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RELATIONSHIP OF ASCORBIC ACID METABOLISM WITH THE CYTOPLASMIC MALE STERILITY IN PEPPER (*CAPSICUM ANNUUM* L.)

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Abstract

To elucidate the metabolic mechanism of Ascorbic Acid (ASA) in the CMS-pepper anthers, the metabolism changes in the reactive oxygen species (ROS) contents, antioxidants contents and ROS scavenging enzymatic activities were investigated in the anther mitochondria of CMS-9704A and maintainer-9704B. At the abortion stage, anthers of CMS-9704A had higher contents of ROS than those of the maintainer. Simultaneously, there were lower contents of ASA and glutathione (GSH) in stage 2 and 3, and the lower activities of ascorbate peroxidase (APX) and glutathione perodixase (GPX) in stage 3 in scavenging ROS in the anthers of the CMS line than maintainer. The expression level of APX and GPX in stage 3 in anthers of CMS-9704A was obviously inhibited when ROS produced with a great deal during anther stage; however the gene expression kept normal in the maintainer. Excessive accumulation of ROS, significant reduction activities and gene expression level of ROS-scavenging enzyme were coinstantaneous with CMS.

Key words: *Capsicum annuum* L., Mitochondrion, Cytoplasmic male sterility, Metabolism of ascorbic acid, Gene expression.

Introduction

In aerobic tissues, mitochondria provide energy for cell activities and response to cell signaling such as reactive oxygen species (ROS) stress (Bartoli *et al.*, 2000, Gueguen *et al*., 2000, Balk & Leaver, 2001, Sweetlove *et al*., 2002). At the same time, mitochondria are the main source of forming ROS (Green & Reed, 1998). In vivo, if ROS are not promptly cleared, the organism will suffer oxidative stress, resulting in protein and nucleic acid damage, lipid peroxidation and even necrocytosis (Foyer & Noctor, 2000, Esposito *et al*., 1999). Living tissues have antioxidant defense systems for protection against oxidative stress.

Ascorbate (ASA) is the most important antioxidant compound played vital role in inactivating most of the ROS (Noctor & Foyer, 1998). Glutathione (GSH) is the major low molecular weight thiol compound in most plants, which regenerates ascorbate. Furthermore reacts with single oxygen and hydroxyl radicals, acting as disulphide reductant to protect thiol groups on enzymes, (Noctor & Foyer, 1998). Ascorbate peroxidase (APX) reduced H_2O_2 into H_2O (Jiménez *et al*., 2002). Similarly glutathione perodixase (GPX) transformed H_2O_2 into H_2O and lipid peroxides into their respective alcohols (Flohe & Gunzler, 1984). Glutathione reductase (GR) catalyzed the oxidized form of glutathione to reduced form of glutathione using NADPH (Foyer & Halliwell, 1976).

Cytoplasmic male sterility (CMS), a maternally inherited trait that is associated with abnormal recombination of mitochondrial genome (Schnable & Wise, 1998, Budar & Pelletier, 2001, Touzet & Budar, 2004, Linke & Börner, 2005). CMS unable to produce or release functional pollens has been described over 150 plants species which plays an important role in utilization of hybrid vigor. The CMS-9704A bred selected from a natural pepper mutant of cytoplasmic male-sterility by Institute of Vegetable Crops, Hunan Academy of Agricultural Science. It has been known as one of the three types of CMS in pepper and has also been used extensively in hybrid pepper production in China (Zou, 2002). Our previous results showed that anthers of CMS line had higher contents of $O_2^{\cdot-}$, $H_2O_2$and MDA than those of maintainer during the abortion stage (Fig. 1) (Deng *et al*., 2012). In order to further understand the roles of ROS on the damage of mitochondria in CMS-9704A line of pepper, we studied the dynamic changes on the antioxidants contents and ROS scavenging enzymic activities in the anther mitochondria of CMS line and maintainer.

