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# Differential salt stress responses and the role of polyamine metabolism in *Lotus japonicus* ecotypes

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## Abstract

Salt stress represents a critical challenge to global agriculture, reducing productivity and threatening food security. Its impacts are particularly severe in legumes, vital for sustainable agriculture. In this study, we investigated the differential responses to salt stress in two *Lotus japonicus* ecotypes, MG-20 (tolerant) and Gifu (sensitive), focusing on polyamine metabolism. Plants were exposed to 100 mM NaCl for two weeks, and various physiological and biochemical parameters were measured. MG-20 exhibited smaller reductions in biomass and maintained a higher Na<sup>+</sup>/K<sup>+</sup> ratio and chlorophyll stability compared to Gifu, indicating greater salt tolerance. Polyamine (putrescine, spermidine, spermine) analysis revealed significant increases in putrescine levels in MG-20 roots and leaves, while Gifu showed inconsistent accumulation patterns. Gene expression analysis indicated enhanced *LjADC1* expression in MG-20 roots, correlating with higher putrescine levels and salt tolerance. These findings suggest that MG-20's superior tolerance may be attributed to its ability to modulate polyamine metabolism more effectively under salt stress conditions. These findings highlight the critical roles of polyamine metabolism and specific biosynthetic pathways in conferring salt stress resilience in *Lotus japonicus*, providing insights for the development of stress-tolerant crops.

**Keywords** Salt stress, Polyamines, Na<sup>+</sup>/K<sup>+</sup>, *Lotus japonicus*

## 1 Introduction

Climate change is accelerating soil salinization, and experts anticipate a further increase shortly due to expanded irrigation practices [1]. It is predicted that these salt effects will impact 50% of arable land by 2050 [2]. This salt accumulation poses a significant threat to agricultural productivity [3]. Soil salinization not only directly harms plants and reduces their productivity but also diminishes the amount of arable land, threatening sustainable agriculture [4]. This issue demands urgent attention and research to mitigate its effects on agricultural sustainability and global food security.



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Salt stress caused by direct exposure to salinity imposes severe physiological challenges on plants [5]. When the concentration of sodium ions ( $\text{Na}^+$ ), chloride ions ( $\text{Cl}^-$ ), and other associated ions in the soil exceeds normal levels, it significantly disrupts cellular functions in plants [6, 7]. Salinity elevates soil osmotic pressure, leading to modifications in essential metabolic processes such as seed germination, photosynthesis, and nutrient uptake [8]. These alterations ultimately threaten plant growth and survival by inducing salt stress [9]. This stress predominantly impacts plants through two major mechanisms: osmotic stress and ion toxicity [6, 10]. Osmotic stress limits the plant's ability to absorb water due to high salt concentrations in the soil [8], resulting in a "physiological drought" that prevents adequate water uptake [11]. Consequently, cell expansion is inhibited, leaf expansion decreases, and stomatal closure occurs [12], reducing photosynthetic rates and impeding overall growth [13]. Meanwhile, ion toxicity arises from the excessive accumulation of  $\text{Na}^+$  within plant tissues.  $\text{Na}^+$  competes with potassium ions ( $\text{K}^+$ ), a vital nutrient with similar size and charge; leading to disruptions in the  $\text{Na}^+/\text{K}^+$  balance [7]. This imbalance interferes with numerous physiological processes, including enzyme activation, protein synthesis, and membrane stability [14]. Furthermore, excessive  $\text{Na}^+$  accumulation impairs the functions of chloroplasts and mitochondria, adversely affecting energy production [15]. The combined physiological disruptions caused by salt stress drastically reduce plant productivity.

To mitigate the severe physiological damage caused by salt stress, plants have evolved a range of defense mechanisms. These mechanisms operate at physiological, biochemical, and molecular levels, playing crucial roles in plant survival and adaptation [10]. Broadly, these defense strategies include osmotic regulation, maintenance of ion homeostasis, and enhancement of antioxidant defense systems [6]. Osmotic regulation involves the accumulation of compatible solutes such as proline, glycine betaine, and polyamines [5, 16]. These compounds adjust cellular osmotic potential to maintain water balance while protecting cellular structures [17]. Ion homeostasis is achieved by regulating the activity of membrane transporters, such as  $\text{Na}^+/\text{H}^+$  antiporters and  $\text{K}^+$  channels, to limit intracellular  $\text{Na}^+$  concentration and optimize the  $\text{Na}^+/\text{K}^+$  ratio [18]. The enhancement of antioxidant defense systems addresses the increased production of reactive oxygen species (ROS) under salt stress. This involves the upregulation of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), as well as the production of non-enzymatic antioxidants [19]. These integrated defense mechanisms collectively determine the plant's tolerance to salt stress.

Among these functional defense mechanisms, polyamines play a multifaceted and pivotal role in conferring salt stress tolerance [20]. Polyamines stabilize cellular membranes, scavenge harmful free radicals, and protect the structural integrity of DNA and RNA [21, 22]. They also contribute to maintaining ionic balance by regulating protein synthesis and ion channel activity [23]. These functions enable plants to sustain normal physiological processes even under high salinity conditions. Notably, polyamines are particularly significant in maintaining ion balance and safeguarding the photosynthetic apparatus [24]. Intriguingly, polyamine accumulation and activity vary with environmental sodium levels [25], and plant species or varieties with higher polyamine levels generally exhibit greater salt stress tolerance. Exogenous application of polyamines has also been shown to enhance salt stress tolerance [26–30], suggesting that polyamines serve as key indicators and regulators of salt stress tolerance. Given their species-, organ-, and

developmental stage-specific roles, further studies on polyamines in different plant systems are necessary [31].

