

Enzymes Activities Analysis Involved in AsA-GSH Cycle of Yellow-flesh Kiwifruit Genotypes

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Abstract. In order to investigate the activity of AsA-GSH cycle related enzymes in yellow flesh kiwifruits, we studied the five kinds of yellow flesh kiwifruits and measured their related enzymes activities by ultraviolet spectrophotometry. The result showed that ‘Jinnong’ had higher activity of enzymes involved in AsA-GSH cycle compared to other kiwifruits, indicating its stronger regeneration ability of ascorbic acid.

1. Introduction

Kiwifruit belongs to *Actinidiaceae Actinidia* Lindl, which riches in Vc and has high nutritional value, known as “the king of fruits” [1]. The AsA content in plants is regulated by the ability of synthesis and regeneration. The AsA-GSH cycle is the main route of AsA regeneration. In this pathway, ascorbate peroxidase (APX) oxidizes AsA to monodehydroascorbic acid (MDHA), while H₂O₂ is scavenged with AsA as the electron donor. A portion of MDHA can be reduced to AsA by the catalysis of monodehydroascorbate reductase (MDHAR), a portion of which can be generated by non-enzymatic disproportionation reactions to AsA and dehydroascorbic acid (DHA), while DHA can be reduced to AsA with the participation of dehydroascorbate reductase (DHAR) and glutathione (GSH). If DHA cannot be reduced in time, it will be further oxidatively degraded to oxalic acid (OA) and tartaric acid (TA) and lost [2]. In this study, we used ultraviolet spectrophotometry to determine these enzymes activity of five yellow flesh kiwifruit.

2. Materials and methods

2.1. Plant material

Five yellow kiwifruit genotypes used in this study were harvested from a kiwifruit resource orchard in Shifang (104°16'N, 31°13'E), Chengdu, China. Guihai 4, Jinshi 2, Fengyue, Jinnong and Hort 16A kiwifruit all belong to *A. chinensis*. Fruits were selected according to the uniformity of the shape when samples have reached physiological maturity (total soluble solid content was 7-8%). At least 10 fruits were harvested for every sample. Prior to preparation of the test samples, the fruit samples were exposed to room temperature to reach easting maturity (total soluble solid content was 10-11%). These fruits were chopped and homogenised under liquid nitrogen in a high-speed blender for 1 min, then



immediately frozen in liquid nitrogen and stored at -80°C until use.

2.2. Assays of APX, GR, DHAR and MDHAR activities

For extraction of enzyme solution, samples were homogenized with 8 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 0.3% (v/v) Triton X-100, 2% (w/v) PVP and 2% (w/v) mercaptoethanol. The homogenates were centrifuged at 16,000 g for 20 min at 4°C and the supernatants were collected for enzyme assays.

APX activity was assayed by measuring the decrease in AsA concentration at 290 nm according to Nakano and Asada [3]. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0) supplemented with 0.1 mM EDTA and 0.5 mM sodium ascorbate. The reaction was triggered by adding 0.1 mM H_2O_2 . One unit (U) of APX activity is defined as 1nmol ascorbate oxidized min^{-1} .

GR activity was calculated by measuring the decrease in absorbance at 340 nm due to oxidation of NADPH as described by Ma and Cheng [4]. The 1mL reaction mixture contains 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM GSSG, and 0.2 mM NADPH. The reaction was initiated by adding NADPH. One unit (U) of GR activity is defined as 1nmol NADPH oxidized min^{-1} .

DHAR and MDHAR activities were assayed using the method of Ma and Cheng [4]. DHAR activity was measured at 265 nm in 3 ml of assay solution containing 100 mM Hepes-KOH (pH 7.0), 1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA, and 0.1 ml of the supernatants. The reaction was initiated by adding DHA. MDHAR activity was assayed at 340 nm in 3 ml reaction mixture containing 50 mM Hepes-KOH (pH 7.6), 0.1 mM NADH, 0.25 mM AsA, 0.25 units AsA oxidase, and 0.1 ml of the supernatants. The reaction was initiated by adding AsA oxidase. One unit of MDHAR activity was calculated in terms of mmol of NADH oxidized per minute, while that of DHAR was expressed as mmol of AsA produced per minute.

AO activity was measured based on a previously described protocol [5]. Flesh (approximately 1.0g) was homogenized with ice-cold 50 mM phosphate buffer (pH 6.5), containing 1 mM EDTA, 4% (w/v) polyvinylpyrrolidone (PVP) and 0.3% (v/v) Triton X-100. The homogenate was centrifuged at 10,000 g for 15 min at 4°C . The extraction of the soluble fraction was centrifuged again at 10,000 g for 30 min at 4°C after the addition of 35% ammonium sulfate to avoid the high AsA content of extract influencing on determination. The pellet was resuspended in 50 mM phosphate buffer (pH 6.5) and the supernatant was recovered. AO activity was determined from the decrease in A_{265} at 25°C in 3ml reaction mixture containing 0.1M sodium phosphate (pH 5.6), 0.5 mM EDTA, 0.5 mM AsA and 0.1ml supernatant. One unit of AO activity was defined as the oxidation of $1\mu\text{mol}$ AsA min^{-1} at 25°C .

