

Overexpression of *Arabidopsis* H⁺-pyrophosphatase improves the growth of alfalfa under long-term salinity, drought conditions and phosphate deficiency

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Abstract: Alfalfa planting is threatened by limited arable land, salinization, water shortage, and soil nutrient deprivation. To deal with this challenge, we previously introduced the *Arabidopsis* type I H⁺-pyrophosphatase gene *AVP1* into alfalfa and found that transgenic lines exhibited enhanced tolerance to short-term salinity or drought. In this study, the growth performances of two transgenic lines were further investigated under long-term salinity or drought conditions, as well as under phosphate deficiency (low-Pi). Compared with wild-type (WT) plants, the transgenic alfalfa showed better growth performance with taller plants and more biomass accumulation after being treated with either long-term salinity, long-term drought, or low-Pi. Most importantly, the overexpression of *AVP1* significantly increased the root dry weight and the root/shoot ratio of transgenic alfalfa. A more robust root system facilitates the transgenic alfalfa to absorb nutrients, and in turn promotes the growth of the plants. Whether being treated with low-Pi or not, transgenic plants showed higher total phosphorus concentrations by 16.5–35.5% than WT plants. This study laid a foundation for breeding alfalfa cultivars adapted to saline, arid and nutrient-deprived marginal land.

Keywords: *AVP1* gene; growth performance; phosphorus nutrition; stress tolerance; transgenic alfalfa

Alfalfa (*Medicago sativa* L.) is an important legume forage all over the world (KUMAR *et al.* 2018), and has been bringing in considerable profits for farmers and companies in northern China in recent years, since it is an important foundation for the Chinese dairy industry. From 2001 to 2013, alfalfa planting areas in China were significantly extended by 74% and about 70% of the areas are distributed in five northwestern provinces which belong to arid or

semi-arid regions with salinization and soil nutrient deprivation (SHI *et al.* 2017). In these areas, the productivity of alfalfa is limited by saline and arid environments because of weak salinity and drought tolerance in the existing cultivars (KUMAR 2011; SHI *et al.* 2017). Moreover, it is reported that phosphate (Pi) deficiency is one of the limiting factors that reduce the yield and persistence of alfalfa (BERG *et al.* 2009), since soil Pi deficiency is common in legume crop

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growing areas (including northern China) (MA *et al.* 2012; BARGAZ *et al.* 2017). To cope with this problem, the increasing application of phosphate fertilizer has resulted in increasing costs as well as environment damage (GAXIOLA *et al.* 2016). Therefore, under the background of limited arable land, for alfalfa production in northwestern China it is necessary to develop alfalfa cultivars suitable for growing on saline, arid and nutrient-deprived marginal land.

Lots of previous studies have demonstrated that the overexpression of type I H^+ -pyrophosphatase (H^+ -PPase) genes significantly prompted the plant growth under various abiotic stresses (e.g., salinity, drought and nutrient deficiency) and non-stressed conditions, since H^+ -PPase plays important roles in many physiological and biochemical processes including vacuolar compartmentation of cations, intracellular ions and pH homeostasis, plant development, nutrient use efficiency and transport of photosynthates (FERJANI *et al.* 2011, 2012; PIZZIO *et al.* 2015; GAXIOLA *et al.* 2016; KHADILKAR *et al.* 2016). *Arabidopsis* H^+ -PPase (AVP1) has recently been considered as “one protein with many roles” depending on its different tissue-specific localizations (SCHILLING *et al.* 2017), suggesting that the genetic manipulation of H^+ -PPases is extremely beneficial to the improvement of stress tolerance and yield in crops.

In a previous study, we generated the transgenic alfalfa lines overexpressing the *Arabidopsis* H^+ -PPase gene *AVP1* and found that these lines exhibit enhanced tolerance to short-term salt (100 and 200 mM NaCl for 7 days) or drought (water withheld for 8 days) treatments (BAO *et al.* 2009). To incorporate these transgenic alfalfa lines into new breeding materials adapting to marginal land, here we further investigated their growth performance under long-term salinity or drought stress, as well as in phosphate deficiency conditions.

