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Research article

The expression of alfalfa *MsPP2CA1* gene confers ABA sensitivity and abiotic stress tolerance on *Arabidopsis thaliana*

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ABSTRACT

Although clade A phosphatase 2Cs (PP2CAs) are well known to regulate abscisic acid (ABA) signaling, few members of this family have been identified in alfalfa so far. Here, the isolation and characterization of the gene *MsPP2CA1* from alfalfa is described. Its transcription was found to be highly inducible by treatment with abscisic acid, salt, hydrogen peroxide and polyethylene glycol. The constitutive expression of *MsPP2CA1* in *Arabidopsis thaliana* seedlings mitigates root growth imposed by either salinity or oxidative stress, while also raising the level of sensitivity to ABA during germination and early seedling development, and promoting stomatal closure. In transgenic plants, many ABA-dependent stress-responsive genes were activated, and the expressions of catalase and peroxidase which involved in reactive oxygen scavenging were promoted. *MsPP2CA1* is suggested as a candidate for the genetic manipulation of salinity tolerance in legume species.

1. Introduction

A number of abiotic stress agents act to restrict plant growth and hence crop productivity. Plants have evolved an elaborate system of signaling to first sense stress and then to trigger a response. A mechanism for signal transduction commonly used by plants exploits the reversible phosphorylation of proteins, since the addition or removal of a phosphate group from a protein can have a profound effect on its structure and thus also its function (Luan, 2003). Phosphorylation is achieved by the action of protein kinases, while dephosphorylation is carried out by protein phosphatases (You et al., 2014). The ability of proteins to be converted readily from one phosphorylation state to another is exploited to achieve a rapid response to stress.

The plant response to most abiotic stresses involves the phytohormone ABA. It has been convincingly established in the model plant species *Arabidopsis thaliana*. Within the family of type 2C phosphatases (PP2Cs), members of clade A have been shown to inhibit ABA signaling (Lee and Luan, 2012). The core ABA signaling module includes the three components PYR/PYL/RCAR (pyrabactin resistance/PYR-like protein/regulatory component of ABA receptor), clade A PP2C and SnRK2 (SNF1-related protein kinase 2) (Ma et al., 2009). The proposition is that in the absence of ABA, the PP2Cs interact with SnRK2s to inhibit SnRK2 kinase activity, while in its presence, the binding of ABA to PYR/PYL/RCAR releases SnRK2, thereby activating the genes required for the response to external stress (Lee and Luan, 2012).

The PP2CAs are well conserved at the sequence and structural level across a range of plant species (Xue et al., 2008; Sun et al., 2011; Wang et al., 2012), although in alfalfa this phenomenon is still less clear. As yet, there is only scanty evidence to support their active contribution to stress tolerance. Tobacco plants constitutively expressing maize *PP2C* have been reported as highly tolerant of low temperature (Hu et al., 2010); the expression of the rice gene *PP108* in *A. thaliana* appears to enhance the plants' tolerance to both salinity and mannitol (Singh et al., 2015); finally, expressing the *Brachypodium distachyon* PP2CA gene *BdPP2CA6* in *A. thaliana* promotes stomatal closure and salinity tolerance (Zhang et al., 2017).

The productivity of alfalfa (*Medicago sativa*), an important perennial leguminous forage species, is severely limited by salinity stress (Sun et al., 2018). A number of genes likely associated with its albeit limited capacity to tolerate salinity have been isolated and characterized. These have encoded various transcription factors (Winicov and Bastola, 1999; Chen et al., 2012), microRNAs (Long et al., 2015), and the synthesis of certain metabolites (Palma et al., 2013). However, there is still little information about the role of PP2Cs in the abiotic stress response of alfalfa. Here, the isolation of the gene *PP2CA1* is described, and its contribution to stress tolerance when constitutively expressed in *A.*

Abbreviations: ABA, Abscisic acid; PEG6000, Polyethylene glycol; GFP, Green fluorescent protein; qPCR, quantitative real-time polymerase chain reaction; WT, Wild-type; MDA, Malondialdehyde; ROS, Reactive oxygen species; SOD, Superoxide Dismutase; CAT, Catalase; POD, Peroxide; DAB, Diaminobezidin

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thaliana assessed.

2. Materials and methods

2.1. Plant materials and growing conditions

Alfalfa seeds were germinated on moist filter paper in the dark for 24 h at 4 °C, then in the light for 48 h at 20 °C. Germinated seeds were potted into a 1:1 mixture of perlite and sand, and irrigated with half strength Hoagland's solution three times a week. The plants were maintained in a cabinet delivering a relative humidity of 80%, a constant temperature of 25 °C, and a photoperiod of 12 h (light intensity 300 μmol m^{-2}·s^{-1}). Four different stress treatments were given to seedlings by including one of 200 mM NaCl, 20% w/v PEG6000, 10 mM H_2O_2, or 100 μM ABA in the irrigation solution. Each treatment was replicated three times. Plant tissue required for RNA extraction was snap-frozen in liquid nitrogen and stored at −80 °C until required.

