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Research paper

Genome-wide identification of genes involved in polyamine biosynthesis and the role of exogenous polyamines in *Malus hupehensis* Rehd. under alkaline stress

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ABSTRACT

Polyamines (PAs) in plants are growth substrates with functions similar to phytohormones. Although they contribute to diverse processes, little is known about their role in stress responses, especially for perennial woody plants. We conducted a genome-wide investigation of 18 sequences involved in PA biosynthesis in the genome of apple (*Malus domestica*). Further analysis was performed to construct a phylogenetic tree, analyze their protein motifs and gene structures. In addition, we developed their expression profiles in response to stressed conditions. Both *MDP0000171041* (*MdSAMDC1*) and *MDP0000198590* (*MdSPDS1*) were induced by alkaline, salt, ABA, cold, and dehydration stress treatments, suggesting that these genes are the main contributors to activities of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermidine synthase (EC 2.5.1.16) in apple. Changes in PA biosynthesis under stress conditions indicated that spermidine and spermine are more essential than putrescine for apple, especially when responding to alkaline or salt stress. When seedlings of *M. hupehensis* Rehd. were supplied with exogenous PAs, their leaves showed less chlorosis under alkaline stress when compared with untreated plants. This application also inhibited the decline in SPAD levels and reduced relative electrolyte leakage in those stressed seedlings, while increasing their concentration of active iron. These results suggest that the alteration in PA biosynthesis confers enhanced tolerance to alkaline stress in *M. hupehensis* Rehd.

1. Introduction

Throughout their life cycles, sessile plants are continually challenged by various adverse environment conditions, including biotic and abiotic stresses, which not only affect their natural distribution but also threaten crop yields worldwide. Thus, it is critical for plants to sense stress signals and adapt to adverse environments by utilizing sophisticated mechanisms at all levels of organization (Krasensky and Jonak, 2012). For example, at the cellular level, plants alter their membrane system, modify cell wall architecture, and even change the cell cycle (Cui and Lee, 2016). They also accumulate compatible metabolites to activate stress signals, maintain cell turgor, and stabilize cellular structures (Xu et al., 2017). At the molecular level, gene expressions are modified in response to stresses (Ohama et al., 2016).

Plant hormones, e.g., auxins, cytokinins, ethylene, gibberellin, and abscisic acid (ABA), are essential for plant growth and stress responses. And some compounds are also known as important growth substance for plants. For example, brassinosteroid (BR), protect plants against environmental hazards; a mutation in the BR-signaling pathway in *Arabidopsis* leads to a salt-sensitive phenotype (Nawaz et al., 2017). Strigolactone (SL) acts as a positive regulator of plant responses to drought and salt stress. In *Arabidopsis*, SL-deficient and SL-response mutants exhibit hypersensitivity to those stresses, while exogenous SL rescues the sensitive phenotype of SL-deficient mutants (Ha et al., 2014). Changes in the expression of genes involved in the jasmonic acid (JA)-signaling pathway result in altered plant immunity responses, and an over-accumulation of JA decreases plant resistance to necrotrophic fungi (Kwon, 2016; Caarls et al., 2017). Modulation of salicylic acid functions in the tradeoff between plant growth and stress responses (Meng et al., 2017). Increasing the level of inositol in *Ipomoea batatas*

Abbreviations: ADC, arginine decarboxylase; BR, brassinosteroid; JA, jasmonic acid; ODC, ornithine decarboxylase; PA, polyamine(s); Put, putrescine; qPCR, quantitative polymerase chain reaction; SAMDC, *S*-adenosylmethionine decarboxylase; SL, strigolactone; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine; SPMS, spermine synthase

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enhances resistance to stem nematodes and tolerance to salt stress and drought under field conditions (Zhai et al., 2016). Finally, proline and sugars serve primarily as important osmoprotectants in the cells, where the biosynthesis of those components is activated under adverse growing conditions (Antoniou et al., 2017).

Polyamines (PAs) are small, flexible, nitrogen-containing compounds found in almost all living cells. In plants, they function in biological processes throughout the entire lifecycle (Tiburcio et al., 2014; Guo et al., 2018). Modifications to PA metabolism can have a positive effect on stress tolerance. For example, accumulations of one PA – putrescine (Put) – confer increased drought tolerance in transgenic plants of tobacco (*Nicotiana tabacum*) (Gong et al., 2015), while suppression of Put biosynthesis can lead to decreased drought tolerance in plants (Wu et al., 2016; Li et al., 2018). Treatment with another PA – spermidine (Spd) – is effective in alleviating salinity stress-induced damage to zoysiagrass (*Zoysia japonica* Steud.) (Li et al., 2017). Furthermore, exogenous pretreatment with a third PA – spermine (Spm) – enhances the tolerance of *Vigna radiata* L. (cv. BARI Mung-2) seedlings to high temperatures and drought stress, individually and in combination (Nahar et al., 2017). All of these PAs are polycations that can regulate gene expression and/or translation (Venkataramanan and Floor, 2018). They are accumulated in plant cells as osmoprotectants under stress conditions (Gong et al., 2015). And they promoted the levels of ROS and NO that can serve as stress signals to activate cascade reactions in plant cells (Agurla et al., 2018).

Salt-alkaline soils are distributed in arid and semi-arid regions of the world and have detrimental effects on plant growth and development (Campestre et al., 2016). In northwestern China, the arid Loess Plateau supports approximately 40% of the total production of apple (*Malus domestica*) in that country. Aside from its arid climate, soil alkalization could be the most severe natural environmental stress affecting orchards in that area because it frequently occurs with soil salinity, which is even more hazardous to plant growth (G. Hu et al., 2015; Zhao et al., 2016). In general, soil alkalization is linked with high pH, osmotic stress, and sodium toxicity caused by excess Na_2CO_3 and $NaHCO_3$ (Gong et al., 2014b). Although the plant response to salt stress has been widely studied, less attention has been paid to the consequences of alkaline stress.

Polyamines are involved in the plant response to alkaline stress (Gong et al., 2014a, 2014b; Zhang et al., 2015; Gong et al., 2017). Here, we conducted a genome-wide analysis of apple genome, isolating all of the genes that function in PA biosynthesis and analyzing their expression patterns. We also utilized a hydroponics system to confirm that PAs have a role in the response to alkaline stress in *M. hupehensis*. Our results should benefit future studies of PAs and their functioning under such stress conditions.

2. Materials and methods

2.1. Identification of genes involved in polyamine synthesis

Genes involved in PA synthesis in *Arabidopsis* were obtained from TAIR (http://www.arabidopsis.org/) and used as queries in a BLAST against the apple genome database in GDR (https://www.rosaceae.org/). All of the sequences identified for PA genes in apple were then subjected to a Batch CD-search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) and SMART (http://smart.embl-heidelberg.de/) to verify their reliability as target PA genes. We also examined the protein properties of these apple PA genes, determining the molecular weight (MW) and isoelectric point (pI) for each via ExPASy (https://www.expasy.org/). Subcellular localization of PA proteins was predicted with Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc/) (Chou and Shen, 2008; Chou and Shen, 2010). The duplication mode for each gene was analyzed with MCScanX software (Wang et al., 2012).

2.2. Phylogenetic analysis of PA genes

To compare the phylogenetic relationship of PA genes in apple with their counterparts in other plant species, we obtained PA genes of *Arabidopsis* from TAIR, rice (*Oryza sativa*) from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html), and orange (*Citrus sinensis*) from its genome database (http://citrus.hzau.edu.cn/orange/). These genes were also applied to the Batch CD-search and SMART to assess their reliability. Their isolated sequences included coding, genomic, and amino acid (aa) sequences. We utilized the GSDS program (http://gsds.cbi.pku.edu.cn/) to confirm the exon/intron structure of each PA gene by comparing coding sequences with corresponding genomic sequences (B. Hu et al., 2015). Protein motifs of the PA genes were analyzed with MEME (http://meme-suite.org/index.html) and illustrated with IBS software (Liu et al., 2015). Multiple alignments were conducted with Clustal X software, and the phylogenetic trees were constructed with MEGA 5.2 software, using the Neighbor-Joining (NJ) method.

2.3. Plant growth and stress treatments

The experiments were conducted from March to July. Tissue-cultured plants of rootstock M26 (*M. domestica*) were used for analyzing gene expression and PA accumulations. They were first transferred to plastic pots and grown for one month in a greenhouse with regular watering before the treatments began. For the alkaline, salt, or ABA treatment, we added 200 mM Na_2CO_3/$NaHCO_3$, 200 mM NaCl, or 100 mM ABA, respectively, to the irrigation solutions for selected plants and sampled their leaves at 0, 1, 3, 6, 12, and 24 h. For inducing chilling stress, another group of plants was transferred to an incubator set at 4 °C for 24 h, and the leaves were sampled at 0, 1, 3, 6, 12, and 24 h. For dehydration treatment, the plants were taken from the pots and washed before the excess water was removed. They were then placed in an empty flask, and their leaves were sampled at Hours 0.0, 0.5, 1.0, 3.0, and 6.0 of treatment. The leaf samples from all stress treatments were immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. Polyamine applications and alkaline stress treatment

Malus hupehensis Rehd. is a triploid species, typically apomixis, and showing superior consistency during its development. We cultivated seedlings of this species as described by Li et al. (Li et al., 2012). Briefly, seeds were cold-stratified for 50 d in Winter. After germination, the seedlings were planted in plastic pots and grown for two months in a greenhouse with regular watering. Afterward, a hydroponics system was set up for polyamine treatments and to test the effects of alkaline stress (Li et al., 2012). For this, plastic basins (35 cm × 25 cm × 10 cm), each containing 5 L of ½-stength Hoagland's nutrient solution, were painted black to protect the roots from light exposure, and covered with foam boards to support the plants. The hydroponics solution was continuously supplied with oxygen, using an air pump, and was refreshed every 3 d. During the two-week pre-culture period, the pH of the solution was adjusted to between 6.0 and 6.5 with sodium hydroxide pellets or 85% phosphoric acid. Polyamine applications were first added to the nutrient solution one week before the stress treatment began, using four different concentrations of Put, Spd, and Spm (5, 10, 50, and 100 μM). These PAs were refreshed every 3 d along with the nutrient solution. Alkaline stress (AK) was induced by adding 2 mM Na_2CO_3/$NaHCO_3$ (1:1) to the solution. The detected pH was between 8.5 and 8.8, which is similar to that of alkaline orchard soils found in northwestern China. In all, our study comprised 14 different treatments (30 plants each), i.e., one group each for the CK and AK plants, plus a group for each of the four concentrations of Put, Spd, and Spm (Table 1). The stress period ended after 15 d, and the youngest four leaves were collected from selected plants in each treatment group for different measurement.

