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Research Article

Influences of sea water on the ethylene-biosynthesis, senescence-associated gene expressions, and antioxidant characteristics of *Arabidopsis* plants

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Abstract

We evaluated the physiological and antioxidant characteristics of *Arabidopsis thaliana* (At) plants grown in different sea water (SW) products containing trace elements, namely RO3, 300K, and 340K, at various dilutions. The synthetic water (namely 300K-Test), a mixture of the main ions of SW including 143.08 mg L^{-1} Mg^{2+}, 5.74 mg L^{-1} Na^{+}, 170 mg L^{-1} K^{+}, and 33.5 mg L^{-1} Ca^{2+} with equal concentrations to those in 300K SW without trace elements, was also used to culture At plants and study the influences that the major ions had on regulating ethylene production. The ethylene-biosynthesis (ACS7 and ACO2) and senescence-associated (NAP, SAG113, and WRKY6) gene expressions in SW- and ionic-treated At plants in response to transcriptional signaling pathways of ethylene response mechanisms were also investigated. Our results show that down-regulation of the ACS7 gene in 300K-treated plants significantly reduced the ethylene content but remarkably increased chlorophyll, total phenol, and DPPH radical scavenging accumulations and strengthened the salt tolerance of 300K-treated plants. The expression of the ACS7 gene of At plants under 300K, Ca^{2+}, Mg^{2+}, and Na^{+} treatments was correlated with decreases in NAP, SAG113, and WRKY6 gene expressions. The application of Ca^{2+} increased total phenol content and reduced the accumulation of superoxide, which in combination decreases plant aging brought on by ethylene. However, K^{+} treatment inhibited SGA113 gene expression, resulting in reducing ACS7 gene expression and ethylene content. The characterization and functional analysis of these genes should facilitate our understanding of ethylene response mechanisms in plants.

Keywords: calcium; ethylene; leaf senescence; salinity; sea water; signal transduction

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From **Volume 49, Issue 1, 2021,** Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal will use article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

Introduction

Sea water (SW) contains abundant essential minerals (*i.e.* Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}), along with minute amounts of many trace elements, and has attracted attention in accordance with a rise of the consciousness of health from the standpoint of preventive medicine (Nakagawa *et al.*, 2000).Studies have shown that SW exerts diverse biological activity, such as regulating the immune system and antioxidant activity in rats (Jung and Joo, 2006). Thus, it has therapeutic effects on lipid metabolism and IgA production (Kang *et al.*, 2015; Shiraishi *et al.*, 2017) and also has been applied in the food, cosmetic, health, and medical fields (Nani *et al.*, 2016; Higgins *et al.*, 2019). Moreover, SW can be favorable for agriculture under certain circumstances, being used as an additional nutrient supplement at different concentrations to improve the nutritional quality of fruits and vegetables (Yudi *et al.*, 2007; Saito *et al.*, 2009; Yamada *et al.*, 2015). Turhan *et al.* (2014) reported that low concentrations of SW are suitable for lettuce production, which can be successfully grown using SW diluted to concentrations of 2.5% and 5%.The effects of salt stress induced by SW treatments evaluated in red lettuce showed that tested plants grown with dilute SW accumulated more chlorophyll compared to those grown in NaCl solutions, thus increasing their quality and nutritional value (Sakamoto *et al.*, 2014). The use of SW has the potential to achieve horticultural crop biofortification, meaning the endogenous nutrient fortification of food (Ding *et al.*, 2016). Atzori *et al.* (2016) concluded that SW can be used in hydroponics, allowing freshwater savings and increasing certain mineral nutrient concentrations. Furthermore, Caparrotta *et al.* (2019) also showed that the use of SW treatments in hydroponic spinach cultivation has positive effects on growth parameters.

Senescence is the final phase of leaf development, characterized by key processes in which resources trapped in deteriorating leaves are degraded and recycled to sustain the growth of newly formed organs. As the gaseous hormone ethylene exerts a profound effect on the progression of leaf senescence, both the optimal timing and amount of its biosynthesis are essential for controlled leaf development (Sun *et al.*, 2017). The ethylene biosynthetic pathway in higher plants has been well documented (Yang and Hoffman, 1984). The rate-limiting step is the conversion of S-adenosylmethionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase (ACS), and finally ethylene is produced through the oxidation of ACC by ACC oxidase (ACO). The regulation of these enzymes is therefore essential for controlling the rate and level of ethylene production. The application of ethylene improves plant tolerance to high salinity, largely by enhancing the expression of reactive oxygen species (ROS) scavengers (Peng *et al.*, 2014). Several key enzymes in ethylene biosynthesis have been addressed as affected by salinity stresses, in which ACS7 is one of the major contributors to the synthesis of ethylene (Dong *et al.*, 2011; Lyzenga *et al.*, 2012). Moreover, ethylene production by ACO2 appears to be a key regulatory step in *Arabidopsis* plants (Linkies *et al.*, 2009; Sekeli *et al.*, 2014). NAP and WRKY proteins are leaf senescence-associated transcription factor (TF) gene families based on their DNA-binding conserved domains of 60 amino acids with an N terminus and a C_2H_2zinc-finger motif at the C terminus (Zhang and Gan, 2012; Kou *et al.*, 2012; Gu*et al.*, 2019). Senescence-associated gene113 (SAG113), a gene encoding a Golgi-localized protein phosphatase 2C family protein phosphatase, mediates abscisic acid (ABA)-regulated stomatal movement and water loss specifically during leaf senescence. Previous studies showed that high accumulations of the ACS7 protein lead to precocious leaf senescence as well as greatly up-regulating the expressions of NAP, WRKY6, and SAG113 genes (Robatzek and Somssich, 2002; Sun *et al.*, 2017).The study presents the expressions of these ethylene-biosynthesis and senescence-associated genes involving the signaling transduction pathways for delaying senescence in *Arabidopsis* plants treated with SW and its major ionic solutions.

Previously, we reported that various dilution rates of commercial SW applied to pakchoi (*Brassica rapa* subsp. Chinensis) and tomato (*Solanum lycopersicum* var. *cerasiforme*) in hydroponic cultivation conditions enhanced cell viability by increasing 1,1-diphenyl- 2-picrylhydrazyl (DPPH) scavenging ability and 2.3.5-triphenyl tetrazolium chloride (TTC) activity and decreasing malondialdehyde (MDA) content in tested plant leaves compared with plant leaves without SW treatment (Xie *et al.*, 2020). TTC activity was used as a

quantitative method in the evaluation of cell viability, while the higher TTC activity the higher cell viability. MDA is a final decomposition product of lipid peroxidation and has been used as an index for the status of lipid peroxidation, the lower the MDA content the higher cell viability. In the present study, *Arabidopsis* plants grown in different concentrations of SW were evaluated for their physiological and antioxidant characteristics. The influences that the major ions (Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}) contained in SW have on regulating ethylene production were determined. Salt ion analysis revealed significantly different accumulations of Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+} between bent grass cultivars in response to salt stress (Krishnan and Merewitz, 2015). Understanding how ethylene profiles change will help elucidate the mechanisms governing salt-stress tolerance in plants, in which ethylene inhibits receptors, suppresses salt sensitivity conferred by ethylene receptors, and promotes ethylene-responsive salt tolerance (Cao *et al.*, 2007).In addition, in order to test whether ethylene is involved in transcriptional regulation signaling pathways during SW and ionic treatments, expression patterns among the ethylene-biosynthesis and senescence-associated genes in SW- and ionic-treated At plants in response to ethylene production are also discussed to facilitate our understanding of ethylene response mechanisms, the physiological and molecular aspects of salt stress sensing functions, and improve plant stress tolerance, all of which are critical for plant growth and productivity.

