulated under high-concentration salt stress. Maybe because of the pretreatment, miR399 was still up-regulated under 1% NaCl treatment. miR319 was also up-regulated, which was consistent with creeping bentgrass [40]. miR535 and miR844 improved plant resistance to pathogenic bacteria by targeting *QUAMOSA promoter binding protein-like 4* and cytidinephosphate diacylglycerol synthase3 (CDS3) mRNAs, respectively [40, 42, 43]. While the up-regulation of miR535 and miR844 indicated that they were also associated with salt stress. Under salt stress, the expression of miR156, miR159, miR160, miR162 and miR396 were almost unchanged in switchgrass seedlings after inoculation with *A. caulinodans* ORS571, indicating that inoculation with *A. caulinodans* ORS571 did not affect the expression of these miRNAs. The significant up-regulation of miR399 indicated that inoculation with *A. caulinodans* ORS571 enhanced the salt tolerance of switchgrass seedlings. However, miR535 and miR844 were significantly decreased, probably because salt tolerance was alleviated. miR169 and miR393 may have similar functions. miR171 and miR393 were involved in legume nodulation and arbuscular mycorrhizal symbiosis [58]. The down-regulation of miR171 and miR393 may be related to *A. caulinodans* ORS571 colonization of switchgrass. Therefore, *A. caulinodans* ORS571 enhanced the salt tolerance of switchgrass seedlings by regulating the expression of miRNAs, which also reflected that the inoculation with *A. caulinodans* ORS571 alleviated the toxicity of salt stress to switchgrass seedlings.

Conclusions

In this study, we investigated the function of inoculation with *A. caulinodans* ORS571 by analyzing the growth parameters, physiological indicators and expression patterns of miRNAs in switchgrass seedlings under normal and salt conditions. Under normal condition, inoculation with *A. caulinodans* ORS571 significantly improved fresh weight, chlorophyll a content, protein content and POD activity in switchgrass seedlings. Under salt stress, the fresh weight, dry weight, the length of shoots and roots as well as chlorophyll content were all significantly enhanced and even recovering to normal levels after inoculation with *A. caulinodans* ORS571. In addition, the of contents soluble sugar and protein as well as POD and SOD activities were also significantly increased, which was contrast with proline. It was suggested that inoculation with *A. caulinodans* ORS571 could enhance the salt tolerance of seedlings by

increasing the water content, photosynthetic efficiency and scavenging ability of ROS. Meanwhile, *A. caulinodans* ORS571 may alleviate salt stress by regulating miRNAs. Our results revealed that the inoculation with *A. caulinodans* ORS571 with switchgrass significantly improves the salt tolerance as well as proposes an environmentally friendly solution to improve the competitiveness of seedlings and increase biomass.

Declarations

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Authors' contributions

HL and BZ were the principal investigators and took primary responsibility for this study. PC and QW conceived and designed the experiments. PC, QW and YY performed the experiments, wrote the paper and prepared figures. LQ and JW prepared experiment materials and analyzed the data. All the authors read and approved the final manuscript.

Competing interests

The authors declare that there is no conflict of interest.

