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Seasonal variation of some antioxidant enzymes in apple (red delicious) leaves and buds during transition to flowering

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Key words: Apple, Ascorbate peroxidase (APX), Floral initiation, Glutation reductase (GR), Super oxide dismutase (SOD).

Abstract

The apple (*Malus domestica* Borkh) is an important fruit crop and a focus of horticultural research. Flower formation in apple involves a long multi-step process, which can be recognized on the basis of histological and morphological differences of shoot apex. In the present study, buds and spur leaves were harvested from 6-year-old uniform-sized apple (cv. Red Delicious) trees grafted on MM-106 rootstock and trained to a central leader. Buds and spur leaves were sampled at 45, 60, 75, 90, 105 days after full bloom (DAFB). Superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) specific activities were determined in crude protein extracts prepared from 0.1 g dry weight of plant material. According to the microscopic and biochemical results, the highest activity in APX, GR and SOD was achieved in 60 days after full bloom, and therefore this time is very important in transition to flowering. On the basis of these results, we suggest that antioxidant enzymes are one of the candidate factors for transition to flowering.

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Introduction

The apple (*Malus domestica* Borkh) is one of the important commercial fruit crops in the world. It is among the fruit trees that can be cultivated in cold districts. Flower buds in apple are produced terminally on fruiting spurs and brandy. The development of flowers extends over two consecutive seasons and consists of several stages including flower induction, flower initiation and flower differentiation during the first season, and terminates with blooming during the second season. The flower initiation occurred in the early summer of the previous year affects directly the yield of apple crops in the autumn of the following year (Foster *et al.* 2003). Therefore, the understanding of the flowering mechanisms is crucial to improve breeding efficiency and achieve a proper return bloom in the apple. Undoubtedly, the first and main step in crop formation is flower initiation, which followed by flower differentiation, fertilization, fruit set and fruit development. Each of these processes may be a limiting factor for crop formation. Flower set and fruit set are the main components of yield in apple. Instability of flower formation from year to year during the main cropping period of an apple tree is defined as the main reason for alternative bearing in this fruit crop (Guitton *et al.* 2012).

Generally, floral induction in some plant species could be fulfilled in response to stress factors. During the transition of a plant from vegetative to reproductive phase, the level of antioxidants and antioxidant enzymes increases, suggesting that plants experience stressful conditions during the flowering process (Hirai *et al.* 1995, Badiani *et al.* 1996, Gielis *et al.* 1999, Wada and Takeno 2010). Lukhande *et al.* (2003) proposed that H_2O_2 is one of the possible factors in flower induction. Superoxide dismutase, catalase and peroxidase could be considered as the effective scavengers which protect cells from active oxygen damage (Beyer and Fridovich 1988). Based on the previous reports, it seems that these enzymes play an essential role in protecting plant tissue from active oxygen damage during growth, development and senescence. However, very little is known about the activities of these enzymes during flower development. Wang and Faust (1994) and Wang *et al.* (1991) studied changes in some antioxidant enzymes activities during the vegetative bud development of 'York Imperial' apple. They observed an increase in SOD and catalase activities during bud swelling, then a decline in the activities of these enzymes at bud break. Nevertheless, due to lack of sufficient data the relationship between the floral initiation and the level of oxidative enzymes has remained unclear. Therefore, in this study we tried to evaluate changes in SOD, APX and GR activities during floral transition in buds and spur leaves of apple (cv. Red delicious) to validate their functions in the floral inception and development.

Material and methods

Plant material

Apple (cv. Red Delicious) buds and leaves were harvested from spur of 6-year-old uniform-sized trees grafted on MM-106 rootstock and trained to a central leader. The experimental orchard is located at East Azerbaijan, Iran. Agricultural practices, such as fertilization, irrigation, pruning, thinning and pest control were according to standard cultural practices. Uniform limbs and two-year-old spurs were tagged on 50 trees for subsequent sampling. Collection of samples was precisely performed 60 days after full bloom (DAFB) until 105 (DAFB) with an interval of 15 days. In each sampling, a number of 100 well-developed buds and spur leaves on the southern side of trees were randomly selected and divided to two groups with 50 buds.