Legumes are crucial components of agricultural systems and ecosystems, serving as primary sources of protein essential for human and animal nutrition [32, 33]. These plants account for 27% of global crop production and are cultivated on 12–15% of arable land worldwide [32]. However, the majority of legumes exhibit high susceptibility to soil salinity, posing a significant threat not only to crop productivity but also to global food security and sustainable agriculture [9]. The acceleration of soil salinization due to climate change has elevated the enhancement of salt tolerance in legumes to a critical priority in agricultural research. Consequently, elucidating the mechanisms of salt stress response in legumes and developing salt-tolerant varieties based on these findings is imperative for ensuring future food security. Such research is expected to contribute significantly to the establishment of a sustainable global food system, transcending mere improvements in agricultural productivity.

Among legumes, *Lotus japonicus* has garnered particular attention. This species not only serves as a model plant in laboratory settings but also plays a vital role in grassland ecosystems [34]. Notably, the genus *Lotus* has demonstrated superior tolerance to soil-derived stresses compared to other legumes such as alfalfa and clover [35]. This characteristic underscores the necessity for an in-depth investigation into the salt stress response mechanisms of *Lotus japonicus*. The present study aims to prevent crop productivity decline due to salinization and contribute to the stabilization of the global food supply. Furthermore, it is anticipated to provide crucial scientific insights for addressing the progressively worsening issue of soil salinization.

## 2 Materials and methods

### 2.1 Experimental plants

*Lotus japonicus* seeds for these experiments were provided by the National BioResource Project (NBRP), University of Miyazaki, Japan (<https://legumebase.nbrp.jp/lotus/>). In this study, the experimental lines *Lotus japonicus* ecotype Miyakojima (MG-20) and Gifu B-129 (Gifu) were used to examine the differential responses to salt treatment based on tolerance; *L. japonicus* ecotype MG-20 was utilized as the tolerant line and Gifu as the sensitive line. These two lines are genetically fixed and considered experimental lines that were native to different latitudes [36]. *L. japonicus* MG-20 was collected in Okinawa, Japan. The plant material was identified by Dr. Masayoshi Kawaguchi, and a voucher specimen was deposited at NBRP with ID Miyakojima MG-20. *L. japonicus* Gifu B-129 was collected in Gifu, Japan. The plant material was collected by Dr. Isao Hirayoshi and identified by Dr. Jens Stougaard, and a voucher specimen was deposited at NBRP with ID Gifu B-129.

### 2.2 Growth condition and salt treatment

Seeds were scarified with sandpaper and sown in pots (60 mm diameter) filled with vermiculite. After germination, plants were grown in a growth chamber (FLI-2000, EYELA, 23.0 °C, 16-h day/8-h night, 190–200  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for three weeks. The nutrient solution used was B&D Nitrogen-free nutrients [37] supplemented with  $\text{NH}_4\text{NO}_3$  to a final concentration of 5 mM. For the salt treatment, 100 mM NaCl was added to the nutrient

solution for two weeks. Control plants were grown in the nutrient solution without NaCl.

### 2.3 Bioassays for growth and biomass analysis

On the 14th day after salt treatment, plants were removed from the pots. Fresh weight, shoot length, and root length were measured. After drying at 80°C for 48 h, dry weight was measured.

### 2.4 Chlorophyll content measurement

On the 14th day after treatment, lower leaves were excised and cut into appropriate sizes. The samples were immersed in 10 mL of dimethyl sulfoxide (DMSO) and extracted at 30°C for 48 h. Chlorophyll a and chlorophyll b were extracted and their absorbances were measured at 664 nm and 648 nm using a spectrophotometer (UV mini-1240, SHIMADZU, Kyoto, Japan). Chlorophyll concentrations were calculated using the formulas by Chappelle et al. [38]. Results were expressed as the sum of chlorophyll a and b per gram of fresh weight.

### 2.5 Inorganic ion content measurement

On the 14th day after salt treatment, leaves and roots were sampled. Samples were frozen in liquid nitrogen and freeze-dried in a lyophilizer. Based on Kim et al. [39], inorganic ions were quantified. Samples (30 mg DW each for leaves and roots) were decomposed by adding 3 mL of nitric acid and heating on a hot plate at 120°C. After cooling, the volume was adjusted to 50 mL with 0.1 M HNO<sub>3</sub>, filtered through a 0.20 µm sterilized filter, and analyzed using inductively coupled plasma emission spectrometry (ICPS-8100, SHIMADZU, Kyoto, Japan) to measure inorganic ions (Na, K).

### 2.6 Polyamine content measurement

Polyamine analysis was performed with slight modifications to the method by Flores and Galston [40]. Freeze-dried samples (30 mg DW) were ground and 1.2 mL of HClO<sub>4</sub> was added. After incubation at 65°C for 16–17 h, samples were centrifuged at 4°C, 10,000 × g for 15 min, and the supernatant was collected as the polyamine extract. Internal standard 1,6-diaminohexane (40 nmol) was added to 200 µL of the extract. The sample was alkalinized with 2 mL of 2M NaOH, followed by the addition of 10 µL benzoyl chloride to derivatize polyamines. After standing in the dark at room temperature for 40 min, 2 mL of saturated NaCl solution and 2 mL of diethyl ether were added, mixed, and 1 mL of the ether phase was collected. The ether phase was concentrated to dryness under nitrogen gas, and dissolved in 80 µL of methanol for HPLC analysis. Polyamines were separated and quantified using HPLC (LC10A, SHIMADZU, Kyoto, Japan) with a C18 reverse-phase column (ODS-3 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan). Benzoylated polyamines were detected at 254 nm. The column temperature was 35°C, eluent was 55% (v/v) methanol, flow rate was 0.8 mL/min, and injection volume was 10 µL. The gradient program used was: 0–20 min: 55–60%; 20–40 min: 60–70%; 40–45 min: 70–90%; 45–50 min: 90–55%. Polyamine amounts were calculated based on the area ratio to the internal standard, with total polyamine content expressed as the sum of putrescine (Put), spermidine (Spd), and spermine (Spm) per gram of dry weight.