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Figures

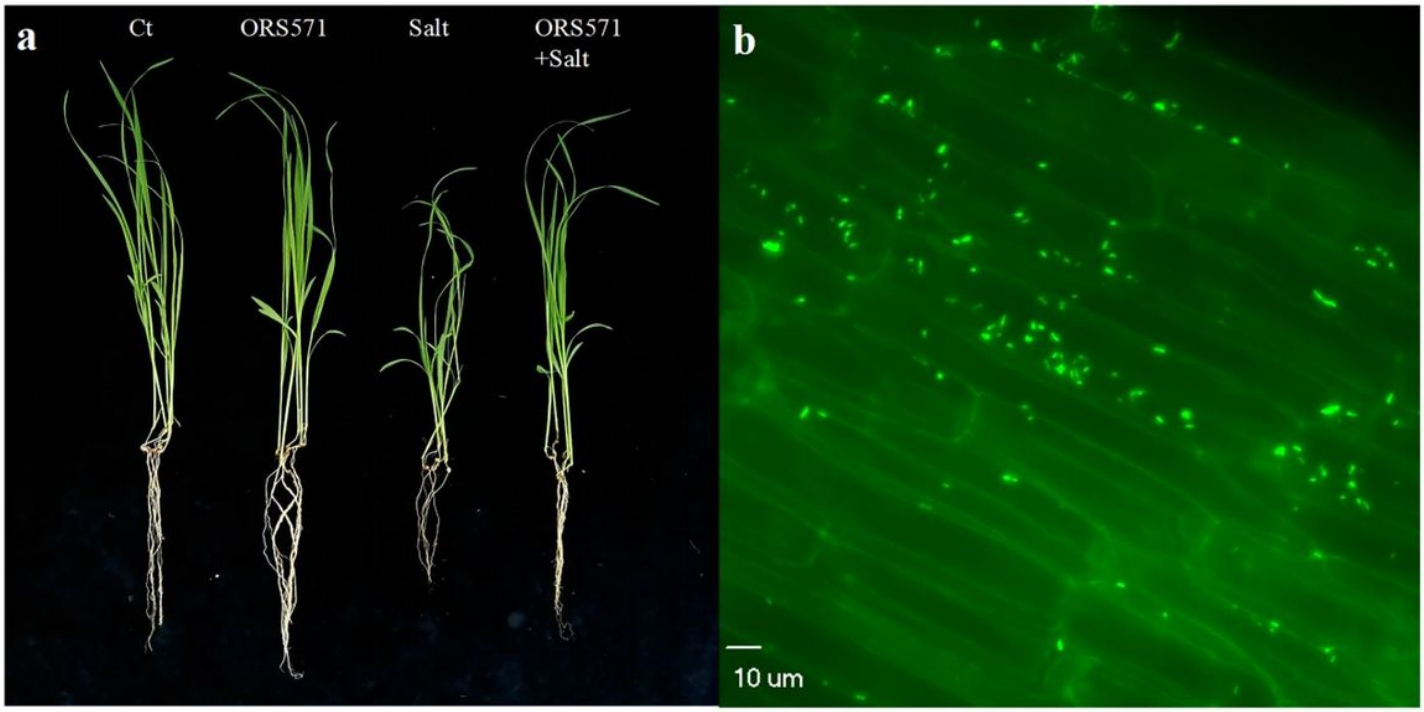

Figure 1

Colonization of *A. Caulinodans* ORS571 in switchgrass.

a Effects of *A. Caulinodans* ORS571 on switchgrass seedlings under normal and salt stress;

b The colonization of *A. Caulinodans* ORS571 in the roots of switchgrass seedlings.