Materials and Methods

Germination test and growth conditions

One hundred seeds of *Arabidopsis thaliana* (At) L. ecotype Columbia were sterilized with 1.5% sodium hypochlorite and rinsed with distilled deionized (dd)H_2O. Seeds were then germinated and grown in half strength Murashige-Skoog (MS, from Sigma-Aldrich Co., San Jose, CA, USA) in Petri dishes for two weeks after sowing in a growth chamber under 200 μmol m^{-2} s^{-1} light with a 16 h photoperiod at a temperature of 23 °C, and a relative humidity of 80% for a week. The germination rate (%) was then calculated (Chiang *et al.*, 2014). Three commercial SW products (namely RO3, 300K, and 340K), each in three different dilution ratios, were applied to determine the optimal dilution rate (X) without influencing seed germination and seedlings by comparing with those plants without SW treatment (control). Uniformly sized three-week old seedlings were individually transferred to 3-inch (7.6 cm) plastic pots, and treated with 100 mL of each SW and complete nutrient solution (Millero *et al.*, 2008; Caparrotta *et al.*, 2019) once per week. Pots were randomly placed in the above-mentioned growth chamber under the same growing conditions for one week.

Table 1 lists the characteristics of the three commercial SW products at various dilutions and 300K-Test synthetic water suitable for plant growth: RO3 (2,000 X, high Na^{+}-containing SW with trace elements), 300K (540X, high Mg^{2+}, K^{+}, and Ca^{2+}-containing ocean water with trace elements), 340K (6,180 X, high Mg^{2+}, K^{+}, and Na^{+}-containing SW with trace elements), and 300K-Test (major ions with equal concentrations to that in 300K SW without trace elements) compared to those non-SW treated plants. Electrical conductivity (EC) and pH values of three SWs and 300K-Test solution were measured by an EC meter (DEC-2, Atago Co., Tokyo, Japan) and pH meter (DPH-2, Atago Co.), respectively. Their values were calculated by using the concentrations of the major constituents (Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}) in the commercial SW (Sakamoto *et al.*, 2014). In addition, the mixture of Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+} synthetic water (same as 300K but free of trace elements, namely 300K-Test) was also used to culture At plants in order to assess the effects of these major ions on the physiological and antioxidant characteristics on the plants compared to 300K (540X) SW. The "300K-Test" ion synthetic water contained equal amounts and concentrations of the major ions Mg^{2+} (143.08 mg L^{-1}), Na^{+} (5.74 mg L^{-1}), K^{+} (170 mg L^{-1}), and Ca^{2+} (33.5 mg L^{-1}) as in the commercial 300K (540X) SW but free of trace elements (Sohrin *et al.*, 1998). The pH of the 300K-Test was adjusted to 5.7, identical to the three commercial SW products. The EC value of 300K-Test was 1.082 (ms/cm), with obtained values being reported in Table 1.

Table 1. Constituent, pH value, and electrical conductivity (EC) value of three commercial sea water products [SW - RO3 (2,000X), 300K (540X), and 340K (6,180X)] and a mixture of Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+} ions only (namely 300K-Test, free of trace elements)

Solution (concentration)	RO3 (2,000X) *	300K (540X) *	340K (6,180X) *	300K-Test
Mg^{2+} (mg L^{-1})	1.125	143.08	3.8	143.08
Na^{+} (mg L^{-1})	8.65	5.74	4.7	5.74
K^{+} (mg L^{-1})	3.3	170	41.2	170
Ca^{2+} (mg L^{-1})	0.38	33.5	0.0017	33.5
Trace elements	+	+	+	-
Salinity ratio	1	0.27	3.09	-
pH value	5.7	5.7	5.7	5.7
EC value (ms/cm)	0.1184	1.458	0.01746	1.082

*The three commercial SW was obtained from LOHA Water Tech Co., Taipei, Taiwan, and processed though electro-deionization and vacuum concentration.RO3 was used as the basic level, set as 1 to calibrate the salinity ratios of 300K (0.27) and 340K (3.09). Trace elements such as zinc, manganese, vanadium, chromium, and selenium are negligible in these three commercial SW solutions.

+: with trace elements; −: without trace elements.

The SW was obtained from LOHA Water Tech Co., Taipei, Taiwan, and processed though electro-deionization and vacuum concentration. However, high salinity from the non-diluted SW had a harmful effect on seed germination. RO3 was then used as the basic level, set as 1 to calibrate the salinity ratios of 300K (0.27) and 340K (3.09). Afterward, three various diluted rates (X) of each SW were established according to the salinity ratio and diluted with dd water to the same salinity. For example, the three dilution rates (500X, 1,000X, and 2,000X) of RO3 were multiplied by 0.27 and 3.09 to obtain the three dilution rates (135X, 270X, and 540X) of 300K and (1,545X, 3,090X, and 6,180X) 340K, respectively (Table 1). RO3 (500X) was used in equal concentration to that of 300K (135X) and 340K (1,454X) SW for studying the influences of the main elements and trace elements in SW on *Arabidopsis* plants.

Each different dilution rate of each SW treatment was applied to 100 seedlings or plants in the experiment in a completely randomized design. The At plants grown under 1/2 MS medium without SW and 300K-Test treatments served as controls. Following each treatment, young, fully expanded leaves from each plant were clipped, frozen in liquid nitrogen, and stored at -80 °C in an ultra-freezer until used for the analyses.

Determination of total chlorophyll, phenolic, and MDA contents, DPPH scavenging capacity, enzyme activity, and in situ ROS staining

The total Chl content of leaves from three-week-old potted At plants from each treatment were determined using methods described by Arnon (1949). The analysis of DPPH radical scavenging activity in At leaf extracts was determined according to Shimada *et al.* (1992). The DPPH scavenging capacity was calculated as the percentage of free radical-scavenging activity. The measurement of MDA content using the thiobarbituric acid (TBA)-trichloroacetic acid (TCA) method was described by Kosugi and Kikugawa (1985). Ten plants per treatment were used for all analyses.

Cut leaves from each treatment were prepared for superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione reductase (GR, EC 1.6.4.2), and ascorbate peroxidase (APX, EC 1.11.1.11) activity analyses. SOD activity was analyzed using a SOD assay kit (Dojindo Molecular Technology, Gaithersburg, MD, USA). CAT activity was determined as described by Hwang and VanToai (1991). APX

activity was determined according to Nakano and Asada (1981). GR activity was measured by the GSH-dependent oxidation of NADPH according to Foyer *et al.* (1997).

The total phenolic content of leaves in each treatment was described in Dewanto *et al.* (2002). The total phenolic concentration is expressed as gallic acid equivalents per gram of dry weight (GAE, mg gallic acid/g sample) using a calibration curve with gallic acid. The calibration curve ranged from 20 to 500 mg/ml (R^2 = 0.996).

ROS were stained *in situ* utilizing the principle of nitroblue-tetrazolium (NBT) reduction to blue formazan by superoxide radicals. The intracellular concentration of superoxide radicals was directly proportional to the development of the intensity of blue color in the leaves and previously described in Shafi *et al.* (2014).

Expression analysis of ethylene-synthesis and senescence-associated genes, amplification of cDNA, and quantification of RNA levels

Three-week-old plant leaves (0.1 g) with SW and ionic one-week treatments were used for total RNA isolation using an Ambion Kit (San Francisco, CA, USA). Paired specific primers for *AtActin8*, *ACS7*, A*CO2*, *NAP, SAG113,* and *WRKY6* were used for amplification (Table 2). Gene amplification was described in our previous study (Lin *et al.*, 2019). The products were electrophoretically separated on 1.5% agarose gels, and predicted sizes of 140, 88, 166, 84, 130, and 84bp of AtActin8 (accession no. At1g49240), ACS7 (accession no. AT4G26200), ACO2 (accession no. AT1G62380), NAP (accession no. AT1G69490), SAG113 (accession no. AT5G59220), and WRKY6 (accession no. AT1G62300) genes, respectively, were verified with a 100bp DNA ladder marker.