Materials and Methods

Plant materials: A typical CMS line, 9704A and its fertile maintainer line, 9704B were grown in experimental fields of Yunnan Agricultural University on campus in the summer. Anthers at four developmental stages were picked out from the corresponding young bud and collected for experiments. The whole anther development was divided into four stages according to the microsporogenesis progress by checking under the microscope, i.e., (stage 1) sporogenous cell division stage, (stage 2) pollen mother cell (PMC) meiosis stage, (stage 3) uninucleate microspore stage and (stage 4) mature pollen stage.

Isolation of mitochondria: Mitochondria were isolated according to the method of Bergman *et al*. (1980) and the protein concentration was determined using the method of Bradford (1976).

Determination of Non-enzymatic antioxidants: Ascorbate (ASA) and dehydroascorbate (DHA) were assayed according to the method of de Pinto *et al.* (1999). The concentrations of reduced and oxidized forms of glutathione were determined spectrophotometrically using an enzymatic cycling assay (Griffith, 1980).

Enzyme activity determinations: Ascorbate peroxidase was spectrophotometrically assayed following a decrease in the absorbance at 265 nm (Chen & Asada, 1989). Glutathione peroxidase activity was measured over a fixed time by means of glutathione (GSH) consumption (Flohe & Gunzler, 1984). Glutathione reductase activity was determined by following the rate of NADPH oxidation as measured by the decrease in the absorbance at 340 nm (Foyer & Halliwell, 1976). γ-Glutamylcysteine synthetase (γ-GCS) activity was determined as previously described (Seelig & Meister, 1985, Lee *et al*., 2003).

Expression level of *APX*, *GPX*, *GR* and *γ-GCS* gene by Semi-quantitative PCR assays: For RNA isolation, anthers were harvested at 4°C and ground in liquid nitrogen. Total RNA was extracted by Trizol procedure (TaKaRa, CHINA). cDNA synthesis was prepared using High Fidelity PrimeScrip® TR-PCR Kit (TaKaRa, CHINA) according to the manufacturer's protocol. Gene-specific RT-PCR primers are designed by Primer 5.0 for RT-PCR analysis. Prime pairs were shown in Table 1. The RT-PCR mixture consisted of 4 µl 5×PrimeSTAR PCR Buffer, 0.4 µl dNTP mixture (10 mM each), 0.2 µl of forward and reverse primer (10 µM), 0.2 µl of PrimeSTAR HS DNA Polymerase (2.5 U/µl)(TAKARA), 2 µl cDNA, and 13 µl RNase Free dH_2O water. The products of amplification were checked on a 1.5% agarose gel and visualized with ethidium bromide. After the densitometric analysis of three independent gels, the relative transcript amount was expressed as a percentage of control (*β-ACTIN*) that represents 100%.

Statistical analyses: Each treatment was analyzed with at least three replicates andstandard deviation (S.D.) was calculated. Statistical analysis was performed using the Student's t-test; $p<0.05$ and $p<0.01$ were con-sidered statistically significant and highly significant, respectively.