MATERIAL AND METHODS

Plant material, growth conditions, and treatments. Two transgenic alfalfa (*M. sativa*) T_0 lines overexpressing the *Arabidopsis* *AVP1* gene [L1 and L8, which showed the highest and the lowest expression level of *AVP1*, respectively, Figure S1 in Electronic Supplementary Material (ESM)], wild-type (WT) plants as well as their propagation procedure were the same as in our previous work (BAO *et al.* 2009; Supplementary Information in ESM). The plants

were cultured in a greenhouse under a photoperiod of 16/8 h (light/dark, the light density during the light period was 800 mmol/m²/s) at $26 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ of relative humidity.

For the long-term salinity treatment, transgenic lines (L1 and L8) and WT plants were transplanted into plastic pots (cylindrical pots of 10 cm in diameter and 12 cm in height, three plants per pot) containing the loessal soil, and watered with 1/8 Hoagland nutrient solution for 15 days. Then the nutrient solution was supplemented with 0, 50 and 100 mM NaCl for 20 days, respectively.

For the long-term drought treatment, the transgenic and WT plants were transplanted into plastic pots (cylindrical pots of 15 cm in diameter and 20 cm in height, six plants per pot) containing the loessal soil, and watered with 1/8 Hoagland nutrient solution with 90% of field water capacity (FWC) for 20 days. The shoots were trimmed away with 5 cm stubble, and the soil water content was controlled to 80% of FWC by weighing for 5 days. Then the pots were divided into three groups: control (80% of FWC), mild drought (60% of FWC) and severe drought (40% of FWC). After 35 days of drought treatment, the plants were sampled for assay.

For phosphate deficiency treatment, the transgenic and WT plants were transplanted into plastic pots (cylindrical pots of 8 cm in diameter and 10 cm in height, one plant per pot) containing perlite (the main constituent is quartz and it does not contain any Pi), and irrigated with Hoagland nutrient solution containing 1 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (control) or 0.01 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (low-Pi, the reduction of nitrogen was supplied with extra NH_4NO_3). The corresponding solutions were refreshed every 2 days. After being treated for 21 days, the plants were harvested for assay.

Determination of growth indicators. The shoot height was measured by a flexible rule. Then root and shoot were separated and dried in an oven at 80°C for 72 h for weighing the dry weight (DW), respectively. Finally, the root/shoot ratio was calculated.

Measurement of total phosphorus concentration. Total phosphorus (P) concentration was determined according to the method described by KANG *et al.* (2016). The dry samples were ashed in a muffle furnace (TNX1700-30; Shinbae Industrial Co. Ltd, Shanghai, P.R. China) at 600°C for 6 h. The ash samples were dissolved in 10 ml of 1 M HCl and a few drops of HNO_3 . After filtration, total Pi concentration was determined using a spectrophotometer (UV-6100PCS; Mapada Instruments Co. Ltd, Shanghai, P.R. China).

Statistical analysis. Data were subjected to One-way Analysis of Variance (ANOVA) using SPSS (Ver. 16.0, 2007). Duncan's multiple range tests were used to detect significant differences between the means at a significance level of $P < 0.05$. There are at least three replicates in each experiment. The data were presented as mean $\pm$ SD ($n = 18$).

RESULTS

Overexpression of *AVP1* improves the growth performance of transgenic alfalfa in long-term NaCl treatments. Under 100 mM of NaCl, both L1 and L8 lines grew better, while WT almost died (Figure S2 in ESM); the shoot height and shoot dry weight of both transgenic lines were 56.9% and 40.8% (Figure 1a), 42.0% and 33.4% (Figure 1b), higher than for WT, respectively, although they decreased progressively from all plants with increasing external NaCl concentration. Interestingly, transgenic lines showed higher root dry weight compared with WT under either control or NaCl treatments (Figure 1c), and correspondingly, the root/shoot ratios of both transgenic lines were significantly higher than those of WT plants (Figure 1d).