2.2. Gene isolation and sequence analysis

A partial alfalfa *PP2CA* homolog identified via our previous RNA-Sequencing analysis (Dong et al., 2018) was used as a BlastN query sequence against the set of alfalfa and *M. truncatula* ESTs represented in GenBank. The resulting hits were assembled using CAP3 software (Huang and Madan, 1999), and the assembled sequence was used to design a pair of PCR primers (Table S1) able to amplify both the full length cDNA and genomic DNA of *MsPP2CA1* (Table S2). Polypeptide sequences homologous to MsPP2CA were obtained from GenBank, and were aligned using the MegAlign program implemented within DNAStar (www.dnastar.com). The neighbor-joining method was used to conduct a phylogenetic analysis, using routines implemented in ClustalX (www.clustal.org) and MEGAv6.0 (www.megasoftware.net), employing 1000 bootstrap replicates to support individual branches.

2.3. Transcriptional analysis using quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen). The cDNA first strand was synthesized using an M-MLV kit (Invitrogen). Each 20 μL qRT-PCR contained 10 μL 2x SYBR Premix Ex Taq mix (TaKaRa), 0.2 mM of each primer, and 1 μL of a 1:10 dilution of the cDNA first strand reaction. The cycling regime comprised an initial denaturation (95 °C/2 min), followed by 45 cycles of 95 °C/10 s, 60 °C/20 s, and 72 °C/20 s. A melting curve analysis was performed over the range 80–95 °C at 0.5 °C intervals. The *MsACTIN* gene was used as the reference (Wang et al., 2015), and relative transcript abundances were derived using the $2^{-\delta\delta Ct}$ method (Livak and Schmittgen, 2001). Three technical replicates were run for each of the three biological replicates.

2.4. A. thaliana transformation and the assessment of the stress response of transgenic plants

The *MsPP2CA1* coding region was inserted into the pStart vector (De Amicis et al., 2007), placing it under the control of the CaMV 35S promoter. The resulting construct (or an empty pSTART vector) was transformed into *A. thaliana* ecotype Col-0 using the floral dip method (Clough and Bent, 1998). Seeds harvested from transgene homozygotes (OE lines) and from lines carrying the empty pSTART vector (VC line) were surface-sterilized and plated on solidified medium containing half strength MS salts. The plates were held at 4 °C in the dark for two days, and then moved to a chamber delivering a 16 h photoperiod and a constant temperature of 22 °C. After three days, the seedlings were transferred to a fresh plate containing the same medium, but supplemented with various concentrations of either NaCl or mannitol (for two weeks) or of ABA or H_2O_2 (for ten days or three weeks). Each experiment was run in triplicate. For the germination assay, about 100–150 seeds of each wild type (WT), the VC line and the two selected OE lines were surface-sterilized and plated on solidified medium containing half strength MS salts containing 10% (w/v) sucrose plus a variable concentration of ABA. The plates were held in the dark at 4 °C for three days, and then moved to the light at 21 °C. Germination was deemed as successful when the radical had visibly emerged, and was scored at various time points.

2.5. Sub-cellular localization and stomatal aperture assay

The coding sequence of *MsPP2CA1* (lacking its stop codon) was fused to the N terminus of GFP represented in the construct pCaMV35S:MsPP2CA1. The fused vector was transformed into *A. thaliana* and the roots of transgenic lines were examined to assess the sub-cellular localization of MsPP2CA1. Epidermal peels required for the analysis of stomatal aperture were following Singh et al. (2015). Each experiment was run in triplicate.

2.6. The quantification of reactive oxygen species (ROS), ion content and malondialdehyde (MDA), and the measurement of ROS scavenging ability

The malondialdehyde (MDA) content of intact two week old *A. thaliana* seedlings, along with its activity of the ROS scavenging enzymes superoxide dismutase (SOD), catalase (CAT), and peroxide (POD), were assessed using commercially available kits (Jiancheng, Nanjing, China). Measurements were made of the ion content in the leaf of seedlings raised for one week on MS medium or MS medium containing 100 mM NaCl. The contents were quantified using a ContrAA 700 atomic absorption spectrometer (Analytik Jena AG, Jena, Germany). Each measurement was repeated three times, and the Student's *t*-test was used to compare means. The level of ROS in the leaf was assessed by DAB staining, according to Asselbergh et al. (2007).

2.7. Statistics

Values are presented as means of three replicates and standard errors (SEs). Experimental data were analyzed by SPSS software followed by one-way ANOVA to determine the significant differences among treatments.

