Plant Science 261 (2017) 18–27

Contents lists available at ScienceDirect

Plant Science

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

# Functions of two *Malus hupehensis* (Pamp.) Rehd. *YTP*s (*MhYTP1* and *MhYTP2*) in biotic- and abiotic-stress responses

Na Wang, Tianli Guo, Xun Sun, Xin Jia, Ping Wang, Yun Shao, Bowen Liang, Xiaoqing Gong, Fengwang Ma*

*State Key Laboratory of Crop Stress Biology for Arid Areas, College of Horticulture, Northwest A & F University, Yangling, Shaanxi 712100, China*

ARTICLE INFO

*Keywords:*
YT521-B homology
*Malus*
Marssonina apple blotch
Oxygen-deficient stress
Drought

ABSTRACT

RNA binding proteins play important roles in plant responses to biotic and abiotic stresses. The YT521-B homology (YTH) domain-containing RNA binding protein (YTP) was first found in *Rattus norvegicus* and is related to oxygen-deficient stress. The *Malus* YTP gene family has 15 members. Results from their functional analysis will help researchers improve stress tolerance and fruit quality in apple. We cloned two homologous YTP family members in *M. hupehensis* – *MhYTP1* and *MhYTP2* – and identified their promoter regions that contain many *cis*-elements related to biotic and abiotic stresses. Both *MhYTP1* and *MhYTP2* can be induced by various treatments, e.g., methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), water-logging, water deficits, and high salinity. When compared with the wild type (WT), transgenic plants of 'GL-3' ('Royal Gala') apple that over-express *MhYTP1* or *MhYTP2* are more sensitive to *D. mali* infection, heat stress, and high salinity, more resistant to water-logging, chilling, drought and nutrition deficient conditions. All of these findings indicate that *MhYTP1* and *MhYTP2* participate in various biotic- and abiotic-stress responses.

## 1. Introduction

Plants are challenged by both biotic and abiotic stresses, including repeated exposure to bacteria, fungi, viruses, oomycetes, and insects that compromise their survival and reproduction [1]. They are also subjected to intense illumination, ultra-violet light, high and low temperatures, drought, salinity, heavy metals, and hypoxia. These adverse conditions can retard growth, hasten senescence, reduce crop yields, and even cause death [2]. Because plants are sessile and cannot escape these sources of stress, they utilize complex mechanisms to react to continuously changing environmental factors [3].

Stress responses involve numerous physiological, molecular, and cellular adaptations. For example, plants can produce and accumulate phytohormones such as abscisic acid (ABA), Jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) [4]. By reprogramming their genetic machinery, they can implement adequate defense reactions and increase their stress tolerance to minimize the effects of any accompanying biological damage [5]. Thus, genetic regulation networks, e.g., post-transcriptional control of gene expression, can serve as powerful strategies in these stress responses [6].

Within such networks, RNA binding proteins (RBPs) are active both during and after transcription [7], interacting with target RNAs via RNA binding domains (RBDs). Approximately 40 types of RBDs have been found in various RBPs. The YT521-B homology (YTH) RBD was first described in rat (*Rattus norvegicus*) YT521-B protein [8]. Related to oxygen-deficient stress, YT521-B is a splicing factor that changes the selection of alternative splicing sites in a concentration-dependent manner [8,9]. Its novel RBD was originally identified by comparing all known protein sequences with the splicing factor YT521-B, and was named the YTH (for YT521-B homology) domain [10]. Since then, YTH domain-containing RNA binding proteins (YTPs) have been described in yeast, animals (including humans), and plants. Researchers are now focusing on their functions in binding with the methylated N6 position of selected internal adenines in mRNAs and noncoding RNAs, which

