



Functional analysis of apple *MhYTP1* and *MhYTP2* genes in leaf senescence and fruit ripening



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ABSTRACT

RNA binding proteins participate in many plant development processes and stress responses. Members of the YTH domain-containing RNA binding protein (YTP) family have been identified in apple (*Malus × domestica* Borkh.), and their stress responses have been investigated via quantitative real time PCR (qRT-PCR) methods. However, their functions in plant senescence have not been verified. Leaf senescence and fruit ripening can affect apple fruit quality. Thus, we cloned two homologous YTP family members in *M. hupehensis*, *MhYTP1* and *MhYTP2*. These Group A1 members are localized to the entire cell. Expressions of *MhYTP1* and *MhYTP2* are induced during natural leaf senescence in *Malus*. The overexpression of either one can promote leaf senescence in *Arabidopsis* and *Malus* and promote fruit ripening in tomato. In addition, *MhYTP2* interacts with an ethylene biosynthesis-related protein, acireductone dioxygenase 4 (*MhARD4*). Therefore, expression of *MhYTP1* or *MhYTP2* is positively correlated with plant senescence, suggesting that they can be used as marker genes for plant senescence. These results provide new information about senescence and can serve as a basis for further research that will help improve apple fruit quality.

1. Introduction

The phenomenon of senescence is an evolutionarily acquired and programmed developmental process. The most prominently studied in plants is leaf senescence (Sobieszczuk-Nowicka 2017). This regulated process of programmed cell death is coordinated with the development of other plant organs such as seeds, more apical leaves, or additional storage structures. It is highly controlled and active, requiring massive transcriptional and metabolic reprogramming geared toward the organized breakdown and remobilization of valuable resources in an orderly manner (Himelblau and Amasino, 2001; Maillard et al., 2015). The most obvious change is leaf color due to the breakdown of chlorophylls (Woo et al., 2013). Leaf senescence is a fundamental part of normal plant development as well as at times when adaptations to adverse environmental conditions are necessary (Liebsch and Keech 2016). Fruit ripening, coordinated with leaf senescence, represents a summation of physiological and biochemical processes that include de-greening, the accumulation of colored pigments for increased attractiveness,

and changes in texture and metabolism. Such changes not only aid in seed dispersal, but also ensure that the fruits provide essential nutrition to the diets of humans and other animals (Klee and Giovannoni 2011).

Regulation of leaf senescence and fruit ripening involves multiple layers of control. In the initial phase, hormonal cues occur, e.g., ethylene, jasmonic acid, salicylic acid, abscisic acid, and cytokinins, with ethylene having a prominent role as a positive regulator (Jibran et al., 2013). Application of ethylene to leaves stimulates senescence, but inhibitors of ethylene perception or biosynthesis delay leaf senescence (Koyama 2014). Furthermore, downregulation of an ethylene biosynthesis gene led to a decrease in ethylene production and substantially delayed leaf senescence, clearly suggesting that ethylene, produced as plants age, accelerates leaf senescence (John et al., 1995). When comes to the relationships between ethylene and fruit ripening, the plant hormone ethylene is the major cue that controls most aspects of ripening in climacteric fruit (like tomato and apple); by contrast, the ripening of nonclimacteric fruit (like orange) does not strictly depend on ethylene, and the nature of the triggers of ripening in this type of

Abbreviations: RBP, RNA binding protein; RBD, RNA binding domain; YTH domain, YT521-B homology domain; YTP, YTH domain containing RNA binding protein; *M. hupehensis*, *Malus hupehensis*; *MhYTP1*, *Malus hupehensis* YTP1; *MhYTP2*, *Malus hupehensis* YTP2; CPSF30, cleavage and polyadenylation specificity factor 30; AP, Alkaline phosphatase; EMSA, electrophoretic mobility shift assay; SAG12, senescence associated gene 12; PAO, pheide a oxygenase; BiFC, biomolecular fluorescence complementation; CDS, coding sequence; X-α-gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside; GST, glutathione S-transferase; ARD4, Acireductone dioxygenase; PCD, programmed cell death; qRT-PCR, quantitative real time-polymerase chain reaction; MTA, methylthioadenosine

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fruit remains yet to be elucidated (Lelièvre et al., 1997; Liu et al., 2015). The involvement of ethylene in fruit ripening was initially reported a long time ago (Burg and Burg, 1962), and since then, direct evidences have accumulated to demonstrate that ethylene mediates fruit ripening at the physiological, biochemical, and molecular levels. Altering ethylene at the level of its biosynthesis, perception, signal transduction, or gene transcription was shown to impact fruit ripening (Hamilton et al., 1990; Oeller et al., 1991; Lanahan et al., 1994; Tieman et al., 2001; Lee et al., 2012; Liu et al., 2014).

In the complex regulatory net of leaf senescence and fruit ripening, numerous regulatory genes are also involved. Post-transcriptional regulation of gene expression is a powerful strategy by which plants develop and adapt to their environment (Huh and Paek 2014). This expression is regulated by RNA binding proteins (RBPs), which are active both during and after transcription (Chen and Varani 2013). RBPs interact with target RNAs via RNA binding domains (RBDs). Approximately 40 types of RBDs have been found in various RBPs (Kishore et al., 2010). The novel YT521-B homology RBD was first reported in rat (*Rattus norvegicus*) as being related to oxygen-deficient stress (Imai et al., 1998). This novel RBD was identified by comparing all known protein sequences with the splicing factor YT521-B and was named the YTH (for YT521-B homology) domain (Stoilov et al., 2002). Since then, YTH domain-containing proteins have been described in yeast, animals (including humans), and plants. First cloned from a library of the rat brain, YT521-B can change the selection of alternative splicing sites in a concentration-dependent manner (Hartmann et al., 1999). Researchers are now focusing on functions of human YTPDF2 in binding via methylation of the N6 position of selected internal adenines (m6A) in mRNAs and noncoding RNAs, which affect the translation status and lifetime of mRNA (Li et al., 2014b; Zhu et al., 2014).