The relative changes in ethylene-synthesis (ACS7 and ACO2) and senescence-associated (NAP, SAG113, and WRKY6) gene expressions in response to various diluted SW and their four major ionic (Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}) treatments were monitored by real-time quantitative (q)PCR and quantification of RNA levels. To test whether NAP, SAG113, and WRKY6gene expressions were induced by ethylene, two-week-old At plants were foliar sprayed by 1mM ethephon aqueous solution (Sigma-Aldrich Cat.# C0143) for one week as previously described (Wen *et al.*, 2015). A real-time qPCR was performed based on our previous study (Lin *et al.*, 2019). To normalize the total amount of cDNA in each reaction, *AtActin-8* from *Arabidopsis* was co-amplified asan internal control. The relative amounts of RNA were calculated by the ratio of the abundance of 300K- and major ionic-treated plants to*AtActin-8* (Livak and Schmittgen,2001).

Table 2. Pairedprimers for *AtActin8* (internal control), *ACS7* and *ACO 2* (ethylene-biosynthesis genes), *NAP, SAG113,* and *WRKY6* (senescence-associated genes) used in the study

Primers	Sequence (5' to 3')	Size (pb)
AtActin8	F: 5'CCCAA AAGCC AACAG AGAGA3' R: 5'CATCACCAGAGTCCAACACAAT3'	140
ACS7	F: 5'TCGTGACGCGAACATTAGAG3' R: 5'TCTAGAACCTTCTTTTGGACC3'	88
ACO2	F: 5'CCAGCTACTTCGCTTGTCGAC3' R: 5'GTCTCTACGGCTGCTGTAGGA3'	166
NAP	F: 5'TTACATGGGACCCGTCTCTC3' R: 5'CCGAACCAACTAGACTCCGA3'	84
SAG113	F: 5'AACTGCATGTAGCGTCGTTC3' R: 5'CTGGCAAATCTCCTCCTCC3'	130
WRKY6	F: 5'CAGTTCTCTGGTGGCTCTCC3' R: 5'GTCAGCTGTGAGTGCCGTTA3'	84

Ethylene emission measurements

The rate of evolution of ethylene was determined on four-week-old At plants (10 different leaves of one rosette and ten plants per treatment) with a portable ethylene gas analyzer (CI-900; CID Bio-Science, Camas, WA, USA). Pots were kept inside the airtight chamber of the instrument for 2 min and their rates of ethylene evolution (ppm) (Krishnanand Merewitz, 2015) were read.

TTC activity determination of At seeds

The data on seed viability by TTC activity test were also recorded up to seven days after sowing and ten plants were used for each treatment. TTC analysis was performed based on Hussain and Reigosa (2014) using ELISA Reader (Spectrophotometer U-2900, Hitachi Tokyo, Japan) and expressed as A_{485} µg per plant weight (g) per hour (h).

Statistical analysis

The measurements of physiological and antioxidant parameters were performed using a paired *t*-test and one-way analysis of variance (ANOVA), with the least significant difference (LSD) test at $p < 0.05$ using the SAS program ver. 9 (SAS Institute, Cary, NC, USA).

Results and Discussion

Morphology of At seedlings

The At seedlings grown in 1/2 MS medium with RO3 (500X, 1,000X, and 2,000X), 300K (135X, 270X, and 540X), and 340K (1,545X, 3,090X, and 6,180X) treatments were impaired, epinastic, senescent, yellowish, and smaller in size relative to all of the control plants (photos not shown). On the other hand, no obvious differences were observed in the colors and sizes of seedlings cultivated in the highest dilution ratios (lowest concentrations) of SW treatments and controls. However, higher concentrations of SW displayed inhibitory effects on growth from salinity, and salt stressed seedlings suffered a changed cell water relation that displayed a cost for osmotic adjustment, which generally reduced the absorption and translocation of water (Munns, 2002). In fact, salinity negatively affects plant growth and physiology through different mechanisms, such as water and osmotic stress (Garcia-Sanchez and Syvertsen, 2009; Balal *et al.*, 2012; Gonzalez *et al.*, 2012). It is generally known that when SW is provided to plants during cultivation, minerals contained in the seawater may stimulate growth. Islam *et al.* (2010) reported that eggplant variety 'Ryoma' plants grown with the applications of 2% mineral controlled sea water to the standard nutrient solution under greenhouse condition had larger vegetative growth rate than with the control. Furthermore, with the 2% mineral controlled sea water treatment the plants increased 14% of fruit yield compared to the control. For *Arabidopsis* tolerance to salinity stress, Alet *et al.* (2012) reported that the inhibitory effects were observed when plants were grown under > 50 mM NaCl conditions. Throughout the duration of the experiment, seedlings appeared healthy and sported green and larger leaves when cultivated in the higher dilution ratios of RO3, 300K, and 340K treatments, withstanding the low osmotic pressure of the growing medium, in comparison to the above-mentioned lower dilution ratios. Therefore, RO3, 300K, 340K, and 300K-Test treatments were used for the following experiments. Moreover, these identified dilution systems could be used for the rapid monitoring and early detection of salt injury in the seedling stage. This means that hundreds of individual plants might be screened per day, providing for the large-scale discovery of individuals that exhibit tolerance to salt stress.

Physiological and antioxidant characteristics of At seeds and plants

No significant differences in the germination rates of At seeds were observed among all treatments and controls, with an average of 95.5% germination (Figure 1A), suggesting that diluted SW does not affect At seed germination due to its tolerance to low salinity stresses. TTC activity responded differently to diluted SW

treatments (Figure 1B), and TTC activity of *Arabidopsis* seeds cultivated in 300K and 340K at an average of 7.3 μg/g·h was significantly higher than in controls, which averaged 6 μg/g·h, whereas seeds under RO3 treatment displayed similar TTC activity to controls. Thus, 300K and 340K SW improved cell activity in seeds compared to RO3 and controls, suggesting that SWs with different concentrations of Mg^{2+}, K^{+}, and Ca^{2+} (Table 1) may influence the tissue cell viability of At plants. The MDA content of all leaves grown in all SW treatments and controls did not show any significant differences (Figure 2A). Therefore, the SW concentrations used in the present experiment were unable to induce any change in MDA content - in other words, decreased lipid peroxidation- in the leaves of At plants, suggesting the possibility of cultivation at the tested SW concentrations. Thus, SWs with different Mg^{2+}, K^{+}, and Ca^{2+} concentrations (Table 1) would not cause the tissue cells of At plants to produce lipid peroxidation. Alternatively, SW treatments to *Arabidopsis* maintained the integrity of the plasma membrane, and the integrity of the cell wall reflected on reduced lipid peroxidation status in the At plants, which might be related to reducing leaf MDA content.

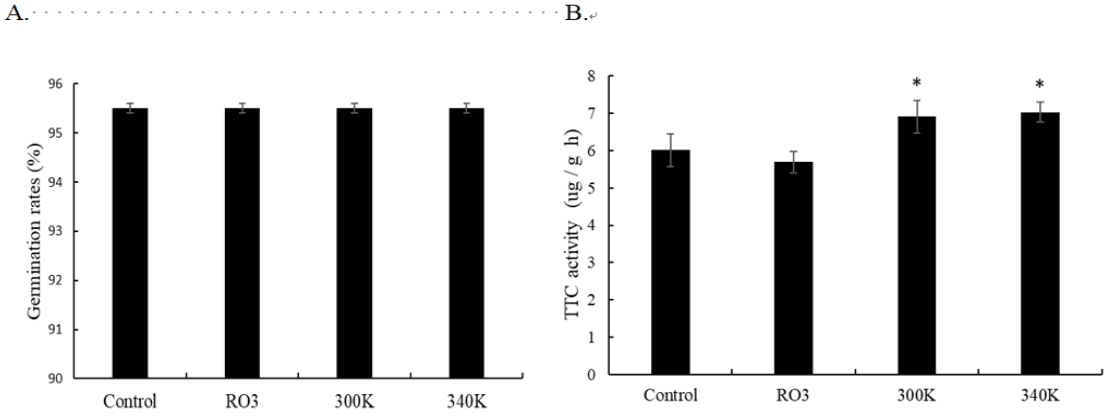

Figure 1. Seeds of *Arabidopsis thaliana* treated with diluted RO3, 300K, and 340K treatments for a week followed by the germination test (A) and TTC activity determination (B)
Arabidopsis thaliana plants grown in 1/2 MS medium without SW treatments were controls. Values are the means of eight replicates with corresponding standard deviations. The germination rate (%) and TTC activity (μg/g·h) are compared to control plants and an asterisk indicates a significance level of $p \leq 0.05$.