### 2.7 RNA isolation and qRT-PCR analysis of polyamine synthesis genes

Fourteen days after salt treatment, approximately 100 mg of leaf and root tissues were frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis. Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN). Genomic DNA was eliminated, and complementary DNA (cDNA) synthesis was performed using the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Bio) according to the manufacturer's instructions. Relative expression levels were measured with SYBR Premix Ex Taq™ (Perfect Real Time) (TaKaRa Bio) on a Thermal Cycler Dice Real Time System (TaKaRa Bio). The relative expression levels were calculated using the delta-delta CT method ( $\Delta\Delta\text{CT}$ ). The primers used for qRT-PCR analysis are listed in Table S1.

### 2.8 Experimental design and statistical analysis

The treatments were arranged in a completely randomized design within a factorial experiment. All values were derived from two independent experiments, each with three replicates, and are presented as means  $\pm$  standard deviation. Differences among treatment groups were analyzed using Tukey's Honest Significant Difference (HSD) test and t-tests.

## 3 Results

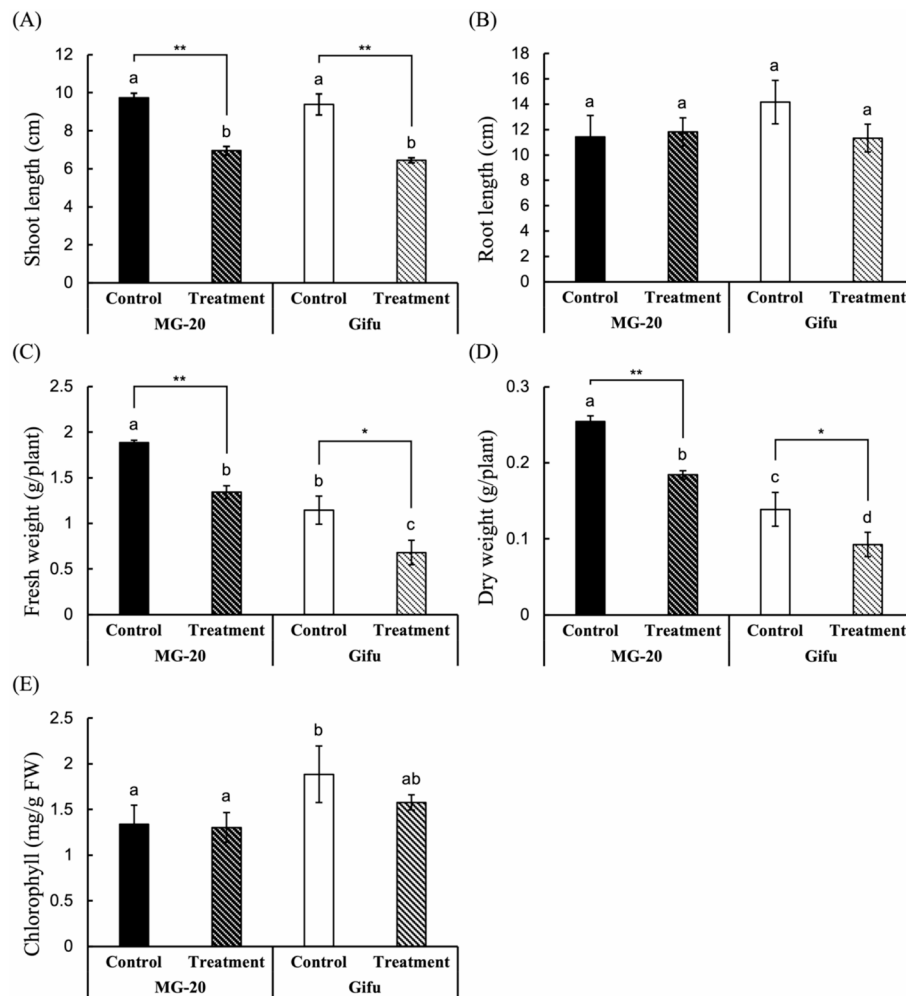
### 3.1 Growth responses of *Lotus japonicus* Ecotypes under salt stress

The result shows the effects of NaCl treatment on growth indexes (Fig. 1). 100 mM NaCl treatment affects a significant decrease in comprehensive growth in both MG-20 and Gifu. The shoot length decreased significantly in both ecotypes (28.6% and 31.4%,  $p < 0.01$ ) (Fig. 1A). But root length doesn't show a significant decrease, even a slight decrease in Gifu (20.0%) (Fig. 1B). Fresh weight significantly decreased due to NaCl treatment in each ecotype (Fig. 1C). While MG-20 decreased by 29.8% ( $p < 0.01$ ), Gifu showed a greater decrease of 40.5% ( $p < 0.05$ ). Additionally, dry weight also decreased due to NaCl treatment, with Gifu showing a greater decrease of 33.2% ( $p < 0.05$ ) than MG-20 of 27.6% ( $p < 0.01$ ) (Fig. 1D). Although the decrease in chlorophyll concentration was not statistically significant, Gifu exhibited a relatively large reduction (16.3%) in leaf chlorophyll concentration.

### 3.2 Ion homeostasis: $\text{Na}^+$ accumulation and $\text{Na}^+/\text{K}^+$ balance

The result shows the effects of NaCl treatment on inorganic ion contents in MG-20 and Gifu (Fig. 2). The sodium concentration significantly increased in both ecotypes and both organs, leaves, and roots, with a higher increase observed in the leaves (Fig. 2A, B). In MG-20, the increase was about 18.8 times in the leaves and about 3.4 times in the roots ( $p < 0.01$ ), while in Gifu, the increase was about 44.3 times in the leaves and about 16.2 times in the roots ( $p < 0.05$ ). In contrast to  $\text{Na}^+$ , the  $\text{K}^+$  content decreased in both ecotypes and organs (Fig. 2C, D). In MG-20, nearly half of the amount decreased (44.5 and 52.9%,  $p < 0.01$ ), whereas Gifu showed a relatively smaller decrease (11.0 and 24.3%, in the roots at  $p < 0.05$ ).

