Pak. J. Bot., 53(1): 23-30, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-1(4)

LeERF-1, AN ETHYLENE RESPONSE FACTOR GENE FROM *LITHOSPERMUM ERYTHRORHIZON*, CONFERS ENHANCED TOLERANCE TO COLD AND SALT STRESSES IN *ARABIDOPSIS*

HUA ZHAO[1,5†], RONGJUN FANG[1,3†], ZHAOYUE WANG[1], AIQIAN LI[2], SHOUCHENG HUANG[1], JIANGYAN FU[1], ZHONGLING WEN[1], MINKAI YANG[1], BAO LIU[4], GUIHUA LU[1], RUNAN TIAN[2*], JINLIANG QI[1,2*] AND YONGHUA YANG[1,2*]

[1]*State Key Laboratory of Pharmaceutical Biotechnology, Institute of Plant Molecular Biology, School of Life Sciences, Nanjing University, Nanjing 210023, P. R. China*
[2] *College of Landscape Architecture, Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, P. R. China*
[3]*Jiangsu University of Science and Technology, Zhenjiang 212003, P. R. China*
[4]*Key Laboratory of Molecular Epigenetics of the Ministry of Education (MOE), Northeast Normal University, Changchun 130024, P. R. China*
[5]*School of Life Sciences, Nantong University, Nantong 226019, P. R. China*
**Corresponding author's email: yangyh@nju.edu.cn; qijl@nju.edu.cn; tianrunan@njfu.edu.cn*
[†]Theses authors contributed equally to this work

Abstract

The ethylene responsive transcription factors (ERFs) play various functions in the processes of plant growth, development and myriad stress responses. We previously reported one light-regulated gene, *LeERF-1*, possibly paticipated in biosynthesis of secondary metabolites in medicinal plant *Lithospermumerythrorhizon*. Here, we further reported the function of *LeERF-1* in cold and salt stress resistance by heterologous overexpressing in *Arabidopsis*. After cold treatment, the *LeERF-1* overexpression lines (OE) exhibited better phenotype, higher survival rates, highersuperoxide dismutase (SOD) and peroxidase (POD) activities than that of the wild type (WT); while the malondialdehyde (MDA) concentrations of OE lines were significantly lower than that of WT. Exposing to 200 mM NaCl stress treatment, the germination and survival rates of OE lines were also remarkably higher than that of WT; The leaves of OE lines maintained relative lower proline content and had significantly lower Na^+/ K^+ ratio compared to that of WT. These findings collectively indicate that over-expression of *LeERF-1* confers improved cold and salt resistance in transgenic *Arabidopsis.*

Key words: *L. erythrorhizon, Arabidopsis*, *LeERF-1*, Heterologous overexpression, Stress tolerance.

Introduction

Cold and soil salinity are two major abiotic stresses that limit the growth, productivity, and geographical distribution of plants. The cold-induced plant injury is a consequence of cell membrane lesions which are primarily caused by cellular dehydration and protein denaturation (Steponkus, 2003; Yamazakia *et al*., 2003; Huo *et al.,* 2016). The harmful effects of high salt on plants are thought to result from osmotic stress induced water deficit and critical biochemical processes caused by excess sodium ions (Vogelien *et al*., 1996; Apse *et al*., 1999; Ma *et al*., 2012). The nonmotile plants have evolved themselves precise and complicated regulatory mechanisms to cope with various environmental stimuli at tissue-specific, cellular, physiological, cellular and molecular levels. To present, much efforts have been devoted toward understanding the plant adaptive mechanism to cold and salt tolerance.

Activation of multiple stress-specific genes is considered as a pivotal mechanism in promoting environmental adaptation, where the genes confer the metabolic adjustments of plants under unfavorable growth environments ultimately (Khan *et al.*, 2013; Mishra *et al*., 2018; Liu *et al*., 2019). The apetala2/ethylene response factor (AP2/ERF) belongs to one of the largest transcription factor families possessing diverse functions during the plant life cycle. As a subfamily of AP2/ERF, ethylene response factor (ERF) is plant specific transcription factor with a highly conserved region DNA-binding domain about 70 amino acids (Okamuro *et al*., 1997; Mizoi *et al*., 2012). To date, ERFs have been proved to exert the roles in regulating the growth and development of plants, as well as responding to vairous abiotic stresses including drought, salt and cold, and also responding to biotic stresses including pathogen infections (Dietz *et al*., 2010; Zhang *et al*., 2012; Cheng *et al*., 2013; Debbarma *et al*., 2019; Fang *et al*., 2019). In *Arabidopsis*, *AtERFs* are differentially regulated by abiotic stress conditions and positively or negatively regulate GCC box–mediated gene expression through responding to extracellular signals (Fujimoto *et al*., 2000).

We previously cloned a full-length cDNA of *LeERF-1* which was classified as a member of B3 subfamily of AP2/ERF family from *Lithospermumerythrorhizon*. We also found that *LeERF-1* was light-regulated and might be involved ethylene regulated biosynthesis of shikonin, a kind of useful medical secondary metabolite specifically accumulated in the roots of *L. erythrorhizon* (Zhang *et al*., 2011; Fang *et al*., 2016).

Evidences indicate that the production of secondary metabolites was co-related with the protection of plants against a wide variety abotic and biotic stresses (Bartwal *et al*., 2013). However, no significant evidence has been provided for the role of *LeERF-1* in stress responses. Therefore, deciphering the regulatory role of *LeERF-1* on abiotic stress is of fundamental importance to further understand the function of *LeERF-1* on the ethylene-regulated formation of shikonin in *L. erythrorhizon.* However, the exact role of *LeERF-1* in the stress response has still not been fully understood yet. In this paper, the role of *LeERF-1* involving in cold and salt stresses in transgenic *Arabidopsis* plants was further clarified. Our work might facilitate the understanding of the complex relationship between stress-associated genes and shikonin

formation in *L. erythrorhizon* and lay a foundation for improving shikonin production through a transgenic strategy in future.