Transgenic alfalfa overexpressing *AVP1* exhibits enhanced resistance to long-term water deficiency. Under either normal watering (control) or

drought stress conditions, the transgenic lines (especially L1) exhibited faster growth than WT plants (Figure S3 in ESM). The shoot heights of L1 and L8 were 21.6%–35.8% and 15.5%–27.4%, respectively, higher than those of WT under control and moderate drought (60% of FWC) conditions; in severe drought (40% of FWC) conditions, however, the shoot height of L1 was significantly higher than that of L8 and WT, while there was no significant difference between L8 and WT plants (Figure 2a). Determination of biomass showed consistent results with the data of shoot height: both transgenic lines showed higher shoot and root dry weight than WT plants under either control or moderate drought conditions (Figure 2b, c). Similar to the situation of salt treatment, the root/shoot ratios of both transgenic lines were significantly higher than that of WT plants under either normal or water deficit conditions. For instance, after being treated with severe drought stress, L1 and L8 maintained the higher root/shoot ratio by 43.5% and 23.9% compared with WT plants, respectively (Figure 2d).

Overexpression of *AVP1* improves the tolerance to phosphate deficiency (Low-Pi) in transgenic alfalfa. Under control (1 mM Pi), there was no growth difference between transgenic and WT plants (Figure S4a in ESM). When subjected to low-Pi (0.01 mM) for 21 days, the growth of all plants was significantly

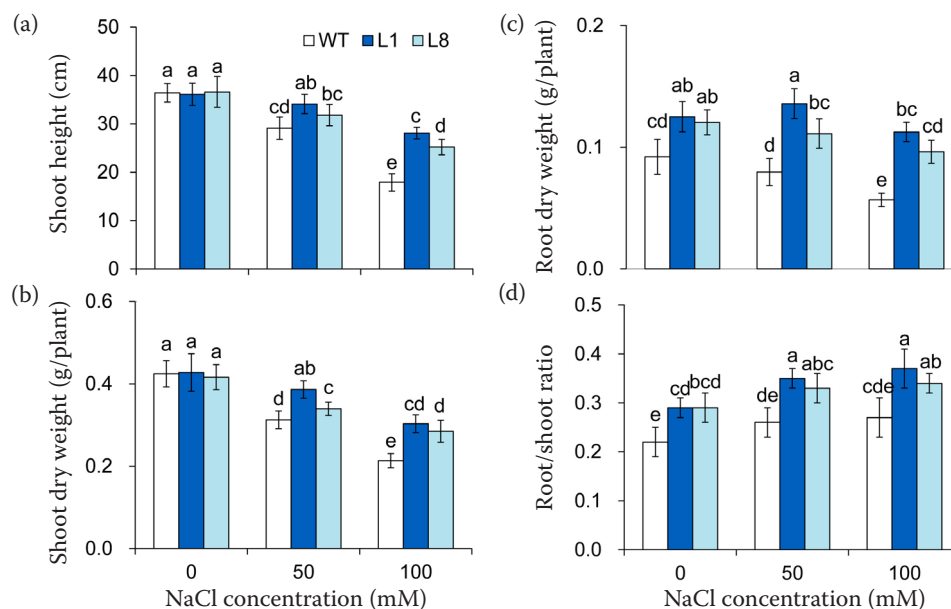


Figure 1. The growth indicators of wild-type plants and transgenic alfalfa treated with long-term salinity (50 and 100 mM NaCl) for 20 days: shoot height (a), shoot dry weight (b), root dry weight (c), root/shoot ratio (d). Values are means $\pm$ SD ($n = 18$); different letters indicate significant differences ($P < 0.05$) between columns; WT – wild-type plants; L1, L8 – transgenic lines overexpressing *AVP1*