The  $\text{Na}^+/\text{K}^+$  ratio increased significantly in all organs and ecotypes (Fig. 2E, F). In MG-20, the ratio increased 33.8-fold in leaves ( $p < 0.05$ ) and 7.4-fold in roots ( $p < 0.01$ ), while in Gifu, it increased 20.8-fold in leaves ( $p < 0.05$ ) and 5.3-fold in roots ( $p < 0.01$ ). Notably, MG-20 exhibited a higher  $\text{Na}^+/\text{K}^+$  ratio in roots than Gifu.



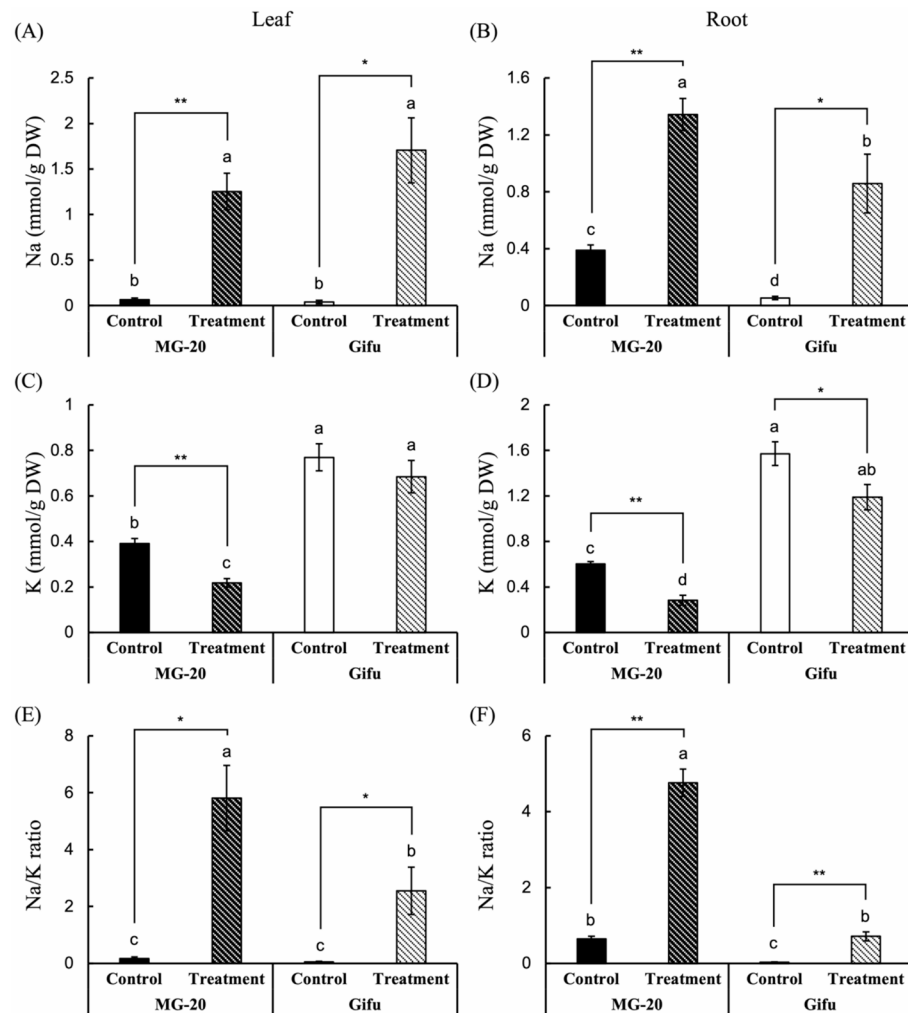
**Fig. 1** Effects of NaCl treatment on plant growth characteristics, including shoot length (A), root length (B), fresh weight (C), dry weight (D), and chlorophyll content (E). Vertical bars indicate the mean  $\pm$  standard deviation (SD) ( $n=3$ ). Different lowercase letters denote significant differences between treatments based on Tukey's HSD test ( $p < 0.05$ ). Asterisks (\*) indicate significant differences between control and treatment groups determined by t-test ( $p < 0.05$ , \*\*  $p < 0.01$ )

### 3.3 Polyamine accumulation patterns under salt stress

Put showed significant changes due to salt treatment (Fig. 3A, B). In the leaves, Put increased by approximately 76.7% and 2 times respectively in both ecotypes (Fig. 3A). However, in the roots, Put increased by about 2 times in MG-20 ( $p < 0.05$ ), but slightly decreased in Gifu (Fig. 3B). In each control group, MG-20 shows higher Put content.

Spd showed a decrease in Gifu (Fig. 3C, D) ( $p < 0.05$ ). It also decreased in the roots of both ecotypes (Fig. 3D). In the leaves, Spd did not show a significant difference between the control and treatment groups in MG-20. In the roots, Gifu showed a decrease, but there was no significant difference in the amount in the control group.

Spm increased both in leaves and roots in MG-20 and Gifu (Fig. 3E, F). In MG-20, it increased by 39.3% in leaves and 32.3% in roots. In Gifu, the increase was even greater. Spm in leaves increased by 66.3% ( $p < 0.05$ ) and roots by approximately 2.5 times following salt treatment ( $p < 0.01$ ) (Fig. 3E). In the control group, Spm showed a higher accumulation in Gifu and also showed a higher increase due to salt treatment.