3. Results

3.1. Isolation of MsPP2CA1 cDNA and analysis of its sequence

An *MsPP2CA1* CDS sequence (Gene Bank accession: MK225609) comprised a 999 nt open reading frame, predicted to encode a 332 residue protein of molecular mass 36.43 kDa. When its matching genomic sequence was amplified from a genomic DNA template, the coding sequence was shown to be split into three exons. A domain analysis of the *MsPP2CA1*-encoded protein confirmed the presence of a PP2C catalytic domain (residues 67–328) (Fig. 1A). A phylogenetic analysis suggested *MsPP2CA1* to be a clade A PP2C, closely related to the *A. thaliana* PP2CA genes *ABI1* and *ABI2* (Fig. S1). A multiple sequence alignment showed that the MsPP2CA1 sequence was highly homologous with that of members of clade A PP2Cs (Fig. 1B). A phylogenetic analysis of the set of clade A PP2Cs revealed that *MsPP2CA1* fell within subgroup b of the clade A PP2Cs (Fig. 1C).

3.2. The inducibility by stress agents and the sub-cellular localization of MsPP2CA1

MsPP2CA1 was strongly induced by a 3 h exposure to ABA, such that the abundance of its transcript reached about fifteen fold of the base level in non-treated seedlings; the level fell to ten fold after 12 h and to six fold after 24 h (Fig. 2A). Salinity stress was also effective in inducing the gene, with its transcript abundance rising to nine fold of the base level after a 3 h exposure, declining to about six fold after 6 h and to