Table 1
Polyamine and alkaline stress treatments performed with apple seedlings in hydroponics system.

Group	Treatment
CK	Normal conditions, pH 6.0–6.5
AK	Alkaline stress treatment with 2 mM Na_2CO_3/$NaHCO_3$, pH 8.5–8.8
Putrescine (Put)	
P1	2 mM Na_2CO_3/$NaHCO_3$ + 5 μM Put
P2	2 mM Na_2CO_3/$NaHCO_3$ + 10 μM Put
P3	2 mM Na_2CO_3/$NaHCO_3$ + 50 μM Put
P4	2 mM Na_2CO_3/$NaHCO_3$ + 100 μM Put
Spermidine (Spd)	
S1	2 mM Na_2CO_3/$NaHCO_3$ + 5 μM Spd
S2	2 mM Na_2CO_3/$NaHCO_3$ + 10 μM Spd
S3	2 mM Na_2CO_3/$NaHCO_3$ + 50 μM Spd
S4	2 mM Na_2CO_3/$NaHCO_3$ + 100 μM Spd
Spermine (Spm)	
M1	2 mM Na_2CO_3/$NaHCO_3$ + 5 μM Spm
M2	2 mM Na_2CO_3/$NaHCO_3$ + 10 μM Spm
M3	2 mM Na_2CO_3/$NaHCO_3$ + 50 μM Spm
M4	2 mM Na_2CO_3/$NaHCO_3$ + 100 μM Spm

2.5. Quantitative PCR analysis

Quantitative PCR (qPCR) was conducted to analyze the transcript levels of PA genes in apple under different stress treatments (Table 2, Supplemental Table 1). Total RNA was extracted from the sampled leaves with RNAiso for Polysaccharide-rich Plant Tissue (Takara, Japan), and 1 μg of total RNA was used to synthesize the first-strand cDNA, using a PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Japan) according to the manufacturer's instructions. Afterward, qPCR was performed on a Stepone Plus System (ABI, USA). The reaction mixture, in a total volume of 10 μL, contained 5 μL of SYBR Advantage qPCR Premix (2×; Takara), 50 ng of cDNA, and 0.25 μM for each primer. The reaction cycles were as follows: 95 °C for 30 s; then 40 cycles of 95 °C for 5 s, 58 to 60 °C for 10 s, and 72 °C for 15 s. Each sample was analyzed in four replicates, and the $^{\triangle\triangle}$CT method was applied to calculate relative expression. We used *MDH* as our internal control to normalize the relative expression levels of all examined genes (Supplemental Table 1).

2.6. Measurements of chlorophyll, relative electrolyte leakage, and active iron

Chlorophyll concentrations were determined from leaves sampled at the end of the treatment period, using a portable chlorophyll meter (SPAD-502; Minolta, Japan). The results were presented as SPAD values, ranging from 0 to 100 (Kendal, 2015). Relative electrolyte leakage (REL) in these young leaves was measured as described by Wang et al. (2011). The active iron was extracted with weak acids and assayed according to the method of Zou et al. (1998). Briefly, 1 g of leaf sample was cut into 5 mm × 5 mm pieces with stainless steel scissors before 10 mL of 1 M HCl was added. After the samples were shaken overnight, each extract was passed through a 0.45 μm filter membrane, and 1 mL of solution was assayed at 522 nm by atomic absorption spectrophotometry (PinAAcle500, USA).

2.7. Quantification of free polyamines

Free PAs were extracted and derived as described by Gong et al. (Gong and Liu, 2017), using 1, 6-hexanediamine as an internal standard. Briefly, 0.1 g of frozen leaf sample was ground to a fine powder and homogenized with 5% perchloric acid (PCA) buffer on ice for 30 min. After centrifugation, the supernatant was collected and the deposition layer was re-extracted with 5% PCA buffer. The crude extracted supernatant was derived with benzoyl chloride at 37 °C for 25 min. The benzoyl-PAs were then leached with ethyl ether and centrifuged at 8000 ×*g* for 5 min before the upper phase was collected and vacuum-dried in a concentrator (Eppendorf, Germany). The dried extracts were re-dissolved with 400 μL of HPLC-grade methanol (Fisher, USA) and filtered. From this, 20-μL aliquots were loaded into an Agilent 1260 Infinite HPLC system (Agilent, USA) equipped with a C18 reversed-phase column (4.6 mm × 250 mm; particle size 5 μm) and a diode array detector. The mobile phase was composed of HPLC-grade methanol (eluent A) and water (eluent B), and was ramped from 65%: 35% (A: B, *v*/v) to 95%: 5% (A: B) over 15 min, at a flow rate of 1.0 mL min^{-1}.

2.8. Statistical analysis

All data were examined with the SAS statistical software package (version 8.1). We used an analysis of variance to compare values, based on Duncan's multiple range tests. Differences among treatments were considered significant at $p < 0.05$.

Table 2
Sequences involved in polyamine biosynthesis in the apple genome[a].

Name	ID in GDR	Chromosome location	CDS length (bp)	Protein length (aa)	pI	MW (Da)	Subcellular localization	Duplication
ADC1	MDP0000813339	chr10:11244847..11247039	2193	730	5.23	78,548.60	Chloroplast	Segmental
ADC2	MDP0000228682	unanchored:7183817..7186003	2187	728	5.34	77,995.67	Chloroplast, nucleus, peroxisome	Segmental
ODC1	MDP0000914975	chr11:31102025..31103302	1278	425	6.37	46,450.28	Chloroplast	Dispersed
ODC2	MDP0000264258	chr9:23285342..23286606	1182	393	6	42,840.24	Chloroplast	Dispersed
SAMDC1	MDP0000171041	chr9:4884404..4885948	1362	453	5.51	49,529.17	Chloroplast	Segmental
SAMDC2	MDP0000185362	chr17:5466810..5468341	1311	436	5.28	47,519.91	Cell membrane, chloroplast, nucleus	Segmental
	MDP0000757066	chr17:5423556..5424680	1125	374	4.79	40,790.30	Cell membrane, chloroplast, nucleus	Proximal
SAMDC3	MDP0000292444	chr17:5470824..5472355	1260	419	5.21	45,551.46	Cell membrane, chloroplast, nucleus	Segmental
SAMDC4	MDP0000151417	chr13:3324812..3326538	1242	413	4.93	45,948.47	Cell membrane, chloroplast, nucleus	Segmental
	MDP0000211726	chr13:3356916..3358633	1233	410	5.04	45,561.13	Cell membrane, chloroplast, nucleus	Proximal
SAMDC5	MDP0000120546	chr16:2236503..2238230	1248	415	5.07	46,052.12	Chloroplast, nucleus	Proximal
	MDP0000148289	chr16:2242811..2244538	1248	415	5.07	46,052.12	Chloroplast, nucleus	Proximal
SPDS1	MDP0000198590	chr13:5954559..5957988	1293	430	4.92	47,280.64	Cytoplasm	Segmental
SPDS2	MDP0000027925	chr16:4218146..4221276	1008	335	4.79	36,391.61	Cytoplasm	Segmental
SPDS3	MDP0000521365	chr1:28172725..28176594	927	308	8.59	34,123.66	Cytoplasm	Segmental
SPDS4	MDP0000294813	chr1:28183509..28186848	1110	369	6.03	40,695.63	Cytoplasm	Proximal
SPDS5	MDP0000788247	chr7:25220028..25223197	1110	369	5.83	40,578.49	Cytoplasm	Segmental
SPDS6	MDP0000162897	unanchored:68389683..68397929	936	311	5.5	34,280.12	Cytoplasm	Dispersed

[a] All sequences were obtained from apple genome database in GDR (https://www.rosaceae.org/). CDS, coding sequence; pI, isoelectric point; MW, molecular weight.F.

3. Results

3.1. Identification of genes involved in polyamine biosynthesis

The PA genes in the apple genome were identified from the GDR database using corresponding protein sequences of *Arabidopsis* as queries. All of the candidates were further confirmed with their protein domains, resulting in 18 putative sequences, including two that encode arginine decarboxylase (ADC), two for ornithine decarboxylase (ODC), eight for *S*-adenosylmethionine decarboxylase (SAMDC), and six for spermidine synthase (SPDS) (Table 2). Their open reading frames ranged in size from 927 to 2193 bp, encoding proteins with lengths of 308 to 730 aa. The MW values for these 18 sequences ranged from 34.28 to 78.55 kDa while pI values were 4.79 to 8.59. They were predicted to localize in all cellular compartments. In particular, MdADCs and MdODCs were predicted to localize in chloroplasts. Three MdSAMDCs – MDP0000171041, MDP0000148289, and MDP0000120546 –were also localized to chloroplasts while five other MdSAMDCs were found primarily in the cell membranes. The MdSPDSs were predicted to localize in the cytoplasm.

3.2. Chromosome localization and duplication of PA genes

We could not assign two of the 18 sequences to any of the 17 apple chromosomes, but found that the other 16 sequences were distributed to eight different chromosomes, including chr 1 (2 sequences), chr 7 (1), chr 9 (2), chr 10 (1), chr 11 (1), chr 13 (3), chr 16 (3), and chr 17 (3) (Table 2). In addition, *MdADCs* were segmentally duplicated while the *MdODCs* showed dispersed duplication. For the *MdSAMDCs*, *MDP0000171041*, *MDP0000185362*, *MDP0000292444*, and *MDP0000151417* were segmentally duplicated while the other four were proximally duplicated. Within the *MdSPDSs*, *MDP0000294813* was also proximally duplicated, *MDP0000162897* showed dispersed duplication, and the other four were segmentally duplicated (Table 2).