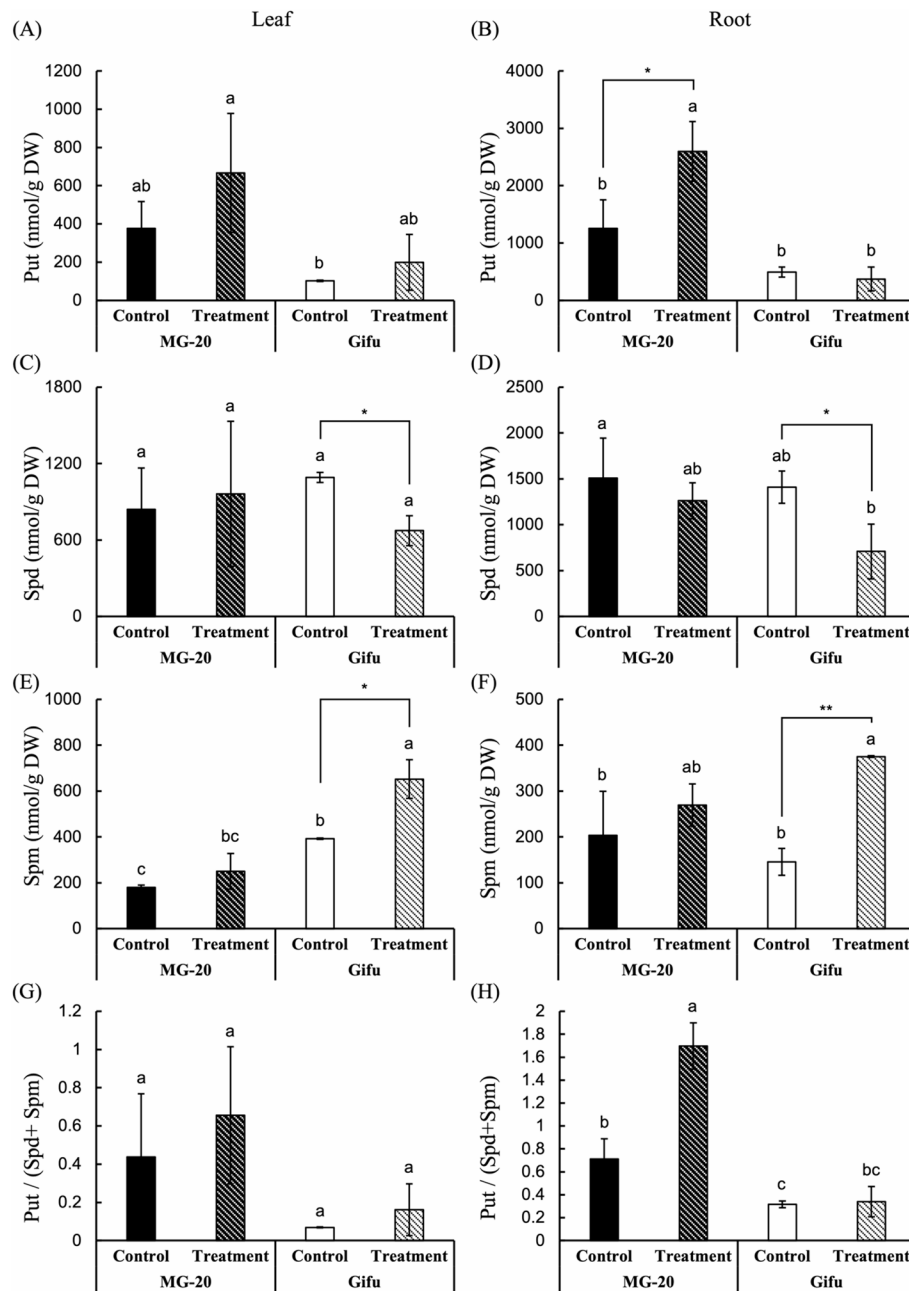


**Fig. 2** Effects of NaCl treatment on sodium ( $\text{Na}^+$ ) concentration (A, B), potassium ( $\text{K}^+$ ) concentration (C, D), and  $\text{Na}^+/\text{K}^+$  ratio (E, F) in leaves and roots of *Lotus japonicus* ecotypes MG-20 and Gifu. Vertical bars represent the mean  $\pm$  standard deviation (SD) ( $n=3$ ). Different lowercase letters indicate significant differences between treatments ( $p < 0.05$ ) based on Tukey's HSD test. Asterisks (\*) indicate significant differences between control and treatment groups determined by t-test (\* $p < 0.05$ , \*\* $p < 0.01$ )

The Put/(Spd + Spm) ratio in leaves showed no significant changes due to salt treatment (Fig. 3G, H). However, MG-20 exhibited a notable 50% increase, indicating a stronger response compared to Gifu. In roots, MG-20 showed a significant 2.3-fold increase, while Gifu displayed a non-significant 7% increase (Fig. 3H). The Put/(Spd + Spm) ratio did not show significant differences in leaves between ecotypes, but in roots, MG-20 displayed a clear increasing trend, while Gifu showed a relatively smaller response, reflecting differing tendencies between the two ecotypes.

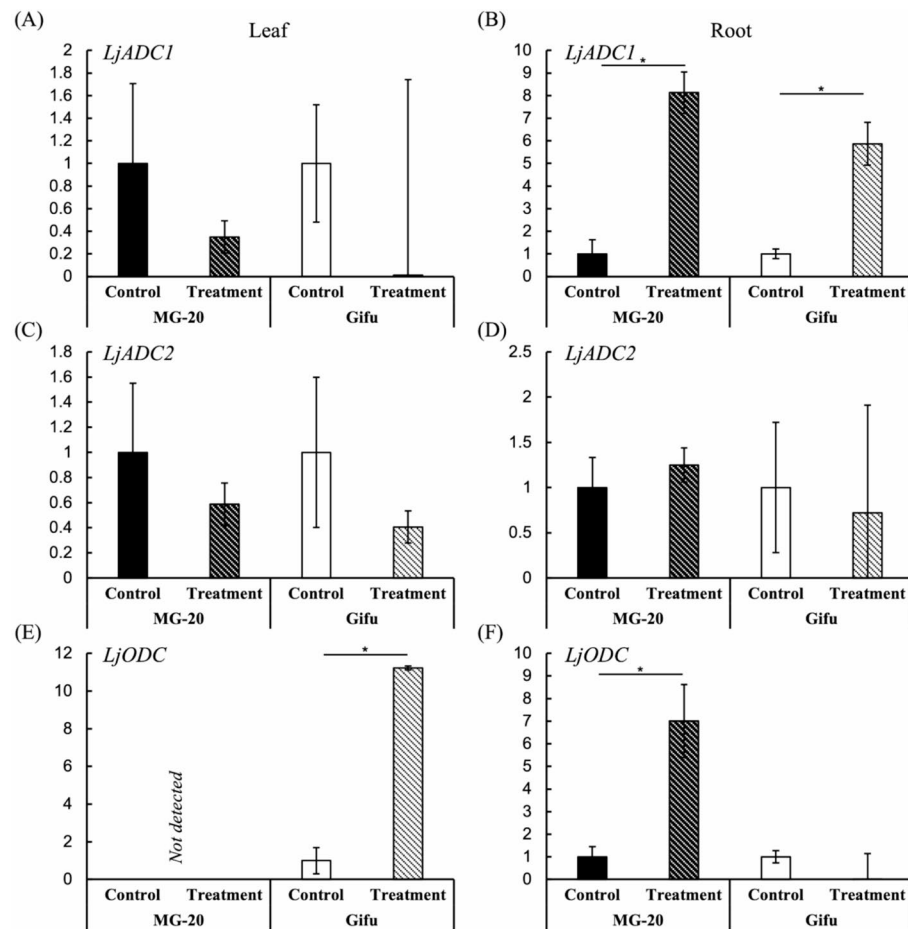
### 3.4 Expression changes of polyamine biosynthesis related genes