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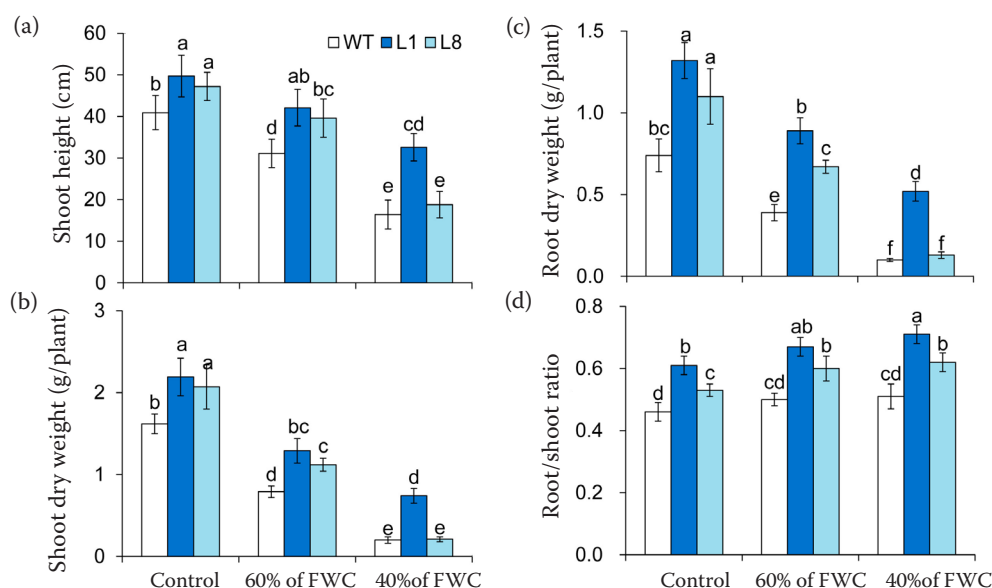


Figure 2. The growth indicators of wild-type plants and transgenic alfalfa treated with long-term drought (60% and 40% of FWC) for 35 days: shoot height (a), shoot dry weight (b), root dry weight (c), root/shoot ratio (d)

Values are means ± SD ($n = 18$); different letters indicate significant differences ($P < 0.05$) between columns; WT – wild-type plants; L1, L8 – transgenic lines overexpressing *AVP1*

inhibited, while transgenic lines showed a lower growth reduction compared with WT (Figure S4b in ESM). The plant height and shoot dry weight of L1 and L8 were higher by 18.4–36.2% and 20.7–39.8% than for WT, respectively (Figure 3a, b). Of note, the transgenic alfalfa developed more robust roots,

whether treated with low-Pi or not (Figure 3c, d). The root dry weights and root/shoot ratios of transgenic lines were 1.3–1.5 and 1.3–1.4 times higher than in WT plants under control, and 1.5–1.9 and 1.2–1.4 times higher under low-Pi conditions, respectively (Figure 3c, d).

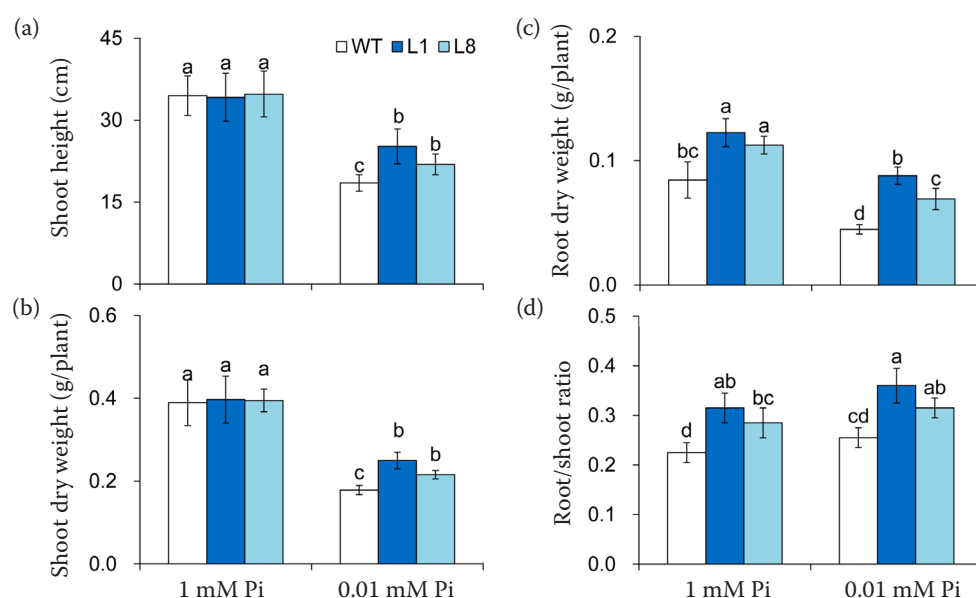


Figure 3. The growth indicators of wild-type plants and transgenic alfalfa treated with phosphate deficiency (0.01 mM Pi) for 21 days: shoot height (a), shoot dry weight (b), root dry weight (c), root/shoot ratio (d)