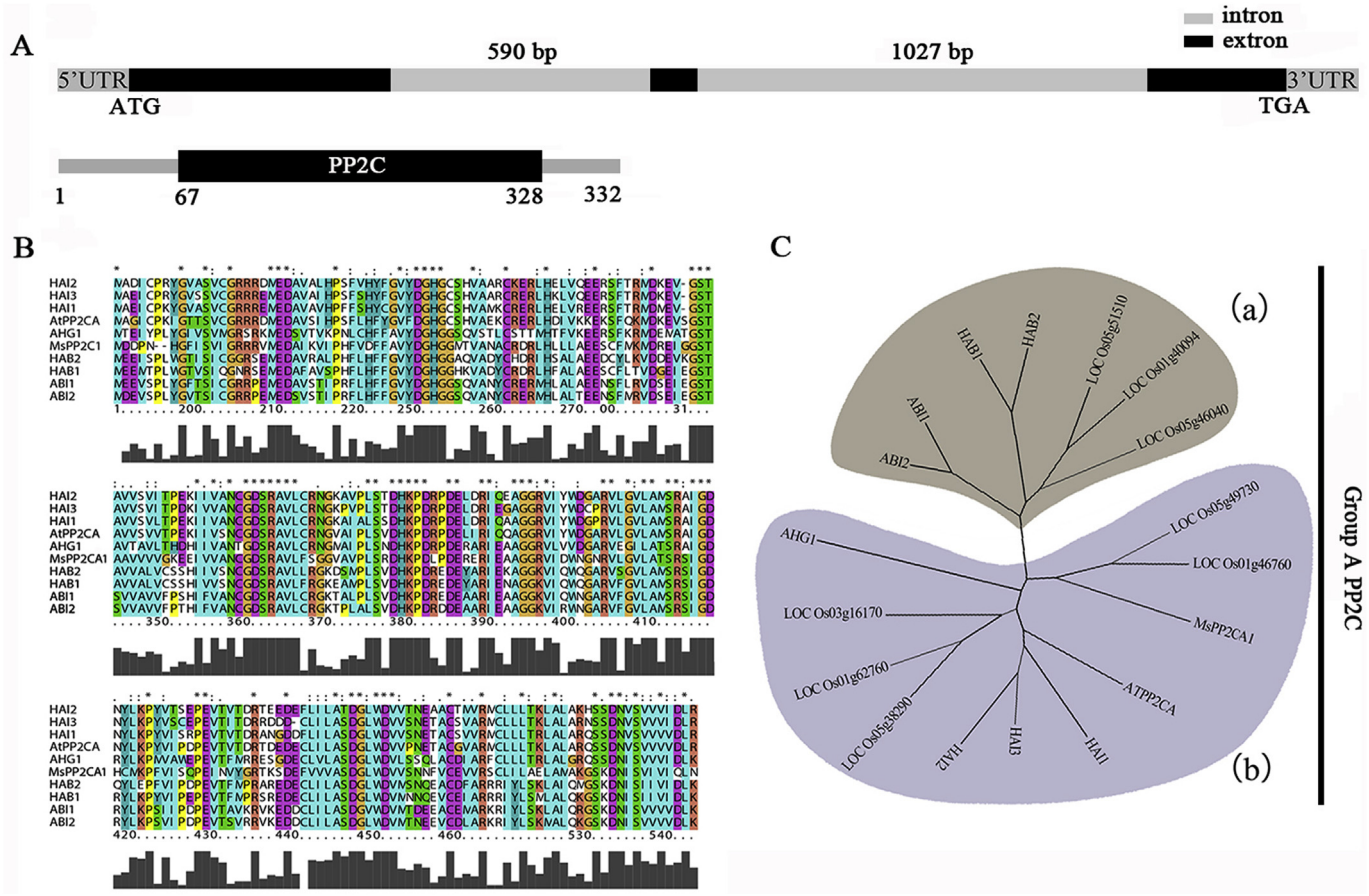

Fig. 1. Sequence and phylogenetic analysis of *MsPP2CA1*. (A) The structure of coding sequence of the gene. (B) A multiple sequence alignment illustrates the extensive inter-specific conservation of the sequence. (C) A phylogenetic analysis of clade A PP2Cs encoded by rice and *A. thaliana*.

three fold after 24 h (Fig. 2B). Similarly, moisture stress up-regulated the gene eight fold after 1 h of stress, 15 fold after 3 h and 35 fold after 6 h (Fig. 2C). The gene responded rapidly to the H_2O_2 treatment, with its transcript abundance rising by about five fold within 1 h, thereafter declining (Fig. 2D). Confocal microscopy analysis of the root cells of *A. thaliana* plants constitutively expressing *MsPP2CA1* revealed that the transgene product was deposited exclusively in the nuclei (Fig. S2A).

3.3. The constitutive expression of MsPP2CA1 raised the ABA sensitivity of A. thaliana

Six independent OE lines (transgenic *A. thaliana* lines constitutively expressing *MsPP2CA1*) were generated, along with a control line harboring the empty vector (VC line). No *MsPP2CA1* transcript was detectable in the VC plants, while the OE line plants varied with respect to the abundance of *MsPP2CA1* transcript present; lines OE1 and OE2 were retained on the basis of the abundance of the transgene transcript (Fig. S2B). There was no difference between any of the lines with respect to germination in the absence of ABA, but the germination of the seed of both OE lines was more strongly inhibited than that of either VC or WT seeds (Fig. 3A–E). In the presence of 0.75 μM ABA, > 80% of WT and VC seeds germinated successfully within three days, but the equivalent proportion for OE line seeds was only about 20%; the respective proportions in the presence of 0.5 μM ABA were nearly 90% and 50% (Fig. 3A,D,E). When these seedlings were submitted to the medium containing 5 μM ABA, the ability to elongate the primary root was lower for the OE line plants than for VC plants (Fig. 3F and G). In addition, under ABA-free growing conditions, there was no significant difference in stomatal aperture between VC and OE seedlings (Fig. 3H and I). The effects of the ABA and NaCl treatments was a reduction in the stomatal aperture of all seedlings, but the decrease was more marked in the transgenic plants than in VC ones (Fig. 3H and I).

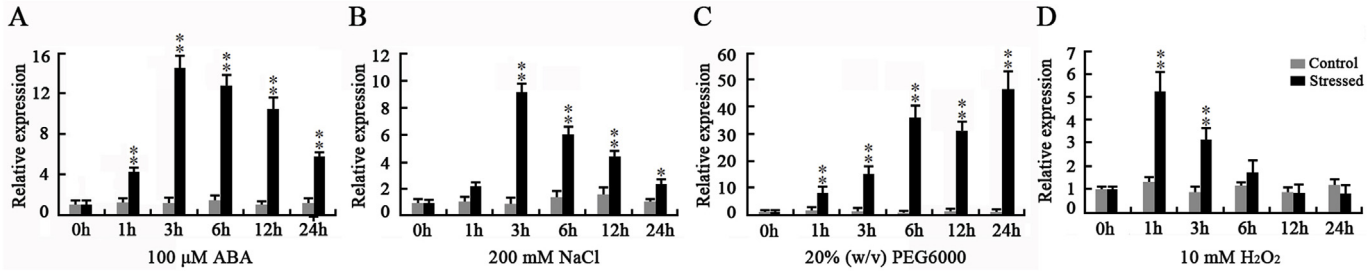

Fig. 2. qRT-PCR analysis of *MsPP2CA1* transcription in alfalfa plants exposed to different stresses. Values are presented as means of three replicates and standard errors (SEs); *, **: means differ from the control value at, respectively, $p < 0.05$ and $p < 0.01$.

