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# Sodium extrusion associated with enhanced expression of SOS1 underlies different salt tolerance between *Medicago falcata* and *Medicago truncatula* seedlings

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ABSTRACT

*Medicago falcata* L. is a perennial legume species native to semiarid grasslands in northern China, and distinguished by its superior tolerance to abiotic stresses such as drought and cold. But there have been few studies to investigate the physiological mechanism underlying salt tolerance in *M. falcata*. In the present study, we compared the response of non-nodulated *M. falcata* with legume model plant *Medicago truncatula* Garetn at physiological and molecular level by submitting them to a salt stock. *M. falcata* exhibited higher survival rate and less chlorosis than *M. truncatula* seedlings upon exposure to salt stress, suggesting that *M. falcata* is more tolerant to salt stress than *M. truncatula*. The ability to maintain a high $K^+$ and low $Na^+$ contents by plants is an important trait for tolerance to salt stress. *M. falcata* accumulated less amount of $Na^+$ in their shoots than *M. truncatula* when exposed to NaCl, leading to a higher $K^+/(K^+ + Na^+)$ ratio in *M. falcata* than in *M. truncatula* under saline conditions. There was a similar difference in $Na^+$ concentration and $K^+/(K^+ + Na^+)$ ratio in roots and xylem sap between the two species under saline conditions. Results from grafting experiments revealed that seedlings grafted with *M. falcata* either as scions or rootstocks can significantly reduce $Na^+$ accumulation and increase $K^+/(K^+ + Na^+)$ ratio in both shoots and roots under saline conditions. Expression levels of *SOS1* that encodes a $Na^+$–$H^+$ antiporter responsible for $Na^+$ extrusion from cells in *M. falcata* were much higher than in *M. truncatula* under both control and saline conditions. Abundance of *SOS1* transcripts in seedlings grafted with *M. falcata* as either rootstocks or scions was significantly higher than in seedlings self-grafted with *M. truncatula*. These results strongly suggested that SOS1-dependent $Na^+$ extrusion is an important mechanism to maintain a relatively low $Na^+$ concentration and high $K^+/(K^+ + Na^+)$ ratio in non-nodulated *M. falcata* under saline conditions, thus conferring tolerance to salt stress.

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## 1. Introduction

As one of the major abiotic stresses, salinity negatively affects the productivity and quality of crops (Munns and Tester, 2008). Plants exposed to salt stress often suffer from osmotic stress, $Na^+$ toxicity and associated oxidative stress, leading to reduction in photosynthesis and inhibition of growth (Zhu, 2001; Munns and Tester, 2008). To cope with the osmotic stress, plants often accumulate osmolytes such as proline (Pro), soluble sugars and $Na^+$ ions to reduce their osmotic potentials, a phenomenon referred to as osmoregulation (Greenway and Munns, 1980; Tester and Davenport, 2003). In addition, Pro can also protect plants from damage by scavenging reactive oxygen species (ROS) produced by salt stress and stabilizing the structure of proteins as a molecular chaperone (Szabados and Savoure, 2010). Although accumulation of $Na^+$ can facilitate osmoregulation under saline conditions, excess accumulation of $Na^+$ in cytosol can be toxic to plants by inhibiting $K^+$ uptake, disturbing $K^+$-dependent enzymatic processes (Tester and Davenport, 2003). To mitigate the toxic effect of $Na^+$, plants can compartmentalize $Na^+$ into the vacuoles (Pardo et al., 2006), and/or efflux $Na^+$ from cells (Shi et al., 2002). Three major transporters, NHX, HKT and SOS1, are involved in the plant $Na^+$ tolerance by mediating $Na^+$ transport, translocation and intracellular compartmentation (Yamaguchi et al., 2013). $Na^+/H^+$ antiporters belonging

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to NHX family are involved in compartmentalization of $Na^+$ into the vacuoles by utilizing the $H^+$ gradient as a driving force (Blumwald, 2000). HKT1 was first discovered in wheat to mediate $K^+$ uptake (Schachtman and Schroeder, 1994). But further studies demonstrated that HKT1 can also mediate $Na^+$ uptake into plant cells (Yamaguchi et al., 2013).

SOS (salt overly sensitive) system has been identified to play an important role in mediation of efflux of cytosolic $Na^+$ from plant cells (Zhu, 2000). SOS1 is a putative $Na^+/H^+$ antiporter in the plasma membrane capable of mediating $Na^+$ extrusion from cytoplasm and regulating long-distance $Na^+$ transport between shoots and roots (Shi et al., 2002). *SOS2* encodes a serine/threonine kinase with a catalytic domain at the N terminus and a regulatory domain at the C terminus (Liu et al., 2000). The catalytic domain is similar to the SNF1/AMPK (yeast sucrose non-fermenting/mammalian AMP-activated protein kinase) kinase which protects cells against nutritional or environmental stresses (Liu et al., 2000). SOS3 is a $Ca^{2+}$-binding protein with one myristoylation and three $Ca^{2+}$-binding EF-hand sites, respectively (Liu and Zhu, 1998). SOS3 activates and interacts with SOS2 after sensing the salt stress-elicited calcium signal, and subsequently up-regulates the expression of *SOS1* by interacting with the SOS2-SOS3 kinase complex, leading to efflux of $Na^+$ from cells (Zhu, 2002). Among the three SOSs, *SOS1* is the most important loci in control of plant salt tolerance, such that overexpression of *AtSOS1* improves salt tolerance and the *sos1* mutant is more sensitive to $Na^+$ (Shi et al., 2003).