The significantly higher DPPH radical scavenging effect (41.2%) and total Chl content (0.58 mg/g FW) in tested leaves were observed with 300K compared to the control (40.4% and 0.48 mg/g FW, respectively), whereas no remarkable differences in DPPH radical scavenging effects and total Chl content were detected among RO3, 340K, and controls (Figures 2B,C).These results demonstrate that only 300K treatment could increase antioxidant content and scavenge DPPH radicals, and also suggests that the major ions contained in 300K SW may enhance the synthesis and accumulation of Chl. Magnesium is a main constituent of the Chl molecule bound by four pyroll groups. Consequently, using a remarkably high concentration (143.08 mg L^{-1}) of Mg^{2+} in the 300K solution could critically influence the additional synthesis of Chl in the leaves. Furthermore, 300K-treated At plants exhibited lower accumulations of superoxide ($\bullet O^{2-}$) in leaves compared to other SW treatments and controls in *in situ* ROS staining (Figure 2D), speculating that plants treated with diluted 300K had slightly lower ROS accumulations compared to the control. In addition, Mg^{2+}, Na^{+}, K^{+}, Ca^{2+}, and 300K also displayed lower levels of $\bullet O^{2-}$ accumulation in leaves as evidenced by the lower intensity of a fuscous precipitant (blue color) compared to the control (Figure 2E). Therefore, 300K, Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}solutions were applied to tested plants for antioxidant capacity analysis.

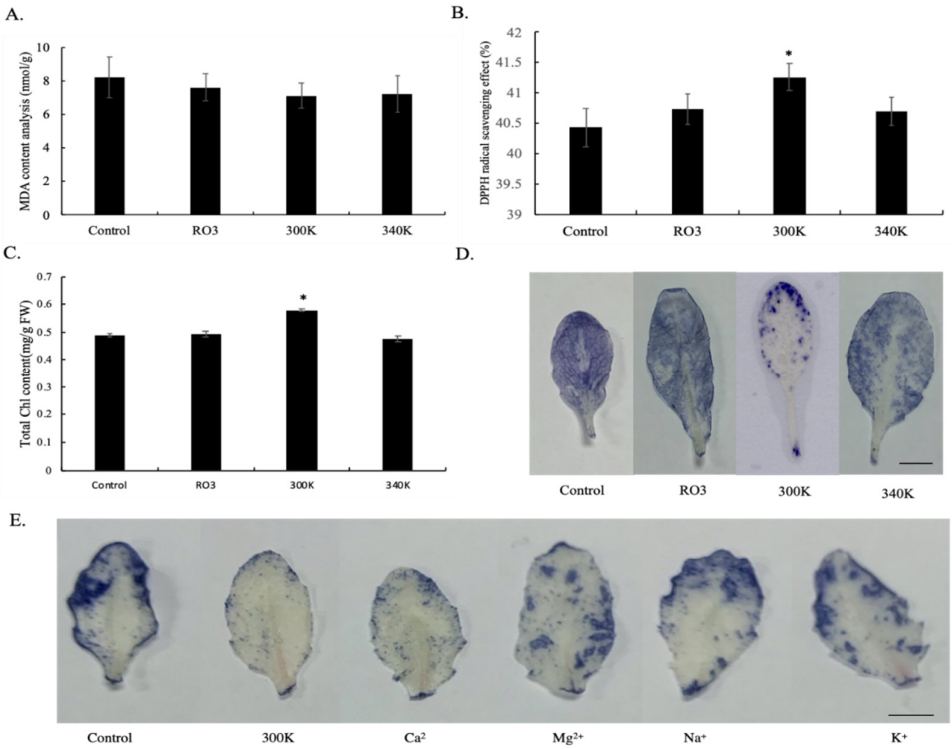

Figure 2. Three-week old potted *Arabidopsis thaliana* plants treated with diluted RO3, 300K, and 340K SW for one week followed by the measurement of MDA content (A), DPPH scavenging ability (B), total chlorophyll content (C), and NBT staining of leaves (D)

Panel E is NBT staining in leaves of the same plants treated for one week with diluted 300K, Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}. Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium without SW or ionic treatments. Values are the means of eight replicates with corresponding standard deviations. The MDA content (nmol/g), DPPH scavenging ability (%), and total chlorophyll content (mg/g FW) are in comparison to control plants and an asterisk indicates a significance level of $p \leq 0.05$. Scale bar size is 0.5 cm.

Plants offset the initial osmotic components of salt stress by adjusting the osmotic gradient, although the accumulation of Na^{+} can lead to toxic effects in the long term (Alvarez-Aragon and Rodriguez-Navarro, 2017). Na^{+} affects the hydration shell of other molecules, causes damage to the cell wall, disturbs the K^{+}/Na^{+} ratio of cells by several mechanisms, and impairs plant physiology (Julkowska and Testerink, 2015). Generally, plants that have the ability to excrete, exclude, or tolerate high levels of salt are salt-resistant, the differential expression genes of proton pumps or antioxidant capacity could play a role in causing the differential accumulation of Na^{+} in plant leaves and roots between cultivars (Janicka-Russak *et al.*, 2013; Pérez-López *et al.*, 2013; Krishnan and Merewitz, 2015). In addition, the maintenance of all K^{+} ion transporters and channels across the plasma membrane is essential for proper K^{+} homeostasis in plants (Zhang *et al.*, 2018). Calcium can also improve K^{+} transport under salt stress conditions (Maathuis, 2006). Although plants rely on a sufficient supply of Mg^{2+} and other elements for normal growth and development, excessive Mg^{2+} accumulation often causes toxicity to plant cells (Niu *et al.*, 2018). Salt movement and accumulation in roots and leaves of At plants subjected to SW and ionic treatments for their contrasts in salt tolerance are worthy of further investigation.

Arabidopsis plants treated with 300K and Ca^{2+} cultures showed significantly higher total phenolic content (18 mg of GAE/g and 22 mg of GAE/g, respectively) compared to the control (13 mg of GAE/g) (Figure 3E). Nevertheless, no significant differences were observed between Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+} alone or combined (300K and 300K-Test) in cultures and controls. The four antioxidant activities of At leaf extracts from 300K and all individual ionic cultures were non-significantly different from controls (Figures 3A-D), suggesting that CAT, SOD, APX, and GR did not participate in active ROS reduction irrespective of the plant

growth period (three weeks after sowing) when treated with 300K and those four major ions.ROS production and scavenging are interactive, maintaining relative stability in plants.