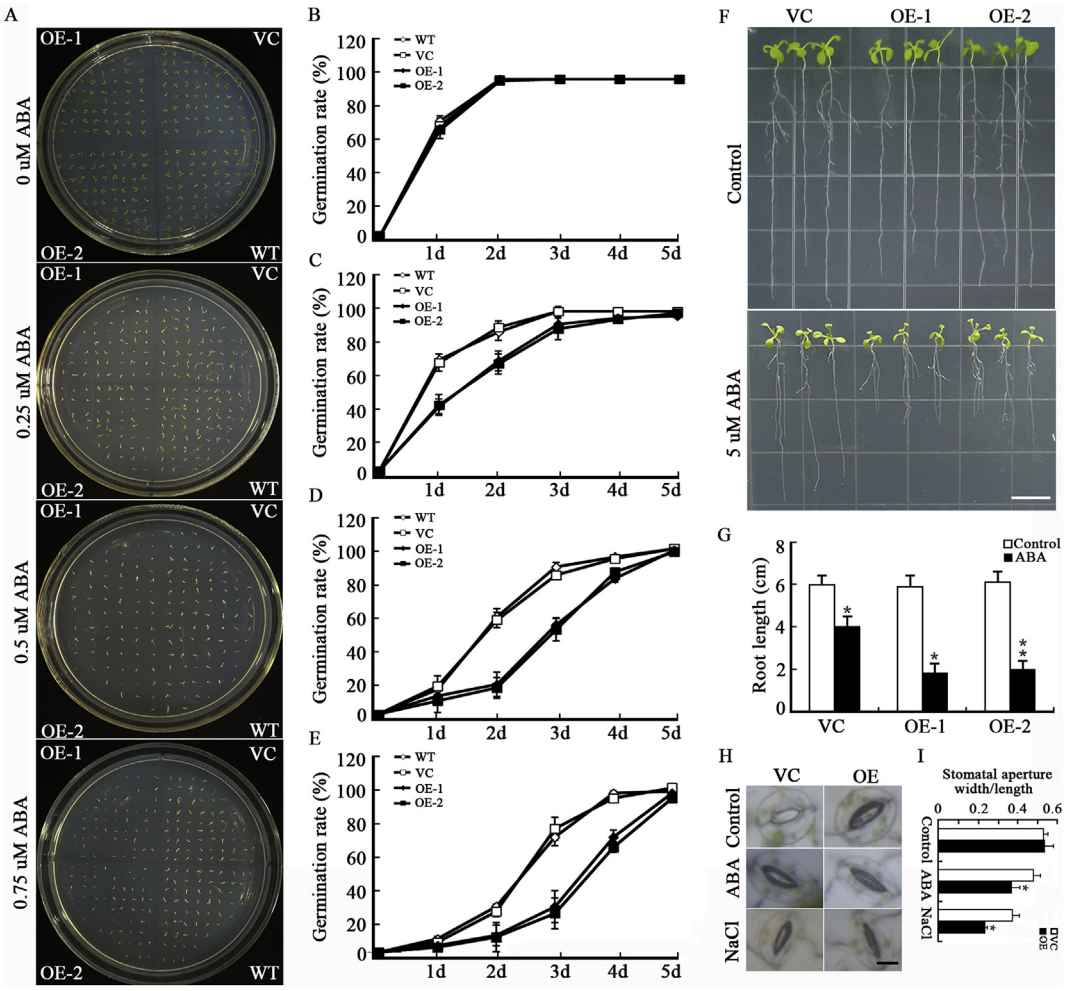

Fig. 3. The constitutive expression of *MsPP2CA1* increases the sensitivity of *A. thaliana* to ABA treatment. (A–E) The germination of OE, VC and WT seeds when challenged by (B) 0 μM, (C) 0.25 μM, (D) 0.50 μM and (E) 0.75 μM ABA. (F) Seedling growth is more severely suppressed in OE than in VC plants exposed to 5 μM ABA for ten days, bar = 1.5 cm. (G) Root lengths of the seedlings shown in (F). (H) Typical stomata in the leaves of VC and OE plants and the response to ABA and NaCl treatment, bar = 10 μm. (I) The width to length ratio of the stomata (n = 20–30), in (G). Values are presented as means of three replicates and standard errors (SEs); *, **: means differ from the control value at, respectively, $p < 0.05$ and $p < 0.01$.