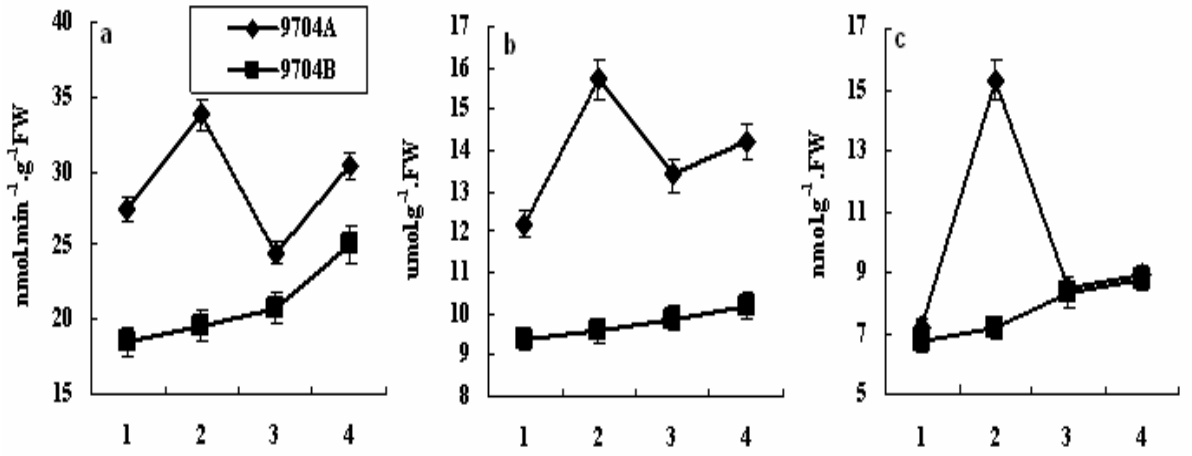

Fig. 1. Change of the generation rate of superoxide ($O_2^{\cdot-}$), contents of malondialdehyde (MDA) and content differences of hydrogen peroxide (H_2O_2) in the anther mitochondria of 9704A (CMS) and 9704B (maintainer) during different development stages. a: the generation rate of $O_2^{\cdot-}$; b: contents of MDA; content of H_2O_2. 1: sporogenous cell division stage; 2: pollen mother cell (PMC) meiosis stage; 3: uninucleate microspore stage; 4: mature pollen stage (the same below).

Table 1. Specific primers used for RT-PCR.

Gene	Primer sequence	T_a	GenBank accession number
APX	F: TCC TAT TAT GCT CCG TCT CG	57	DQ002888 AY078080
	R: AAC AGG TGG TTC TGG CTT G		
GPX	F: CTG ACT AAT TCA AAC TAC ACC GAC AT	60	AJ973135
	R: ACC ATC ACC AAA GAA CCC ACC T		
GR	F: ATG GAT GGG AAG TGA ATG AGA	57	AY547351
	R: TTA GAA TGT GCT TTG CCG AAT		
γ-GCS	F: GAT GTC TCA GGC AGG TTC TT	60	DQ444219
	R: ATC AAA TCG CTC GGC AAT AC		
β-ACTIN	F: TGC AGG AAT CCA CGA GAC TAC	57	DQ252512
	R: TAC CAC CAC TGA GCA CAA TGT T		

Results

Contents of Non-enzymatic antioxidants: The contents of ASA, DHA and total of ASA (ASA+DHA), were determined during the four stages of anther development in the CMS and maintainer lines (Fig. 2). Anthers of the CMS lines had a remarkably lower total of ASA contents compared to the maintainer lines at all four stages ($p<0.05$) (Fig. 2a). Among the four stages in the CMS lines, stage 2 revealed least total of ASA contents which are 33% less than that of the maintainer. Furthermore ASA contents in stage 1 were highest in CMS while was the lowest in maintainer lines at all four stages of anther development (Fig. 2b). ASA contents in the CMS line were the lowest of the four stages, at 29% and 19% more than that of the maintainer respectively in stage 2 and 3. The anthers of the CMS line had a noticeable lower DHA content than those of maintainer at all four stages (p 0.05) (Fig. 2c). The present results showed that the variation trends of ratio of ASA/ (ASA+DHA) were different inCMS lines and maintainer line at the four watched stages (Fig. 2d). The ratio of ASA/ (ASA+DHA) in CMS lines increased and then gently decreased, a peak occurred at stage 2. While the ratio in maintainer line firstly declined and then followed by a slight rise.

The abundance of GSH, GSSH and total of GSH (GSH+GSSH) in isolated mitochondria were analyzed (Fig. 3). Our results showed that the content of GSH reduced continuously in anthers of the CMS and maintainer lines in all four stages (Fig. 3a). CMS was only able to produce high GSH contents at 1st stage of anther development as compare to maintainer. During the four stages of anther development, content of GSSH increased in the first two stages but decreased in the last two stages in CMS lines, while in maintainer lines, content of GSSH at 2nd was the lowest and at 4th was the highest (Fig. 3b). At stage 2, GSSH content was the highest of the four stages in CMS lines, at 42% more than that of the maintainer liners. Total GSH content decreased at all stages in two different genotypes (Fig. 3c). The abundances of total GSH in CMS lines kept higher than those in maintainer lines at the first two stages, but opposite results were watched at the next two stages. The anthers of the CMS line had a noticeable lower the ratio of GSH/ (GSH+GSSH) than those of maintainer at stage 2 and 3 (Fig. 3d).