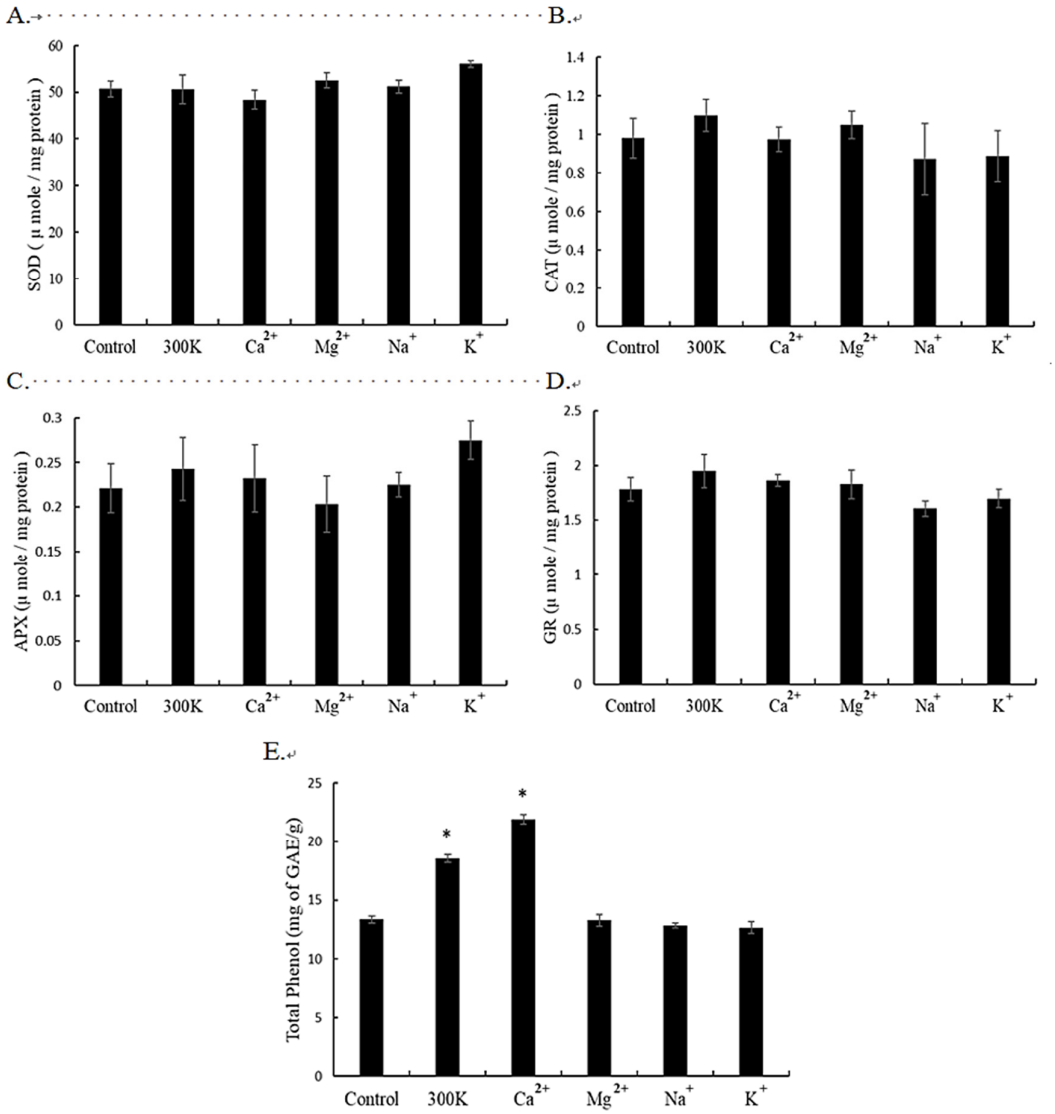

Figure 3. Three-week old potted *Arabidopsis thaliana* plants treated for one week with diluted 300K, Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}, followed by analysis of SOD activity (A), CAT activity (B), APX activity (C), GR activity (D), and total phenol content (E)

Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium without SW or ionic treatments. Values are the means of eight replicates with corresponding standard deviations. The SOD, CAT, APX, and GR activities (U/mg protein) and total phenol contents (mg of GAE/g) are in comparison to control plants, and an asterisk indicates a significance level of $p \leq 0.05$.

Presumably, the accumulation of antioxidant system components and ROS formation are favored in salt tolerance. Increased levels of ROS in salt-stressed plants could lead to an increased capacity of the ROS scavenging system. Salt stress induces the production of ROS such as singlet oxygen ($1O_2$), superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$). These ROS are necessary for inter- and intracellular signaling, but at high concentrations they seriously disrupt normal metabolism in plants through the oxidation of membrane lipids, proteins, and nucleic acids (Hoque *et al.*, 2007). Numerous studies have

indicated that antioxidant systems are correlated with plant tolerance to salt stress, these enzymes and/or non-enzymes are required to maintain redox homeostasis, and the induction of antioxidants and osmolytes is part of an integrated strategy for salt stress defense (Lin and Pu, 2010). Pre-treating with SW and ionic solutions may influence the ability to maintain a balance between the formations and de-oxidation of ROS, leading to leaf vulnerability against oxidative stress. Salt stresses induce the production of ROS, which are necessary for inter-and intracellular signaling, but under stress conditions they seriously disrupt normal metabolism in plants through the oxidation of membrane lipids, proteins, and nucleic acids in the absence of protective mechanisms (Nguyen *et al.*, 2018). In our study, total phenolic content is markedly accumulated in At leaves exposed to salinity stress, suggesting that total phenol content may be useful in screening salt-tolerant plants.

Increased DPPH radical scavenging activity was observed in the extracts of At grown in cultures with 300K SW, which may be due to the contribution of phenolics accumulated in the leaves. Supplementation of 300K SW increased the salinity of the nutrient solution and subsequently might increase the Ca^{2+} uptake; thus, leaves contained more total phenolic content. The phenolic compound alteration due to salinity stress is critically dependent on the salt sensitivity of the plant. In fact, salt stress creating both ionic as well as osmotic stress in plants resulting in increased polyphenol concentration indifferent tissues have been reported in a number of plants (Parida and Das, 2005). We assume that At plants were subjected to osmotic stress by the addition of Ca^{+2} to the cultures, and as a result, phenolics are produced and accumulate in leaf cells and function as osmolytes, and are believed to facilitate osmotic adjustments by acting as osmoprotectants. Errabii *et al.* (2006) reported that growth, proline and ion accumulation in sugarcane callus cultures under drought-induced osmotic stress and its subsequent relief, and a sudden osmotic up shift in the medium causes a water efflux from the cells, loss of turgor pressure, and concomitant reduced growth. It is known that hyperosmolality and various other stimuli trigger increases in cytosolic free calcium concentration. Environmental water deficiency triggers an osmotic stress signaling cascade, which induces short-term cellular responses to reduce water loss and long-term responses to remodel the transcriptional network and physiological and developmental processes (Yuan *et al.*, 2014). The cell wall also contains phenolics, enzymes, proteins, and Ca^{2+}, and osmotic stress can lead to the accumulation of ROS in the cell wall (Tenhaken, 2014). The calcium ion acts at a convergence point for integrating different signals, and may have a role in providing salt tolerance to plant cells. Some oxidant systems use Ca^{2+} to stimulate oxidative bursts in leaf cells, but some do not. Manipulating Ca^{2+} homeostasis by altering the concentration of Ca^{2+} could be an important strategy to alter the behavior and survival of plants under salt stress (Lin *et al.*, 2008). As a consequence, Ca^{2+} may play an important role in the antioxidant system under salt stress. It is possible that both calcium and ROS could be important modulators of the cellular signaling of transduction events following salt-stress injury. Perhaps a higher level of Ca^{2+} under non-stressed conditions allows for enhanced stress perception, signaling, or Ca^{2+}-induced stabilization of cell structure at the onset of salt stress. The development of salt stress in leaves was more gradual or perhaps delayed by Ca^{+2} with 33.5 mg L^{-1} treatment.