Materials and Methods

Plant materials and growth conditions: All of the *LeERF-1* overexpression lines (OE) in this study were derived from the wild-type (WT) *Arabidopsis thaliana* Columbia ecotype (Col-0) (Zhang *et al*., 2011). Three randomly selected transgenic *Arabidopsis* lines exhibited 100% kanamycin-resistant progenies in the T3 or T4 generation (OE-9, OE-29, and OE-57) were considered homozygous and used as materials in this study. Seeds of the WT and OE lines were surface-sterilized using 70% ethanol, and then rinsed with sterile distilled water for more than three times. The sterilized seeds were stratified under 4°C for 2 days and germinated on 1/2 MS medium in growth chambers under 16-h-light/8-h-dark cycle at 23°C with 80% relative air humidity. *LeERF-1* overexpression of thetransgenic plants was confirmed by both PCR analysis and subcellular localization of LeERF-1 protein analysis as we previously reported (data not shown) (Zhang *et al*., 2011). Adult plants were cultivated in a chamber under continuous illumination at 23°C with a photoperiod of 16h light/8h dark.

Cold tolerance assay: The WT and OE seedlings of about four-week-old were transferred into cold stress conditions at -10°C for 0 h, 2 h, 4 h, 6 h and 8 h. Leaves were collected for measurement of physiological parameters. The SOD activity assay was based on calculated according to inhibition of nitrobluetetrazolium (NBT) reduction (Durak *et al*., 1993). The POD enzyme activity was determined using guaiacol as a phenolic substrate (Maksimović *et al*., 2012; Wang *et al*., 2019*a*). A thiobarbituric acid (TBA) method for determining free MDA (Schmedes & Holmer, 1989; Janero, 1990).

Seedlings in the low temperature incubator of -10°C at the time points of 0 h and 4 h were then transferred into the normal growth condition of *Arabidopsis* for 6 d for phenotype observation and the calculation of survival ratio.

Salt tolerance assay: The WT and OE seedlings of about four-week-old grown in potting soil were treated with 200 mM NaCl for13 d, followed by resumption of normal conditions for 7 d, and the phenotypes of WT and OE seedlings under salt stress treatment were observed.

Seeds of WT and OE lines were germinated on 1/2 MS medium containing 200 mM NaCl, and the germination rates were calculated from 1 d to 5 d.

The seedlings growing in soil were collected to detect the proline content and analyze the ion of Na^+/K^+ (Bates *et al*., 1973; Wang *et al*., 2019a). WT plants and OE lines were irrigated with 200 mM NaCl solution or ddH_2O (CK) for 10 d, then the fresh leaves were collected. For the measurement of proline content, 0.2 g~0.3 g sample was homogenized in 6 ml of 3% salicylsulfonic acid and centrifuged at 3000 g for 5 min. The proline content was determined according to Bates' method. (1973). The proline contents were quantified by using the standard curve based on peaks of standard proline （R^2=0.992）and determined at 520 nm. All contents of proline were from at least three replicates.

To determine the contents of Na^+ and K^+, 0.2 g samples above were selected and dried. Then Na^+ and K^+ of samples were released by using 10 ml of 0.1 M HCl (Zhang *et al*., 2012). The Na^+ and K^+concentrations of the final solutions were determined on inductively coupled plasma (ICP, Perkin Elmer, Optima 5300DV).

Results

Overexpression of LeERF-1 confers enhanced cold tolerance: To test whether the transgenic *Arabidopsis* of *LeERF-1* could tolerate low temperature stress, we performed phenotype observation for the seedlings of WT and three OE lines after subjected into the cold stress condition (-10°C).

Under cold treatment, the WT seedlings exhibited more serious damage compared to the OE lines, which displayed enhanced freezing tolerance (Fig. 1a). A higher survival rate was observed in OE lines compared to WT after transferred the plants from cold stress conditions into normal growth condition of *Arabidopsis* for 2 days (Fig. 1b). When plants were exposed to treatment at -10°C for 4 h, followed by recovery at normal growth condition of *Arabidopsis* for 6 days, the OE seedlings grew well and displayed significantly higher survival rates in comparison to WT (by 1.6 to 2.8-fold) (p <0.01).

The activities of antioxidant enzymes of SOD were determined in WT and OE lines during the cold treatment. The activity of SOD in leaves increased when transferred these plants into the low temperature incubator by exposure to -10°C and reached highest level at the time point of 4 h, then it decreased gradually in all lines, reached the lowest level at the time-point 8 h-cold exposure. On the other hand, there was no significant difference between WT and OE lines in the control conditions (0 h), whereas the activities of SOD were sharply higher in the OE lines when the plants were exposed to cold stress at 2 h, 4 h, 6 h and 8 h (p <0.01) (Fig. 1c).

The activities of POD in leaves exhibited much similar changes to SOD under cold stress (Fig. 1d). We also noticed that the OE lines displayed higher POD activities relative to WT. The highest POD enzyme activity values were also observed when plants were transferred into the low temperature incubator at -10°C for 4 h. The activities of POD were remarkably higher in the OE lines in comparison to WT when the plants experienced cold stress at 2 h, 4 h, 6 h and 8 h (p<0.01). Thus, SOD and POD activities observed in cold stress-tolerance might be an indicator of these genotypes. It seems possible that *LeERF-1* may play important role in the acclimation or protection of plant under cold stress condition.

MDA contents were also investigated using the leaves of WT and three transgenic plants above. Before the cold stress treatments, MDA contents did not show significant differences in OE lines compared to WT (p＞ 0.05) (Fig. 1e). The MDA concentrations kept on increasing with the prolongation of low temperature stress. At each time point of 2h, 4h, 6h and 8h, the transgenic plants OE-9, OE-29, and OE-57 showed lower MDA content under cold stress in comparison to WT (p<0.01). This result was much similar to that in other reports (Tian *et al*., 2011; Dahro *et al*., 2016), indicating that the accumulation of MDA might closely related to the adaptability of plants under cold stress condition.