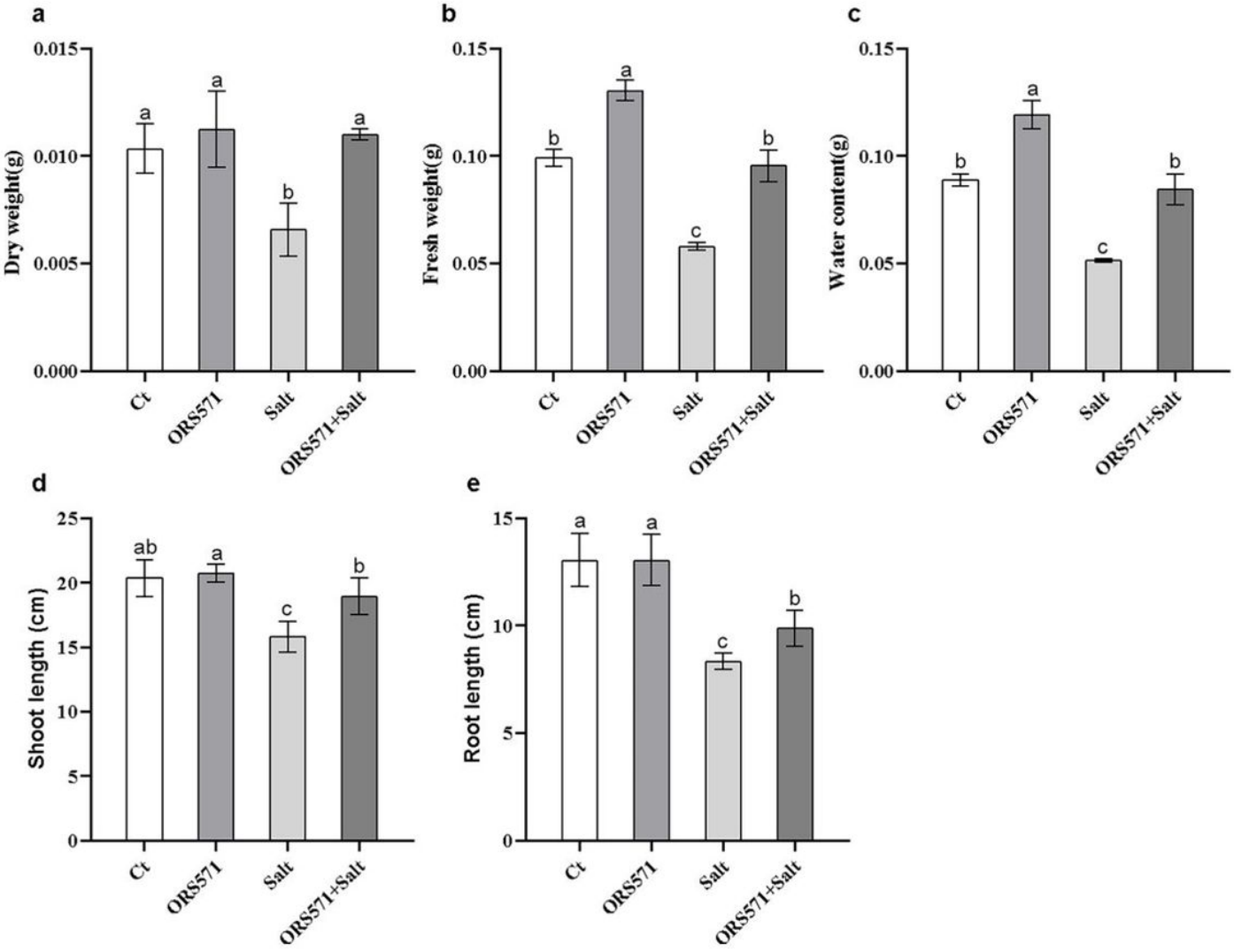

Figure 2

Effects of *A. Caulinodans* ORS571 on the growth status of switchgrass seedlings.

a Dry weight; **b** Fresh weight; **c** Water content; **d** Shoot length; **e** Root length.

The same letters indicate that the result is not significant (P<0.05) according to One-way ANOVA, error bars are standard error (SE), n=3.