3.3. Analyses of evolution and structure of PA genes

To gain insight into the evolutionary relationship among plant PA genes, we compared the full-length PA protein sequences from *Malus domestica*, *Arabidopsis*, rice, and orange. Multiple alignments revealed high similarity in their conserved domain but divergence in the N- and C-terminals (Supplemental Fig. 1–4). Among the four species, the ADCs displayed 67.93% similarity in protein sequences, while those percentages were 55.32% for ODCs, 44.30% for SAMDCs, and 46.77% for SPDSs. In addition, multiple alignment of the MdSAMDCs alone (data not shown) indicated that three pairs had the same sequences. This was true for the pairings of MDP0000151417 and MDP0000211726, and for MDP0000120546 and MDP0000148289. MDP0000757066 was the same as MDP0000185362, except for a 62-aa deletion in the *N*-terminal, encoding the SAMDC leader peptide.

Our phylogenetic trees demonstrated that PA proteins were highly conserved in the four species, with the *ADCs*, *ODCs*, *SAMDCs*, and *SPDSs* being clustered into one group, respectively. The *MdADCs* and five *ADCs* in our three comparison species shared a highly conserved protein motif in the sequences, and none of the ADC genes had introns (Fig. 1A–C). Although the ADC and ODC pathways work the same in plants to produce Put, *Arabidopsis* mainly relies upon the former, and the ODC gene is deleted in its genome (Hanfrey et al., 2001). The eight *ODCs* in apple, rice, and orange also clustered into one group with highly similar protein motif constructions, and none of those genes had more than one intron (Fig. 2A–C).

For the eight *SAMDC* sequences found in the apple genome, they clustered, pair-wise, into four branches on the phylogenetic tree. Three of those pairs showed high similarity in their protein motifs and differed only slightly in gene structure (Fig. 3A–C). However, although the SAMDC leader peptide is highly conserved in plant species (Franceschetti et al., 2001) (Fig. 3B, protein motif in pale blue), it is deleted in *Arabidopsis* and orange, as well as in five rice sequences. Nevertheless, MdSAMDCs contained the leader peptide, and MDP0000757066 was the same as MDP0000185362 except for the leader peptide.

The *SPDSs* clustered into two obvious groups based on the presence of similar protein motifs. Those in Group I (top) were involved in the synthesis of Spm while those in Group II (bottom) functioned in the production of Spd. Our examination of structure showed that these genes contained seven to ten introns in their sequences, which is much more than those of other PA genes (Fig. 4A–C).

3.4. Expression profiles of PA genes under different stresses

Because the *MdSAMDCs* had three sequence pairs that were the same, we used one sequence from each pair in the qPCR analysis. We designated these apple PA genes as *MdADC1* through *MdSPDS6* (Table 2) and examined their expression profiles under different stress conditions. As shown in Fig. 5A, expression of *MdADC1* and *MdODC2* was slightly changed in response to alkaline stress while that of *MdADC2* and *MdODC1* rapidly responded, i.e., within the first hour. Whereas the expressions of *MdSAMDC1* and *−3* increased under alkaline treatment, which of *MdSAMDC2*, *−4*, and *−5* were suppressed during the first 6 to 12 h before rising up until Hour 24. Among the six *MdSPDSs*, expressions of *MdSPDS2* and *−3* increased over 24 h, while that of *MdSPDS1*, *−4*, *−5*, and *−6* first increased and then decreased during this stress period.

Expression of *MdSAMDC3* was slightly altered under salt stress, while others in that gene group showed more distinct changes in expression patterns (Fig. 5B). For example, expression of *MdADC1* peaked in 6 h, to a level > 20-fold higher than that measured at Hour 0. *MdSPDS1* also peaked in 6 h, by approximately 8-fold. Expression of *MdODC2* and *MdSPDS4* rose during the stress period, while others declined after first being induced.

Very different expression patterns were noted under cold stress. Almost all of the PA genes were induced in response to chilling (Fig. 5C). The exception was *MdODC1*, for which the transcript level was only slightly changed by cold condition. In particular, seven of our target genes were first induced and then down-regulated, including *MdADC1* and *−2*; *MdODC2*; *MdSAMDC2*, *−4*, and *−5*; and *MdSPDS1*. For the other seven, expressions rose consistently throughout the 24-h stress period. They included *MdSAMDC1* and *−3*; and *MdSPDS2*, *−3*, *−4*, *−5*, and *−6*. Among these 14 PA genes, *MdADC1* and *MdODC2* were most strongly up-regulated by cold stress.

MdADC1 and *−2* responded quickly to dehydration, with both showing high levels of expression. *MdODC1* was also induced by dehydration, whereas expression of *MdODC2* was repressed. Among the five *MdSAMDCs*, *MdSAMDC1* and *−2* were induced by dehydration while the other three were inhibited. Transcript levels of *MdSPDS1*, *−2*, and *−3* were increased under dehydration, which of *MdSPDS4* was decreased, and levels of *MdSPDS5* and *−6* showed little change under such stress (Fig. 5D).

The four genes (*MdADC1* and *−2*, and *MdODC1* and *−2*) involved in Put synthesis were induced by ABA treatment. Expression of *MdSAMDC1* and *−2* also increased for 24 h after ABA exposure. *MdSAMDC3* rapidly responded to cold stress, but its expression declined after the first hour. Expression of *MdSAMDC4* and *−5* was inhibited by ABA. Among the six *MdSPDSs*, only *MdSPDS1* and *−3* were induced by ABA treatment, while *MdSPDS2*, *−4*, *−5*, and *−6* were only slightly affected (Fig. 5E).

3.5. Accumulation of free polyamines under different stresses

Consistent with the expression patterns of PA genes under various types of stress, the accumulation of PAs was also changed under such conditions (Fig. 6). The alkaline stress treatment caused levels of total

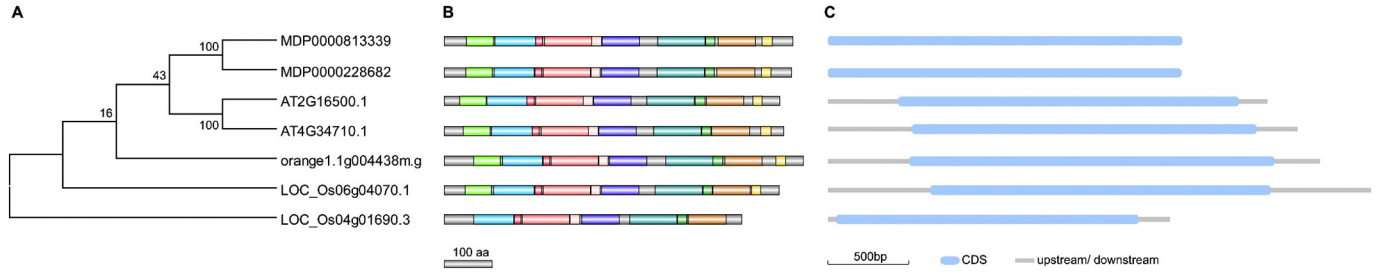

Fig. 1. Comprehensive analysis of genes encoding arginine decarboxylase from genomes of apple (MD), *Arabidopsis* (AT), orange, and rice (Os). A) Phylogenetic tree. B) Protein motif constructions. C) Gene structures analysis.

free PAs to increase by 1.8-fold over time when compared with those at 0 h (Fig. 6A), with Spd contributing to half of the total PAs (Fig. 6B). Similar results were found with Spd and Spm under salt stress, their levels accounting for nearly 80% of the total free PAs. In contrast, the accumulation of Put was only 510 nmol g^{-1} FW after 24 h of salt stress, much lower than that of Spd and Spm (Fig. 6C-D). Concentrations of free PAs increased in response to cold stress, peaking by 2.5-fold over CK levels (Hour 0) at Hour 24 (Fig. 6E). Whereas accumulations of Spm were only slightly modified by chilling, the level of Put was dramatically elevated, from 238 to 868 nmol g^{-1} FW. While the accumulation of Spd declined since Hour 12 (Fig. 6F). Dehydration stress also induced the accumulation of free PAs (Fig. 6G), with Put and Spd accounting for most of that response and Spd itself contributing approximately 50% of the total. The concentration of Spm declined after the first hour (Fig. 6H). Although Put accumulations were distinctly changed under ABA treatment, the response was greater by Spd and Spm (Fig. 6I–H).

3.6. Effects of polyamines on apple phenotype under alkaline stress

As presented above, we showed that transcription of PA genes was altered in M26 seedlings under different types of stress, leading to changes in PA accumulations. To determine whether exogenous PAs would help alleviate the effects of alkaline stress, we added 2 mM Na_2CO_3/$NaHCO_3$ (pH = 8.5–8.8) to the hydroponics system used for cultivation of *Malus hupehensis* Rehd. After 15 d of treatment, the young leaves from stressed plants showed obvious yellowing when compared with the CK (Fig. 7A). Among the plants in the Put (P1–4) groups, young leaves from the P1 and P2 treatments were less chlorotic than those from the P3 and, especially, the P4 treatments. Those leaves from P1 and P2 also displayed wrapped edges. For the Spd (S1–4) groups, leaves from the S1 and S2 treatments were less yellow than those from S3 and S4. For leaves in the Spm (M1–4) groups, M2 leaves had the least amount of yellowing. To quantify the degree of yellowing, we measured individual SPAD levels from the youngest four leaves, from the apex to the stem base in each treatment, and found that those values were obviously lower in the AK group than in the CK group (Fig. 7B). Furthermore, the P2 leaves had much higher SPAD levels when compared with the other three Put treatments, with values being nearly the same as those obtained from the CK leaves. Among the Spd groups, SPAD levels were much higher in the S1 leaves. For the Spm treatment, SPAD levels were higher for Leaf 1 and Leaf 2 in M2 than those in M1/3/4, but those differences were negligible for Leaf 3 and Leaf 4. In fact, for Leaf 4, the SPAD levels were almost the same for all four Spm treatments.