Epi-illumination light microscopy

For morphological study the first group of buds was immediately fixed in FAA (5 parts formalin, 5 parts glacial acetic acid, 90 parts 50% ethanol). After a fixation period of 24 hours, the samples were rinsed, dehydrated in 70% ethanol and further dehydrated in 95% ethanol prior to being stained with alcohol soluble nigrosin (0.4% nigrosin in 95% ethanol) for at least 2 days according to the method reported by

Charlton *et al.* (1989). The specimens were washed in 95% ethanol before investigation using epi-illumination light microscopy. Prepared samples were observed with a Nikon E600 reflected light microscope (Nikon, Tokyo, Japan) in dark field mode. Image (z-stack) acquisition was performed with a Nikon DXM1200F high resolution digital camera (Nikon). For this purpose, series of consecutive images from different focal planes of the sample were taken and then superimposed automatically to improve the depth of focus using Image J 1.41 software (freely available from http://rsbweb.nih.gov/ij/) in accordance with Dadpour *et al.* (2008, 2011). Outputs from the z stack acquisition were trimmed and saved as TIF format images.

Protein extraction

For biochemical study the spur leaves and second group of buds were immediately frozen in liquid nitrogen and transferred to laboratory where they lyophilized and stored at -80^{oC} until analysis. Superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) specific activities were determined in crude protein extracts prepared from 0.1 g dry weight of plant material stored and frozen at $-80^{\circ C}$. Each sample was ground in a mortar in the presence of liquid nitrogen and proteins were extracted with 2 mL of extraction buffer [20 mM Hepes, pH 7.5, 50 mM KCl, 1 mM EDTA, 0.1 % (v/v) Triton X-100, 0.2 % (w/v) polyvinylpyrrolidone, 0.2 % (w/v) polyvinylpolypyrrolidone and 5 % (v/v) glycerol], followed by addition of 0.2 mL of 'high salt buffer' (225 mM Hepes, pH 7.5, 1.5 M KCl and 22.5 mM MgCl2). Homogenates were centrifuged and after removing precipitated material, the final protein extracts were aliquoted, flash frozen by liquid nitrogen and stored at $-80^{\circ C}$ until used for enzyme assays. The protein concentration in the extracts was determined by the method of Bradford (1976), using the Bio-Rad reagent and bovine serum albumin as a standard.

Measurements of Superoxide Dismutase (SOD) activity

Total SOD activity was determined according to Beyer and Fridovich (1987) by monitoring the inhibition of nitroblue tetrazolium (NBT) photoreduction using the following colorimetric assay; The reaction mixtures (1 mL) contained 50 mM potassium phosphate buffer, pH 7.8, 9.9 mM l-methionine, 58 μM NBT, 0.025 % (v/v) Triton X-100, 2.4 μM riboflavin (as the source of superoxide radicals) and the protein extract. After adding riboflavin, the reaction mixtures were irradiated (300 μmol m−2 s−1, provided by three 23 W Osram DULUX PRO compact fluorescent lamps) for 10 min at $25^{\circ C}$, and the absorbance at 560 nm was measured, using a non-irradiated reaction mixture as a blank. One SOD unit was defined as the amount of enzyme that causes 50 % inhibition of NBT photoreduction under assay conditions.

Measurements of ascorbate peroxidase (APX) activity

The activity of APX is determined by the measurement of the diminution of the absorbance of oxidized ascorbate at 290 nm (using quartz cuvets) according to (Nakano and Asada, 1981). The volume of the reaction is 2 ml containing 0.5 mM of ascorbate, 0.1 mM EDTA, 1.2 mM H_2O_2, and protein extract to be tested for enzyme activity. Reactions were initiated by adding H_2O_2 and the decrease in O.D. was monitored until it became stable. Control reactions with no protein extract were incubated in parallel to correct for non-enzymatic H_2O_2 reduction. Activity was calculated using the extinction coefficient (2.8 mM-1 cm-1 at 290 nm) for ascorbate.