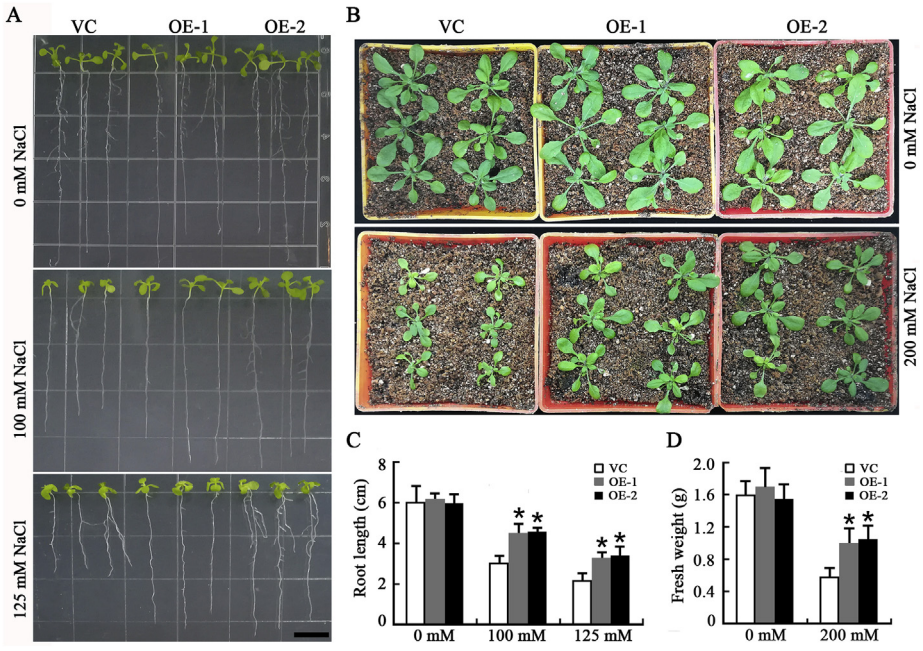

Fig. 4. The constitutive expression of *MsPP2CA1* reduces the sensitivity of *A. thaliana* to NaCl treatment. (A) The effect on root elongation of a 14 day exposure to NaCl on agar medium, bar = 1 cm. (B) The phenotype of VC and OE lines grow in soil, treated with or without NaCl. (C) The root lengths of the seedlings shown in (A). (D) The fresh weight of the seedlings shown in (B). Values are presented as means of three replicates and standard errors (SEs); *: means differ from the control value at, respectively, $p < 0.05$.

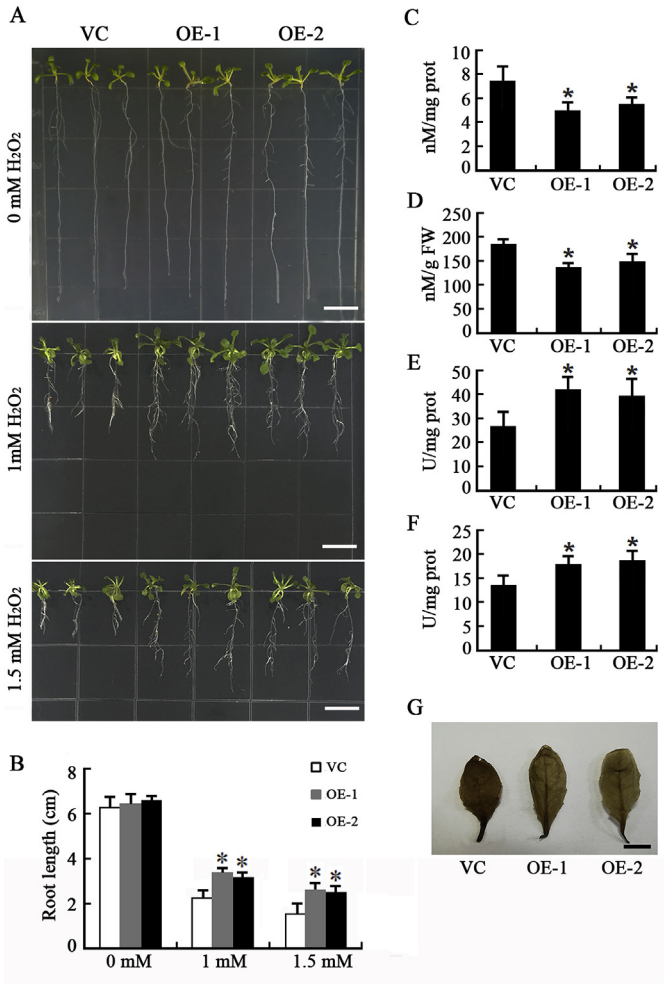

Fig. 5. The constitutive expression of *MsPP2CA1* increase antioxidant capacity of *A. thaliana* (A) The effect on root elongation of a 14 day exposure to H_2O_2 on agar medium, bar = 1 cm. (B) The root length of the seedlings shown in (A). (C)–(F) The MDA content (C), H_2O_2 content (D), POD activity (E), CAT activity (F) of control and OE lines. Values in (B) through (F) are presented as means of three replicates and standard errors (SEs); *: means differ from the VC value at $p < 0.05$. (G) DAB stained leaves of OE and VC plants, bar = 1 cm.