When we examined the severity of leaf damage to the four leaves from each treatment, we found that the PA supplements helped alleviate the adverse effect of alkaline stress. This was reflected by the much smaller REL values obtained from the 12 PA treatments when compared with the AK treatment (Fig. 8). Leakage was lowest in the CK plants. Among individual PA treatment groups, the lowest values were detected for P2 (Put) and S1 (Spd). We found it interesting that, although the young leaves in M4 were much yellower than those in M2, their REL values were almost the same, and were lower than those measured from M1 and M3 leaves.

Excess amounts of OH^- in the soil can result in an iron deficiency, and the level of active iron is considered to be a vital index of the plant response to alkaline stress (Chen et al., 2004). Here, the concentration of active iron in young leaf samples was 36.5 μg g^{-1} FW in the CK group versus 26.1 μg g^{-1} FW in the AK group (Fig. 9). Supplementation with PAs had only a minor impact on those concentrations. In fact, no obvious differences were observed between most PA groups and the AK group, except for P2 (36.0 μg g^{-1} FW), S1 (33.0 μg g^{-1} FW), and M2 (37.0 μg g^{-1} FW) (Fig. 9).

4. Discussion

Polyamines are a class of low-molecular-weight aliphatic polycations in all living organisms. Since their discovery in plants, they have been widely implicated in stress responses (Romero et al., 2018). Exogenous PAs lead to enhanced stress tolerance, for example, heavy metal, $Ca(NO_3)_2$, drought and so on (Soudek et al., 2016; Ebeed et al., 2017; Du et al., 2018; Tajti et al., 2018). Soil alkalization is closely associated with soil salinity, and both cause the same type of ion toxicity in plants. However, tolerance to salinity stress is increased when plants are treated with exogenous PAs such as Spd (Li et al., 2017; Saha and Giri, 2017; Baniasadi et al., 2018). Because alkaline stress

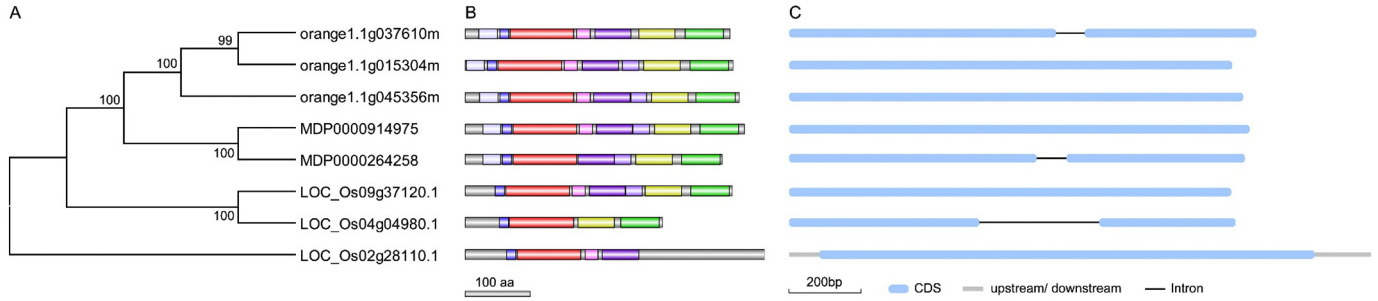

Fig. 2. Comprehensive analysis of genes encoding ornithine decarboxylase from genomes of apple (MD), *Arabidopsis* (AT), orange, and rice (Os). A) Phylogenetic tree. B) Protein motif constructions. C) Gene structures analysis. Note: the ODC gene is deleted in *Arabidopsis* genome.

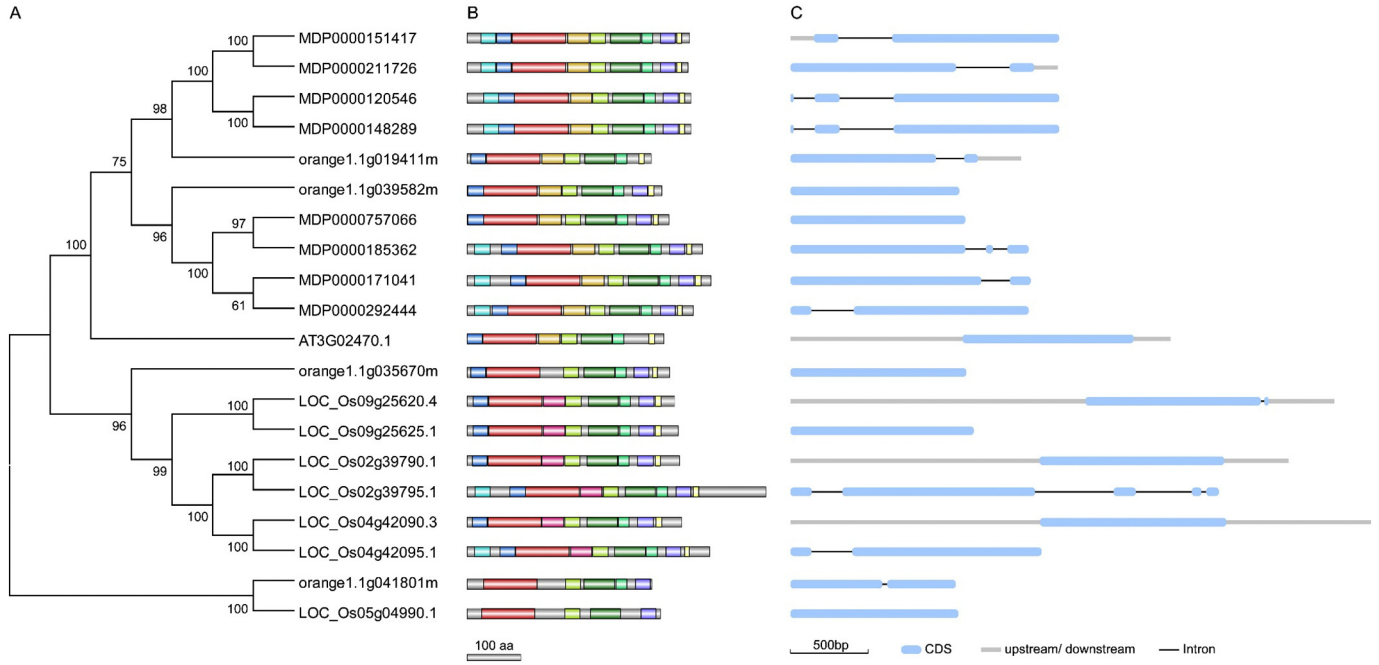

Fig. 3. Comprehensive analysis of genes encoding *S*-adenosylmethionine decarboxylase from genomes of apple (MD), *Arabidopsis* (AT), orange, and rice (Os). A) Phylogenetic tree. B) Protein motif constructions. C) Gene structures analysis.

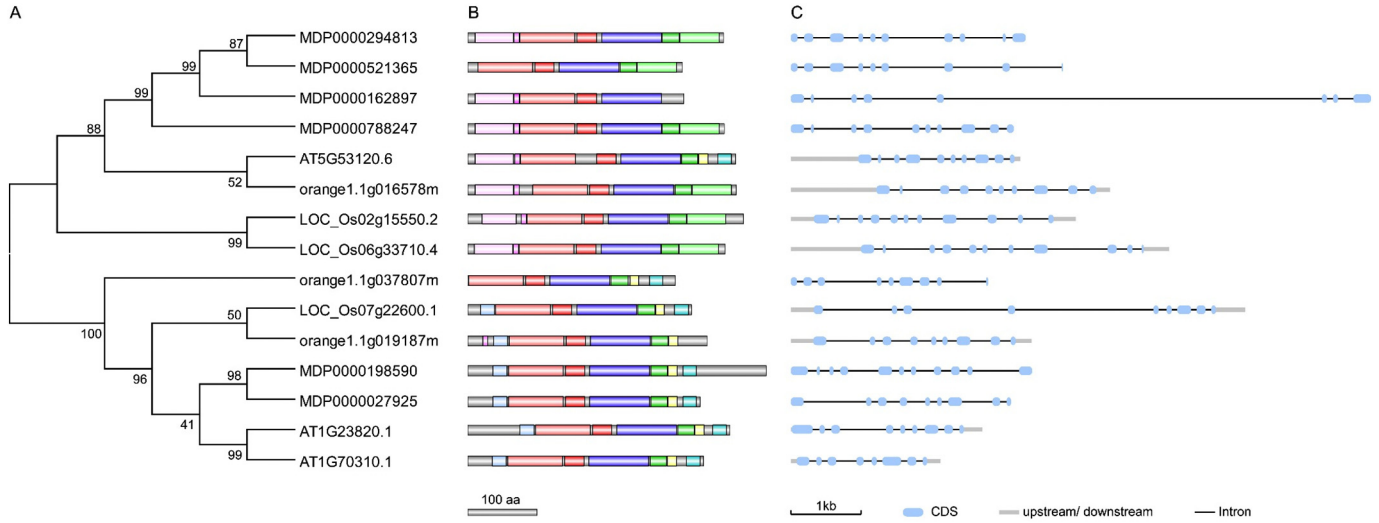

Fig. 4. Comprehensive analysis of genes encoding spermidine synthase decarboxylase from genomes of apple (MD), *Arabidopsis* (AT), orange, and rice (Os). A) Phylogenetic tree. B) Protein motif constructions. C) Gene structures analysis.

inevitably leads to stresses due to high pH and osmotic conditions, the role of PAs in plant responses to alkalinity is now receiving greater research attention. For example, exogenous Spm alleviates the effects of sodic alkaline stress in tomato (*Solanum lycopersicum*) by interacting with nitric oxide (Gong et al., 2014a). We found that, in the woody perennial *Malus hupehensis* Rehd., 10 μM Put (P2) or Spm (M2), or 5 μM Spd (S1), positively regulated the alkaline stress response in those plants. Moreover, SPAD values were higher and REL was lower for plants supplied with P2, S1, or M2.