*Abbreviations:* ABA, abscisic acid; ARE, anaerobic induction *cis*-element; Box-W1, fungal elicitor-responsive *cis*-element; *COI1*, *Coronatine insensitive 1*; dpi, days post-inoculation; ERE, ethylene response *cis*-element; ERF, ethylene response factor; ET, ethylene; GC-motif, anoxic specific induction *cis*-element; GDR, Genomic Database in Rosaceae; HSE, heat stress response *cis*-element; JA, jasmonic acid; K, potassium; *MhYTP1*, *Malus hupehensis YTP1*; *MhYTP2*, *Malus hupehensis YTP2*; MDA, malondialdehyde; MeJA, methyl jasmonate; N, nitrogen; NaCl, sodium chloride; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; Pi, phosphate; *PLD*, *Phospholipase D*; Pn, photosynthetic rate; *PR*, *Pathogen related gene*; Pro, proline; qRT-PCR, quantitative real-time PCR; RBD, RNA binding domain; RBP, RNA binding protein; REL, relative electrolyte leakage; SA, salicylic acid; SOD, superoxide dismutase; TCA-Element, salicylic acid response *cis*-element; TGA-Element, auxin response *cis*-element; W-Box, WRKY transcription factor binding site; WT, wild type; YTH, YT521-B homology; YTP, YTH domain containing RNA binding protein; YTH domain, YT521-B homology domain

* Corresponding author.
*E-mail addresses:* fwm64@sina.com, fwm64@nwsuaf.edu.cn (F. Ma).

http://dx.doi.org/10.1016/j.plantsci.2017.05.002
Received 19 March 2017; Received in revised form 2 May 2017; Accepted 5 May 2017
Available online 15 May 2017

affect the translation status and lifetime of mRNA [11–13].

Unlike their characterizations in yeast and animals, the functions of YTPs in plants are less clear. The first plant YTP − cleavage and polyadenylation specificity factor 30 (CPSF30) − was discovered in *Arabidopsis*, where it participates in oxidative-stress responses, defense responses, and cellular signaling [14,15]. Li et al. [16] have identified *Arabidopsis* YTP gene family members and analyzed their functions in abiotic-stress responses. Fifteen members of the YTP family have been reported in *Malus* [17].

The fruits of apple (*Malus* × *domestica* Borkh.) are some of the most widely cultivated and consumed in the world. Their development and quality are affected by numerous stresses. One of the most severe apple diseases is Marssonina apple blotch, which is caused by the fungus *Diplocarpon mali* [Y. Harada & K. Sawamura (anamorph *Marssonina coronaria* (Ell. & J. J. Davis) J. J. Davis, syn. *Marssonina mali* (Henn.) S. Ito)] (Taxonomy ID: 946123; NCBI) [18]. Infection leads to defoliation during the growing season, thereby reducing the leaf photosynthetic area and affecting the size, color, quality, and quantity of the fruit. It also weakens tree vigor and the capacity to bear fruit in the following years. Abiotic stresses, such as water-logging, extreme temperatures, drought, high salinity, and nutrient deficiencies, are common and all can reduce apple productivity and even cause plant death. Therefore, additional research focused on the responses of apple YTPs to both biotic and abiotic stresses will help breeders improve apple resistance and fruit quality.

The valuable *Malus hupehensis* (Pamp.) Rehd. species in China is primarily used as a rootstock but also occasionally cultivated for its own fruit production. Although functions of *M. hupehensis* YTP1 and YTP2 have been identified in leaf senescence and fruit ripening [19], their roles in responses to stresses have not yet been confirmed. In this study, we cloned the promoter regions of *MhYTP1* and *MhYTP2* and analyzed their *cis*-elements. Expression patterns of *MhYTP1* and *MhYTP2* were investigated in response to exogenous applied phytohormones (MeJA, SA, and ABA), water-logging, drought, high salinity, and nutrient deficiencies. Using wild type (WT) and overexpression apple plants as materials, we compared their phenotypes when subjected to those treatments.

## 2. Material and methods

### 2.1. Promoter cloning and analysis of cis-elements

One-year-old *M. hupehensis* seedlings were produced in the greenhouse at the Horticultural Experimental Station of Northwest A & F University, Yangling, China (34°20′N, 108°24′E). Young leaves were the source of DNA for cloning the promoter regions of *MhYTP1* and *MhYTP2*.

Genomic DNA was isolated by a CTAB-based method [20]. The promoter regions were cloned by PCR, based on the region upstream of *MhYTP1* and *MhYTP2*, i.e., on contig MDC008177.673 and contig MDC011033.286, respectively. All primers are listed in Appendix A.

The PCR-amplified promoter sequences were sequenced on both strands and then compared with those in GDR (Genomic Database in Rosaceae; GDR; http://www.rosaceae.org/), using the BLAST program. Finally, the functions for *cis*-acting elements in those promoters were predicted from the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [21,22].