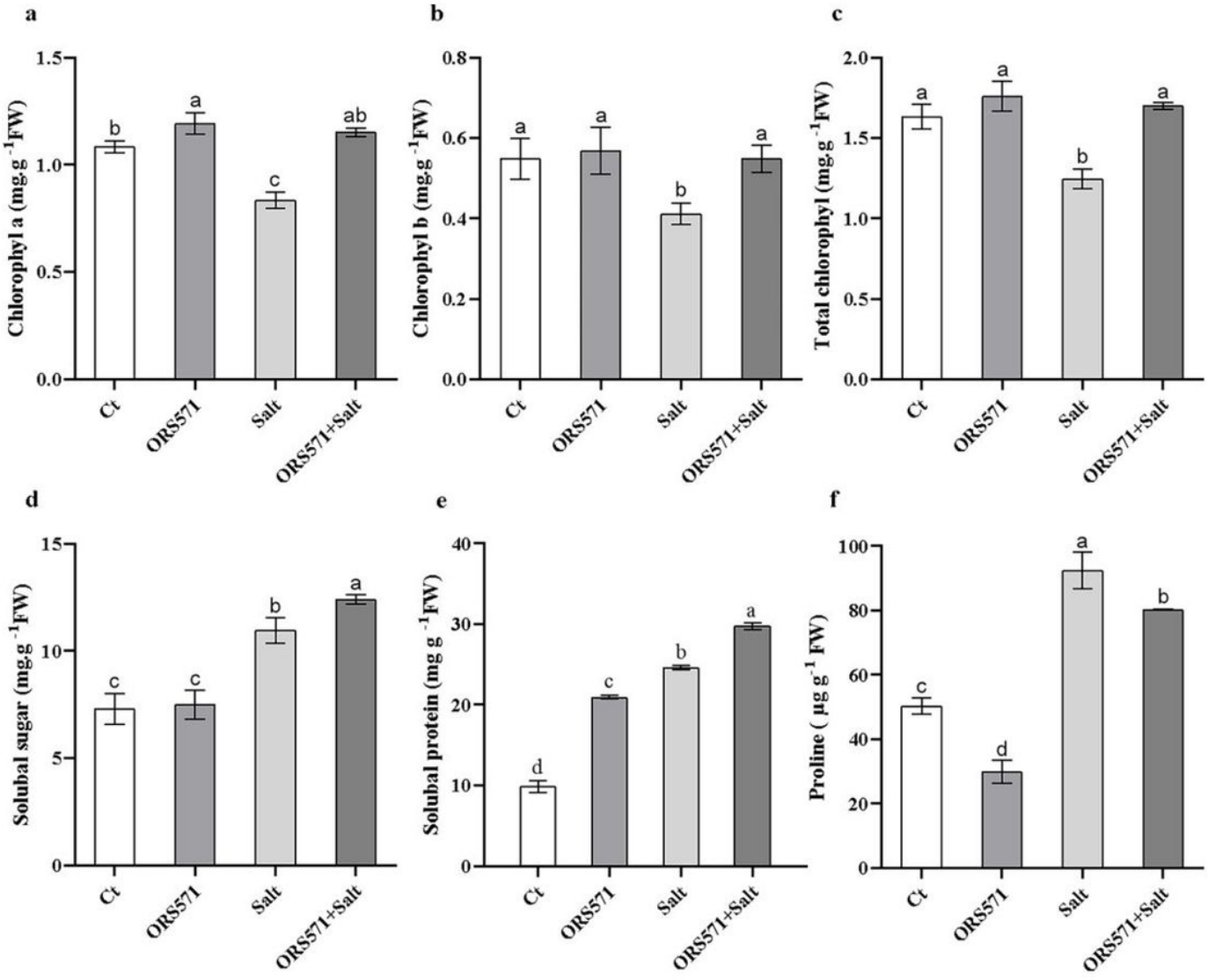

Figure 3

A. caulinodans ORS571 promoted chlorophyll content and soluble solids and decreased proline under salt stress.

a Chlorophyll a content; **b** Chlorophyll b content; **c** Total chlorophyll content;

d Soluble sugar content; **e** Soluble protein content; **f** Proline content.

The same letters indicate that the result is not significant (P<0.05) according to One-way ANOVA, error bars are standard error (SE), n=3.

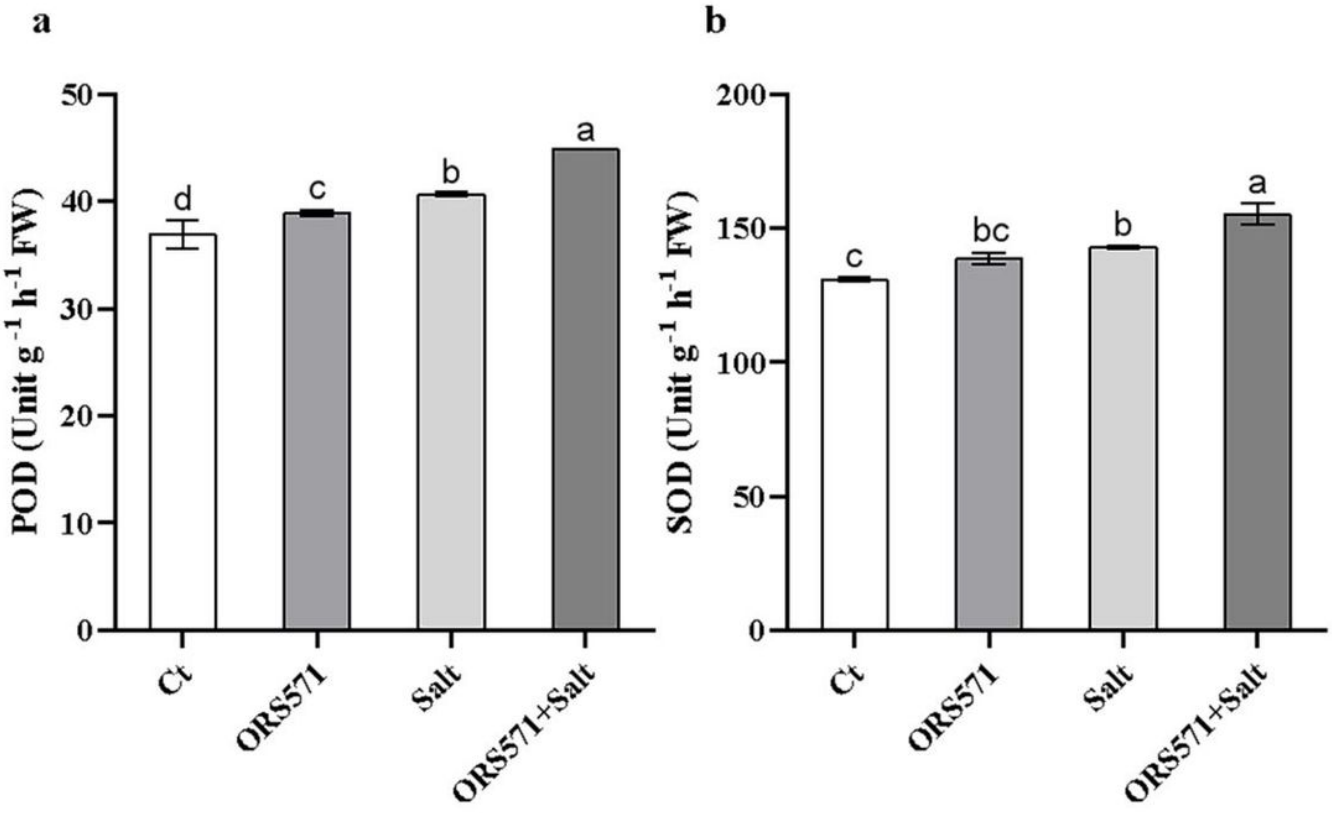

Figure 4

A. caulinodans ORS571 improved activities of POD and SOD.

a Activity of POD; **b** Activity of SOD.