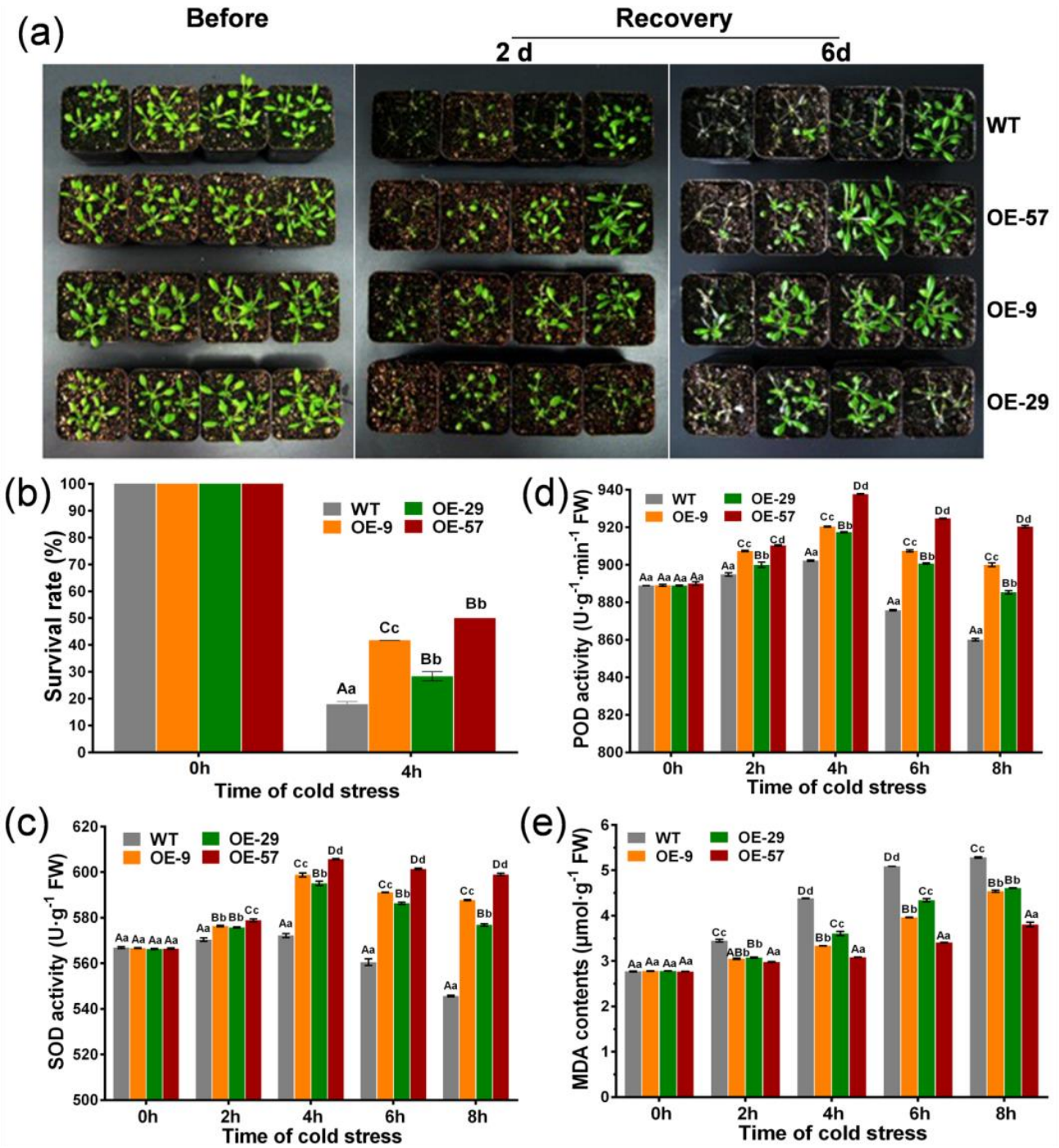

Fig. 1. Cold tolerance treatments of the transgenic plants. (a) The freezing phenotype of wild type and transgenic *LeERF-1 Arabidopsis*. The plants were exposed to treatment at -10°C for 4 h, and then treated at 22°C for 2 d and 6 d. The survival rates (b), SOD activity (c), POD activity (d) and the changes of MDA contents (e) of WT and OE lines under low temperature stress. The values are means ± SD (n =3) and the bars with different letters indicate significant difference at $p<0.05$ (*lower case letters*) or $p<0.01$ (*capital letters*), respectively (Least Significant Difference).

Overexpression of LeERF-1 confers enhanced salt tolerance: Similar to drought, salinity is also a common abiotic factor that causes ionic and osmotic stresses during the plant growth. Recent researches have demonstrated that the AP2/ERF family extensively participated in the regulation of plant response to salt stresses (Cheng *et al*., 2013; Liu *et al*., 2014; Xing *et al*., 2017). We then asked whether *LeERF-1* was related to the salt stress. To examine the effects of *LeERF-1* overexpression in responding to salt stress, seedlings of WT and OE lines of four-week-old were cultured on soil supplemented with 200 mM NaCl. Significant phenotypic differences between WT plants and OE lines were observed after 13 days of 200 mM NaCl treatment. All seedlings of WT failed to survive. By contrast, most OE lines survived and displayed not severe symptoms of salt-associated phenotypes, such as leaf yellowing and wilting (Fig. 2a).

The germination rates of WT and *LeERF-1*transgenic *Arabidopsis* seeds were detected to investigate the role of *LeERF-1* in salt stress. No significant differences of

germination rate between WT and the OE lines on the medium of 1/2 MS were observed (p<0.01) (Fig. 2b). Germination of the wild type was inhibited thoroughly within 5 d under the stress condition of 200 mM NaCl. However, the germination rates of the seeds from OE-9 and OE-57 remained over 74% in the same case, significantly higher than that of the other transgenic line of OE-29, which germination rates ranged from 15% to 22%. Even so, seeds from transgenic lines had evidently higher germination rates on medium containing 200 mM NaCl from 1 d to 5 d compared to that of WT. The results above demonstrate that heterologous overexpression of *LeERF-1* effectively enhanced salt tolerance and greatly increased germination rate upon salt treatment for 5 days.

Since proline accumulation plays a highly protective role in plant cellular structures against osmotic stress, and the proline is an important marker to assess cell membrane integrity caused by salt stress in plants (Ueda *et al*., 2007; Cha-Um & Kirdmanee, 2009; Wang *et al*., 2019*b*), we next examined the proline contents in WT and transgenic *Arabidopsis* seedlings to further explore the role of *LeERF-1* in salt tolerance. The leaves of WT and OE lines demonstrated no significant difference of proline content under normal growth conditions. The levels of proline accumulation increased significantly in all lines under the treatment of 200 mM NaCl (p<0.01), indicating that the accumulation of proline is a consequence of salt stress but lack the direct co-relationship with overexpression of *LeERF-1* (Fig. 3a). However, transgenic *Arabidopsis* seedlings showed significantly lower levels of proline contents compared to wild type plants (p<0.01). This result indicated that the OE lines could maintain relative lower proline content under salt stress compared with that of WT plants.