These PA applications alleviated the pH stress associated with the use of an alkalized nutrient solution, and detected contents of active iron were much more in such plants (Fig. 9), possibly because of the effect that PAs have on ion channels (Zepeda-Jazo and Pottosin, 2018). In addition, the polycation PAs can bind to different anionic macromolecules, either stabilizing or destabilizing those components. In *Arabidopsis*, the mRNA stability of stress-responsive genes can be increased if plants are pre-treated with PAs (Shen et al., 2016). Furthermore, PAs can serve as scavengers of free radicals in the nucleus (Du et al., 2018). Although plants can generate PAs as osmoprotectants when growing in an adverse environment (Gong et al., 2015), this does not seem to be as effective under alkaline stress, as PAs are nitrogen-containing alkali compounds. Therefore, it is reasonable to suggest that the positive role of PAs in *Malus hupehensis* Rehd. under alkaline stress is due to their effects on ion channels, their capacity to bind with stress-responsive genes, and their direct scavenging of free radicals.

Exogenous PAs function in the stress response through diverse pathways, one of which leading to changes in the accumulation of endogenous PAs in the cells (Li et al., 2016; Talaat and Shawky, 2016; Ebeed et al., 2017). We previously demonstrated that the production of PAs, especially Spd and Spm, is altered in *Malus hupehensis* Rehd. seedlings under alkaline stress (Gong et al., 2017). Here, we further analyzed the accumulations of free PAs under different stresses and

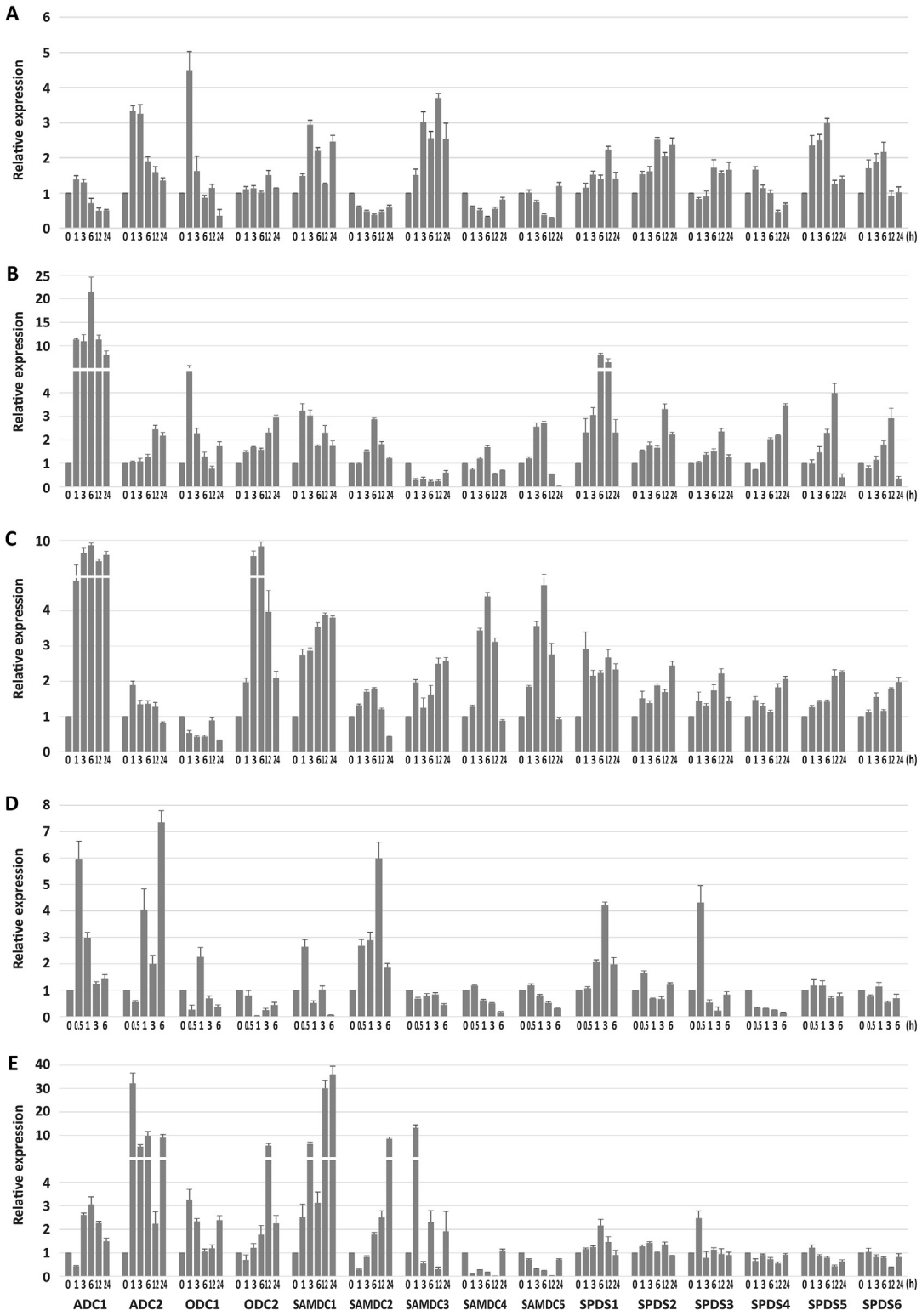

Fig. 5. Expression patterns of genes involved in polyamine biosynthesis in M26 seedlings in response to stressed conditions. A) Alkaline stress. B) Salt stress. C) Cold stress. D) Dehydration treatment. E) ABA treatment.

found that their levels were elevated in response to alkalinity, salt, cold, dehydration, and ABA treatments. However, as shown in other studies, the responses can be distinctly different among Put, Spd, and Spm. For example, Put is accumulated much more than Spd and Spm in drought-stressed plants of *Hordeum vulgare* var. Golden Promise (Montilla-Bascon et al., 2017). This is an important consideration when developing plants that can tolerate water deficits (Gong et al., 2015; Wu et al., 2016). We also noted here that the accumulations of Put did not vary much under stress conditions when compared with the control. The exception was for the cold-stressed M26. In contrast, the concentrations of Spd and Spm were higher than those of Put under alkaline and salt stresses, or after ABA treatment. Dehydration treatment also caused the level of Spd to increase steadily, such that its concentration was greater than the sum of the Put and Spm by the end of

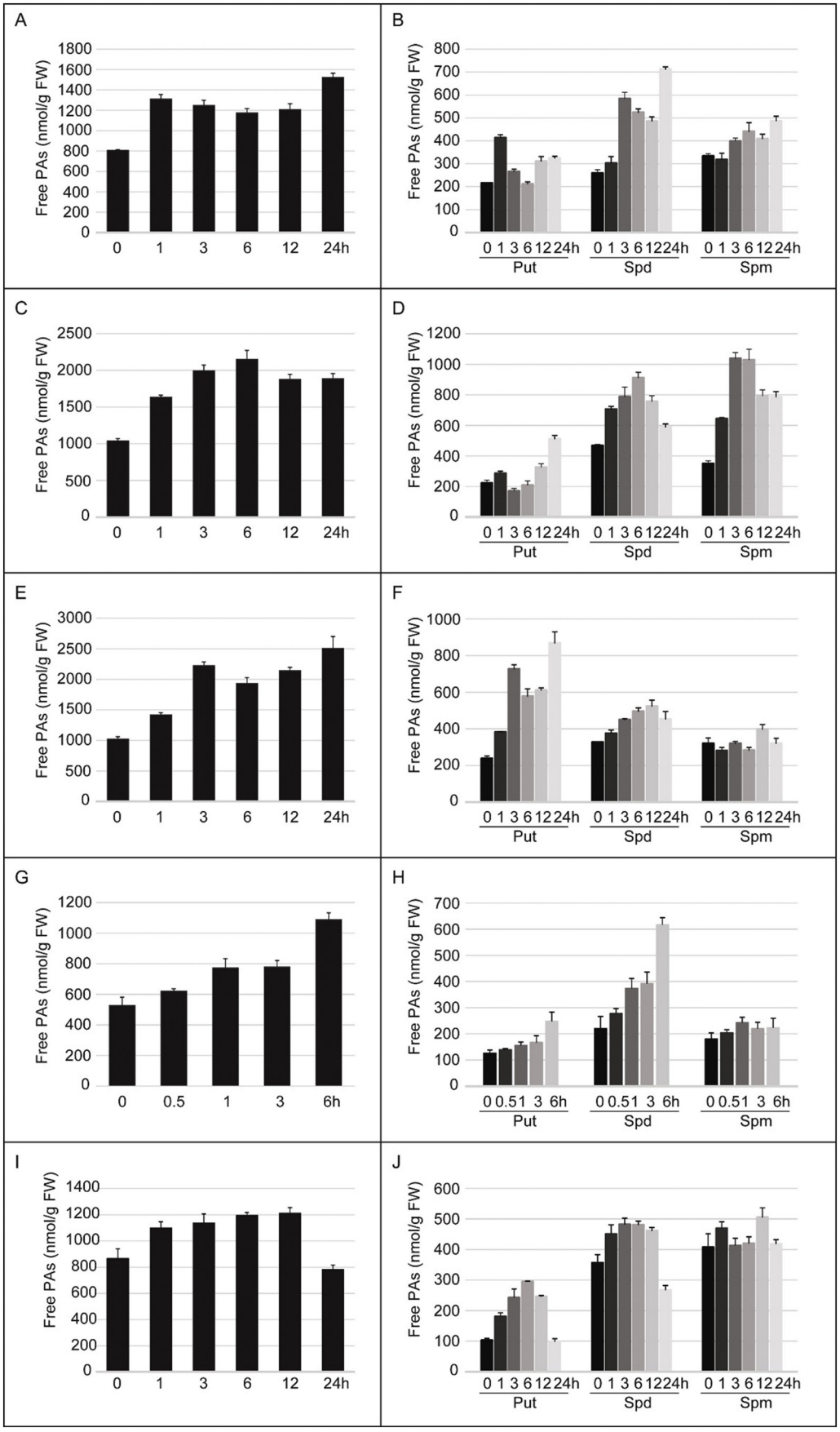

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Fig. 6. Levels of free polyamines in M26 seedlings under stress conditions. A–B) Accumulation of total free PAs (A) and putrescine (Put), spermidine (Spd), and spermine (Spm) (B) under alkaline stress. C–D) Accumulation of total free PAs (C) and Put, Spd, and Spm (D) under salt stress. *E*–F) Accumulation of total free PAs (E) and Put, Spd, and Spm (F) under cold stress. G–H) Accumulation of total free PAs (G) and Put, Spd, and Spm (H) under dehydration stress. I–J) Accumulation of total free PAs (I) and Put, Spd, and Spm (J) under ABA treatment.

that stress period. Therefore, Spd and Spm may be more vital to the stress response by apple because they appear to contribute much more than does Put when plants are challenged by stress conditions, for example, alkaline stress (Gong et al., 2014b, 2017).