Measurements of Glutathione Reductase (GR) activity

Glutathione reductase activity was determined according to Connell and Mullet (1986), following the oxidation of NADPH, the cofactor in the GR-catalysed reduction of oxidized glutathione (GSSG). In a 1-mL final volume, the reaction mixtures contained 100 mM Hepes, pH 7.5, 1 mM EDTA, 3 mM $MgCl_2$, 0.5 mM GSSG and the protein extracts. Reactions were

initiated by adding NADPH to a final concentration of 0.2 mM. Samples were incubated at 25 °C, and the decrease in absorbance at 340 nm (Δε = 6.22 mM−1 cm−1) was measured after 25 min. Control reactions with no protein extract were incubated in parallel to correct for non-enzymatic NADPH oxidation. One GR unit was defined as the amount of enzyme that will oxidize 1 µmol of NADPH per minute at 25°C.

Statistical Analysis

The experiment was arranged as split plots in time on the basis of completely randomized design with three replications. Analysis of variance (ANOVA) carried out with SPSS software. The significance of the differences among treatments was tested by applying a one-way ANOVA, at a confidence level of 95%.

Results and discussion

Morphological changes of SAM during the transition from vegetative to reproductive phase were obtained by reflected light microscopy and divided to two crucial stages: transition phase (Fig. 1.A) and floral initiation (Fig. 1.B).

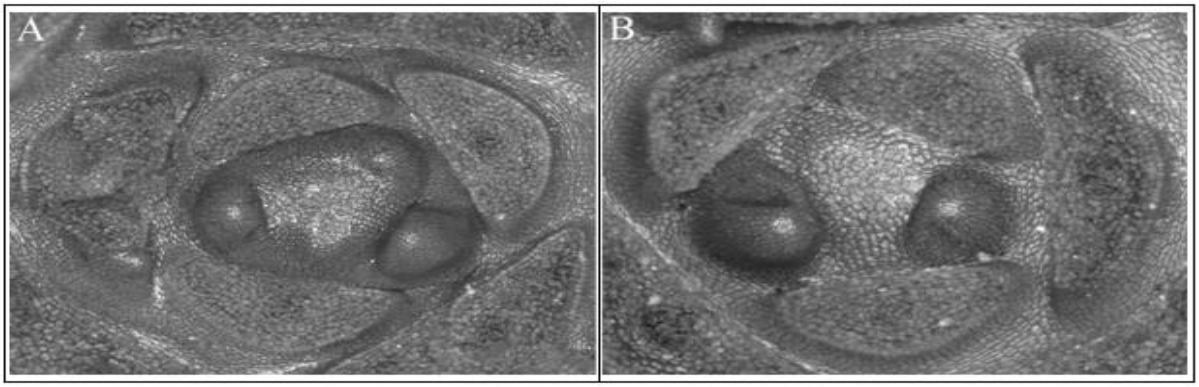

Fig. 1. Developmental stages of apex. A. floral transition phase. B- Floral initiation phase.

Activity of enzymes involved in scavenging ROS changed significantly over time. In all samples SOD activity in leaves was much lower than buds (fig. 2). There was significant difference not only between leaves and bud but also during the growing season. The highest activity was seen 60 days after full bloom in both tissues. Foster *et al* (2003) reported that floral initiation in apple bud accrued 50-60 floral in days after full bloom approximately. SODs catalyze the production of O_2 and H_2O_2 from superoxide (O_2^-), which results in less harmful reactants. H_2O_2 was one of possible factor that affected in floral initiation in *Arabidopsis* (Lukhande *et al,* 2003). In *Chenopodium rubrum*, peroxidase activity increased more in IAA-induced flowering plants than in non-induced flowering plants (Krekule and Machackova 1986). Cathodic peroxidase activity also increased in tobacco epidermal explants during flower formation *in vitro* (Thorpe *et al.* 1978). Similarly, in *Fagus murielia*, superoxide dismutase and peroxidase enzyme increased in the leaves of flowering plants compared to vegetative plants (Gielis *et al.* 1999).