A good strategy to survive under salt stress condition is to maintain cellular Na^+/K^+ homeostasis in plants (Vogelien *et al*., 1996; Ma *et al*., 2012). To evaluate the physiological changes in transgenic plants, the effects of salt stress on Na^+/K^+ of WT and transgenic *Arabidopsis* seedlings were also examined.

Under normal growth conditions, K^+ and Na^+ levels in the OE lines did not show significant differences in OE lines compared to the control of WT (p＞0.05) (Fig. 3b and 3c). Under the treatment of 200 mM NaCl, the K^+ levels declined dramatically in WT plants, and significant difference was found in the comparisons of OE-9 and OE-57 versus WT (p<0.05). Much similar to that of the proline contents, the Na^+ contents significantly increased in all tested plants; moreover, the OE lines had a significantly lower Na^+ and Na^+/K^+ ratio than that of WT (p<0.01) after treatment with 200 mM NaCl (Fig. 3d). This result is much more similar to studies from other plants (Chen *et al*., 2014; Gao *et al*., 2016), indicating that heterologous over expression of *LeERF-1*affects the Na^+ and K^+ contents, as well as the Na^+/K^+ ratio in the leaves of plant.

Discussion

AP2/ERF transcription factors have been widely studied in plant physiological and developmental processes (Dietz *et al*., 2010; Zhang *et al*., 2012; Cheng *et al*., 2013), however, the roles of *LeERF-1* in abiotic stress (eg. cold and salt) responses are largely unknown in the medicinal plant *L. erythrorhizon.*

It has been demonstrated that plants accumulate various compounds such as proline, glucose, polyolsmannitol, etc., for osmoregulation and protecting enzyme activity under extreme stresses (Shulaev *et al*., 2008; Cha-Um and Kirdmanee, 2009). The activation of antioxidant enzyme systems is very important for making plants tolerant to extreme environment (Sarkar *et al*., 2018). Some antioxidant enzymes such as peroxidase (POD) and supperoxide dismutase (SOD) are considered to be involved in plant stresses and frequently used as indicators of cold and salt stresses in plants (Ping *et al*., 2008; Wang *et al*., 2009; Fernández-Ocaña *et al*., 2011; Hu *et al*., 2013; Wei *et al*., 2015). MDA is a lipid peroxidation product (Janero, 1990). Cold stress often causes damage to cell membranes. MDA levels were used to assess the extent of membrane injuries caused by oxidative stress (Weber *et al*., 2004; Meloni & Martínez, 2009; Maiti *et al*., 2012) and other stresses, including cold stress (Distelbarth *et al*., 2013; Liu *et al*., 2013). Therefore, the MDA contents and the activities of antioxidant enzymes of SOD and POD were used as indicators to determine whether *LeERF-1* confers cold tolerance in our current study.

In the present study, differential cold acclimation capacities of seedlings were observed among the tested lines. The OE lines grew well and exhibited significantly higher survival rates in comparison to the WT control after cold treatment (Fig. 1a and 1b). The cold tolerance phenotype of *LeERF-1* transgenic plants was consistent with the following physiological parameters, such as SOD, POD and MDA contents.

In the cold stress tolerance assay, the activities of SOD were notably higher in the OE lines in comparison to WT under cold stress at different time points of 2 h, 4 h, 6 h and 8 h (Fig. 1c). Much more similar changes of POD activities were observed in the leaves of all tested lines (Fig. 1d). The highest POD enzyme activity values were also observed at the time point 4 h when plants cultured in -10°C. The OE lines showed significantly higher activities of POD in comparison with WT when the plants experienced cold stress within 8 h. The MDA concentration kept on increasing with the prolongation of low temperature stress. We noticed that all OE lineshad significantly lower MDA levels at each time point of 2 h, 4 h, 6 h and 8 h compared to WT, implying that the transgenic lines suffered lower degrees of stress damages (Fig. 1e). It was much similar to that in other reports (Tian *et al*., 2011; Dahro *et al*., 2016) and supported the better cold acclimation capacities of *LeERF-1*-overexpression *Arabidopsis* lines compared with WT. These studies collectively suggest that overexpression of *LeERF-1* confers enhanced cold tolerance intransgenic *Arabidopsis.*

By heterologous overexpression of *LeERF-1* in *Arabidopsis*, we also investigated its role in the physiological adaptation mechanisms of salt stress. All seedlings of WT failed to survive after 200 mM NaCl treatment for 13 days. By contrast, most OE lines survived and displayed no severe symptoms of salt-associated phenotypes (Fig. 2a). Moreover, the germination of WT was inhibited thoroughly within 5 days on the 1/2 MS medium supplemented with 200 mM NaCl (Fig. 2b). However, the germination rates of OE lines significantly higher than that of WT within 5 days under the stress condition of 200 mM NaCl.