The biosynthetic pathways for PAs are conserved among organisms, from bacteria to animals and plants (Tabor and Tabor, 1984). As the first product, Put in plants can be derived from both the ADC and ODC pathways. With the aid of SAMDC, Spd and Spm are generated by SPDS and SPMS (Tiburcio et al., 2014). However, SPDS and SPMS are not distinct from each other (Vuosku et al., 2018). For example, *AtSPMS* in the *Arabidopsis* genome is also designated as *AtSPDS3*. We found 18 sequences involved in PA biosynthesis in the apple genome, including two encoding ADC, two encoding ODC, eight encoding SAMDC, and six encoding SPDS. Because the number of PA genes varies among species, i.e., 6 in *Arabidopsis*, 15 in rice, 11 in orange, and 14 in tomato (Liu et al., 2018), versus 18 in apple, this implies that duplication events occurred during the evolution of the apple genome. Gene duplication, as well as inversion and translocation, leads to different modes of genome structure modifications and gene family expansion (Wang et al., 2012). Duplicated events have been reported for many gene families, such as the G-box factor 14–3-3 genes in the *Brachypodium distachyon* genome (Yang et al., 2017), and the *TIFY/JAZ* gene family from tomato (Chini et al., 2017). Our 18 apple sequences also presented

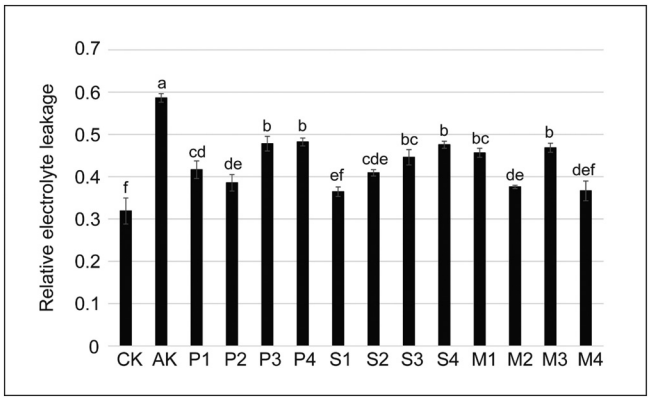

Fig. 8. Effects of alkaline stress and PA treatments on relative electrolyte leakage in young leaves from *Malus hupehensis* seedlings. Bars not labeled with same lower-case letters indicate significant differences among treatments, based on Duncan's multiple range test ($p < 0.05$).

different types of duplications, with most being segmental. This meant that the same sequences could be detected in the PA genes. Even so, the apple PA genes had high similarity with those from *Arabidopsis*, rice,

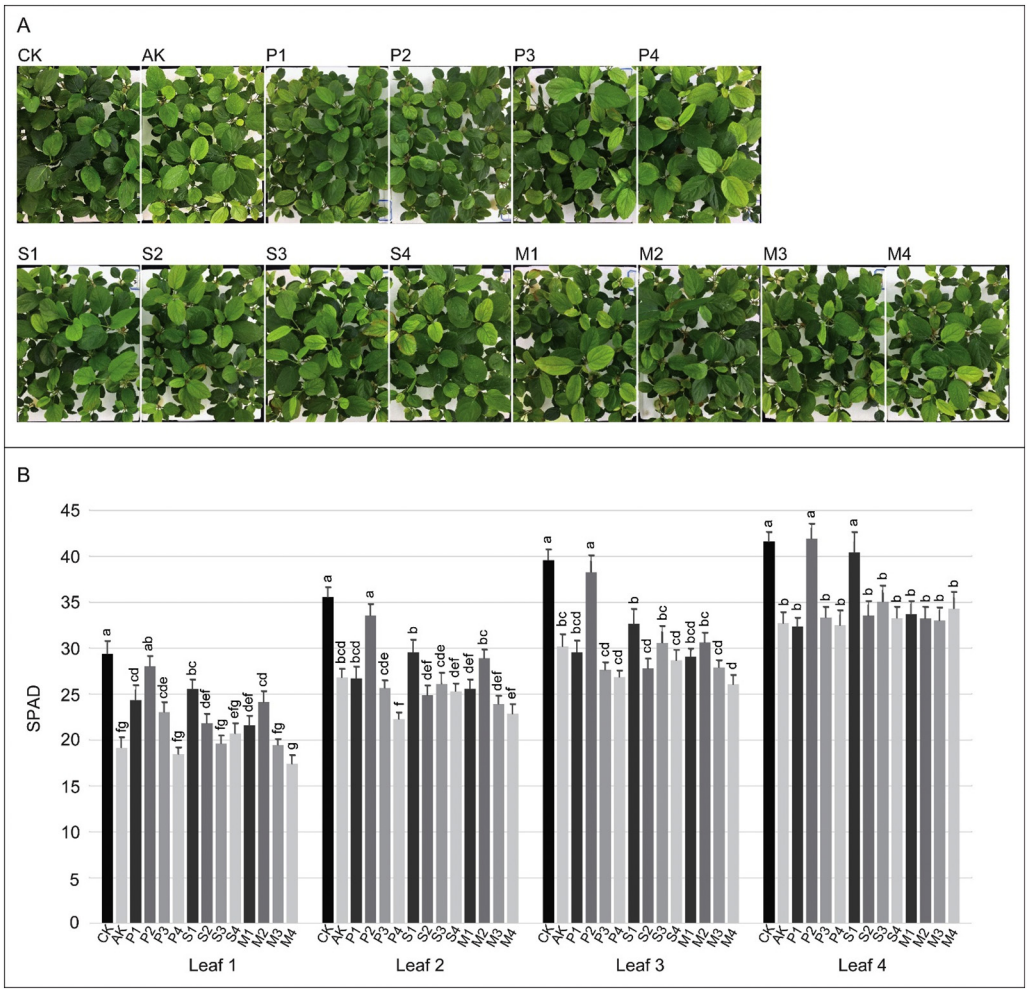

Fig. 7. Response of *Malus hupehensis* leaves to alkaline stress and/or PA treatment. A) Phenotypes of leaves under alkaline stress; B) SPAD levels measure from 4 youngest leaves sampled from each treatment. CK: normal growing conditions, pH 6.0–6.5, no PA supplement. AK: alkaline stress treatment with 2 mM Na_2CO_3/$NaHCO_3$ (1:1), pH 8.5–8.8. P1–P4: 2 mM Na_2CO_3/$NaHCO_3$ plus 5, 10, 50, or 100 μM Put. S1–S4: 2 mM Na_2CO_3/$NaHCO_3$ plus 5, 10, 50, or 100 μM Spd. M1–M4: 2 mM Na_2CO_3/$NaHCO_3$ plus 5, 10, 50, or 100 μM Spm. In panel B, for each numbered leaf, bars not labeled with same letter indicate significant differences among treatments, based on Duncan's multiple range test ($p < 0.05$).

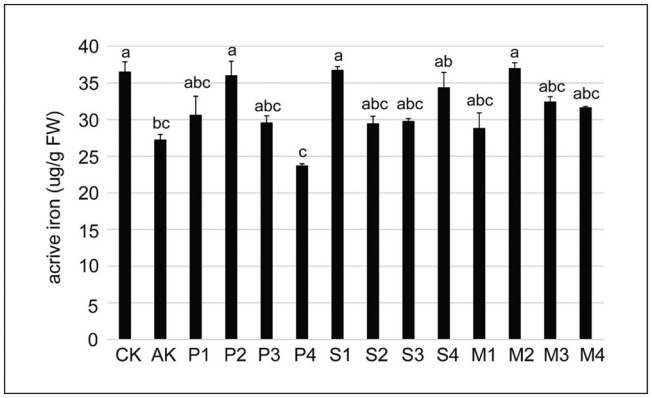

Fig. 9. Effects of alkaline stress and PA treatment on active iron concentrations in young leaves from *Malus hupehensis* seedlings. Bars not labeled with same lower-case letters indicate significant differences among treatments, based on Duncan's multiple range test ($p < 0.05$).

and orange, sharing a conserved functional protein domain and almost the same protein motif constructions. This also confirmed that the PA biosynthetic pathway is highly conserved in plants.