Unlike their characterizations in yeast and humans, 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 (Addepalli and Hunt 2007; Chakrabarti and Hunt 2015). Since then, Li et al. (2014a) have identified YTP gene family members in *Arabidopsis* and analyzed their functions in abiotic stress responses. Fifteen members of the YTP family have been reported in *Malus* (Wang et al., 2014). Although their expressions have been investigated via quantitative real-time PCR (qRT-PCR) experiments under various abiotic stress conditions, little is known about their potential roles in leaf senescence and fruit ripening.

Fruits of apple (*Malus × domestica* Borkh.) are some of the most widely cultivated and consumed in the world. Additional research on its leaf senescence and fruit ripening will help breeders improve fruit quality. In China, plants of *Malus hupehensis* (Pamp.) Rehd. are valuable rootstocks but are also occasionally cultivated for their own fruit productions. Moreover, *M. hupehensis* seedlings are usually used as experiment materials because of their apomixes reproduction mode, which makes the seedlings uniform. Therefore, we cloned two homologous YTP family members in *M. hupehensis*, *MhYTP1* and *MhYTP2*. We then conducted comparative analyses with transgenic *Arabidopsis*, ‘GL-3’ (‘Royal Gala’) apple, and tomato (*Lycopersicon esculentum*) plants to examine the relationships among these YTPs, leaf senescence, and fruit maturation.

2. Material and methods

2.1. Gene cloning and sequence analysis

We grew one-year-old *M. hupehensis* plants in pots in the greenhouse at the Horticultural Experimental Station of Northwest A & F University, Yangling, China (34°20'N, 108°24'E). Standard horticultural practices were followed for disease and pest control.

The open reading frames of *MdYTP1* (MDP000012404) and

MdYTP2 (MDP0000488588) were obtained through EST assembling, and RNA was extracted from the fully unfolded *M. hupehensis* leaves by a CTAB-based method (Gambino et al., 2008). First-strand cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. Primers for cloning were designed using Primer Premier 6.0 (Appendix A in Supplementary material).

2.2. Sub-cellular localization

Onion epidermal cell layers were placed on an MS medium, with the interior of the layer facing upward. Gene gun-mediated methods were applied to transfer pA7-*MhYTP1*-GFP, pA7-*MhYTP2*-GFP, or pA7-GFP (negative control) into those cells. Conditions for bombardment with gold microcarriers included a vacuum pressure of 94.818 kPa, helium pressure of 7.584–8.963 MPa, and 6 cm of target distance. Afterward, the bombarded tissues were incubated on an agar-solidified MS medium for 24 h at 22 °C. Samples were then transferred to glass slides and observed under a fluorescence microscope (Olympus; Japan), using a 20× objective. The excitation wave length was set at 480 nm. Exposure time was adjusted according to the picture status. The light field and corresponding fluorescent field photos were taken under the same view.

2.3. Prokaryotic protein expression and western blotting

Reconstructed pET32a plasmids containing *MhYTP1* or *MhYTP2* were transformed into *Escherichia coli* strain BL21 (DE3) to express proteins by induction with 1 mM isopropyl-β-D-thiogalactopyranoside at 18 °C for 10 h. The bacterial cells were centrifuged and re-suspended in lysis buffer [50 mM Tris-HCl (pH7.5) containing 100 mM NaCl and 1 mM phenylmethanesulfonyl fluoride]. The resuspension solutions were sonicated (40% power; on5 s, off10 s, for 30 min) in ice by using an ultrasonic cell disruptor (FB120220, Thermo Fisher Scientific, USA), and then centrifuged at 12000 × g. Afterward, the supernatant and precipitate were collected separately and analyzed by SDS-PAGE electrophoresis.

For western blot analysis, we used a biosynthesis antibody of *MhYTP1* and *MhYTP2*. Because both are very similar in their amino acid sequences we then selected the amino acid region that was most similar between them but which could also be used to distinguish them from other *Malus* YTPs. That sequence was ‘CQTKGPDDIPDATA’. Primary antibody was produced in rabbit at Genescript Company (<http://www.genescript.com.cn/>). Traditional western blot analysis was conducted via SDS-PAGE electrophoresis, membrane transformation, antibody incubation, high strict washing, and developing. Our secondary antibody was Goat anti Rabbit IgG tagged with alkaline phosphatase (AP) (A0208; Beyotime Biotechnology, <http://www.beyotime.com/>).

2.4. Prokaryotic protein purification and RNA electrophoretic mobility shift assay (EMSA)

The soluble portion of the total protein was purified with a Millipore His-tag protein purification kit (<http://www.emdmillipore.com/>). A sequence of minimum theoretical length (516 bp) containing every possible 5-nt long RNA binding motif was designed computationally and created as six double stranded, 100-bp long, overlapping sequences were termed Pentaprobos (PPs) (Bendak et al., 2012). These oligonucleotides were synthesized as 12 ssDNAs (PP1–PP12). PP1 is complementary to PP7, PP2–PP8 and so on. DNAs of probes (PP1–PP12; Bendak et al., 2012) were artificially synthesized in Invitrogen Biotechnology Co., Ltd. Afterward, the corresponding probe RNAs were transcribed and labeled with a digoxigenin labeling kit (Roche) according to the manufacturer's instructions. The labeled RNA probes were mixed and loaded without *MhYTP1* protein, which acted as our negative control. We also mixed those 12 probes with *MhYTP1*,

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