*LjADC1* expression in leaves decreased in both ecotypes due to salt treatment (Fig. 4A). However, in roots, expression increased by more than five times under the same treatment ( $p < 0.05$ ) (Fig. 4B). *LjADC2* expression in leaves was halved in both varieties following salt treatment, while no significant change was observed in the roots (1.25, 0.72) (Fig. 4C, D). In the MG-20 ecotype, *LjODC* was not detected in either the control or



**Fig. 3** Effects of NaCl treatment on polyamine content, including putrescine (Put) (A, B), spermidine (Spd) (C, D), spermine (Spm) (E, F), and Put/(Spd + Spm) ratio (G, H) in leaves and roots of MG-20 and Gifu. Vertical bars indicate the mean  $\pm$  standard deviation (SD) ( $n=3$ ). Different lowercase letters denote significant differences between treatments based on Tukey's HSD test ( $p < 0.05$ ). Asterisks (\*) indicate significant differences between control and treatment groups determined by t-test (\* $p < 0.05$ , \*\* $p < 0.01$ )

treatment groups, while its expression significantly increased in the leaves of Gifu under salt stress ( $p < 0.05$ ) (Fig. 4E, F). In the roots, MG-20 showed a significant increase in *LjODC* expression due to salt treatment ( $p < 0.05$ ), whereas Gifu exhibited a decrease (Fig. 4F).



**Fig. 4** Relative expression levels of polyamine biosynthesis-related genes (*LjADC1*, *LjADC2*, and *LjODC*) in leaves (left) and roots (right) of MG-20 and Gifu under control and NaCl treatment conditions. Vertical bars represent the mean  $\pm$  standard deviation (SD) ( $n = 3$ ). Asterisks (\*) indicate significant differences between control and treatment groups determined by t-test (\* $p < 0.05$ , \*\* $p < 0.01$ )

## 4 Discussion

### 4.1 Physiological changes under salt stress in *Lotus japonicus*

Salt stress damage in plants primarily stems from two factors: the loss of osmotic homeostasis, which impedes water absorption, and ionic stress caused by chloride accumulation [41]. From a physiological perspective, these stressors inhibit nutrient uptake, disrupt normal cellular functions, and impair photosynthesis [9, 11]. Collectively, these effects manifest in various ways to hinder normal plant growth.

In our study, treatment with 100 mM NaCl resulted in comprehensive growth inhibition in both ecotypes (Fig. 1). Notably, the salt treatment elicited differential growth responses between the ecotypes (Fig. 1C, D). MG-20 exhibited comparatively smaller reductions in fresh and dry weights, indicating less severe damage under 100 mM NaCl treatment compared to Gifu. This implies that MG-20 possesses relatively higher salt tolerance than Gifu.

The chlorophyll concentration exhibited no statistically significant reduction in the leaves, although a 16.3% decrease was observed in Gifu (Fig. 1E). This suggests that the physiological stress induced by 100 mM NaCl did not significantly impact chlorophyll levels in MG-20. However, the slight decrease observed in Gifu implies a concomitant

reduction in photosynthetic capacity. This differential response between genotypes indicates varying degrees of salt tolerance and its effects on photosynthetic apparatus.

#### 4.2 Differential $\text{Na}^+$ and $\text{K}^+$ accumulation in *Lotus japonicus* ecotypes

Under salt stress conditions, plants experience excessive accumulation of sodium ions [9].  $\text{Na}^+$  influx occurs through non-selective cation channels, which subsequently inhibits potassium uptake.  $\text{K}^+$  absorption is mediated by active transport mechanisms such as high-affinity potassium transporters (HKTs). However, elevated  $\text{Na}^+$  levels impede  $\text{K}^+$  uptake, resulting in decreased intracellular  $\text{K}^+$  concentrations [42]. This disruption of  $\text{Na}^+/\text{K}^+$  homeostasis leads to increased  $\text{Na}^+/\text{K}^+$  ratios, which adversely affect osmotic regulation, charge balance, and enzymatic activities, ultimately causing widespread physiological disturbances in plants [43].

Our results show these findings, demonstrating a significant increase in  $\text{Na}^+$  accumulation and a concomitant decrease in  $\text{K}^+$  levels (Fig. 2). Notably, the magnitude of  $\text{Na}^+$  accumulation was markedly higher in Gifu compared to MG-20. This aligns with previous studies indicating that chloride accumulation under salt stress is associated with salt tolerance in *Lotus* spp. [44, 45], suggesting that MG-20 exhibits superior salt tolerance compared to Gifu. Also, the two ecotypes have been reported to originate from distinct natural habitats [46], indicating that differences in environmental conditions, particularly those influenced by geographic factors, may contribute to the phenotypic and physiological variations observed between MG-20 and Gifu. MG-20 is native to the coastal areas of Southwest Islands of Japan, while Gifu is native to the inland region of central Japan. These differing native environments are likely to have influenced their adaptive traits. The ecological and geographical characteristics of their native habitats suggest that the differences between the ecotypes may serve as a valuable genetic resource for stress response studies [47].

