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Cytokinin-mitigation of salt-induced leaf senescence in perennial ryegrass involving the activation of antioxidant systems and ionic balance

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ABSTRACT

Leaf senescence is one of typical symptoms of salt stress in higher plants. The objectives of this study were to examine whether salt-induced leaf senescence could be alleviated by exogenous cytokinin and to elucidate on the regulatory mechanisms of cytokinin for mitigating salt stress in plants. Perennial ryegrass (*Lolium perenne* cv. Pinnacle) plants were exogenously treated with 6-benzylaminopurine (25 μM) for 3 d prior to salt stress imposition and every 7 days during salt stress for 28 days (250 mM NaCl) in growth chambers. Physiological indicators of leaf senescence, including visual turfgrass quality, leaf photochemical efficiency, leaf chlorophyll content, electrolyte leakage, malondialdehyde content, percentage of cell death, and reactive oxygen species production rate and content were evaluated. Leaf relative water content was also measured to indicate leaf hydration status. Salt stress caused significant declines in turfgrass quality, leaf photochemical efficiency, leaf chlorophyll content, and leaf relative water content, and significantly increased electrolyte leakage, malondialdehyde content, percentage of cell death, and reactive oxygen species rate and content. 6-benzylaminopurine application alleviated the adverse physiological effects of salt stress, which was associated with reactive oxygen species scavenging by increased activities of superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, and glutathione reductase and up-regulating gene expression levels for ascorbate peroxidase and glutathione reductase. Salt stress significantly decreased cellular K^+/Na^+ ratio and 6-benzylaminopurine application suppressed Na^+ accumulation to maintain a higher K^+/Na^+ ratio associated with increased high-affinity K^+ transporter expression. The results demonstrate that 6-benzylaminopurine effectively reduced salt-induced cellular damages by suppressing oxidative and ionic stresses in perennial ryegrass.

1. Introduction

Salt stress is a major abiotic factor limiting plant growth and development particularly in areas with salt-affected soils or with poor-quality irrigation water. Salt damages are characterized by chlorophyll degradation inducing leaf senescence and also by changes to various physiological and biochemical processes, including photosynthetic inhibition caused by ionic Na^+ toxicity and oxidative stress (Munné-Bosch and Alegre, 2004; Munns and Tester, 2008). Na^+ accumulation can disrupt cellular membrane stability causing ion leakage or directly damage macromolecules such as proteins, both of which accelerate leaf senescence (Hasegawa et al., 2000; Munns, 2005). Salt stress also induces oxidative damages by accelerating production of reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), single oxygen, and hydroxyl radical (Bhattacharjee, 2005; Dat et al., 2000; Wu et al., 2014). In order to detoxify ROS and mitigate cellular oxidative damages, plants utilize enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), glutathione reductase (GR) and dehydroascorbate reductase (DR) as well as non-enzymatic antioxidants including ascorbate (AsA) and glutathione (GSH) (Dat et al., 2000; Mittler, 2002); gene expression level or activity of these antioxidant components typically increases during short-term stress periods whereas longer durations of salt stress may reduce antioxidant effectiveness or efficacy (Arghavani et al., 2012;

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Bernstein et al., 2010; Hu et al., 2012a). Therefore, greater insight is needed regarding mechanisms which mitigate those salt-induced ionic or oxidative damages contributing to leaf senescence in order to identify stay-green traits for improved salt tolerance.

Cytokinins (CKs) are a class of plant hormones regulating a wide range of growth and developmental processes in plants and have direct effects on chlorophyll status during leaf senescence (Choi and Hwang, 2007; Li, 1998; Lim et al., 2007). Exogenous CKs applications have been shown to enhance salt tolerance in various plant species, such as eggplant (*Solanum melongena*), which displayed increased photosynthetic activity, biomass accumulation of roots and shoots, and stem width along with decreased O_2^- production rate and malondialdehyde (MDA) content following 6-benzylaminopurine (BAP) application during salt stress (Wu et al., 2014). It was reported that CKs application in cereal crops increased seed germination rate, early seedling growth, and grain yield in wheat (*Triticum durum*) (Iqbal et al., 2006) and grain weight, grain yield and filled-grain percentage in rice (*Oryzal sativa*) (Javid et al., 2011). Overexpression of the isopentenyl transferase gene (*IPT*) which catalyzes the rate-limiting step in the cytokinin biosynthetic pathway improved germination rate and dry mass of leaves in cotton (*Gossypium hirsutum*) (Liu et al., 2012), increased chlorophyll content (Chl) and ROS scavenging capability, and lowered MDA content in tobacco (*Nkotiana abacum*) and tomato (*Solanum lycopersicum*) (Qiu et al., 2012; Žižková et al., 2015); however, cytokinin synthesis-deficient mutants (*ipt1,3,5,7* and *35s:CKXs*) and cytokinin signaling-deficient mutants (*AHKs* and *ARRs*) in *Arabidopsis* exhibited stronger salt tolerance phenotypes, as demonstrated by higher survival rates and leaf relative water content (RWC), and lower electrolyte leakage (EL) compared with WT (Mason et al., 2010; Nishiyama et al., 2011; Tran et al., 2007). The results above regarding *Arabidopsis* show discrepancies, which suggest the complexity of mechanisms of CKs-regulation on salt responses that deserve further investigation. Furthermore, while most previous studies have focused on the growth and yield effects of CKs on model plants or annual crops, limited information is known about how CKs may regulate salt-induced leaf senescence in perennial grasses that are used as forage or turf, for which stay-green phenotypes to maintain photosynthetically active leaves is critically important.