### 2.2. Expression patterns of MhYTP1 and MhYTP2 under various stress treatments

After the *M. hupehensis* seedlings were grown outdoors in sand-filled pots for approximately four months, they were transferred to a chamber for hydroponic culture. Standard ½-strength Hoagland solution was used to provide nutrients and was renewed every 3 d. The treatment period began after the seedlings had been in the chamber for 15 d. For studying the response to phytohormones, we sprayed the leaves and stems with 50 μM MeJA, 50 μM SA, or 50 μM ABA (Sigma, http://www.sigmaaldrich.com/). In addition, we treated collected samples of newly formed lateral roots with 50 μM MeJA, 50 μM SA, or 50 μM ABA. In a separate experiment, we added NaCl (Sigma) to the ½-strength Hoagland solution to obtain a final concentration of 50 μM. To represent nitrogen (N)-deficiency conditions, we replaced the Ca $(NO_3)\cdot 4H_2O$ and $KNO_3$ entirely with KCl. For low-phosphate (Pi) treatment, the $KH_2PO_4$ in the original Hoagland solution was reduced from 0.5 mM to 5 μM, with the difference substituted by KCl. For inducing a potassium (K) deficiency, the $KH_2PO4$ and $KNO_3$ in the solution was replaced by $(NH_4)_3PO4$ and reduced to 5 μM. During these stress experiments, application of standard ½-strength Hoagland solution was used as the control. Each solution, whether stress-related or control, was renewed every 3 d.

The *M. hupehensis* seedlings were grown outdoors in sand-filled pots for approximately four months, which were ready for water-logging treatment. For simulating water-logged conditions, entire pots were submerged in water for 3 d, and then returned to normal growing conditions for recovery for 1 d (R1).

The *M. hupehensis* seedlings were firstly grown outdoors in sand-filled pots for approximately four months. Then they were transferred to soil-filled pots for two months. Drought stress was induced by withholding water after the designated plants had previously been fully irrigated.

### 2.3. Production of overexpression 'GL-3' apple plants and treatments

The *Agrobacterium*-sensitive *Malus* genotype 'GL-3' ('Royal Gala') was obtained from Dai et al. [23]. WT GL-3 plants, *MhYTP1* overexpression GL-3 plants, and *MhYTP2* overexpression GL-3 plants are the same with we previously used in leaf senescence experiments [19].

#### 2.3.1. Hormone-, high salinity-, and nutrient-stress treatments

After the WT GL-3 plants, *MhYTP1* overexpression GL-3 lines, and *MhYTP2* overexpression GL-3 lines were grown on a rooting medium for 15 d, they were transferred to either normal MS media, or MS media supplemented with 5 μM MeJA, 5 μM SA, 2 μM ABA, or 50 μM NaCl for the hormone experiments and high salinity treatment. For inducing nutrition deficient conditions for 20 d, plants were placed on low-N, low-Pi, low-K, and normal MS media (PhytoTechnology Laboratories). For all stress treatments, at least 5 seedlings per line were used for evaluating physiological parameters (fresh weight, height, stem diameter, leaf number, root number, and root length).

#### 2.3.2. Temperature- and waterlogged-stress treatments

After 35 d on rooting media, WT GL-3 plants, *MhYTP1* overexpression GL-3 plants, and *MhYTP2* overexpression GL-3 plants were transferred to pots and placed in phytotrons for 30 d under constant (24-h) illumination (photosynthetic photon flux density, or PPFD, of 270 μmol $m^{-2}$ $s^1$) and 25 °C.

To test their response to extreme temperatures, we took two approaches. First, leaf discs from WT and overexpression GL-3 plants were placed in tubes containing 4 mL of ultrapure water. And then these tubes were put into a water bath (Thermo-Fisher). After treatments under a range of temperatures for 1 h, relative electrolyte leakages (RELs) were measured with a conductivity meter (Leci; DDS-307). The second method involved transferring whole seedlings to phytotrons (24-h illumination and PPFD of 270 μmol $m^{-2}$ $s^{-1}$). Temperature stress was induced by adjusting the chambers to either low-temperature (4 °C) or high-temperature (40 °C), while the normal temperature (25 °C) was used as the control. A SPAD-502 (Konika-Minolta) was used to obtain SPAD values. REL was also tested, as described above.

For the water-logging treatment, entire pots were submerged. After 4 d, the equivalents of malondialdehyde (MDA), proline (Pro) content,