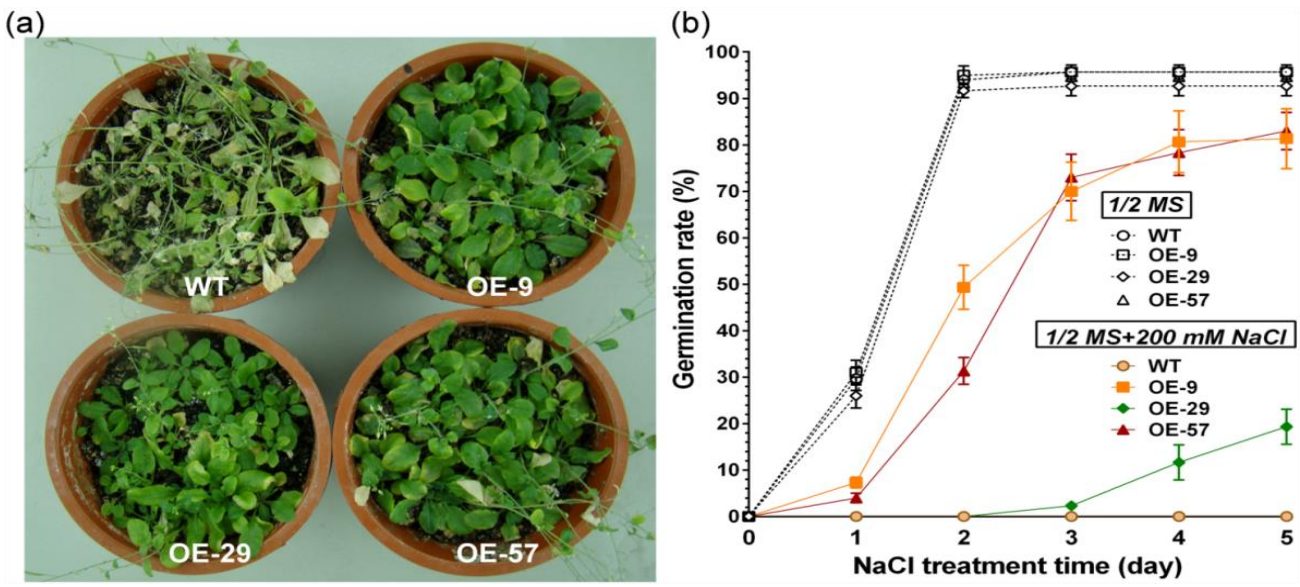

Fig. 2. Salt stress tolerance assay of the transgenic plants. (a) The phenotypes of WT and OE lines under salt stress treatment. The seedlings of four-week-old were treated with 200 mM NaCl for 13 d, followed by normal growth condition of *Arabidopsis* for 7 d (recover). (b) The germination rates of WT and *LeERF-1*transgenic *Arabidopsis* seeds under normal or salt stress. The values are means ± SD (n =3); No remarkable difference of germination rate was found between WT and OE lines from 1 d to 5 d (Least Significant Difference, $p>0.05$) when seedlings cultured in 1/2 MS medium. Significant difference was found in the comparisons of OE-9 and OE-57 versus WT from 1 d to 5 d, and OE-29 versus WT from 3 d to 5 d (Least Significant Difference, $p<0.01$) when seedlings cultured in 1/2 MS medium supplemented with 200mM NaCl (Least Significant Difference, $p<0.01$).

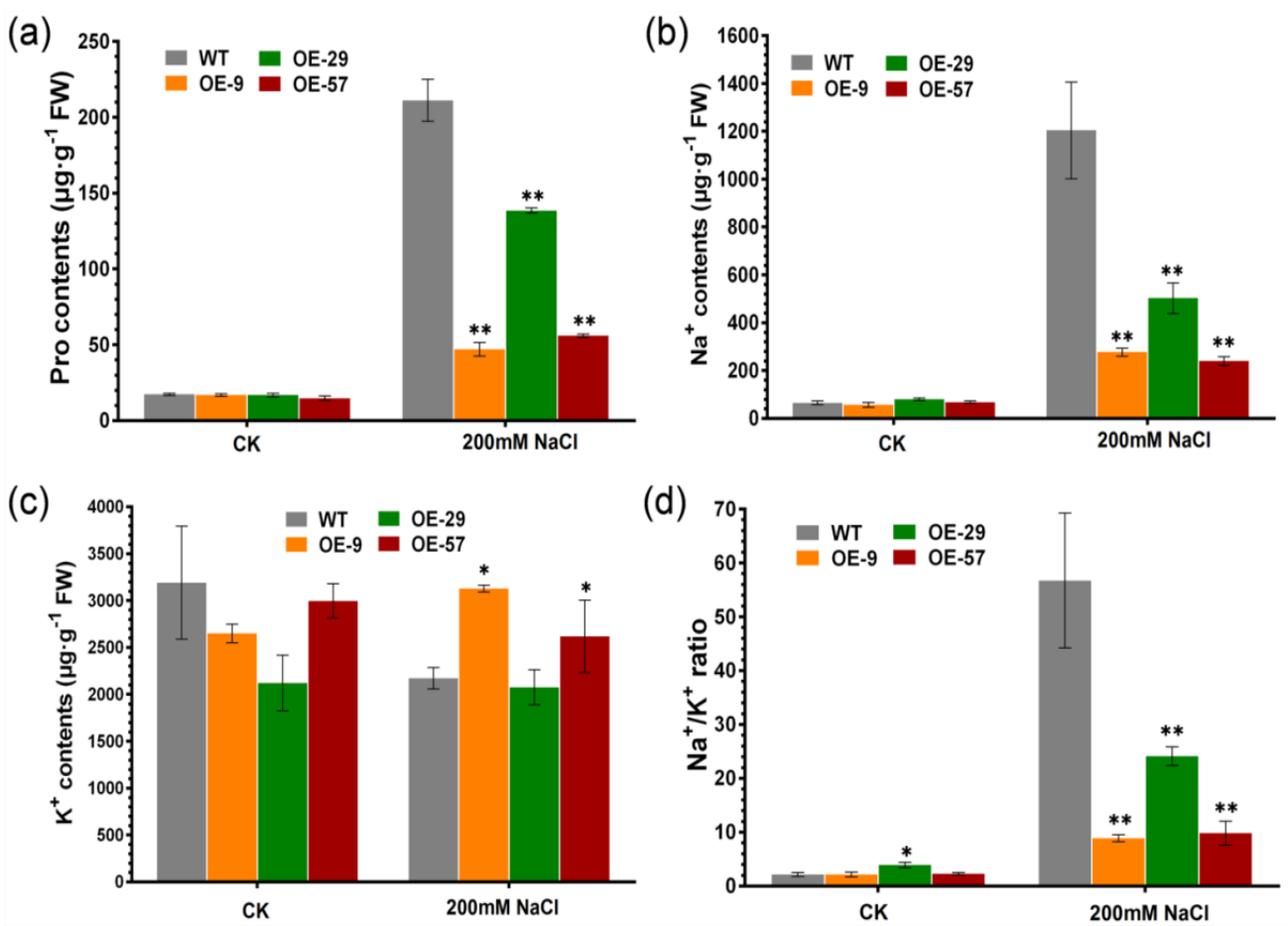

Fig. 3. Determination of proline accumulation and Na^+/K^+ contents under the treatment of 200 mM NaCl. (a) The comparison of proline content in WT and *LeERF-1* transgenic *Arabidopsis* plants with or without salt treatment. The effects of heterologous overexpression of *LeERF-1* on the changes of Na^+ content (b), K^+ content (c), and Na^+ to K^+ ratio (d) under salt stress. Four-week-old WT and OE plants grown under normal condition were irrigated with 200 mM NaCl solution for 10 d. The asterisk indicates that the mean values in the OE lines were sharply different from that of WT (Student's t-test, ** $p<0.01$).