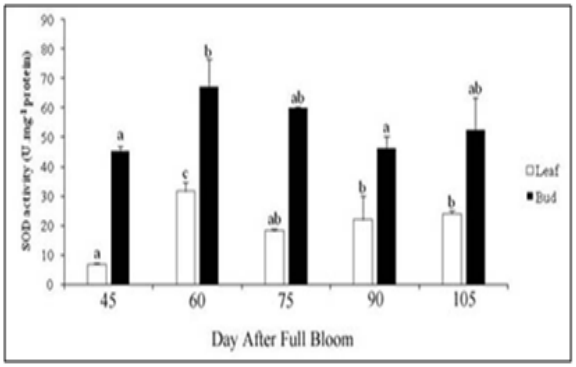

Fig. 2. SOD activity in leaves (white bars) and buds (black bars) of apple cv. Red delicious. Values carrying different letters are significantly different at $P \leq 0.05$.

APX is one of the important antioxidant enzymes and scavenges hydrogen peroxide (H_2O_2) using ascorbate (AsA) as an electron donor in the chloroplast and cytosol through the AsA-glutathione (GSH) cycle

(Asada 1992). Our result showed that the activity of APX in 45 DAFB was lowest and then significant increase in 60 DAFB. The highest activity was seen at the second stage such as SOD activity and then enzyme activity were decrease in the next stages during the growing season (fig. 3). Our result showed that antioxidant enzyme in 60 DAFB has been significantly different during bud development and these data were similar to the result of APX activity on transition phase in *A. taliana* (Lakhunde *et al.* 2003).

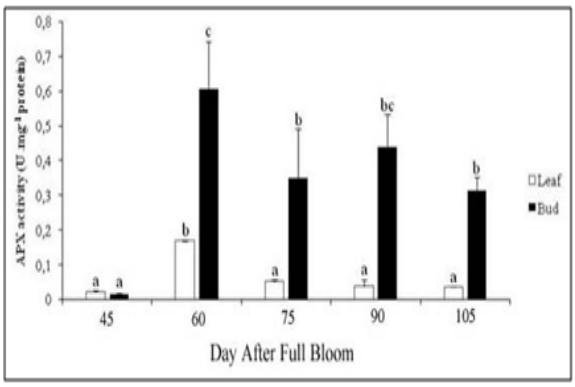

Fig. 3. APX activity in leaves (white bars) and buds (black bars) of apple cv. Red delicious. Values carrying different letters are significantly different at P ≤0.05.

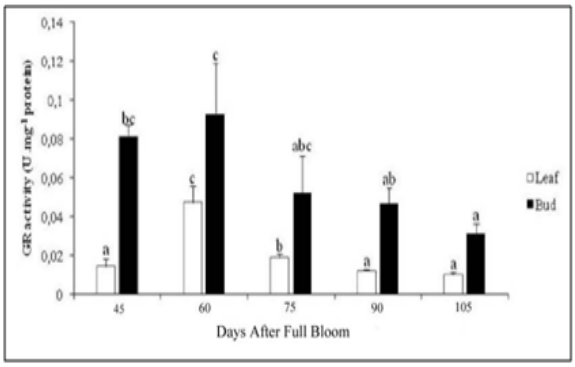

Fig. 4. GR activity in leaves (white bars) and buds (black bars) of apple cv. Red delicious. Values carrying different letters are significantly different at P ≤0.05.

The ascorbate–glutathione-cycle which uses reduced glutathione (GSH) as an electron donor to regenerate ascorbate from its oxidized form, dehydroascorbate, is considered the main pathway of superoxide and H_2O_2 removal in chloroplasts (Tausz *et al.* 2004). Study of GR activity showed that the in the first of season there were low activity and in 60 DAFB activity was increased significantly then decreased in 75,90 and 105 DAFB, respectively (fig. 4). In *A. thaliana*, the level of GSH that is involved in the ROS scavenging system is associated with flowering (Ogawa *et al.* 2001). In wheat, the level of antioxidant enzymes increased during the transition from the vegetative to reproductive phase (Badiani *et al.* 1996). In conclusion, according to microscopic and biochemical study, 60 days after full bloom chronologically was very important in transition to flowering showing the highest activity in APX, GR and SOD. Based on these results, we suggest that antioxidant enzymes are one of the candidate factors for transition to flowering.

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