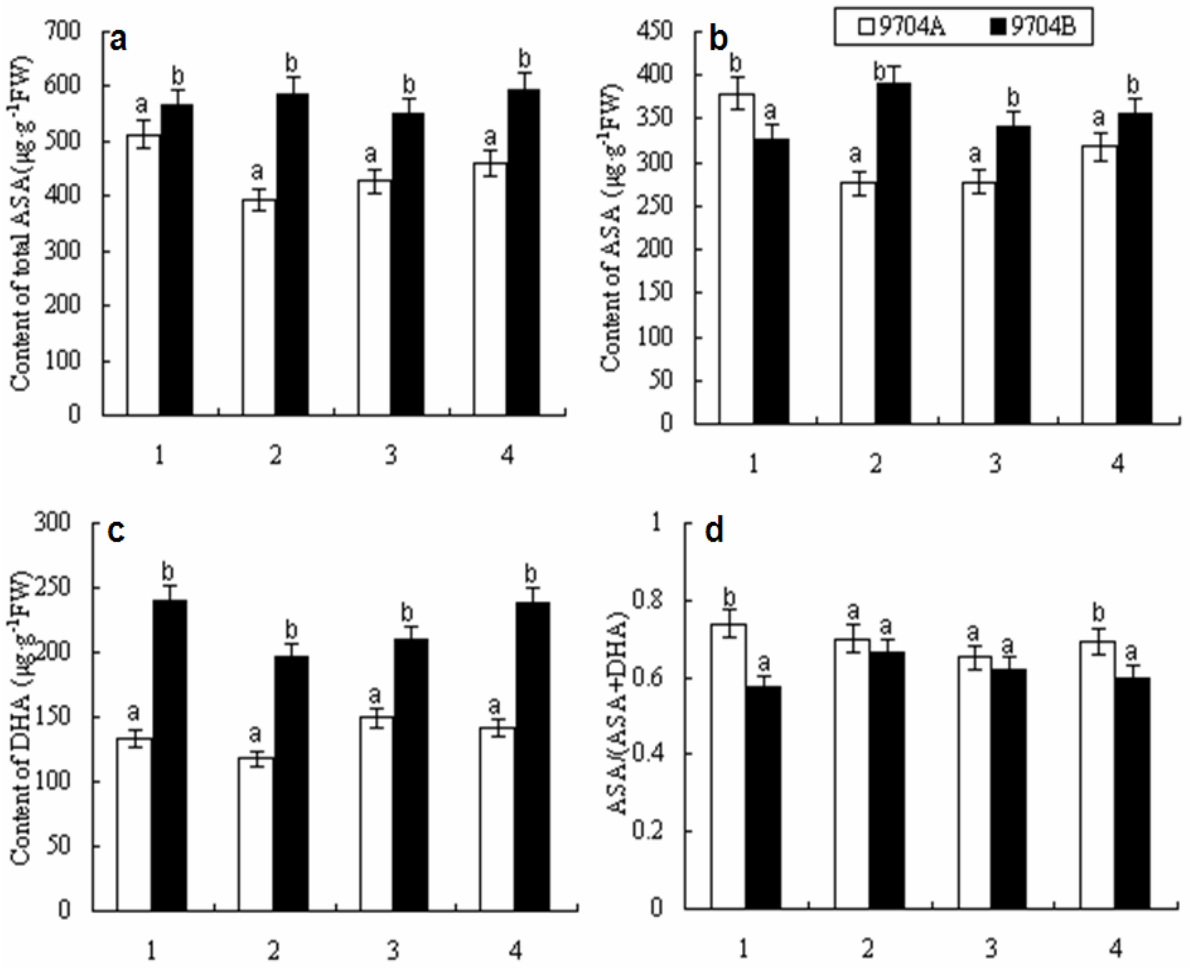

Fig. 2. Changes of total ascorbate (ASA+DHA), reduced ascorbate (ASA), oxidized ascorbate (DHA) and ASA/(ASA+DHA) ratio in the anther mitochondria of 9704A (CMS) and 9704B (maintainer) during different development stages. a: ASA+DHA; b: ASA; c: DHA; d: ASA/(ASA+DHA) ratio.