Determination of ACS7 and ACO2 genes expression and ethylene emission in At plants with ethephon treatment

Ethylene biosynthesis may have resulted from gene activation and/or up-regulation of ethylene-induced ACS7 and ACO2 genes. To investigate the regulation and expression of ACS7 and ACO2 genes in At plants, a real-time qPCR analysis was performed with extracted RNA from two-week-old plants subjected to diluted SW and 300K-Test solutions for one week. Data were normalized with respect to the RNA level of AtActin8, a housekeeping gene that is consistently expressed in plants. Figures 4A and B show that RNA abundances of *ACS7* and*ACO2* were significantly up-regulated in RO3 and 340K treatments in comparison to controls. However, RNA expressions of *ACS7* were significantly lower in 300K, 300K-Test, and individual Ca^{2+}, Mg^{2+}, and Na^{+} in 300K solutions than in controls (Figures 4A and C). Moreover, the ACS7 gene was significantly and highly expressed in K^{+}-treated plants compared to control plants.

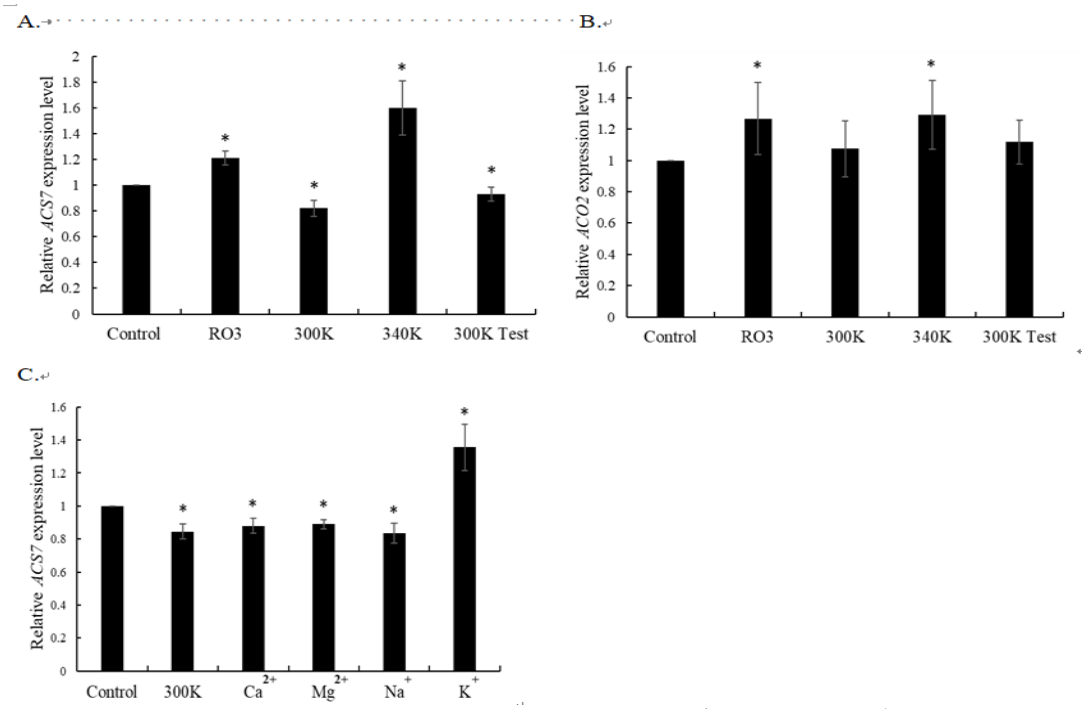

Figure 4. Relative RNA expressions of ethylene-biosynthesis genes (*ACS7* and *ACO2*) in *Arabidopsis thaliana* plants under various SW and ionic treatments

Total RNA in all tested plants was extracted from leaves of two-week-old plants subjected for one week to diluted RO3, 300K, 340K, and 300K-Test, followed by ACS7 (A) and ACO2 (B) gene expressions.

Panel C is of two-week-old plants treated for one week with diluted 300K, and individual Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+} ions, followed by the relative RNA expression of the ACS7 gene.

Relative amounts were calculated and normalized with respect to the AtActin-8 gene. Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium without SW and ionic treatments. Values are the means of eight replicates with corresponding standard deviations. The relative ACS7 and ACO2 gene expressions are in comparison to control plants, and an asterisk indicates a significance level of $p \leq 0.05$

Figure 5A shows that the ethylene level of three-week-old plants under 300K treatment (0.085 ppm) was significantly lower than in controls (0.105 ppm). Furthermore, ethylene levels in all tested plants in all ionic treatments (0.09 ~ 0.1 ppm) were significantly lower in controls (0.12 ppm) after exogenously applied 1μM ethephon treatment for one week, whereas ethylene levels in all plants in all ionic treatments were close to the levels of controls (0.105 ppm) without ethephon treatment (Figure 5B).These results suggest that leaf senescence depends on the balance between ACS7-generated ethylene and ionic-dependent ethylene accumulation in *Arabidopsis*. Ethephon is an ethylene production inducer and is chemically converted to ethylene by oxidation. Ethylene biosynthesis occurs in all plant tissues and throughout all stages of leaf development, but endogenous ethylene levels vary according to the stage of leaf growth and development (Iqbal *et al.*, 2017), which can be promoted or inhibited by ethylene, coupled with an increase or decrease in ACC synthesis, respectively (Ceusters and VandePoel, 2018). Plants at different developmental stages or with different genetic backgrounds express ACO at different levels, leading to regulation of ethylene production (Kim *et al.*, 2003). Sun *et al.* (2017) reported that ACS7 degradation is highly regulated by senescence signals to enable optimal ethylene production at the appropriate times during At leaf development.

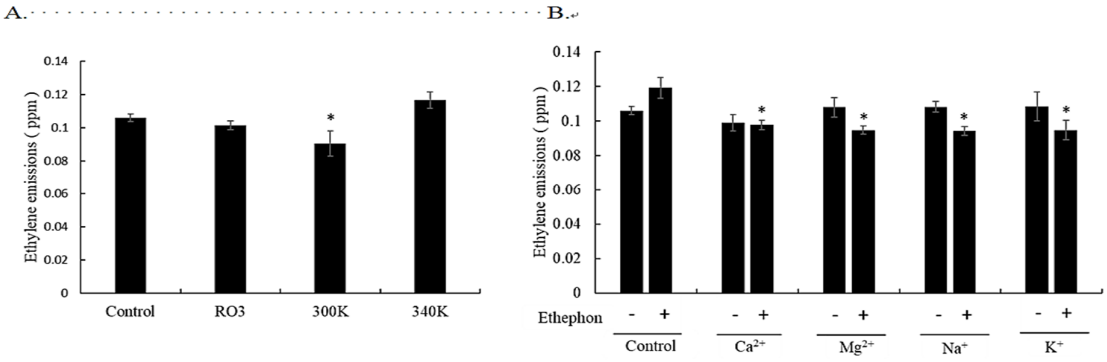

Figure 5. Ethylene emissions of four-week old potted *Arabidopsis thaliana* plants treated for one week with diluted RO3, 300K, 340K and individual Mg^{2+}, Na^{+}, K^{+}, and Ca^{2+}

(A) Ethylene emissions within 2 min after diluted SW treatments. Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium without SW treatments.

(B) Ethylene emissions within 2 min after individual ionic treatments with (+) or without (-) 1 mM ethephon. Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium with or without ethephon treatments.

Values are the means of eight replicates with corresponding standard deviations. Ethylene emission is compared to control plants and an asterisk indicates a significance level of $p \leq 0.05$.