$\text{K}^+$  concentrations were consistently higher in both leaves and roots of Gifu compared to MG-20 (Fig. 2C, D). This suggests that Gifu maintains higher  $\text{K}^+$  accumulation regardless of stress conditions and exhibits relatively lower inhibition of  $\text{K}^+$  uptake under stress. However, Gifu demonstrated greater growth inhibition under salt stress, indicating a potentially weaker stress resistance response in terms of  $\text{Na}^+$  uptake, sequestration, and transport mechanisms.

A substantial increase in  $\text{Na}^+/\text{K}^+$  ratios was observed across all organs and ecotypes (Fig. 2E, F). Gifu exhibited higher  $\text{Na}^+$  accumulation relative to the increase in  $\text{Na}^+/\text{K}^+$  ratio, which can be attributed to its lower  $\text{K}^+$  efflux. Interestingly, despite showing a greater increase in  $\text{Na}^+/\text{K}^+$  ratio, MG-20 appeared to possess higher salt tolerance. This suggests that the differential salt tolerance between these ecotypes may not primarily stem from  $\text{Na}^+/\text{K}^+$  ratio regulation but rather from disparities in stress mitigation capabilities mediated by other biochemical compounds.

#### 4.3 Differential polyamine accumulation in *Lotus japonicus* under salt stress

Polyamines have many functions to cope with environmental stress in plants. Polyamines affect ion channels especially potassium channel on plasma membrane [48, 49]. This suggests that polyamines play a role in regulating stomatal closure. Also polyamines could enhance cell wall and plasma membrane stability through conjugate with membrane [21, 50]. And polyamines have acid-neutralizing abilities and strong antioxidant

activities [50]. Additionally, polyamines could serve as nitrogen and carbon sources under stress conditions in plants [51, 52]. The functions of polyamines can exhibit variability across different plant species, as well as among various organs within a single plant species, depending on the environmental stress [53, 54]. However, it is generally observed that plants with higher polyamine concentrations demonstrate enhanced salt tolerance [16]. However, the accumulation patterns vary depending on the type of polyamine, indicating differential responses to stress conditions among different polyamine species. Furthermore, this variability in accumulation patterns underscores the distinct roles and behaviors of various polyamines under stress conditions.

Put is known to accumulate in response to abiotic stresses [52, 55]. Our results showed that Put levels increased after treatment with 100 mM NaCl (Fig. 3A, B). This increase was more pronounced in MG-20, which is considered to have enhanced salt tolerance compared to Gifu. Notably, the increase in Put concentration was more significant in the roots than in the leaves. In plants exposed to environmental stress, Put tends to accumulate at higher levels in the roots compared to the shoots [56, 57], indicating that stress-induced increases in Put concentration primarily occur in the root system. Furthermore, the higher Put content observed in the roots of MG-20 indicates greater stress tolerance, while the stable Put levels in Gifu's roots imply a relatively lower resistance to stress (Fig. 3B). These findings highlight both the direct and indirect roles of Put in enhancing stress resistance. Put accumulation helps mitigate stress by maintaining ionic homeostasis and scavenging reactive oxygen species [30, 58]. Shu et al. [28] found that exogenous Put treatment under salt stress protected thylakoid membranes and preserved photosynthetic efficiency. Consequently, the increased Put levels in MG-20 enhanced its resistance to salt stress and reduced associated damage. Interestingly, MG-20 consistently exhibited higher Put levels than Gifu in all treatment conditions and tissues (Fig. 3A, B). This elevated baseline Put content in MG-20 before exposure to stress is noteworthy. Plants with high stress resistance typically maintain higher basal levels of Put, indicating preparedness for rapid stress responses [16, 59]. Therefore, the higher accumulation of Put in MG-20 suggests a more proactive approach to stress resistance.

Spd levels remained relatively stable in MG-20 but decreased in Gifu under 100 mM NaCl treatment (Fig. 3C, D). Environmental stresses such as salt and drought often induce a reduction in Spd, attributed to its metabolism or conversion into other compounds [60]. The substantial increase in Spm concentration in Gifu suggests that the observed Spd decrease may be due to its conversion to Spm (Fig. 3E, F). Conversely, MG-20 showed minimal changes in Spm levels, Spd and Spm often exhibit less pronounced fluctuations compared to Put [61, 62]. Spm also plays crucial roles in stress responses. Exogenous Spm application in cucumber has been shown to enhance chloroplast stability and antioxidant enzyme activity [29]. This suggests that the increase in Spm in Gifu may have contributed to mitigating damage despite the lack of change in Put levels.

Put/(Spd + Spm) ratio exhibited distinct responses to salt stress depending on ecotype and tissue type (Fig. 4G, H). In MG-20, the ratio showed a clear increasing trend in both leaves and roots following salt treatment, whereas Gifu exhibited no significant changes. This differential response underscores the importance of the Put/(Spd + Spm) ratio as a key indicator of plant stress responses [27]. The findings suggest that MG-20 is more sensitive to salt stress compared to Gifu, potentially employing a stress response

strategy characterized by rapid Put accumulation. The pronounced increase in the Put/(Spd + Spm) ratio observed in MG-20 roots further highlights tissue-specific metabolic differences. Given that this ratio is primarily driven by changes in Put levels, it is likely that Put synthesis and metabolism play a central role in shaping the overall polyamine profile under salt stress conditions [63].

#### 4.4 *LjADC1* upregulation in MG-20 roots enhances Put accumulation

Polyamine biosynthesis in plants primarily occurs through two distinct pathways, both originating from arginine. The predominant pathway involves the enzyme arginine decarboxylase (ADC) [64], which plays a crucial role in polyamine metabolism [16]. ADC catalyzes the decarboxylation of arginine to produce agmatine, which is subsequently converted to Put, the precursor of other polyamines. The secondary pathway, less prevalent in plants but present in certain species [65], involves a two-step process. Initially, the enzyme arginase converts arginine to ornithine. Subsequently, ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine, directly synthesizing Put.