Values are means ± SD ($n = 18$); different letters indicate significant differences ($P < 0.05$) between columns; WT – wild-type plants; L1, L8 – transgenic lines overexpressing *AVP1*

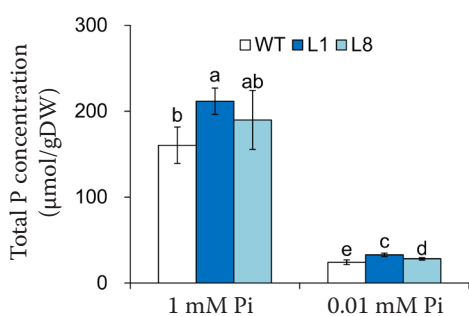


Figure 4. Total phosphorus (P) concentration in wild-type plants and transgenic alfalfa treated with phosphate deficiency (0.01 mM Pi) for 21 days

Values are means $\pm$ SD ($n = 18$); different letters indicate significant differences ($P < 0.05$) between columns; WT – wild-type plants; L1, L8 – transgenic lines overexpressing *AVP1*

Under normal conditions, the transgenic alfalfa accumulated more P. The total P concentration of L1 was 31.9% higher than that of WT plants. After being treated with low-Pi for 21 days, P accumulations significantly declined in all plants, but L1 and L8 still showed the higher total P concentration by 35.5% and 16.5% in comparison with WT, respectively (Figure 4).

DISCUSSION

We previously found that the transgenic alfalfa overexpressing *AVP1* exhibited better growth performance than WT under short-term NaCl or water deficit treatments (BAO *et al.* 2009). To investigate whether the transgenic alfalfa possesses persistent resistance to salinity or drought, here we tested the transgenic lines with long-term salinity or drought treatments, and got similar results to previous work, further suggesting that the overexpression of *AVP1* enhanced the salt and drought tolerance of transgenic alfalfa. In general, the above salt- and drought-tolerant phenotypes of transgenic alfalfa might be ascribed to constant intracellular ionic homeostasis and enhanced vacuolar osmoregulatory capacity in transgenic plants by sequestering excess cytosolic Na^+ into vacuoles, since tonoplast H^+ -PPase is considered as an efficient proton pump across the vacuole membrane (GAXIOLA *et al.* 2016).

In addition, studies in recent years also revealed other functions of H^+ -PPase, such as increasing the cell number (ASAOKA *et al.* 2016), stimulating the heterotrophic growth (FERJANI *et al.* 2012; PIZZIO *et al.* 2015; ASAOKA *et al.* 2016; FUKUDA *et al.* 2016; KHADILKAR *et al.* 2016), facilitating the transport of