Salt injury to plants can be estimated by the relative contents of proline and Na^+/K^+ ratio. Interestingly, the transgenic *Arabidopsis* seedlings of OE lines showed significantly lower levels of proline contents compared to WT plants ($p<0.01$) (Fig. 3a). This result indicated that the OE lines could maintain relative lower proline content under salt stress compared with that of WT plants. We presumed that there might be a serial of salt-tolerant mechanisms resulting in producing low content of proline in the OE lines, and the accumulation of proline is controlled by multiple factors, such as phytohormones like gibberellins, abscisic acid, salicylic acid and jasmonates (Iqbal *et al*., 2014); it is not the absolute amount of proline, but the homeostasis of proline and other factors that determines a plant's ability to survive under salt stress condition. However, further researches are needed to reveal the correlation between proline level and *LeERF-1* under salt stress condition.

As one of the most important physiological parameters, the effects of salt stress on the contents of Na^+ and K^+ in WT and OE lines were also examined (Fig. 3b and 3c). After treatment with 200 mM NaCl, the Na^+ contents significantly increased in all tested plants; however, the OE lines had a significantly lower Na^+ and Na^+/K^+ ratio than WT plants ($p<0.01$) (Fig. 3d).

It is noteworthy that the Na^+/K^+ homeostasis (Na^+/K^+ ratio) rather than the absolute content of Na^+ or K^+ that determines plants' capicity to survive under salt stress condition. The intracellular Na^+/K^+ homeostasis is crucial for cell metabolism and is closely related to plant salt sensitivity (Ma *et al*., 2012; Peng *et al*., 2014). We also noticed that the OE lines could maintain significantly lower level of Na^+ contents and Na^+/K^+ ratio than that of the WT ($p<0.01$) after treatment with 200 mM NaCl (Fig. 3d), indicating that maintaining relative lower Na^+ contents and Na^+/K^+ ratio may also be another aspect of the salt-tolerant mechanism for the OE lines, which in turn helps them adapt to salt stress. These results implied that overexpression of *LeERF-1* confers enhanced salt tolerance is possibly the consequence of more effectively Na^+/K^+ homeostasis in the OE lines. However, further researches are needed to reveal whether the lower level of Na^+ contents or Na^+/K^+ ratio is a direct occurrence of *LeERF-1*-overexpression in the transgenic *Arabidopsis*.

As one kind of traditional medicinal plant, *L. erythrorhizon* can biosynthesize large amounts of medicinal secondary metabolites, shikonin and its derivatives, in the root tissues. These red naphthoquinone pigments have valuable uses in the treatment of human diseases (Chen *et al*., 2011). The molecular mechanism of shikonin biosynthesis has been well characterized based on the analyses of diverse shikonin biosynthesis-related regulators, such as light (Yazaki*et al*., 1999), mineral elements (Deno *et al*., 1987) and phytohormones (Touno *et al*., 2005).

Previous studies revealed that *LeERF-1* was light-regulated and might be involved ethylene regulated biosynthesis of shikonin (Fang *et al.,* 2016; Zhang *et al*., 2011). Since plant secondary metabolism is also regulated by abotic and biotic stress factors (Bartwal *et al*., 2013), our study on the role of *LeERF-1* under cold and salt stress will contribute to understand the relationship between the genetic control and secondary metabolism in *L. erythrorhizon*. Further research on the definite function of *LeERF-1* in respose to abiotic stresses should be made to provide direct evidences for its role on shikonin biosynthesis.

Conclusion

The heterologous overexpression of *LeERF-1* in *Arabidopsis* reveals it confers enhanced cold and salt tolerance and drought stresses. Our results not only present stress-related gene which have great potential to be used in genetic engineering for improvement of stress tolerance of plant, but also lay a foundation for the understanding of the relationship between stress tolerance and secondary metabolite biosynthesis of shikonin in the non-model plant *L. erythrorhizon* in the future.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (31970321, U1903201, 31771413, 31670298), the Open Project Program from MOE Key Laboratory of Molecular Epigenetics of China, and the Program for Changjiang Scholars and Innovative Research Team in University from the Ministry of Education of China (IRT_14R27), and the Natural Science Foundation of Jiangsu Bureau of Science and Technology (BK20191254).

References

Apse, M.P., G.S. Aharon, W.A. Snedden and E. Blumwald. 1999. Salt tolerance conferred by overexpression of a vacuolar

Bartwal, A., R. Mall, P. Lohani, S.K. Guru and S. Arora. 2013. Role of secondary metabolites and brassinosteroids in plant defense against environmental stresses. *J. Plant Growth Regul.*, 32(1): 216-232.

Bates, L.S., R.P. Waldren and I.D. Teare. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil*, 39(1): 205-207.

Cha-Um, S. and C. Kirdmanee. 2009. Effect of salt stress on proline accumulation, photosynthetic ability and growth characters in two maize cultivars. *Pak. J. Bot.*, 41(1): 87-98.

Chen, H., X. Chen, H. Gu, B. Wu, H. Zhang, X. Yuan and X. Cui. 2014. GmHKT1;4, a novel soybean gene regulating Na^+/K^+ ratio in roots enhances salt tolerance in transgenic plants. *Plant Growth Regul.*, 73(3): 299-308.

Chen, J., J. Xie, Z. Jiang, B. Wang, Y. Wang and X. Hu. 2011. Shikonin and its analogs inhibit cancer cell glycolysis by targeting tumor pyruvate kinase-M2. *Oncogene*, 30(42): 4297-4306.

Cheng, M.C., P.M. Liao, W.W. Kuo and T.P. Lin. 2013. The *Arabidopsis* ETHYLENE RESPONSE FACTOR1 regulates abiotic stress-responsive gene expression by binding to different cis-acting elements in response to different stress signals. *Plant Physiol.*, 162(3): 1566-1582.

Dahro, B., F. Wang, T. Peng and J.H. Liu. 2016. *PtrA/NINV* , an alkaline/neutral invertase gene of *Poncirus trifoliata*, confers enhanced tolerance to multiple abiotic stresses by modulating ROS levels and maintaining photosynthetic efficiency. *BMC Plant Biol.*, 16(76): 1-18.