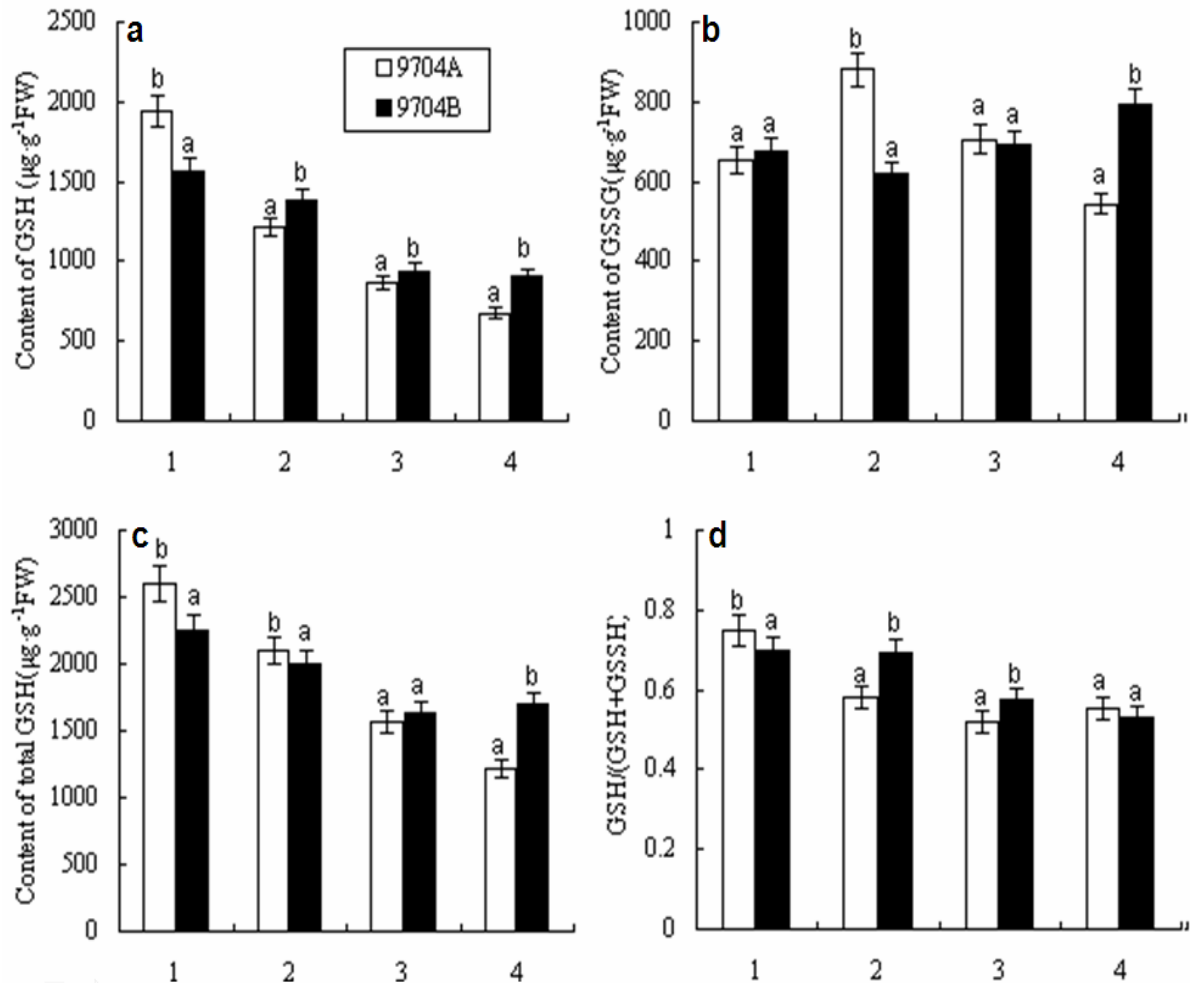

Fig. 3. Change of reduced glutathione (GSH), oxidized glutathione (GSSH), total glutathione (GSH+GSSH) and GSH/(GSH+GSSH) ratio in the anther mitochondria of 9704A (CMS) and 9704B (maintainer) during different development stages. a: GSH; b: GSSH; c: GSH+GSSH; d: GSH/(GSH+GSSH) ratio.

Activities of APX、GPX、GR and γ-GCS enzymes: There were differences in APX、GPX、GR and γ-GCS activities among the anthers of the CMS and maintainer lines (Fig. 4).

APX activities reduced continuously in anthers of the CMS in all four stages (Fig. 4a). In maintainer lines, reduced activities for APX were only noted at 1st stage of anther development while all other stages behaved alike (Fig. 4a). APX activity in the CMS line was significantly higher than that in maintainer line at stage 1 and lower than that in maintainer line at last two stages. There were no differences in APX activities in stage 2 between the two lines.

GPX activities continuously increased and dropped at 4th stage but still higher than the 1st and 2nd stages, while in CMS lines kept decreasing (Fig. 4b). At first two stages, GPX activities in CMS lines was significantly higher than maintainer line, but was noticeable lower than that in maintainer line at last two stages.

GR activities in maintainer's anthers reached a peak at 2nd stage and then declined gradually (Fig. 4c). The general trend for GR activities in CMS was a significant decrease in the early phase (1st and 2nd) and gradual increase in the late stage. GR activity in maintainer was a maximum value of 268% of CMS at 2nd stage.

At stage 3, γ-GCS activities was highest of the four stages in the CMS line, at 180% more than that of the maintainer (Fig. 4d). γ-GCS activities at stage 3 were lowest in the maintainer line. At stage 4, γ-GCS activities CMS was lower than that in the maintainer line. At first two stages γ-GCS activities of CMS and maintainer lines behaved similar.

Expression level of *APX*、*GPX*、*GR* and *γ-GCS* gene by Semi-quantitative PCR assays: To test the expression of the *APX*、*GPX*、*GR* and *γ-GCS* genes, primers were designed according to the cDNA sequences of the enzymes and quantitative RT-PCR was carried out. The amplification results showed that the expressions of *APX*、*GPX*、*GR* and *γ-GCS* were very similar to the activities of their related enzymes (Fig. 5).