sucrose from source to sink as well as improving the nutrient uptake (GAXIOLA *et al.* 2016; KHADILKAR *et al.* 2016; SCHILLING *et al.* 2017). In this study, the transgenic alfalfa overexpressing *AVP1* showed higher shoot biomass under various stresses, and interestingly, developed a more robust root system with increased root dry weight and root/shoot ratio under either normal or stress conditions, compared with WT plants. Similar results were observed in other species overexpressing H^+ -PPase including *Arabidopsis* (YANG *et al.* 2014; KHADILKAR *et al.* 2016) and *Lotus corniculatus* (BAO *et al.* 2014), since H^+ -PPase contributes to enhanced vacuolar acidification and regulation of cytoplasmic PPI levels which are responsible for many traits related to the growth of plants (GAXIOLA *et al.* 2016; SCHILLING *et al.* 2017). On the other hand, a bigger and stronger root system would facilitate plants to absorb nutrients, and in turn promote the growth of plants under different conditions. For example, the overexpression of H^+ -PPase conferred tolerance to nitrogen deficiency (PAEZ-VALENCIA *et al.* 2013; Lv *et al.* 2015) and low-Pi stress (YANG *et al.* 2007) in transgenic plants. The latest study indicated that the upregulation of H^+ -PPase even improved the iron use efficiency of sweet potato (*Ipomoea batatas*) (FAN *et al.* 2017). These findings were further supported by our data, in which the transgenic alfalfa overexpressing *AVP1* exhibited enhanced tolerance to Pi deficiency compared with WT. This phenotype is consistent with increased P accumulation in transgenic lines, suggesting that overexpression of *AVP1* improved the phosphorus nutrition of alfalfa.

In conclusion, the data of our study demonstrate that the transgenic alfalfa overexpressing *AVP1* shows enhanced growth performance under long-term salinity and drought stresses, in particular, overexpression of *AVP1* confers tolerance to phosphate deficiency by increasing P accumulation in transgenic plants. This study laid a foundation for generating the alfalfa cultivar adapting to saline, arid and nutrient-deprived marginal land.

References

- Asaoka M.M., Segami S., Ferjani A., Maeshima M. (2016): Contribution of PPI-hydrolyzing function of vacuolar H^+ -pyrophosphatase in vegetative growth of *Arabidopsis*: evidenced by expression of uncoupling mutated enzymes. *Frontiers in Plant Science*, 7: 415.
- Bao A.K., Wang S.M., Wu G.Q., Xi J.J., Zhang J.L., Wang C.M. (2009): Overexpression of the *Arabidopsis* H^+ -PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Science*, 176: 232–240.