Debbarma, J., Y.N. Sarki, B. Saikia, H.P.D. Boruah, D.L. Singha and C. Chikkaputtaiah. 2019. Ethylene response factor (ERF) family proteins in abiotic stresses and CRISPR–Cas9 genome editing of ERFs for multiple abiotic stress tolerance in crop plants: A review. *Mol. Biotechnol.*, 61(2): 153-172.

Deno, H., C. Suga, T. Morimoto and Y. Fujita. 1987. Production of shikonin derivatives by cell suspension cultures of *Lithospermum erythrorhizon*. *Plant Cell Rep.*, 6(3): 197-199.

Dietz, K.J., M.O. Vogel and A. Viehhauser. 2010. AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signalling. *Protoplasma*, 245(1-4): 3-14.

Distelbarth, H., T. Nägele and A.G. Heyer. 2013. Responses of antioxidant enzymes to cold and high light are not correlated to freezing tolerance in natural accessions of *Arabidopsis thaliana*. *Plant Biol.*, 15(6): 380-383.

Durak, I., Z. Yurtarslanl, O. Canbolat and O. Akyol. 1993. A methodological approach to superoxide dismutase (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. *Clin. Chim. Acta*, 214(1): 103-104.

Fang, R., A. Zou, H. Zhao, F. Wu, Y. Zhu, H. Zhao, Y. Liao, R.J. Tang, Y.Pang, R. Yang, X. Wang, J. Qi, G. Lu and Y. Yang. 2016. Transgenic studies reveal the positive role of *LeEIL-1* in regulating shikonin biosynthesis in *Lithospermum erythrorhizon* hairy roots. *BMC Plant Biol.*, 16(121): 1-12.

Fang, R.J., A.Q. Li, R.N. Tian, W.J. Zhang, A.L. Zou, F.Y. Wu, Y.H. Liao, X.M. Wang, Y.J. Pang, R.W. Yang, G.H. Lu, J.L. Qi and Y.H. Yang. 2019. Heterologous overexpression of *Lithospermum erythrorhizon LeERF-1* gene increases drought and pathogen resistance in Arabidopsis. *Acta Physiol. Plant.*, 41(2): 22-30.

Fernández-Ocaña, A., M. Chaki, F. Luque, M.V. Gómez-Rodríguez, A. Carreras, R. Valderrama, J.C. Begara-Morales, L.E. Hernández, F.J. Corpas and J.B. Barroso. 2011. Functional analysis of superoxide dismutases (SODs) in sunflower under biotic and abiotic stress conditions. Identification of two new genes of mitochondrial Mn-SOD.*J. Plant Physiol.*, 168(11): 1303-1308.

Fujimoto, S.Y., M. Ohta, A. Usui, H. Shinshi and M. Ohme-Takagi. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell*, 12(3): 393-404.

Gao, J., J. Sun, P. Cao, L. Ren, C. Liu, S. Chen, F. Chen and J. Jiang. 2016. Variation in tissue Na^+ content and the activity of *SOS1* genes among two species and two related genera of Chrysanthemum. *BMC Plant Biol.*, 16(98): 1-15.

Hu, K.L., L. Zhang, J.T. Wang and Y. You. 2013. Influence of selenium on growth, lipid peroxidation and antioxidative enzyme activity in melon (*Cucumis melo* L.) seedlings under salt stress. *Acta Soc. Bot. Pol.*, 82(3): 193-197.

Huo, C., B. Zhang, H. Wang, F. Wang, M. Liu, Y. Gao, W. Zhang, Z. Deng, D. Sun and W. Tang. 2016. Comparative study of early cold-regulated proteins by two-dimensional difference gel electrophoresis reveals a key role for phospholipase Dα1 in mediating cold acclimation signaling pathway in rice. *Mol. Cell. Proteomics*, 15(4): 1397-1411.

Iqbal, N., S. Umar, N.A. Khan and M.I.R. Khan. 2014. A new perspective of phytohormones in salinity tolerance: Regulation of proline metabolism. *Environ. Exp. Bot.*,100(2): 34-42.

Janero, D.R. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Rad. Biol. Med.*, 9(6): 515-540.

Khan, F., T. Sultana, F. Deeba and S.M.S. Naqvi. 2013. Dynamics of mRNA of glycine-rich RNA-binding protein during wounding, cold and salt stresses in Nicotiana tabacum. *Pak. J. Bot.*, 45(SI): 297-300.

Liu, M., Y. Li, Y. Ma, Q. Zhao, J. Stiller, Q. Feng, Q. Tian, D. Liu, B. Han and C. Liu. 2019. The draft genome of a wild barley genotype reveals its enrichment in genes related to biotic and abiotic stresses compared to cultivated barley. *Plant Biotechnol. J.*, 18(2): 443-456.

Liu, W., K. Yu, T. He, F. Li, D. Zhang and J. Liu. 2013. The low temperature induced physiological responses of *Avena nuda* L., a cold-tolerant plant species. *Sci. World J.*, 2013(1): 1-7.

Liu, W., Y. Wang and C. Gao. 2014. The ethylene response factor (ERF) genes from *Tamarix hispida* respond to salt, drought and ABA treatment. *Trees–Struct. Funct.*, 28(2): 317-327.

Ma, L., H. Zhang, L. Sun, Y. Jiao, G. Zhang, C. Miao and F. Hao. 2012. NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na^+/K^+ homeostasis in *Arabidopsis* under salt stress. *J. Exp. Bot.*, 63(1): 305-317.

Maiti, S., N. Ghosh, C. Mandal, K. Das, N. Dey and M.K. Adak. 2012. Responses of the maize plant to chromium stress with reference to antioxidation activity. *Braz. J. Plant Physiol.*, 24(3): 203-212.

Maksimović, J.D., M. Mojović, V. Maksimović, V. Römheld and M. Nikolic. 2012. Silicon ameliorates manganese toxicity in cucumber by decreasing hydroxyl radical accumulation in the leaf apoplast. *J. Exp. Bot.*, 63(7): 2411-2420.

Meloni, D.A. and C.A. Martínez. 2009. Glycinebetaine improves salt tolerance in vinal (*Prosopis ruscifolia* Griesbach) seedlings.*Braz. J. Plant Physiol.*, 21(3): 233-241.