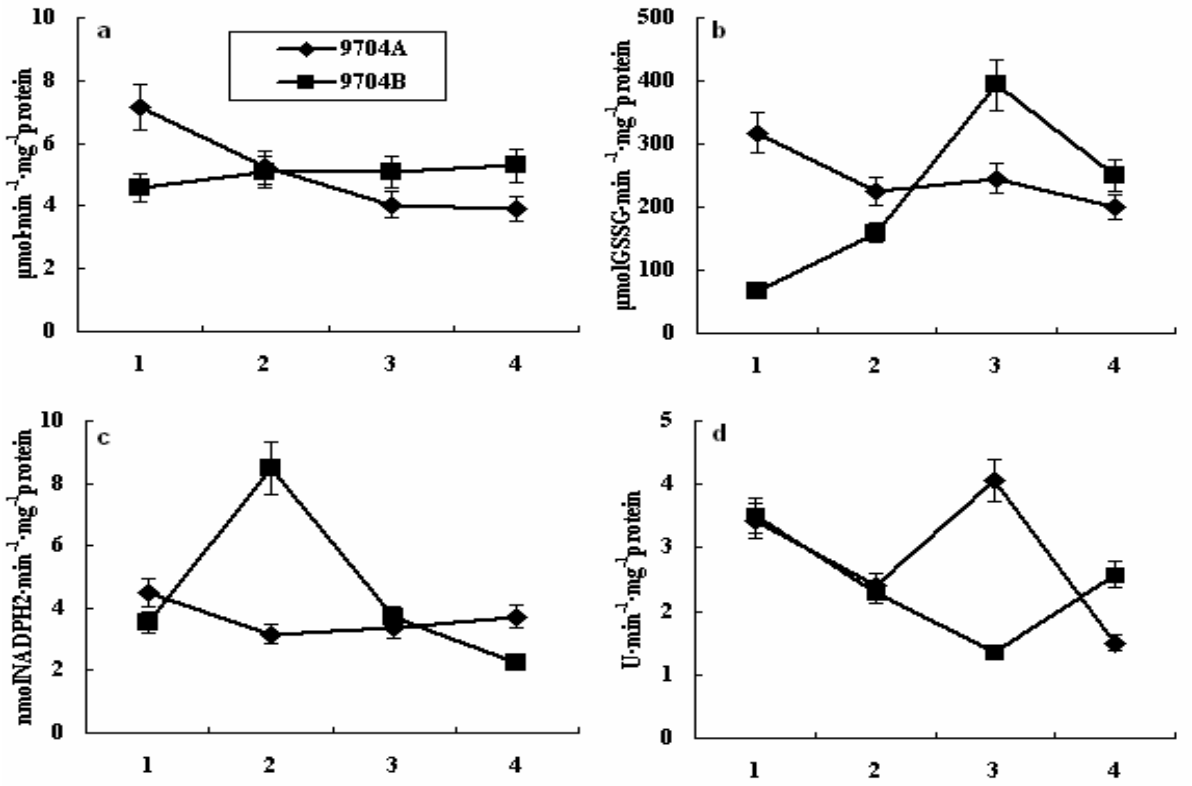

Fig. 4. Change of **a**ntioxidase activitiesin the anther mitochondria of 9704A (CMS) and 9704B (maintainer) during different development stages. a: ascorbate peroxidase (APX); b: glutathione peroxidase (GPX); c: glutathione reductase (GR); d: γ-Glutamylcysteine synthetase (γ-GCS).