<https://doi.org/10.17221/134/2018-CJGPB>

- Bao A.K., Wang Y.W., Xi J.J., Liu C., Zhang J.L., Wang S.M. (2014): Co-expression of xerophyte *Zygophyllum xanthoxylum* *ZxNHX* and *ZxVP1-1* enhances salt and drought tolerance in transgenic *Lotus corniculatus* L. by increasing cations accumulation. *Functional Plant Biology*, 41: 203–214.
- Bargaz A., Genevieve L.N., Georg C., Jessie R.F., Erik J., Driss D., Roberta F., Marney E.I. (2017): Species interactions enhance root allocation, microbial diversity and P acquisition in intercropped wheat and soybean under P deficiency. *Applied Soil Ecology*, 120: 179–188.
- Berg W.K., Cunningham S.M., Brouder S.M., Joern B.C., Johnson K.D., Volenec J.J. (2009): Influence of phosphorus and potassium on alfalfa yield, taproot C and N pools, and transcript levels of key genes after defoliation. *Crop Science*, 49: 974–982.
- Fan W.J., Wang H., Wu Y., Yang N., Yang J., Zhang P. (2017): H⁺-pyrophosphatase IbVP1 promotes efficient iron use in sweet potato [*Ipomoea batatas* (L.) Lam.]. *Plant Biotechnology Journal*, 15: 698–712.
- Ferjani A., Segami S., Horiguchi G., Muto Y., Maeshima M., Tsukaya H. (2011): Keep an eye on PPi: the vacuolar-type H⁺-pyrophosphatase regulates postgerminative development in *Arabidopsis*. *The Plant Cell*, 23: 2895–2908.
- Ferjani A., Segami S., Horiguchi G., Sakata A., Maeshima M., Tsukaya H. (2012): Regulation of pyrophosphate levels by H⁺-PPase is central for proper resumption of early plant development. *Plant Signaling and Behavior*, 7: 38–42.
- Fukuda M., Segami S., Tomoyama T., Asaoka M.M., Nakaniishi Y., Gunji S., Ferjani A., Maeshima M. (2016): Lack of H⁺-pyrophosphatase prompts developmental damage in *Arabidopsis* leaves on ammonia-free culture medium. *Frontiers in Plant Science*, 7: 819.
- Gaxiola R.A., Regmi K., Hirschi K.D. (2016): Moving on Up: H⁺-PPase mediated crop improvement. *Trends in Biotechnology*, 34: 347–349.
- Kang P., Bao A.K., Kumar T., Pan Y.Q., Bao Z., Wang F., Wang S.M. (2016): Assessment of stress tolerance, productivity, and forage quality in T₁ transgenic alfalfa co-expressing *ZxNHX* and *ZxVP1-1* from *Zygophyllum xanthoxylum*. *Frontiers in Plant Science*, 7: 1598.
- Khadilkar A.S., Yadav U.P., Salazar C., Shulaev V., Paez-Valencia J., Pizzio G.A., Gaxiola R.A., Ayre B.G. (2016): Constitutive and companion cell-specific overexpression of AVP1, encoding a proton-pumping pyrophosphatase, enhances biomass accumulation, phloem loading, and long-distance transport. *Plant Physiology*, 170: 401–414.
- Kumar S. (2011): Biotechnological advancements in alfalfa improvement. *Journal of Applied Genetics*, 52: 111–124.
- Kumar T., Bao A.K., Bao Z., Wang F., Gao L., Wang S.M. (2018): The progress of genetic improvement in alfalfa (*Medicago sativa* L.). *Czech Journal of Genetics and Plant Breeding*, 54: 41–45.
- Lv S.L., Jiang P., Nie L.L., Chen X.Y., Tai F., Wang D.L., Fan P.X., Feng J.J., Bao H., Wang J.H., Li Y.X. (2015): H⁺-pyrophosphatase from *Salicornia europaea* confers tolerance to simultaneously occurring salt stress and nitrogen deficiency in *Arabidopsis* and wheat. *Plant, Cell and Environment*, 38: 2433–2449.
- Ma X.F., Tudor S., Butler T., Ge Y., Xi Y., Bouton J., Harrison M., Wang Z.Y. (2012): Transgenic expression of phytase and acid phosphatase genes in alfalfa (*Medicago sativa*) leads to improved phosphate uptake in natural soils. *Molecular Breeding*, 30: 377–391.
- Paez-Valencia J., Sanchez-Lares J., Marsh E., Dorneles L.T., Santos M.P., Sanchez D., Winter A., Murphy S., Cox J., Trzaska M., Metler J., Kozic A., Facanha A.R., Schachtman D., Sanchez C.A., Gaxiola R.A. (2013): Enhanced proton translocating pyrophosphatase activity improves nitrogen use efficiency in romaine lettuce. *Plant Physiology*, 161: 1557–1569.
- Pizzio G.A., Paez-Valencia J., Khadilkar A.S., Regmi K., Patron-Soberano A., Zhang S., Sanchez-Lares J., Furstenu T., Li J.S., Sanchez-Gomez C., Valencia-Mayoral P., Yadav U.P., Ayre B.G., Gaxiola R.A. (2015): *Arabidopsis* proton-pumping pyrophosphatase AVP1 expresses strongly in phloem where it is required for PPi metabolism and photosynthate partitioning. *Plant Physiology*, 167: 1541–1553.
- Schilling R.K., Tester M., Marschner P., Plett D.C., Roy S.J. (2017): AVP1: one protein, many roles. *Trends in Plant Science*, 22: 154–162.
- Shi S.L., Nan L.L., Smith K.F. (2017): The current status, problems, and prospects of alfalfa (*Medicago sativa* L.) breeding in China. *Agronomy*, 7: 1.
- Yang H.B., Knapp J., Koirala P., Rajagopal D., Peer W.A., Silbart L.K., Murphy A.S., Gaxiola R.A. (2007): Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H⁺-pyrophosphatase. *Plant Biotechnology Journal*, 5: 735–745.
- Yang H.B., Zhang X., Gaxiola R.A., Xu G., Peer W.A., Murphy A.S. (2014): Over-expression of the *Arabidopsis* proton-pyrophosphatase AVP1 enhances transplant survival, root mass, and fruit development under limiting phosphorus conditions. *Journal of Experimental Botany*, 65: 3045–3053.

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