Salt damage becomes a major threat for perennial grasses that are irrigated with effluent or recycled water due to the shortage of fresh water for irrigation in many areas (Marcum, 2006; Pessarakli, 2007, 2010). Understanding mechanisms of controlling salt-induced leaf senescence in perennial grass is imperative in developing stay-green phenotypes and stress-tolerant genotypes. It was hypothesize in this study that CKs serve positive roles in suppressing salt-induced leaf senescence in perennial grasses by alleviating ionic and oxidative stresses or by activating antioxidant metabolism. The objectives of this study were to examine whether salt-induced leaf senescence could be alleviated by exogenous application of CKs and to determine the regulatory mechanisms of CKs for mitigating salt stress damages in perennial ryegrass (*Lolium perenne*).

2. Materials and methods

2.1. Plant materials and growing conditions

Perennial ryegrass (Cv. 'Pinnacle') seeds were germinated and seedlings were established in plastic pots (15 cm diameter, 15 cm depth) filled with soil and vermiculite (1:1, v/v). Plants were irrigated weekly with half-strength Hoagland nutrient solution (Hoagland and Arnon, 1950) and trimmed weekly maintaining 7-cm canopy height. Each pot was planted with same number of seeds to ensure uniform plant density during the study. Plants were maintained in a controlled-climate growth chamber (EGC, Chagrin Falls, OH) with 20/15 °C (day/night), 12-h photoperiod, 650 μmol m^{-2} s^{-1} photosynthetically active radiation at the canopy level, and 70% relative humidity.

2.2. Treatments and experimental design

Following 45-days establishment period, plants were subjected to salt stress by irrigating plants with NaCl solution dissolved in half-strength Hoagland nutrient solution or with half-strength Hoagland nutrient solution (refereed as Control in all figures). Plants exposed to salt were irrigated daily with NaCl solution of increasing concentrations at 80 and 160 mM NaCl for three days at each concentration to avoid salt shock, and subsequently irrigated with 250 mM NaCl for 28 days (refereed as Salt in all figures).

Three days prior to salt stress, plants were treated with CKs by spraying leaves with 25 μM BAP solution, and subsequently sprayed with BAP every 7 days during the 28-days period of salt stress (refereed as Salt + BAP in all figures). Treatments (Control, Salt, and Salt + BAP) in this experiment were arranged in a randomized complete block design with four replicates for each treatment. The Salt treatment was compared to the non-stress control to evaluate level of salt stress damages. The Salt + BAP treatment was compared to Salt alone to determine beneficial effects of foliar application of BAP on plants subjected to salt stress. Physiological measurements were made at 7, 14, 21, and 28 day of salt stress or by the end of each 7-days interval of BAP treatment and biochemical analysis was performed for samples collected at 0, 7, and 21 day of salt stress, when no effects and initial and maximal physiological effects of Salt or Salt + BAP were present, respectively.

2.3. Physiological analysis

Several commonly-used parameters for leaf senescence, Chl, turf quality (TQ), leaf photochemical efficiency (F_v/F_m), EL, RWC and MDA were evaluated at 7, 14, 21, and 28 day of salt stress or by the end of each 7 days interval of BAP treatment. In addition, leaf length was measured as the length from the base of leaf sheath to the leaf tip. The ratio of yellow leaf area to green leaf area was calculated from areas of yellow leaves and green leaves measured with an image analysis using magic wand and digimizer tool (http://www.digimizer.com).

Turf quality was evaluated to assess overall plant performance during salt stress. Ratings were based on turf color, uniformity, and density on a scale of 1–9 with 1 being brown and desiccated grass and 9 being green and dense canopy according to Turgeon (1991). Leaf photochemical efficiency was measured as chlorophyll fluorescence based on the ratio of variable (F_v) to maximum (F_m) fluorescence using a fluorescence induction meter (Fim 500; Bio-Scientific Ltd., Herts, UK). Leaf photochemical efficiency was measured following 30 min dark-adaptation period and calculated as F_v/F_m. For EL, about 0.1 g of fresh leaves were excised and rinsed three times, cut into 0.5 cm segments, and put into tubes with 30 ml distilled water. The initial conductivity (C_i) was measured using a conductivity meter (YSI Incorporated, Yellow Springs, OH) after being shaken for 24 h. The maximum conductivity (C_{max}) was measured when the leaves were killed in an autoclave at 121 °C for 30 min, and the EL was calculated as $(C_i/C_{max}) \times 100\%$ (Blum and Ebercon, 1981). For Chl quantification, fresh leaves (0.1 g) were excised and extracted with 10 ml of dimethyl sulfoxide in the dark for 72 h. Chl content (a + b) was measured at 663 nm and 645 nm according to Arnon (1949). For relative water content (RWC), fresh leaves (0.1 g) were detached and immediately weighed as the fresh weight (FW), then soaked in water for 24 h at 4 °C and weighed as turgid weight (TW). Leaf samples were then dried for 72 h in 80 °C