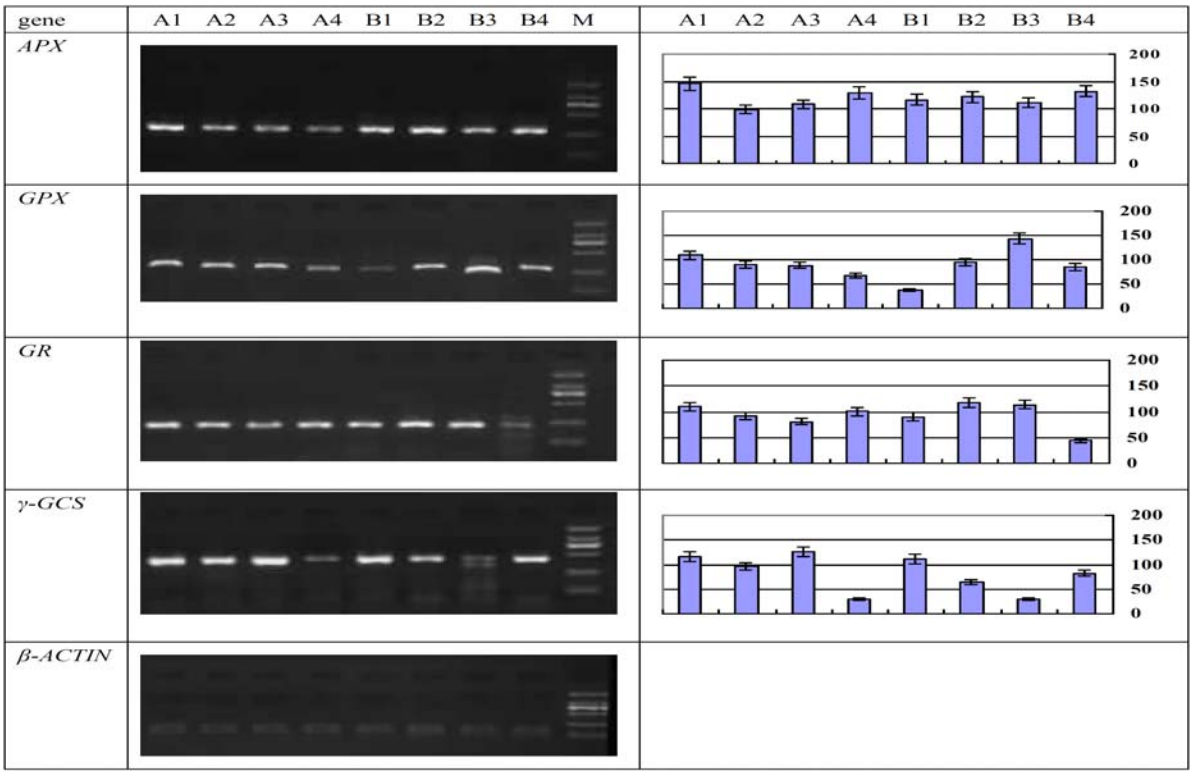

Fig. 5. Expression level of *APX*, *GPX*, *GR* and *β-ACTIN* gene in anthers of 9704A (CMS) and 9704B (maintainer) at different development stages by RT-PCR assays. A: CMS line (9704A); B: maintainer (9704B).

Discussion

It is well known that ROS production by the mitochondrial respiratory chain is a physiological and continuous process in aerobic organisms (Møller, 2001, Sweetlove & Foyer, 2004). ROS damage cellular macromolecules (Asada, 1996). To avoid the damage, it requires multiple mechanisms for keeping ROS accumulation under control by the operation of an antioxidant defense system that is comprised of enzymic (SOD, CAT, GR, APX) and nonenzymic (ascorbate, α-tocopherol, carotenoids, glutathione) components (Van Der Mescht *et al*., 1998, Shigeoka *et al*., 2002, Tewari *et al*., 2002). In our study, ROS contents in CMS 9704A showed a little increase compared with the maintainer during the anther abortion preliminary stage (Fig. 1), which is in accordance with the result of Li *et al*. (2004), Wan *et al*. (2007) and Jiang *et al*. (2007).

Subtle contents of ROS are essential to cell metabolism. However, they would cause a series of damage to cells, including proteins and nucleic acids, chlorophyll and membrane function when ROS generation exceeds normal levels despite the operation of these protective mechanisms (Forsmark-Andree *et al*., 1997, Kellogg and Fridovich, 1975, Helbock *et al*., 998, Li *et al*., 2004). Plants have developed a set of defense system to keep the balance of ROS (Møller, 2001). The small-molecule antioxidants, such as alpha-tocopherol, beta-carotene, ascorbate acid (ASA) and reduced glutathione (GSH) is an important part of the antioxidant defense system inside cells. amonth theses antioxidants, GSH and ASA are two of the most important reducing substrates for ROS detoxification (Mehlhorn *et al*., 1996). ASA can be effective to directly scavenge a wide array of ROS and free radicals such as superoxide, hydroxyl radicals single oxygen and reduce H_2O_2 to H_2O via ascorbate peroxidase reaction (Noctor & Foyer, 1998). GSH is the most important nonprotein thiol and a major antioxidant in plant cells. The central role of GSH in the antioxidative defense is due to its ability to regenerate another powerful antioxidant-ASA via the ascorbate- glutathione cycle.

In our study, ROS contents in CMS 9704A sharply increased with a large scope compared with the maintainer 9704B, which greatly exceeded the normal level during the abortion stage (in stage 2 and 3). The content of ASA and GSH in stage 2 and 3, the transcripts and protein activities of APX GPX and GR in stage 3 were too low to scavenge the toxicity of excessive ROS in sterile line, but they were significantly higher in the anthers of maintainer, which indicated that stable content of ASA, GSH, and stable transcripts of APX and GPX are beneficial to keep the ROS metabolism with a normal level.

Acknowledgements

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