Ethylene production and expressions of senescence-associated genes in At plants with ethephon treatment

Relative RNA expressions of NAP, SAG113, and WRKY6 genes involved in the senescence response were analyzed for the possibility that ethylene is involved in the signaling pathways of NAP, SAG113, and WRKY6genes. Figure 6A shows that significant up-regulated expressions of *NAP, SAG113*, and *WRKY6* were observed in plants treated with ethephon (> 2.2) compared to non-ethephon treatment (= 1). Nevertheless, when those plants were subjected to 300K, Ca^{2+}, Mg^{2+}, and Na^{+} treatments, all expressions of *NAP, SAG113*, and *WRKY6* (< 0.7) were significantly lower than in control plants (= 1) (Figures 6B-D). Moreover, RNA levels of *NAP* and *SAG113* in plants under K^{+} treatment were significantly higher (= 1.2) and lower (0.38), respectively, than in controls (= 1) (Figures 6B, C). These differences in gene expression may be because of the influence that major ions in SW have on the ethylene biosynthesis of At plants. This study used a mixture of these major ions to prepare the SW used to culture At plants in the attempt to understand the influence that the major ions in SW have on regulating ethylene-produced pathways. The results suggest that the combination of the four individual ionic waters may have a synergistic effect on the regulation of ethylene biosynthesis. These findings can serve as a valuable reference for improving electric dialysis and water separation techniques based on SW.

Aging factors *NAP, SAG113,* and *WRKY6* play important roles in delaying senescence, with different signaling pathways in these ionic-treated At plants. After ethephon application and ionic treatments of At plants, the up-regulation of NAP, SAG113, and WRKY6 gene expressions and obviously decreased ethylene content delayed senescence during plant development compared to control plants. The down-regulation of these senescence-associated genes in the ionic-treated plants activated the expression of the downstream target ACS7 gene that was also down-regulated in expression in Ca^{2+}-, Mg^{2+}-, and Na^{+}-treated plants, but was highly expressed in K^{+}-treated plants compared to control plants. Consequently, these ions acted as a signal to NAP, SAG113, and WRK6 genes and activated gene products involved in ethylene acclimation and tolerance of ethylene-downregulated pathway in ionic-treated plants. Both ethylene and Ca^{2+} have been documented to play important roles in plant senescence. A balanced and timely supply of Ca^{2+} sources for fruit and vegetable crops during the growing season and at the postharvest stage improves the shelf life and nutritional quality of horticultural produce (Gao *et al.*, 2019). Furthermore, Ca^{2+} supply to ornamental crops extends the vase-life of flowers by delaying senescence and reducing intensified ethylene production (Aghdam *et al.*, 2012). When the Ca^{2+}concentration changes, plants use a Ca^{2+} effector protein to sense this signal, and then manage external

stimulation by regulating the expression of the plant stress gene. The Ca^{2+} signaling process is activated with the presence of a Ca^{2+} sensor and their target proteins (Zhang *et al.*, 2018).

Monitoring the expressions of plant genes at the transcriptional level is an essential step in their functional analysis. Expression patterns in *ACS7* and *ACO2* in response to SW and ionic treatment stress provide a molecular basis for the ethylene biosynthesis pathway in plants. Thus, down-regulation in the ACS7 gene in 300K-treated plants (Figure 4) reduced ethylene content (Figure 5), but increased Chl, total phenol, and DPPH radical scavenging accumulations (Figures 2 and 3) and strengthened salt tolerance in 300K-treated plants. The expression profile of the ACS7 gene in control plants and with 300K, Ca^{2+}, Mg^{2+}, and Na^{+} treatments (Figure 4C) was correlated with decreases in NAP, SAG113, and WRKY6 gene expressions (Figures 6B-D). These ionic-induced transcriptional activations of senescence-associated genes correspond to decreases in the products of NAP, SAG113, and WRKY6 genes that protect cellular components against the effects of ROS accumulation as a consequence of delaying senescence.

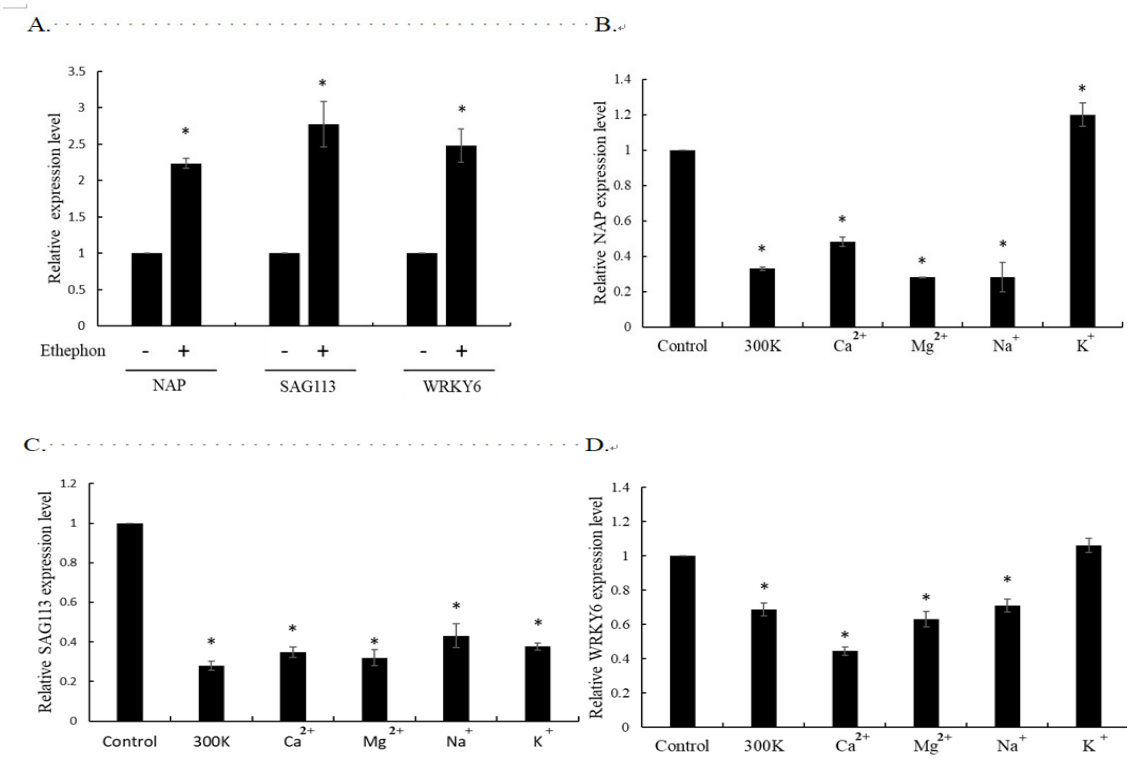

Figure 6. Relative RNA expressions of senescence-associated genes (*NAP, SAG113,* and *WRKY6*) in *Arabidopsis thaliana* plants

(A) Total RNA in all tested plants was extracted from leaves of two-week-old plants with (+) or without (-) ethephon (1mM) treatments for one week followed by NAP, SAG, and WRKY6 gene expressions. (B~D) Total RNA in all tested plants was extracted from leaves of two-week-old plants subjected to 300K, Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} treatments for one week followed by NAP (B), SAG (C), and WRKY6 (D) gene expressions. Relative amounts were calculated and normalized with respect to the AtActin-8 gene. Controls were *Arabidopsis thaliana* plants grown in 1/2 MS medium without SW and ionic treatments. Values are the means of eight replicates with corresponding standard deviations. Relative *NAP*, *SAG113*, and *WRKY6* gene expressions are compared to control plants, and an asterisk indicates a significance level of $p \leq 0.05$.

Ethylene is an important plant growth substance, and ethylene responses may interact with other physiological processes or responses. Hua and Meyerowitz (1998) reported that ethylene treatment results in leaf senescence coupled with a decrease in Chl content and up-regulation of the SAG2 expression. Figure 7 demonstrates a prediction of signaling transduction pathways of delaying senescence in *Arabidopsis* plants treated with 300K DOW. The Mg^{+2}, Na^{+}, and Ca^{2+} in the 300K solution decreased the expressions of NAP, SAG113, and WRKY6, leading to decreased ACS7 gene expression and reduced ethylene production, but expression of the ACO2 gene was not affected by 300K treatment. The alternative pathway of Ca^{2+} in the diluted 300K was to increase total phenol content and reduce the accumulation of free radicals (*i.e.*, superoxide) that decrease plant aging from ethylene. However, the K^{+} in the diluted 300K inhibited SGA113 gene expression, resulting in reducing ACS7 gene expression and reducing ethylene production. Although the main ions in SW induced ethylene biosynthesis via transcriptional regulation, whether the signaling transduction pathways of delaying senescence in At plants were affected by trace elements in the SW in this study remain unknown.