Mishra, P., N. Singh, A. Jain, N. Jain, V. Mishra, G. Pushplatha, K.P. Sandhya, N.K. Singh and V. Rai. 2018. Identification of cis-regulatory elements associated with salinity and drought stress tolerance in rice from co-expressed gene interaction networks. *Bioinformation*, 14(03): 123-131.

Mizoi, J., K. Shinozaki and K. Yamaguchi-Shinozaki. 2012. AP2/ERF family transcription factors in plant abiotic stress responses. *BBA-Gene Regul. Mech.*, 1819(2): 86-96.

Okamuro, J.K., B. Caster, R. Villarroel, M.V. Montagu and K.D. Jofuku. 1997. The AP2 domain of *APETALA2* defines a large new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, 94(13): 7076-7081.

Peng, J., Z. Li, X. Wen, W. Li, H. Shi, L. Yang, H. Zhu and H. Guo. 2014. Salt-induced stabilization of EIN3/EIL1 confers salinity tolerance by deterring ROS accumulation in *Arabidopsis*. *PLoS Genet.*, 10(10): e1004664-e1004664.

Ping, L., W.G. Sang and K.P. Ma. 2008. Differential responses of the activities of antioxidant enzymes to thermal stresses between two invasive *Eupatorium* species in China. *J. Integ. Plant Biol.*, 50(4): 393-401.

Sarkar, J., B. Chakraborty and U. Chakraborty. 2018. Plant growth promoting rhizobacteria protect wheat plants against temperature stress through antioxidant signalling and reducing chloroplast and membrane injury. *J. Plant Growth Regul.*, 37(4 suppl): 1-17.

Schmedes, A. and G. Holmer. 1989. A new thiobarbituric acid (TBA) method for determining free malondialdehyde (MDA) and hydroperoxides selectively as a measure of lipid peroxidation. *J. Amer. Oil Chem. Soc.*, 66(6): 813-817.

Shulaev, V., D. Cortes, G. Miller and R. Mittler. 2008. Metabolomics for plant stress response. *Physiol Plant.*, 132(2): 199-208.

Steponkus, P.L. 2003. Role of the plasma membrane in freezing injury and cold acclimation. *Ann. Rev. Plant Biol.*, 35(1): 543-584.

Tian, Y., H. Zhang, X. Pan, X. Chen, Z. Zhang, X. Lu and R. Huang. 2011. Overexpression of ethylene response factor *TERF2* confers cold tolerance in rice seedlings. *Transgenic Res.*, 20(4): 857-866.

Touno, K., J. Tamaoka, Y. Ohashi and K. Shimomura. 2005. Ethylene induced shikonin biosynthesis in shoot culture of *Lithospermum erythrorhizon*. *Plant Physiol. Biochem.*, 43(2): 101-105.

Ueda, A., Y. Yamamoto-Yamane and T. Takabe. 2007. Salt stress enhances proline utilization in the apical region of barley roots. *Biochem. Biophys. Res. Commun.*, 355(1): 61-66.

Vogelien, D.L., L.G. Hickok and T.R. Warne. 1996. Differential effects of Na^+ , Mg^{2+} , K^+ , Ca^{2+} and osmotic stress on the wild type and the NaCl-tolerant mutants *stl1* and *stl2* of *Ceratopteris richardii*. *Plant Cell Environ.*, 19(1): 17-23.

Wang, J., X. Xu and D. Ma. 2019a. Establishment of an efficient alfalfa regeneration system via organogenesis and the co-expression of *Arabidopsis SOS* genes improves salt tolerance in transgenic alfalfa (*Medicago sativa* L.). *Can. J. Plant Sci.*, 99(3): 348-363.

Wang, W.B., Y.H. Kim, H.S. Lee, K.Y. Kim, X.P. Deng and S.S. Kwak. 2009. Analysis of antioxidant enzyme activity during germination of alfalfa under salt and drought stresses. *Plant Physiol. Biochem.*, 47(7): 570-577.

Wang, Y.G., P. Stevanato, C.H. Lv, R.R. Li and G. Geng. 2019b. Comparative physiological and proteomic analysis of two sugar beet genotypes with contrasting salt tolerance. *J. Agri. Food Chem.*, 67(21): 6056-6073.

Weber, H., A. Chételat, P. Reymond and E.E. Farmer. 2004. Selective and powerful stress gene expression in *Arabidopsis* in response to malondialdehyde. *Plant J.*, 37(6): 877-888.

Wei, S., Z. Du, F. Gao, X. Ke, J. Li, J. Liu and Y. Zhou. 2015. Global transcriptome profiles of 'Meyer' zoysiagrass in response to cold stress. *PLoS One*, 10(6): 1-27.

Xing, L., Z. Di, W. Yang, J. Liu, M. Li, X. Wang, C. Cui, X. Wang, X. Wang, R. Zhang, J. Xiao and A. Cao. 2017. Overexpression of *ERF1-V* from *Haynaldia villosa* can enhance the resistance of wheat to powdery mildew and increase the tolerance to salt and drought stresses. *Front. Plant Sci.*, 8. doi: 10.3389/fpls.2017.01948

Yamazakia, T., Y. Kawamura and M. Uemura. 2003. Extracellular freezing-induced mechanical stress and surface area regulation on the plasma membrane in cold-acclimated plant cells. *Plant Signal Behav.*, 4(3): 231-233.

Yazaki, K., H. Matsuoka, T. Ujihara and F. Sato. 1999. Shikonin biosynthesis in *Lithospermum erythrorhizon*: Light-induced negative regulation of secondary metabolism. *Plant Biotechnol.*, 16(5): 335-342.

Zhang, W., A. Zou, J. Miao, Y. Yin, R. Tian, Y. Pang, R. Yang, J. Qi and Y. Yang. 2011. *LeERF-1*, a novel AP2/ERF family gene within the B3 subcluster, is down-regulated by light signals in *Lithospermum erythrorhizon*. *Plant Biol.*, 13(2): 343-348.

Zhang, Z.J., J. Wang, R.X. Zhang and R.F. Huang. 2012. The ethylene response factor AtERF98 enhances tolerance to salt through the transcriptional activation of ascorbic acid synthesis in *Arabidopsis*. *Plant J.*, 71(2): 273-287.

(Received for publication 20 February 2019)