Plant Science 236 (2015) 283–294

Contents lists available at ScienceDirect

Plant Science

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

Redox homeostasis and reactive oxygen species scavengers shift during ontogenetic phase changes in apple

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ARTICLE INFO

Article history:
Received 11 February 2015
Received in revised form 14 April 2015
Accepted 16 April 2015
Available online 24 April 2015

Keywords:
Malus asiatica
M. domestica
Phase change
Reduction and oxidation

ABSTRACT

The change from juvenile to adult phase is a universal phenomenon in perennial plants such as apple. To validate the changes in hydrogen peroxide (H_2O_2) levels and scavenging during ontogenesis in apple seedlings, the H_2O_2 contents, its scavenging capacity, and the expression of related genes, as well as miR156 levels, were measured in leaf samples from different nodes in seedlings of 'Zisai Pearl' (*Malus asiatica*) × 'Red Fuji' (*M. domestica*). Then *in vitro* shoots were treated with redox modulating chemicals to verify the response of miR156 to redox alteration. The expression of miR156 decreased gradually during ontogenesis, indicating a progressive loss of juvenility. During the phase changes, H_2O_2 and ascorbate contents, the ratio of ascorbate to dehydroascorbate, the ascorbate peroxidase, catalase and glutathione reductase activities, and the expressions of some *MdGR* and *MdAPX* gene family members increased remarkably. However, the glutathione content and glutathione to glutathione disulfide ratio declined. In chemicals treated *in vitro* shoots, the changes in miR156 levels were coordinated with GSH contents and GSH/GSSG ratio but not H_2O_2 contents. Conclusively, the relative reductive thiol redox status is critical for the maintenance of juvenility and the reductive ascorbate redox environment was elevated and sustained during the reproductive phase.

1. Introduction

The loss of juvenility in many perennial higher plants causes the vegetative phase change once a lifecycle during ontogenesis [1,2]. However, the juvenility can be retrieved by certain horticultural or biotechnological approaches such as sequential grafting onto juvenile rootstocks or repeated tissue culturing [1,3,4]. The intrinsic mechanisms of both the vegetative phase change and rejuvenation have been studied extensively in plants [5–7].

miR156 is a key regulator of juvenility in flowering plants. A high level of miR156 expression is a prerequisite of juvenile traits in both herbaceous and woody flowering plant species [8–10]. When miR156 was over-expressed by a transgenic approach in *Arabidopsis thaliana* and maize (*Zea mays*), they retained juvenility and did not respond to floral inducing environmental stimuli [11,12]. In rice (*Oryza sativa*), the over-expression of two *miR156* genes (*miR156b* and *miR156h*) delayed flowering [13]. A maize mutant with an increased miR156 expression level exhibited severe morphological alterations, including enhanced leaf and tiller formation and deformed inflorescence architecture [14]. A miR156 over-expressing tomato (*Solanum lycopersicum*) showed several juvenile traits, such as producing numerous adventitious roots, a significantly increased node number, and the early development of lateral shoots [15]. However, when miR156 expression was repressed by transgenic silencing, the transformants exhibited no juvenile traits [16–18]. During the rejuvenation of adult phase apple rootstocks, M26, M9 and *Malus xiaojinensis*, via shoot stem subcultures, the expression of miR156 increased to the same level as that in juvenile tissue by the 15th subculture and rooted well *in vitro* at the 18th subculture [19].

Recently, it was proposed that the transcription levels of *pre-miR156* genes were regulated by upstream signals derived from the leaf primordia in *Arabidopsis* [10]. Ablating some leaf primordia of *Arabidopsis*, maize and tobacco (*Nicotiana benthamiana*) led to a marked delay in the performance of several phase-specific leaf traits, delayed the vegetative phase change and increased the expression of some *MIR156* primary transcripts, indicating that the

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbic acid; BSO, buthionine sulphoximine; CAT, catalase; CTAB, cetyltrimethylammonium bromide; DEPC, diethyl pyrocarbonate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DPI, diphenyleneiodonium; GGT, γ-glutamyl transpeptidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; MDHAR, monodehydroascorbate reductase; MED, menadione; OTC, L-2-oxothiazolidine-4-carboxylic acid; ROS, reactive oxygen species.

