Gene 669 (2018) 52–62

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

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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ARTICLE INFO

Keywords:
Polyamine
Apple genome
Expression profiles
Alkaline stress
Malus hupehensis Rehd

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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https://doi.org/10.1016/j.gene.2018.05.077
Received 27 February 2018; Received in revised form 9 May 2018; Accepted 21 May 2018
Available online 22 May 2018
0378-1119/

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.