3. Results and discussion

3.1. Activities of APX, AO and H_2O_2

As displayed in Figure 1A, a significant difference of APX activity was found in flesh of 5 yellow kiwifruit genotypes. The activity of APX ranged from 0.52 U/g FW (Fengyue) to 1.42 U/g FW (Hort 16A). The activity of APX of 'Hort 16A' kiwifruit was significantly higher than other kiwifruit genotypes. APX is the first enzyme of the AsA-GSH pathway.

As shown in Figure 1 B, the content of H_2O_2 ranged from 6.49 (Fengyue) to 17.87 $\mu\text{mol/g}$ FW (Jinnong). The content of H_2O_2 of 'Jinnong' kiwifruit was significantly higher than other kiwifruit genotypes, while that of other kiwifruit genotypes did not change obviously. The higher H_2O_2 content may be due to lower APX activity in red-flesh kiwifruit, because APX can prevent the accumulation of toxic levels of H_2O_2 in the cell [6].

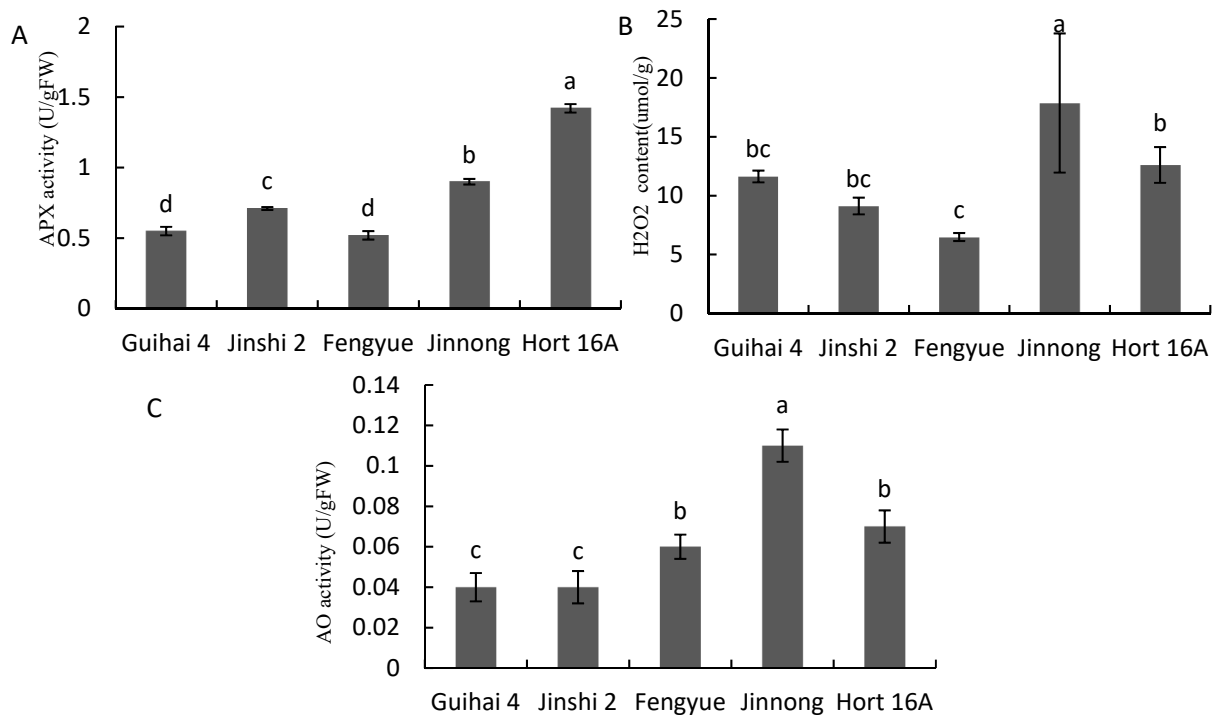


Figure 1 Activities of APX (A), AO (B) and H₂O₂ (C) in 5 yellow flesh kiwifruit genotypes.

As showed in Figure 1 C, AO activity ranged from 0.04 (Cuihai 4, Jinshi 2) to 0.11 U/gFW (Jinnong). AO activity of 'Jinnong' kiwifruit was significantly higher than other kiwifruit genotypes. AO not only apparently works to decrease oxygen content, thus limiting the formation of reactive oxygen species (ROS), but also oxidizes AsA to DHA. These strongly suggest that AO has an actual role in regulating AsA content [7].