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http://dx.doi.org/10.1016/j.plantsci.2015.04.008
0168-9452/

regulating signal of vegetative phase change comes from the leaf primordia [10,12]. What is this signal from the leaf primordia? One hypothesis is that sugar could be the signal upstream of miR156 that regulates the juvenile-to-adult phase transition in *Arabidopsis* [7,20,21]. Exogenous metabolically active sugars, such as fructose, sucrose and maltose, repressed the miR156 level by inhibiting the transcription of *MIR156* genes (*MIR156A* and *MIR156C*), whereas glucose regulates the accumulation of miR156 by degrading some primary miRNAs, which results in the acceleration of phase change and floral differentiation [7]. The mechanism of sugar modulating miR156 is evolutionarily conserved, and has been verified not only in *Arabidopsis*, but also in tobacco, moss (*Physcomitrella patens*) and tomato [7]. However, further experimental evidence is required to sufficiently support this hypothesis.

Several changes in reactive oxygen species (ROS) generation and scavenging were age related in some organisms. Significant increases in mitochondrial hydrogen peroxide (H_2O_2) production rates were observed during the post-reproductive phase of life in various species, including *Drosophila melanogaster*, *Musca domestica* and mice (*Mus musculus*) [22,23]. A progressive shift in the cellular redox state may lead to the over-oxidation of redox-sensitive protein thiols, which disrupts the redox-regulated signaling [23]. Indeed, a strong decrease in the concentration of the reduced form of glutathione (GSH) and the glutathione/glutathione disulfide (GSH/GSSG) ratio during aging have been reported in *Caenorhabditis elegans*, rats (*Rattus norvegicus*) and mice [24–27]. These age-associated changes in antioxidant levels are attributed to the effect of endogenous ROS over-production, indicating a more oxidative state in some tissues during aging [28,29].

Changes in redox homeostasis occur during the vegetative phase change and floral transition in apple seedlings [30–32]. In our previous experiments, five of the six redox enzymes, which are regulated at the transcriptional level, were elevated during the vegetative phase change. The monodehydroascorbate reductase (MDHAR) level was higher in the adult vegetative phase than in the juvenile and reproductive phases [30]. Additionally, several redox-related genes were differentially expressed between the juvenile and adult phases as determined by suppression subtractive hybridization. The level of an ascorbate peroxidase (APX)-encoding gene was up-regulated in the adult phase [31]. There were also remarkable differences in ROS metabolism during the phase change in seedlings of 'Zisai Pearl' × 'Red Fuji'. The levels of H_2O_2 and related scavenger enzyme activity levels, such as those of catalase (CAT), APX and glutathione reductase (GR), were higher in the reproductive phase than in the juvenile and adult vegetative phases [32].

To further validate the phase-related changes in ROS levels and scavenging during the ontogenetic development in apple seedlings, H_2O_2 contents, activities of ROS scavengers, expression levels of some related genes and miR156 levels were measured in leaf samples from different nodes along the trunk of hybrid seedlings derived from 'Zisai Pearl' (*Malus asiatica*) × 'Red Fuji' (*M. domestica*) apple. The observed changes in miR156 in response to redox homeostasis alteration were validated by the treatments of *in vitro* shoots with the activators and inhibitors of H_2O_2 and GSH.