The PA genes function in various stress responses. *CaADC1* in *Capsicum annuum* is induced by *Xanthomonas campestris* pv *vesicatoria* (*Xcv*) infection and acts as a key defense and cell death regulator by mediating the metabolism of polyamines and GABA (Kim et al., 2013). *PtADC* from *Poncirus trifoliata* confers enhanced tolerance to drought and cold stress in transgenic plants (Wang et al., 2011). Overexpression of *SAMDC1* in tomato callus leads to greater tolerance of alkaline stress (Gong et al., 2014b; Gong et al., 2016), while tobacco plants with downregulated *SAMDC* exhibit reduced PA synthesis and are sensitive to salt stress, which suggests that PAs positively regulate plant tolerance (Mellidou et al., 2016). Expression of *MdSPDS1* leads to significant salt tolerance in tomato and pear (*Pyrus communis*) plants (He et al., 2008; Wen et al., 2008; Neily et al., 2011), and it also confers tolerance to heavy metals and osmotic stress in pear (He et al., 2008; Wen et al., 2008, 2010). We also observed increased transcription of *MdSPDSs* under salt stress. Because *MdSPDS1* was more highly expressed in stressed plants, we propose that MdSPDS1 is the main contributor to SPDS activity in apple plants when growing in adverse environments. While *MdADC1* was dramatically induced by salt stress, *MdADC2* responded quickly to ABA treatment. Transcript levels for *MdSAMDC1* were induced by five different stress treatments, which implies that MdSAMDC1 is the main factor in SAMDC activity in apple.

5. Conclusion

In summary, we performed a genome-wide analysis to identify genes involved in polyamine biosynthesis, and obtained 18 sequences from the apple genome. Although each responded to diverse environment stimuli, *MdSPDS1* and *MdSAMDC1*were the most strongly induced by stress treatments. In addition, spermidine and spermine were much more active than putrescine under such conditions. Further investigation demonstrated that exogenous PAs alleviate the effects of alkaline stress in *Malus hupehensis* Rehd. seedlings. These findings will be beneficial to our future study of the functional mechanism by which polyamines help *Malus* species respond to and tolerate alkaline stress.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Xiaoqing Gong and Fangfang Dou contributed same to this paper. Xiaoqing Gong and Fengwang Ma designed all the experiments; Xiaoqing Gong, Fangfang Dou, Jing Zhou and Xi Cheng performed the experiments; Xiaoqing Gong and Fengwang Ma wrote and revised the manuscript; Yangjun Zou provided all financial support for the experiment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2018.05.077.

References

Agurla, S., Gayatri, G., Raghavendra, A.S., 2018. Polyamines increase nitric oxide and reactive oxygen species in guard cells of Arabidopsis thaliana during stomatal closure. Protoplasma 255, 153–162.

Antoniou, C., Chatzimichail, G., Xenofontos, R., Pavlou, J.J., Panagiotou, E., Christou, A., Fotopoulos, V., 2017. Melatonin systemically ameliorates drought stress-induced damage in *Medicago sativa* plants by modulating nitro-oxidative homeostasis and proline metabolism. J. Pineal Res. 62. http://dx.doi.org/10.1111/jpi.12401.

Baniasadi, F., Saffari, V.R., Moud, A.A.M., 2018. Physiological and growth responses of *Calendula officinalis* L. plants to the interaction effects of polyamines and salt stress. Sci. Hortic. (Amsterdam) 234, 312–317.

Caarls, L., Elberse, J., Awwanah, M., Ludwig, N.R., de Vries, M., Zeilmaker, T., Van Wees, S.C.M., Schuurink, R.C., Van den Ackerveken, G., 2017. *Arabidopsis* JASMONATE-INDUCED OXYGENASES down-regulate plant immunity by hydroxylation and inactivation of the hormone jasmonic acid. Proc. Natl. Acad. Sci. U. S. A. 114, 6388–6393.

Campestre, M.P., Antonelli, C., Calzadilla, P.I., Maiale, S.J., Rodriguez, A.A., Ruiz, O.A., 2016. The alkaline tolerance in *Lotus japonicus* is associated with mechanisms of iron acquisition and modification of the architectural pattern of the root. J. Plant Physiol. 206, 40–48.

Chen, L.S., Smith, B.R., Cheng, L., 2004. CO_2 assimilation, photosynthetic enzymes, and carbohydrates of 'Concord' grape leaves in response to iron supply. J. Am. Soc. Hortic. Sci. 129, 738–744.

Chini, A., Ben-Romdhane, W., Hassairi, A., Mam, A.S., 2017. Identification of TIFY/JAZ family genes in *Solanum lycopersicum* and their regulation in response to abiotic stresses. PLoS One 12, e0177381.

Chou, K.C., Shen, H.B., 2008. Cell-PLoc: a package of Web servers for predicting subcellular localization of proteins in various organisms. Nat. Protoc. 3, 153–162.

Chou, K.C., Shen, H.B., 2010. Plant-mPLoc: a top-down strategy to augment the power for predicting plant protein subcellular localization. PLoS One 5, e11335.

Cui, W., Lee, J.Y., 2016. *Arabidopsis* callose synthases CalS1/8 regulate plasmodesmal permeability during stress. Nat. Plants 2, 160354.

Du, J., Guo, S., Sun, J., Shu, S., 2018. Proteomic and physiological analyses reveal the role of exogenous spermidine on cucumber roots in response to $Ca(NO_3)_2$ stress. Plant Mol. Biol. http://dx.doi.org/10.1007/s11103-018-0721-1.

Ebeed, H.T., Hassan, N.M., Aljarani, A.M., 2017. Exogenous applications of polyamines modulate drought responses in wheat through osmolytes accumulation, increasing free polyamine levels and regulation of polyamine biosynthetic genes. Plant Physiol. Biochem. 118, 438–448.

Franceschetti, M., Hanfrey, C., Scaramagli, S., Torrigiani, P., Bagni, N., Burtin, D., Michael, A.J., 2001. Characterization of monocot and dicot plant S-adenosyl-L-methionine decarboxylase gene families including identification in the mRNA of a highly conserved pair of upstream overlapping open reading frames. Biochem. J. 353, 403–409.

Gong, X., Liu, J.H., 2017. Detection of free polyamines in plants subjected to abiotic stresses by high-performance liquid chromatography (HPLC). Methods Mol. Biol. 1631, 305–311.

Gong, B., Li, X., Bloszies, S., Wen, D., Sun, S.S., Wei, M., Li, Y., Yang, F.J., Shi, Q.H., Wang, X.F., 2014a. Sodic alkaline stress mitigation by interaction of nitric oxide and polyamines involves antioxidants and physiological strategies in *Solanum lycopersicum*. Free Radic. Biol. Med. 71, 36–48.

Gong, B., Li, X., VandenLangenberg, K.M., Wen, D., Sun, S.S., Wei, M., Li, Y., Yang, F.J., Shi, Q.H., Wang, X.F., 2014b. Overexpression of S-adenosyl-L-methionine synthetase increased tomato tolerance to alkali stress through polyamine metabolism. Plant Biotechnol. J. 12, 694–708.

Gong, X.Q., Zhang, J.Y., Hu, J.B., Wang, W., Wu, H., Zhang, Q.H., Liu, J.H., 2015. FcWRKY70, a WRKY protein of *Fortunella crassifolia*, functions in drought tolerance and modulates putrescine synthesis by regulating arginine decarboxylase gene. Plant