3.2. Activities of DHAR, MDHAR and GR

As indicated in Figure 2, significant differences in the activity of DHAR, MDHAR and GR were found in flesh of 5 yellow flesh kiwifruit genotypes. The activity of DHAR, MDHAR and GR ranged from 0.17 U/g FW (Jinshi 2) to 0.24 U/g FW (Jinnong), 0.06U/g FW (Jinshi 2, Jinnong) to 0.1U/g FW (Fengyue) and 0.01U/g FW (Jinshi 2) to 1.77U/g FW (Jinnong), respectively. By comparing the activity of DHAR, MDHAR and GR in 5 yellow flesh of kiwifruit genotypes, we observed that the activity of above three enzymes in 'Jinnong' kiwifruits were obviously higher than that in other kiwifruits (Figure 2). Several previous studies had reported MDHAR [8], DHAR [9] and GR [10] played vital roles in AsA level in plant cell.

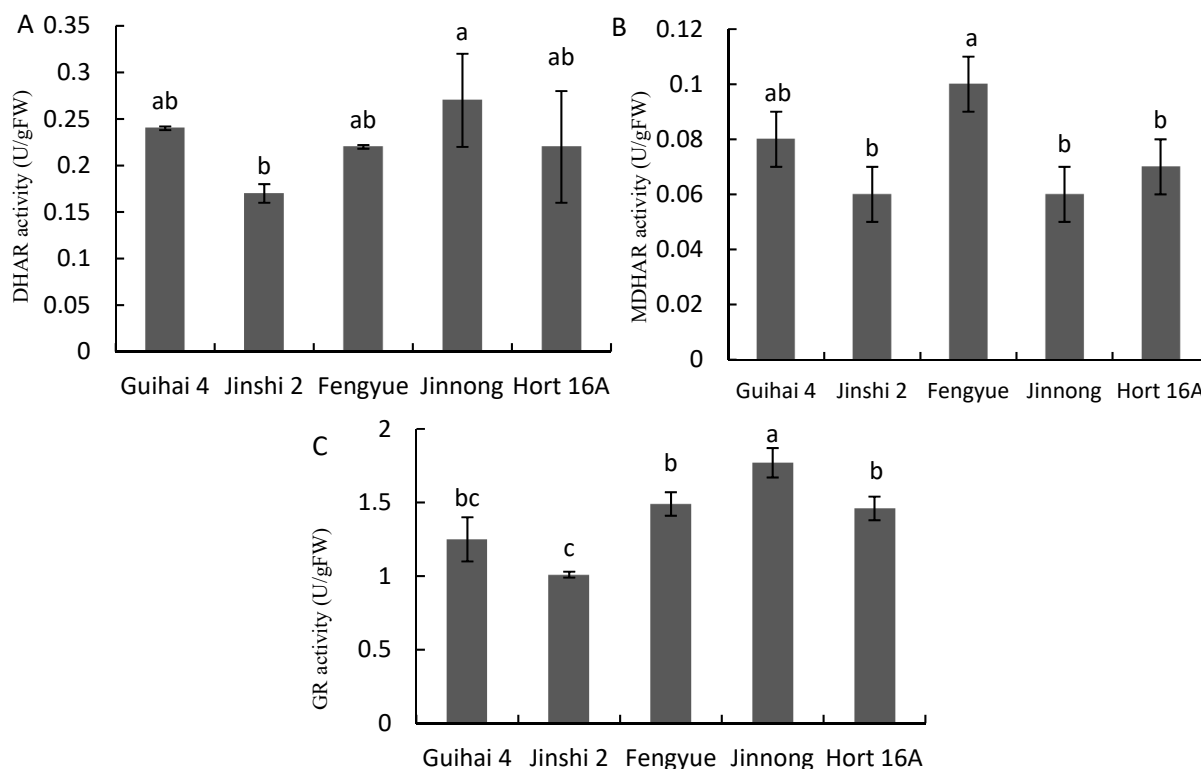


Figure 2 Activity of DHAR (A), MDHAR (B) and GR (C) in 5 yellow flesh kiwifruit genotypes.

4. Conclusion

APX catalyzes the conversion of H_2O_2 to H_2O and O_2 using AsA as specific electron donor. The AO catalyzes a complex reaction, which reduces safely the molecular oxygen into water without the release of ROS. DHAR, MDHAR and GR were key enzymes involved in AsA-GSH cycle to regulate AsA accumulate in plant cell. As a major anti-oxidant in plants, AsA is oxidized to DHA via successive reversible electron transfers with MDHA as a free radical intermediate. MDHAR can recycle MDHA molecules into AsA and DHAR reduce DHA to AsA by with GSH as an electron donor (EC 1.8.5.1). GR regenerate the reduced form of GSH to maintain the cellular redox state. It resulted that these enzymes of 'Jinnong' kiwifruit had higher activity compared to other kiwifruits, with stronger regeneration of ascorbic acid.

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