The same letters indicate that the result is not significant (P<0.05) according to One-way ANOVA, error bars are standard error (SE), n=3.

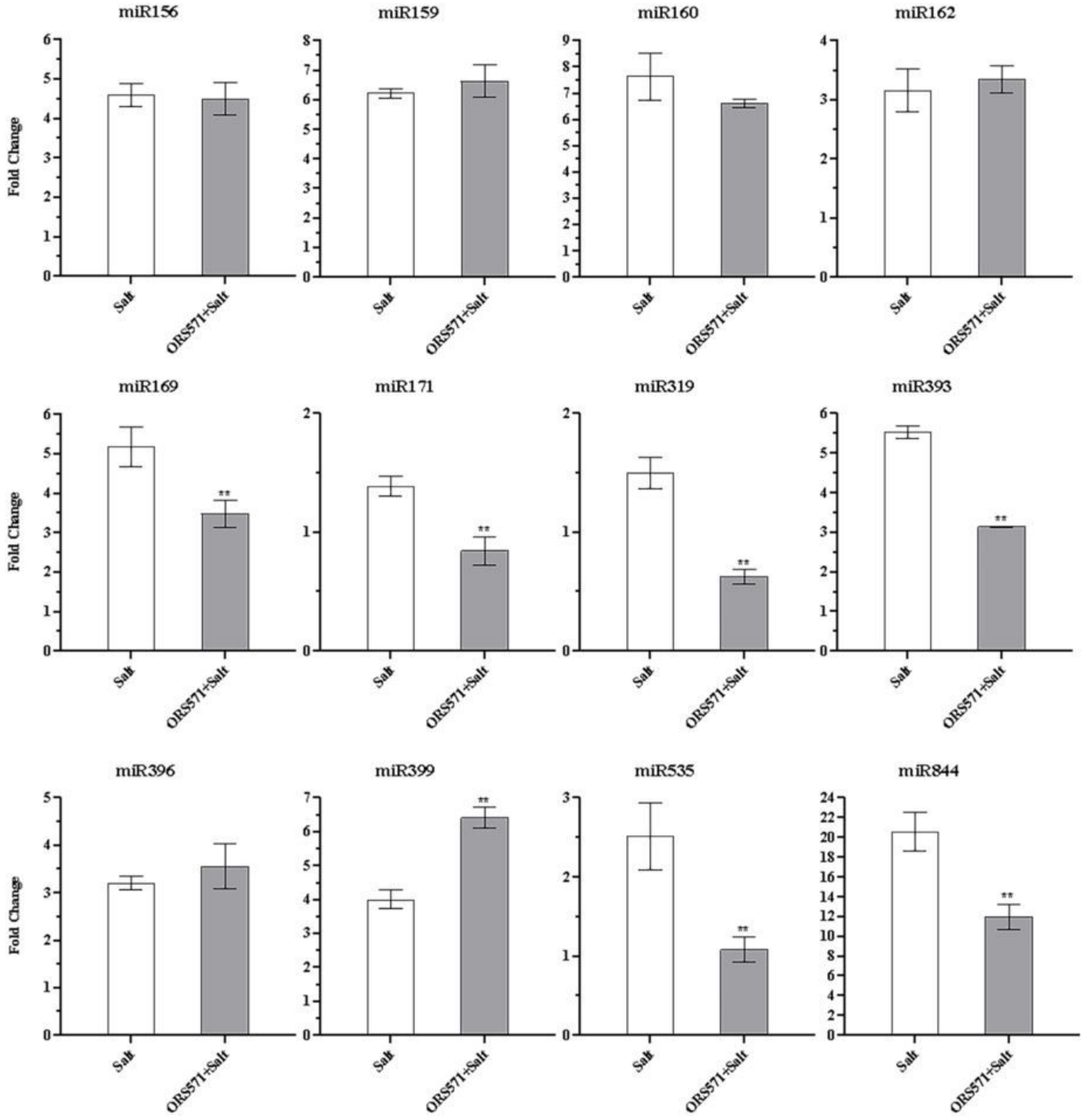

Figure 5

Differential expression of miRNAs.

Significance according to T-test results, *P*<0.05 (*) and *P*<0.01 (**). Error bars are standard error (SE), n=3.