*ADC* serves as a primary regulatory factor in Put accumulation [55]. Environmental stressors have been reported to induce *ADC* expression, leading to increased Put levels [66, 67]. Recent studies have demonstrated that *ADC* overexpression in *Lotus tenuis* resulted in enhanced Put accumulation and improved environmental stress resistance [68]. *ADC* is typically encoded by two genes in plants, with *ADC2* generally exhibiting greater sensitivity to stress compared to *ADC1* [61, 69]. In *Lotus japonicus*, two homologous genes, *ADC1* and *ADC2*, have been reported [70]. However, our findings indicate that both ecotypes exhibited a more pronounced increase in *LjADC1* expression in response to stress compared to *LjADC2* (Fig. 4B, D). Some studies have reported *ADC1* responsiveness to environmental stress [71], with Liu [72] noting that salt stress induced a higher response in *ADC1* compared to other polyamine biosynthesis genes. The expression patterns of *ADC1* and *ADC2* have been shown to vary based on stress duration [55] and may differ among plant species and organs [73, 74]. Consequently, we postulate that the increased Put levels in *Lotus japonicus* MG-20 can be attributed to the upregulation of *ADC1* in the roots. This further suggests that Put synthesis predominantly occurs in the roots and may be transported to other organs. Even under non-stress conditions, *ADC* expression in *Lotus japonicus* has been reported to be higher in roots compared to leaves [70], which explains the elevated Put accumulation in roots under control conditions. Other studies have suggested that the high Put accumulation induced by salt and osmotic stress is due to increased *ADC* activity in roots, with root-synthesized Put being translocated to other parts of the plant [75, 76].

However, in Gifu, despite exhibiting high *LjADC1* expression in roots in response to stress, Put accumulation remained unchanged in roots while showing a slight increase in leaves (Figs. 3, 4). The increased Put accumulation in Gifu leaves, despite decreased *LjADC1* and *LjADC2* expression, suggests that transport from roots to leaves may have been prioritized over root accumulation. This indicates that increased *ADC1* expression does not necessarily correlate with increased Put accumulation.

Polyamine synthesis via *ODC* is absent in some plant species and is not considered essential for all plants [16, 65]. However, *ODC* expression has been reported to increase in response to environmental stress [77]. *LjODC* expression patterns differed between

the two ecotypes, showing a significant increase in Gifu leaves and MG-20 roots in response to salt treatment (Fig. 4E, F). In Gifu, the substantial increase in *LjODC* expression resulted in only a minimal increase in leaf Put concentration, while the decreased expression in roots led to no change in Put accumulation. This may explain the lower Put accumulation in Gifu roots compared to MG-20 and suggests that the differential response in Gifu contributes to its lower salt resistance relative to the resistant MG-20. The increased Put accumulation in Gifu leaves is likely due to a combination of increased Put transport from roots (where *LjADC1* expression was elevated) and increased *LjODC* expression in leaves. In MG-20, *LjODC* expression was undetectable in leaves (Fig. 4E), indicating significantly lower *LjODC* expression in leaves of this ecotype. Flemetakis et al. [70] reported *LjODC* expression in *Lotus japonicus* roots and nodules but not in leaves or other organs, suggesting that *LjODC* is primarily expressed in roots.

#### 4.5 Polyamine enhance chlorophyll stability and salt stress tolerance in *Lotus japonicus*

Polyamines exert significant influence on chlorophyll biosynthesis, accumulation of photosynthetic proteins, membrane reorganization, and the formation and structural alignment of thylakoid membranes. Notably, polyamines play a crucial role in protecting chlorophyll under stress conditions. Despite high salt stress, the chlorophyll concentration in MG-20 remained unchanged, which is thought to be due to the protective action of chlorophyll resulting from elevated Put concentrations. Furthermore, *LjADC* expression is predominantly localized in chloroplasts in the aerial parts suggests that Put synthesis via *LjADC* also contributes to chlorophyll protection.

Polyamines directly impact plant ion balance to mitigate damage under stress conditions. They inhibit non-selective cation channels (NSCCs), preventing excessive  $\text{Na}^+$  influx and  $\text{K}^+$  efflux [17]. In barley, treatment with Spd regulates ion channels, limiting  $\text{Na}^+$  influx and preventing  $\text{K}^+$  loss, thereby modulating osmotic pressure and enhancing plant salt resistance [43]. Additionally, Put treatment in cucumber enhances  $\text{K}^+$  transport, regulating the  $\text{Na}^+/\text{K}^+$  ratio in leaves and improving salt resistance [58]. These findings suggest that polyamines effectively alleviate salt stress-induced damage and imply that the increased salt resistance observed in MG-20 may be attributed to elevated polyamine accumulation. Moreover,  $\text{K}^+$  loss due to salt stress induces polyamine biosynthesis.  $\text{Na}^+$  ion over-accumulation leading to  $\text{K}^+$  loss induces *ADC1* expression [78]. This indicates that  $\text{K}^+$  deficiency resulting from severe salt stress accelerates polyamine biosynthesis, suggesting that the decrease in  $\text{K}^+$  ions in roots influenced the increase in *LjADC1* expression (Fig. 4B).

## 5 Conclusion

*Lotus japonicus* MG-20 and Gifu exhibited markedly different polyamine responses under stress conditions, and these disparities are believed to significantly influence their salt tolerance capabilities. The distinct polyamine accumulation patterns and biosynthetic pathways observed in these ecotypes appear to be key factors in determining their differential resistance to salt stress.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44372-025-00300-6>.

Supplementary Material 1

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### Author contributions

YL, AY and CKK conceived and designed the experiments. YL, AY and CKK performed the experiments. YL, AY, CKK and YS analyzed the data. MH and RA contributed plant materials tools. YL, MR and AY wrote the paper. CKK, MH, RA and YS revised the paper. All authors read and approved the final manuscript.

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### Availability of data and materials

The data used during the current study is available from the corresponding author on reasonable request.

### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

All authors consent for publication.

#### Competing interests

The authors declare no competing interests.

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