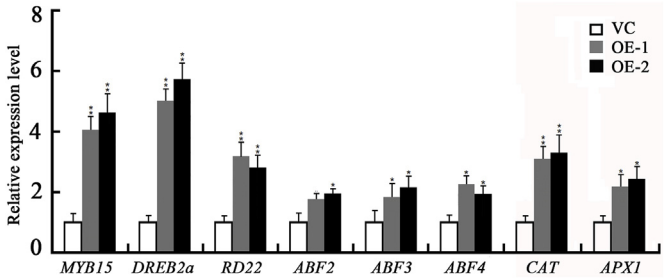

Fig. 6. The transcription of genes associated with the plant stress response in transgenic *A. thaliana* seedlings. Values are presented as means of three replicates and standard errors (SEs). *, **: means differ from the VC value at, respectively, $p < 0.05$ and $p < 0.01$.

3.4. The effect of the constitutive expression of MsPP2CA1 on tolerance to salinity and H_2O_2

The seedling root length attained by the two selected OE lines did not differ from that of VC seedlings when the seedlings were raised on agar for 14 days (Fig. 4A, C). In contrast, when challenged by the inclusion of 100 mM NaCl, the OE seedlings' primary roots grew on average 42% longer than those of the VC seedlings; in the presence of 125 mM NaCl, the advantage fell to 35% (Fig. 4A, C). After 14 days of growth in soil, 200 mM NaCl treatment was applied, and the growth amount of the control was significantly inhibited compared with that of the OE lines (Fig. 4B,D).

In addition, the OE lines also out-performed the VC lines when challenged with either 1.0 or 1.5 mM H_2O_2 for 14 days (Fig. 5A and B). In the absence of any stressors, the OE line plants accumulated less MDA and H_2O_2 than the VC plants, and both POD and CAT activity was higher (the former by approximately 40% and the latter by 20%) (Fig. 5C–F). The tendency of the OE line plants to accumulate a lower level of ROS than VC plants was confirmed by DAB staining the leaf of three week old seedlings (Fig. 5G).

3.5. Transcriptional profiling of the OE line plants

A series of qRT-PCRs conducted on OE line and VC plants grown in the absence of any stress showed that all of *AtMYB15*, *AtDREB2a*, *AtRD22*, *AtABF2*, *AtABF3*, *AtABF4*, *AtCAT* and *AtAPX1* were more strongly transcribed in the former than in the latter plants (Fig. 6). This is consistent with the hypothesis that the enhanced stress tolerance conferred by the constitutive expression of *MsPP2CA1* may achieved by the up-regulation of these (and other) genes associated with the abiotic stress response.

4. Discussion

Soil salinity imposes an important constraint on plant growth and crop productivity, disrupting K^+/Na^+ ionic homeostasis, and encouraging the over-production of ROS (Mittler, 2002). Plants employ both ABA-dependent and -independent signaling to trigger their abiotic stress response (Fujita et al., 2011). The former typically involves various members of the clade A PP2Cs to act as regulators. The present research has sought to characterize an alfalfa member of this family of proteins, and to understand its participation in the abiotic stress response.

When *MsPP2CA1* was constitutively expressed in *A. thaliana,* both the plants' sensitivity to ABA and their tolerance to salinity stress were enhanced. Unlike the *A. thaliana* clade A PP2Cs, which are negative regulators of ABA (Merlot et al., 2001; Saez et al., 2004), this alfalfa PP2C appeared to act as positive regulator of ABA and stress signaling. The presence of MsPP2CA1 also promoted stomatal closure in plants subjected to ABA and NaCl treatment (Fig. 3H and I). The accumulation of ABA in response to external stress is well recognized to induce stomatal closure, a mechanism which has evolved to conserve the plants water content and its ability to survive drought (Schroeder et al., 2001; Kim et al., 2012). The inference was that the observed improved tolerance to abiotic stress resulting from the presence of MsPP2CA1 is, at least in part, derived from ABA-dependent induced stomatal closure. A survey of the transcriptional effect of the presence of the *MsPP2CA1* transgene on a range of stress-responsive genes showed that all of *MYB15*, *DREB2a*, *RD22*, *ABF2*, *ABF3* and *ABF4* were up-regulated (Fig. 6). Transgenic expression of *MYB15* conferred hypersensitivity to exogenous ABA, improved tolerance to drought/salt stresses with changes in the expression levels of a number of ABA- or stress-regulated genes such as *RD22* (Ding et al., 2009) *ABF2-4* are members of a small subfamily of ABRE-binding bZIP proteins designated as ABFs (Choi et al., 2000). It has been demonstrated that ABA-dependent post-translational modification, probably phosphorylation, is required for the maximal transcriptional activity of *ABF2* and *ABF4* (Uno et al., 2000). Overexpression of *ABF3* or *ABF4* in *Arabidopsis* resulted in ABA hypersensitivity and enhanced drought tolerance (Kang et al., 2002; Kim et al., 2004). The constitutive expression of *DREB2A*, a dependency of *ABF3* in *Arabidopsis thaliana* seedlings also resulted in significant drought stress tolerance (Sakuma et al., 2006; Kim et al., 2011). Similarly, *ABF2* overexpression in *Arabidopsis* reduced sensitivity to salt;