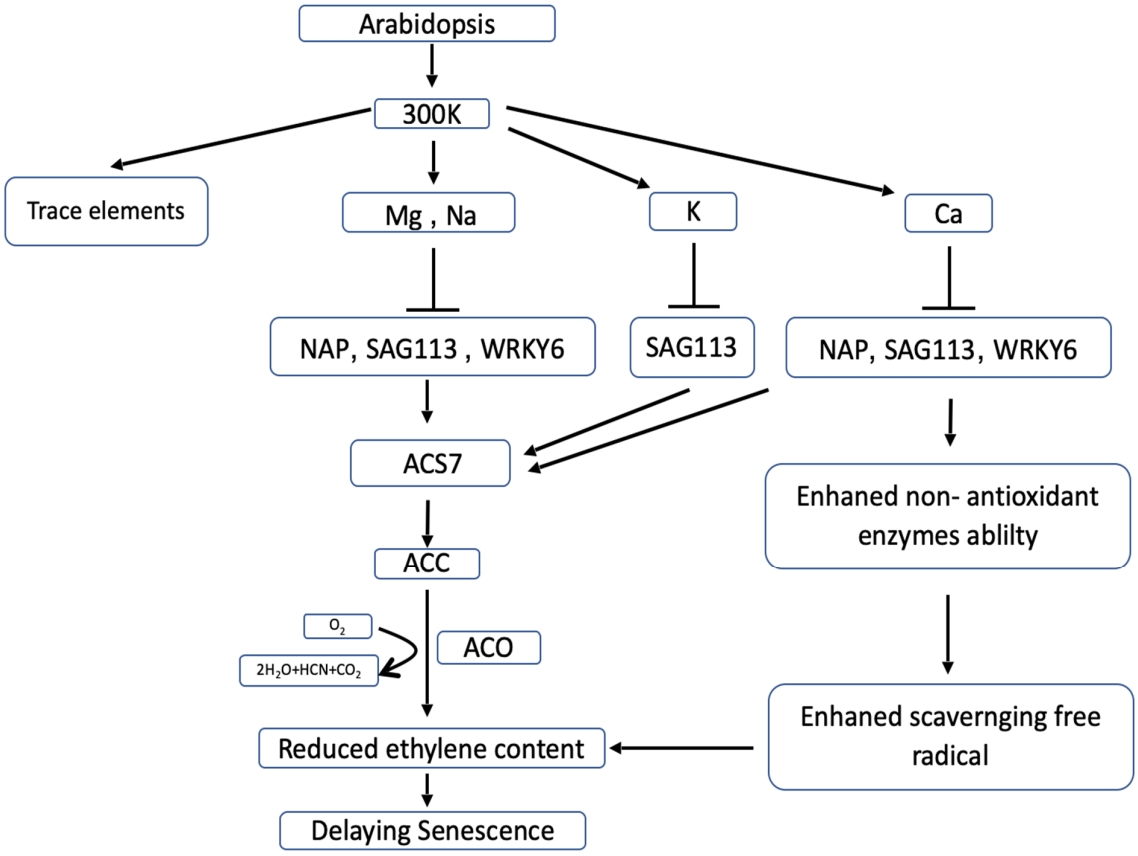

Figure 7. Schematic diagram of signaling transduction pathways for delaying senescence in *Arabidopsis* plants treated with diluted 300K SW

The Mg^{+2}, Na^{+}, and Ca^{2+} in the 300K solution decreased the expressions of NAP, SAG113, and WRKY6, leading to a decrease in ACS7 gene expression and reduction of ethylene production, but expression of the ACO2 gene was not affected. The alternative pathway of Ca^{2+} in the diluted 300K was to increase total phenol content and reduce the accumulation of free radicals (*i.e.*, superoxide) in order to decrease ethylene-caused plant aging. However, the K^{+} in the diluted 300K inhibited SGA113 gene expression, resulting in reducing ACS7 gene expression and reducing ethylene production.

The presence of NAP, SAG113, and WRKY6 transcripts in the 300K-, Ca^{2+}-, Mg^{2+}-, and Na^{+}-treated plants was a rapid response to ethylene content, indicating the importance of these genes in the ROS defense system in plant cells. The 300K-treated plants exhibited stronger resistance to ethylene tolerance due to less $\bullet O^{2-}$accumulation. The senescence stress tolerance caused by Ca^{2+} treatment led to the reduced production of ethylene, consequently increasing total Chl and phenol content. The capacity of a plant to down-regulate the expression of *NAP*, *SAG113*, and *WRKY6* defines that plant's tolerance to Ca^{2+} stress, with enhanced transcription of these genes and subsequently increased total phenolic content and DPPH radical scavenging effects leading to reduced ethylene content and delayed senescence in plants. A high level of total phenol content and DPPH radical scavenging activity may result from the down-regulated expressions of ACS7, NAP, SAG113, and WRKY6 genes, which could eliminate ethylene induced $\bullet O^{2-}$, and these genes in 300K- and Ca^{2+}-treated plants were involved in $\bullet O^{2-}$ detoxification and thus helped overcome ethylene-induced stress. The 300K- and Ca^{2+}-treated plants with higher total phenol levels and DPPH radical scavenging activity were benefitted by an increased capacity in their ROS-scavenging system. The ethylene-induced transcriptional activation of NAP, SAG113, and WRKY6 genes corresponded to an increase in total phenol content and DPPH radical scavenging activity, which protected cellular components against the effects of ROS and ethylene. This will be helpful for precisely controlling ethylene production and signaling to enhance salinity tolerance and improve the agronomic traits of crops. Although *ACS7, NAP, SAG113*, and *WRKY6* play major roles in the biosynthesis of senescence ethylene and elucidate underlying mechanisms, how plants coordinate their ethylene biosynthesis with leaf senescence in the post-transcriptional regulation remains an area for further work.

Conclusions

Many lands and fields are located in coastal areas, and diluted SW can be considered a free local resource for possible use as irrigation. In present study, we found that the increased TTC activity in 300K SW suggests the enhance tissue cell viability of At plants. The 300K SW could down-regulation ACS7 gene and significantly reduced the ethylene content, but remarkably increased Chl, phenol, and DPPH analyses and strengthened the salt tolerance. In addition, Mg^{2+}, Na^{+}, K^{+}, Ca^{2+}, and 300K displayed lower levels of $\bullet O^{2-}$ accumulation in leaves. The induction of ACS7-generated ethylene for leaf senescence was countered by NAP, SAG113, and WRKY6-dependent Ca^{2+}, Mg^{2+}, and Na^{+} accumulation; moreover, phenol content increased and NAP, SAG113, and WRKY6-induced leaf senescence occurred in non-senescing leaves. Nevertheless, K^{+} treatment inhibited SGA113 gene expression, resulting in reducing ACS7 gene expression and ethylene content. The increased tolerance to salt by At plants that down-regulated the ACS7, NAP, SAG113, and WRKY6 genes suggests that these genes may be helpful in decreasing the ethylene content. The use of 300K SW caused a delay in leaf senescence, and this may be the primary cultural use for Ca^{2+}. The characterization and functional analysis of these genes should facilitate our understanding of ethylene response mechanisms in plants.

Authors' Contributions

CMC conceived and designed the experiments; WJC, MYH, and SFP performed the experiments; YSC, HCW, and HHL analyzed and interpreted the data; KHL and CMC prepared, wrote, and reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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