2. Materials and methods

2.1. Plant materials and chemical treatments

Six-year-old seedlings derived from 'Zisai Pearl' × 'Red Fuji' (*M. asiatica* × *M. domestica*) were used in this experiment. 'Zisai Pearl' is a Chinese domestic cultivar originating from Hebei. The hybrid cross was made in 2007, and the seedlings were planted in 2008 and subjected to conventional cultivation management and pest control. Because of the hyper heterozygocity of the parents and violent segregation of the hybrids, to estimate the variation between individuals, three intact seedlings on their own roots, 07-07-115, 07-08-155 and 07-09-141, were sampled as biological replicates. Young leaves were sampled in the middle of spring from 1-year-old suckers and annual branches. Each sampling unit included 20 nodes from the base collar through the canopy top as illustrated by Gao et al. [31]. The leaves collected from the same sampling unit of one seedling were mixed and divided into three experimental replicates. Then, the leaf samples were wrapped with aluminum foil, frozen immediately in liquid nitrogen and stored in a −70 °C freezer.

To validate the changes in miR156 in response to redox homeostasis alteration, *in vitro* micro-shoots were treated with redox modulating chemicals. Because rejuvenation will occur in *in vitro* shoots derived from adult phase explant, the juvenility of the micro-shoot is changing with the passages of subculture [19]. The plant materials used in this experiment were micro-shoots derived from juvenile phase shoot tip meristem of 07-07-115 after 18 passages of *in vitro* propagation by nodal stem segment subculture on Murashige and Skoog media containing 30 g L^{-1} sugar, 7.5 g L^{-1} agar, 0.5 mg L^{-1} 3-indolebutyric acid and 0.5 mg L^{-1} 6-benzylaminopurine (pH 5.5). The redox modulating reagents were added to the culture media after cold sterilization. In the experiments, 25 μM menadione (MED) and 50 μM diphenyleneiodonium (DPI) were used as inducer and inhibitor of H_2O_2, respectively, while 100 μM L-2-oxothiazolidine-4-carboxylic acid (OTC) and 1 mM buthionine sulphoximine (BSO) were used as precursor and inhibitor of GSH biosynthesis, respectively. The concentrations of the reagents used in these experiments were selected from a gradient of concentrations in the preliminary test according to the references [33–36]. Micro-shoots cultured on the medium containing no redox altering reagent were used as control. The experiment was performed as a complete randomized experiment design with three replicates. On day 0, 1, 3, 5 and 7 of the treatments, the micro-shoots were sampled, each sample containing three micro-shoots, and the contents of H_2O_2, GSH, GSSG and the miR156 expression were measured.

2.2. Methods

2.2.1. Determination of H_2O_2 content

H_2O_2 was detected using the method described by Mukherjee and Choudhuri [37]. A leaf sample of 200 mg was ground to a fine powder in liquid nitrogen. The H_2O_2 was extracted thoroughly using 1.9 mL pre-cooled acetone, then centrifuged (3000 × *g*) at 4 °C for 20 min. Then, 5% titanium sulfate and ammonia were added to the supernatant, and it was centrifuged again (3000 × *g*) at 4 °C for 10 min. The precipitant was rinsed three to five times with acetone and vortexed. It was then redissolved in 1.0 M H_2SO_4. The absorbance of the supernatant at 415 nm was measured using an UV spectrophotometer (UV 1800, Shimadzu, Japan) against a blank. The H_2O_2 content was determined based on a standard curve plotted using known H_2O_2 concentrations. The H_2O_2 content was measured as micromoles per gram fresh weight (μmol g^{-1}).

2.2.2. Analysis of ASC and DHA content and ASC/DHA ratio

Total ascorbic acid (ASC) and reduced ASC were assayed using an improved protocol [38]. ASC was extracted from 0.1 g leaf samples using 5% pre-cooled sulfosalicylic acid. After centrifuging at 16,000 × *g* at 4 °C for 20 min, 100 μL supernatant was neutralized with 24 μL 1.84 M triethanolamine and 250 μL phosphate buffer (50 mM, pH 7.5, containing 2.5 mM ethylenediamine tetraacetic acid) was added. Then, 50 μL 10 mM dithiothreitol (DTT) was added to the solution and kept at 25 °C for 10 min to reduce dehydroascorbic acid (DHA) to ASC. After that, 50 μL 0.5% ethylmaleimide was added to remove the remaining DTT, then 200 μL 10% trichloroacetic acid, 44% phosphoric acid, 4% double pyridine