enhanced tolerance to oxidative stress, and hypersensitive of the primary root elongation to ABA (Kim et al., 2004). The likelihood is that the benefit gained by the heterologous expression of *MsPP2CA* operates through an ABA-dependent pathway. Some examples of this class of protein, such as BdPP2CA6 have been documented as acting in an ABA dependent manner (Zhang et al., 2017), as appeared to be the case for MsPP2CA1, while most PP2CAs act as positive regulators of the ABA-independent abiotic stress response (You et al., 2014; Singh et al., 2015; Bhaskara et al., 2012). Notably, according to the phylogenetic analysis, the PP2CA most closely related to MsPP2CA1 was BdPP2CA6 (Fig. S3).

Salinity stress is known to induce the production of ROS, so a component of tolerance is represented by the plant's ability to deal with an excess of these compounds, which would otherwise damage membranes through the peroxidation of lipids. Lipid peroxidation is conveniently assayed by quantifying the tissue content of MDA (Nankivell et al., 1994). As is the case for *A. thaliana* plants constitutively expressing *BdPP2CA6* (Zhang et al., 2017), the MDA content of the OE line leaves was lower than that of the VC leaves, and consequently, the OE plants' ability to grow when challenged by H_2O_2 stress was better than that of the VC plants; this represents a first report of the contribution of a clade A PP2C to the tolerance of oxidative stress. The presence of the *MsPP2CA1* transgene elevated the activities of the ROS-scavenging enzymes CAT and POD, as well as the level of transcription of their encoding genes *CAT* and *APX1* (ascorbate peroxidase 1) respectively (Fig. 6). These observations provided evidence of the ability of MsPP2CA1 to promote ROS scavenging.

The clade A PP2Cs generated by *A. thaliana* are deposited in both the cytosol and the nucleus, and interact with the plasma membrane K^+ transporter AKT2 (Chérel et al., 2002) and the nucleus-located proteins PYL and SnRK2 to form a key component of the ABA-dependent signaling network. The regulation of K^+ channel activity achieved by phosphatases influences the cellular K^+/Na^+ ratio, which is important for plant survival when challenged by salinity stress (Chérel et al., 2002; Lan et al., 2011). The constitutive expression in *A. thaliana* of *BdPP2CA6* has been shown to support the accumulation of K^+, thereby enhancing the plants' tolerance of salinity stress (Zhang et al., 2017). Here, the localization of MsPP2CA1 within the root cells of transgenic *A. thaliana* showed that, unlike BdPP2CA6 (Zhang et al., 2017) and various *A. thaliana* clade A PP2Cs (Xue et al., 2008), but like several rice clade A PP2Cs (Singh et al., 2015; Kim et al., 2012), this alfalfa protein is restricted to the nucleus. No significant difference with respect to the K^+/Na^+ ratio was observed between VC and OE plants, whether or not they were exposed to salinity (Fig. S4).

In conclusion, unlike most PP2Cs, the alfalfa clade A PP2C *MsPP2CA1* overexpression in *Arabidopsis* leads to hypersensitivity to ABA and enhanced salinity tolerance at early developing stage through ABA-dependent pathway. It has been shown to positively regulate ABA signal and salinity stress signaling. Furthermore, we suppose that ABA-dependent enhanced stomatal closure of *MsPP2CA1* overexpression plants attributes to better tolerance under salinity treatment because of the accumulation of ABA under stress conditions leads to stomatal closure, thus conserving water to survive abiotic stress such as drought (Schroeder et al., 2001; Lee and Luan, 2012). Genes which respond positively to salinity and drought stress in a manner which supports plant growth and development are an important resource in the context of breeding for crop resilience. *MsPP2CA1* may represent such a gene, since it had a positive effect on the performance of plants exposed to stress when constitutively expressed in *A. thaliana*. The assumption is –although this needs to be experimentally verified -that the same will be true when this gene is over-expressed and/or knocked down/out in alfalfa.

Conflicts of interest

The authors declare that they have no conflict of interest.

Declarations of interest

None.

Author contribution statement

YG Song designed the research. W Dong, J Lv, XJ Liu and TX Gao conducted the experiment and performed data analysis. YG Song wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.09.004.

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