*Medicago falcata* is a perennial leguminous plant widely occurred in Russia, Mongolia, China and Scandinavia (Lesins and Lesins, 1979). *M. falcata* has been widely used as a general source of germplasm for breeding alfalfa (*Medicago sativa* L.) with enhanced tolerance to environmental stress because of its superior tolerance to abiotic stresses such as drought, cold and soil infertility (Riday et al., 2003). However, few studies have investigated the molecular mechanisms underlying the greater tolerance of *M. facalta* to abiotic stresses due to limited information on its genomics. The completion of whole-genome sequencing of legume model plant *Medicago truncatula*, a close relative of *M. falcata*, provides a valuable tool to study the molecular mechanisms by which *M. falcata* tolerates abiotic stresses (Young et al., 2011). Several studies have investigated the physiological and molecular mechanisms associated with tolerance of *M. falcata* to cold stress (Pennycooke et al., 2008; Zhang et al., 2011), drought stress (Kang et al., 2011) and deficiency in mineral nutrients (Li et al., 2009; Gao et al., 2011; Li et al., 2011, 2014; Wang et al., 2014). In addition, enhanced accumulation of compatible solutes such as soluble sugars, Pro and glycine betaine to prevent water loss is a common adaptive strategy in response to abiotic stress (Ashraf and Foolad, 2007). For instance, a positive correlation between salt tolerance and contents of Pro and glycine betaine has been detected under salt stress in two crop legumes differing in salt tolerance (Ashraf and Bashir, 2003). Tian et al. (2009) investigated the effect of NaCl on seed germination and seedlings growth of several accessions of *M. falcata*. However, no study has evaluated the response of *M. falctata* to salt stress at both physiological and molecular levels.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

*M. truncatula* (cv. Jemalong A17) and *M. falcata* were used in this study. The seeds were dipped in concentrated sulfuric acid for approximately 6 min. and then rinsed with running water. The seeds were then put into Petri plates with 0.7% agar at 4 °C for 1 day and then transferred to 25 °C in dark for 2 days. The seeds with about 2 cm radicles were transferred to plastic buckets filled with 4 L of fully aerated nutrient solution. Each bucket contained two ecotypes for each 6 plants. Plants were grown in a growth chamber with 26 °C (day)/20 °C (night), 14/10 h day/night period with light intensity of 140 $\mu mol\,m^{-2}\,s^{-1}$. After grown in the culture solution for 3 weeks, half of the plants were transferred to culture solution with 100 mM NaCl. The composition of other nutrients in the hydroponic nutrient solution was: 0.5 mM $KH_2PO_4$, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.1 mM Fe-$Na_2$-EDTA, 0.25 mM $K_2SO_4$, 1 mM $NH_4NO_3$, 2.5 mM $KNO_3$, 30 $\mu$M $H_3BO_3$, 5 $\mu$M $MnSO_4$, 1 $\mu$M $ZnSO_4$, 1 $\mu$M $CuSO_4$ and 0.7 $\mu$M $Na_2MoO_4$. The pH of the hydroponic solution was adjusted to 6.0.

### 2.2. Determination of osmolality

Leaves of *M. truncatula* and *M. falcata* from those seedlings grown in the absence and presence of 100 mM NaCl for different durations were collected in 1.5 mL tubes, and frozen in liquid $N_2$. Cell saps were collected by centrifuging at 10,000 × *g* for 1 min after thawing at room temperature as described by Zhang et al. (1996). Osmolality of the cell sap was measured by a VAPRO 5520 vapor pressure osmometer.

### 2.3. Determination of Pro and soluble sugars contents

Shoots of the two species were dried at 80 °C for 48 h and triturated. About 100 mg dried shoot-materials were extracted in 5 mL 80% ethanol at 80 °C for 1 h, then cooled at room temperature. The extract was de-colored with 50 mg active carbon for 30 min and then centrifuged at 6000 × *g* for 10 min. The supernates were used to determine concentrations of Pro and soluble sugars. Pro concentration was determined as described previously (Bates et al., 1973). Briefly, the extract (2 mL) was incubated with 2 mL ninhydrin reagent (2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid, 40% 6 M phosphoric acid) and 2 mL of glacial acetic acid at 100 °C for 1 h, and the reaction terminated in an ice bath. Toluene (4 mL) was added, followed by vibrating and incubation at room temperature. The absorbance was measured at wavelength of 520 nm using a spectrophotometer (SmartSpecTM Plus, BioRad). Concentration of soluble sugars was measured following the methods described by Bailey (1958). Briefly, 1 mL extract was incubated with 5 mL anthrone reagent at 95 °C for 15 min, and then cooled at room temperature. The absorbance was measured at wavelength of 625 nm using a spectrophotometer (SmartSpecTM Plus, BioRad). The soluble sugar content was expressed as $\mu$mol eq. glucose per g DW.

### 2.4. Determination of $Na^+$ and $K^+$ contents in shoots and roots

Shoots and roots of the two species were harvested, and washed with ultrapure water thoroughly, dried at 80 °C for 48 h and triturated. About 20 mg of dry materials were weighed and placed in a digestion tube, and 6 mL of nitric acid and 1 mL of hydrogen peroxide were added for digestion. The digested fluid volume was finalized to 100 mL, and concentrations of $Na^+$ and $K^+$ were measured by ICP-AES (Thermo).

### 2.5. Determination of $Na^+$ and $K^+$ concentration in xylem

Xylem sap was collected by a pressure chamber (PMS, Instruments, Corvallis, OR) following the protocols described by Li et al. (2009). Briefly, the excised roots from the two species grown in medium supplemented with and without 100 mM NaCl for varying periods were put into the chamber. To minimize the diurnal fluctuations in the concentration of xylem sap contents, xylem sap was collected in the morning between 10:00 and 12:00 am, and the collection was made from *M. truncatula* and *M. falcata* seedlings alternatively. A pressure of about 1.1 MPa was applied for 15 min

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