Cell Environ. 38, 2248–2262.
Gong, B., Wan, X.F., Wei, M., Li, Y., Wei, M., Shi, Q.H., 2016. Overexpression of *S-adenosylmethionine synthetase* 1 enhances tomato callus tolerance to alkali stress through polyamine and hydrogen peroxide cross-linked networks. Plant Cell Tissue Organ Cult. 124, 377–391.
Gong, X.Q., Shi, S.T., Dou, F.F., Song, Y., Ma, F.W., 2017. Exogenous melatonin alleviates alkaline stress in malus hupehensis rehd. by regulating the biosynthesis of polyamines. Molecules 22, 1542.
Guo, J., Wang, S., Yu, X., Dong, R., Li, Y., Mei, X., Shen, Y., 2018. Polyamines regulate strawberry fruit ripening by abscisic acid, auxin, and ethylene. Plant Physiol. 177, 339–351.
Ha, C.V., Leyva-Gonzalez, M.A., Osakabe, Y., Tran, U.T., Nishiyama, R., Watanabe, Y., Tanaka, M., Seki, M., Yamaguchi, S., Dong, N.V., Yamaguchi-Shinozaki, K., Shinozaki, K., Herrera-Estrella, L., Tran, L.S.P., 2014. Positive regulatory role of strigolactone in plant responses to drought and salt stress. Proc. Natl. Acad. Sci. U. S. A. 111, 851–856.
Hanfrey, C., Sommer, S., Mayer, M.J., Burtin, D., Michael, A.J., 2001. *Arabidopsis* polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. Plant J. 27, 551–560.
He, L., Ban, Y., Inoue, H., Matsuda, N., Liu, J., Moriguchi, T., 2008. Enhancement of spermidine content and antioxidant capacity in transgenic pear shoots overexpressing apple *spermidine synthase* in response to salinity and hyperosmosis. Phytochemistry 69, 2133–2141.
Hu, B., Jin, J.P., Guo, A.Y., Zhang, H., Luo, J.C., Gao, G., 2015. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics 31, 1296–1297.
Hu, G., Liu, Y., Zhang, X., Yao, F., Huang, Y., Ervin, E.H., Zhao, B., 2015. Physiological evaluation of alkali-salt tolerance of thirty switchgrass (*Panicum virgatum*) lines. PLoS One 10, e0125305.
Kendal, E., 2015. Relationship between chlorophyll and other features in durum wheat (*Triticum turgidum* L. var. *durum*) using SPAD and biplot analyses. J. Agric. Sci. Technol. 17, 1873–1886.
Kim, N.H., Kim, B.S., Hwang, B.K., 2013. Pepper arginine decarboxylase is required for polyamine and *Y*-aminobutyric acid signaling in cell death and defense response. Plant Physiol. 162, 2067–2083.
Krasensky, J., Jonak, C., 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J. Exp. Bot. 63, 1593–1608.
Kwon, T., 2016. A double-stranded RNA binding protein, HYL1, regulates plant immunity via the jasmonic acid pathway. J. Plant Biol. 59, 506–514.
Li, C., Wang, P., Wei, Z.W., Liang, D., Liu, C.H., Yin, L.H., Jia, D.F., Fu, M.Y., Ma, F.W., 2012. The mitigation effects of exogenous melatonin on salinity-induced stress in *Malus hupehensis*. J. Pineal Res. 53, 298–306.
Li, Z., Zhang, Y., Zhang, X.Q., Peng, Y., Merewitz, E., Ma, X., Huang, L.K., Yan, Y.H., 2016. The alterations of endogenous polyamines and phytohormones induced by exogenous application of spermidine regulate antioxidant metabolism, metallothionein and relevant genes conferring drought tolerance in white clover. Environ. Exp. Bot. 124, 22–38.
Li, S., Cui, L., Zhang, Y., Wang, Y., Mao, P., 2017. The variation tendency of polyamines forms and components of polyamine metabolism in zoysiagrass (*Zoysia japonica* Steud.) to salt stress with exogenous spermidine application. Front. Physiol. 8, 208.
Li, Z., Zhang, Y., Peng, D.D., Peng, Y., Zhang, X.Q., Ma, X., Huang, L.K., Yan, Y.H., 2018. The inhibition of polyamine biosynthesis weakens the drought tolerance in white clover (*Trifolium repens*) associated with the alteration of extensive proteins. Protoplasma 255, 803–817.
Liu, W.Z., Xie, Y.B., Ma, J.Y., Luo, X.T., Nie, P., Zuo, Z.X., Lahrmann, U., Zhao, Q., Zheng, Y.Y., Zhao, Y., Xue, Y., Ren, J., 2015. IBS: an illustrator for the presentation and visualization of biological sequences. Bioinformatics 31, 3359–3361.
Liu, T., Huang, B., Chen, L., Xian, Z., Song, S., Chen, R., Hao, Y., 2018. Genome-wide identification, phylogenetic analysis, and expression profiling of polyamine synthesis gene family members in tomato. Gene 661, 1–10.
Mellidou, I., Moschou, P.N., Ioannidis, N.E., Pankou, C., Gemes, K., Valassakis, C., Andronis, E.A., Beris, D., Haralampidis, K., Roussis, A., Karamanoli, A., Matsi, T., Kotzabasis, K., Constantinidou, H.I., Roubelakis-Angelakis, K.A., 2016. Silencing S-adenosyl-L-methionine decarboxylase (SAMDC) in *Nicotiana tabacum* points at a polyamine-dependent trade-off between growth and tolerance responses. Front. Plant Sci. 7, 379.
Meng, Z., Ruberti, C., Gong, Z.Z., Brandizzi, F., 2017. CPR5 modulates salicylic acid and the unfolded protein response to manage tradeoffs between plant growth and stress responses. Plant J. 89, 486–501.
Montilla-Bascon, G., Rubiales, D., Hebelstrup, K.H., Mandon, J., Harren, F.J.M., Cristescu, S.M., Mur, L.A.J., Prats, E., 2017. Reduced nitric oxide levels during drought stress promote drought tolerance in barley and is associated with elevated polyamine biosynthesis. Sci. Rep. 7, 13311.
Nahar, K., Hasanuzzaman, M., Alam, M.M., Rahman, A., Mahmud, J.-A., Suzuki, T., Fujita, M., 2017. Insights into spermine-induced combined high temperature and drought tolerance in mung bean: osmoregulation and roles of antioxidant and glyoxalase system. Protoplasma 254, 445–460.
Nawaz, F., Naeem, M., Zulfiqar, B., Akram, A., Ashraf, M.Y., Raheel, M., Shabbir, R.N., Hussain, R.A., Anwar, I., Aurangzaib, M., 2017. Understanding brassinosteroid-regulated mechanisms to improve stress tolerance in plants: a critical review. Environ. Sci. Pollut. Res. Int. 24, 15959–15975.
Neily, M.H., Baldet, P., Arfaoui, I., Saito, T., Li, Q.L., Asamizu, E., Matsukura, C., Moriguchi, T., Ezura, H., 2011. Overexpression of apple spermidine synthase 1 (*MdSPDS1*) leads to significant salt tolerance in tomato plants. Plant Biotechnol. Nar. 28, 33–42.
Ohama, N., Kusakabe, K., Mizoi, J., Zhao, H.M., Kidokoro, S., Koizumi, S., Takahashi, F., Ishida, T., Yanagisawa, S., Shinozaki, K., Yamaguchi-Shinozaki, K., 2016. The transcriptional cascade in the heat stress response of arabidopsis is strictly regulated at the level of transcription factor expression. Plant Cell 28, 181–201.
Romero, F.M., Maiale, S.J., Rossi, F.R., Marina, M., Ruiz, O.A., Garriz, A., 2018. Polyamine metabolism responses to biotic and abiotic stress. Methods Mol. Biol. 1694, 37–49.
Saha, J., Giri, K., 2017. Molecular phylogenomic study and the role of exogenous spermidine in the metabolic adjustment of endogenous polyamine in two rice cultivars under salt stress. Gene 609, 88–103.
Shen, Y., Ruan, Q., Chai, H., Yuan, Y., Yang, W., Chen, J., Xin, Z., Shi, H., 2016. The Arabidopsis polyamine transporter LHR1/PUT3 modulates heat responsive gene expression by enhancing mRNA stability. Plant J. 88, 1006–1021.
Soudek, P., Ursu, M., Petrova, S., Vanek, T., 2016. Improving crop tolerance to heavy metal stress by polyamine application. Food Chem. 213, 223–229.
Tabor, C.W., Tabor, H., 1984. Polyamines. Annu. Rev. Biochem. 53, 749–790.
Tajti, J., Janda, T., Majlath, I., Szalai, G., Pal, M., 2018. Comparative study on the effects of putrescine and spermidine pre-treatment on cadmium stress in wheat. Ecotoxicol. Environ. Saf. 148, 546–554.
Talaat, N.B., Shawky, B.T., 2016. Dual application of 24-epibrassinolide and spermine confers drought stress tolerance in maize (*Zea mays* L.) by modulating polyamine and protein metabolism. J. Plant Growth Regul. 35, 518–533.
Tiburcio, A.F., Altabella, T., Bitrian, M., Alcazar, R., 2014. The roles of polyamines during the lifespan of plants: from development to stress. Planta 240, 1–18.
Venkataramanan, S., Floor, S.N., 2018. The traffic jam: polyamine prevalence pauses protein production. Mol. Cell 70, 191–192.
Vuosku, J., Karppinen, K., Muilu-Makela, R., Kusano, T., Sagor, G.H.M., Avia, K., Alakarppa, E., Kestila, J., Suokas, M., Nickolov, K., Hamberg, L., Savolainen, O., Haggman, H., Sarjala, T., 2018. Scots pine aminopropyltransferases shed new light on evolution of the polyamine biosynthesis pathway in seed plants. Ann. Bot. http://dx.doi.org/10.1093/aob/mcy012.
Wang, J., Sun, P.P., Chen, C.L., Wang, Y., Fu, X.Z., Liu, J.H., 2011. An arginine decarboxylase gene PtADC from *Poncirus trifoliata* confers abiotic stress tolerance and promotes primary root growth in *Arabidopsis*. J. Exp. Bot. 62, 2899–2914.
Wang, Y.P., Tang, H.B., DeBarry, J.D., Tan, X., Li, J.P., Wang, X.Y., Lee, T.H., Jin, H.Z., Marler, B., Guo, H., Kissinger, J.C., Paterson, A.H., 2012. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res. 40, e49.
Wen, X.P., Pang, X.M., Matsuda, N., Kita, M., Inoue, H., Hao, Y.J., Honda, C., Moriguchi, T., 2008. Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. Transgenic Res. 17, 251–263.
Wen, X.P., Ban, Y., Inoue, H., Matsuda, N., Moriguchi, T., 2010. Spermidine levels are implicated in heavy metal tolerance in a spermidine synthase overexpressing transgenic European pear by exerting antioxidant activities. Transgenic Res. 19, 91–103.
Wu, H., Fu, B., Sun, P.P., Xiao, C., Liu, J.H., 2016. A NAC transcription factor represses putrescine biosynthesis and affects drought tolerance. Plant Physiol. 172, 1532–1547.
Xu, X.X., Hu, Q., Yang, W.N., Jin, Y., 2017. The roles of call wall invertase inhibitor in regulating chilling tolerance in tomato. BMC Plant Biol. 17, 195.
Yang, L., You, J., Wang, Y.P., Li, J.Z., Quan, W.L., Yin, M.Z., Wang, Q.F., Chan, Z.L., 2017. Systematic analysis of the G-box factor 14-3-3 gene family and functional characterization of *GF14a* in *Brachypodium distachyon*. Plant Physiol. Biochem. 117, 1–11.
Zepeda-Jazo, I., Pottosin, I., 2018. Methods related to polyamine control of cation transport across plant membranes. Methods Mol. Biol. 1694, 257–276.
Zhai, H., Wang, F.B., Si, Z.Z., Huo, J.X., Xing, L., An, Y.Y., He, S.Z., Liu, Q.C., 2016. A *myo*-inositol-1-phosphate synthase gene, *IbMIPS1*, enhances salt and drought tolerance and stem nematode resistance in transgenic sweet potato. Plant Biotechnol. J. 14, 592–602.
Zhang, Y., Zhang, H., Zou, Z.R., Liu, Y., Hu, X.H., 2015. Deciphering the protective role of spermidine against saline-alkaline stress at physiological and proteomic levels in tomato. Phytochemistry 110, 13–21.
Zhao, Q., Suo, J., Chen, S., Jin, Y., Ma, X., Yin, Z., Zhang, Y., Wang, T., Luo, J., Jin, W., Zhang, X., Zhou, Z., Dai, S., 2016. Na_2CO_3-responsive mechanisms in halophyte *Puccinellia tenuiflora* roots revealed by physiological and proteomic analyses. Sci. Rep. 6, 32717.
Zou, C., Chen, X., Zhang, F., Mao, D., 1998. Study on the correlation between the active Fe and Fe nutritional status of plants. Plant Nutr. Fertil. 4, 399–406.