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BRIEF COMMUNICATION

Role of antioxidative system during the development and senescence of cucumber fruit

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Abstract

The oxidative processes and antioxidative system in cucumber (*Cucumis sativus* L.) fruit were determined during development and senescence. Four distinct developmental stages could be delineated during fruit maturation: immature (3 - 8 d after anthesis, DAA), mature (9 - 16 DAA), breaker (17 - 22 DAA), and yellow (35 - 40 DAA). The electrolyte leakage, malondialdehyde content, superoxide anion production rate, and hydrogen peroxide content increased continuously during fruit development and senescence. Superoxide dismutase and peroxidase activities consistently increased during fruit maturation, and the catalase activity displayed a single peak at the mature stage. Ascorbate peroxidase and glutathione reductase activities declined during fruit development, but both were activated in yellow fruit. Monodehydroascorbate reductase activity declined and dehydroascorbate reductase (DHAR) activity increased during fruit growth. DHAR was repressed in yellow fruit. Ascorbate dramatically accumulated and its redox state increased, whereas glutathione was degraded and its redox state declined, with fruit maturation.

Additional key words: ascorbate, ascorbate peroxidase, catalase, glutathione, glutathione reductase, oxidative stress, peroxidase, redox state, superoxide dismutase.

The development and senescence of fruits is an irreversible, genetically programmed process involving a series of internal physiological reactions. Reactive oxygen species (ROS) seem to be involved in fruit ripening and senescence that contribute to a general deterioration of cellular metabolism (Huang *et al*. 2007). The antioxidant system likely plays a crucial role during fruit ripening and senescence, although different patterns have been reported in tomato (Jimenez *et al*. 2002), orange (Huang *et al*. 2007), and mango (Zhao *et al*. 2009). The balance between ROS biosynthesis and its removal varies during fruit development and senescence (Jimenez *et al*. 2002, Huang *et al*. 2007, Zhao *et al*. 2009).

The ripening and senescence of cucumber fruits are generally characterized by decreases in fruit surface hue angle and firmness, degradation of chlorophyll and β-carotene (Hurr *et al*. 2009). However, almost all cucumber fruits are harvested at an immature stage prior to achieving full fruit size and physiological maturity. The optimum harvest size and developmental stage of cucumber fruits vary and depend on cultivar and its intended use (*e.g*., pickling, fresh market selling, or slicing; Hurr *et al*. 2009). Limited information on the regulation of the development and senescence of cucumber fruits is available because of their preferred consumption at early developmental stages. The aim of the present work is to investigate the roles of ROS and the antioxidative system on the development and senescence of cucumber fruit.

A Chinese mini-cucumber (*Cucumis sativus* L. cv. Hangcui-1) was grown at the plastic greenhouse in the pilot farm of Zhejiang University. Based on fruit size and color, four distinct developmental stages were chosen:

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Abbreviations: APX - ascorbate peroxidase; ASC - ascorbate; CAT - catalase; DAA - days after anthesis; DHA - dehydroascorbate; DHAR - dehydroascorbate reductase; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; MDHAR - monodehydroascorbate reductase; POX - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.
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immature (3 - 8 days after anthesis; DAA), mature (9 - 16 DAA), breaker (17 - 22 DAA) and yellow (35 - 40 DAA). The immature stage is the phase when the cucumber fruits rapidly lengthen, but the fruit volumes do not increase significantly. Hangcui-1 cucumber is commercially harvested in this period. The mature stage is the period when fruit lengthening slows, radial expansion is rapid, and fruit volume significantly increases. The breaker fruits have an almost negligible growth rate until they finally attain full size. The fruit color at this stage is still light green. The yellow fruits exhibit no growth and their surface color gradually turn to yellow. Fruits corresponding four stages, free of any visible defects and infection, were simultaneously harvested. Mesocarp tissues of five fruits at each stage were immediately sampled and frozen in liquid nitrogen, and then stored at -80 °C for further analysis.

Cylinders of cucumber tissue were excised with a 10 mm diameter stainless steel cork borer. Two pieces of 4 mm thickness were cut from each cylinder for determining electrolyte leakage. Electrolyte leakage and malondialdehyde (MDA) content were measured as previously described (Mao *et al*. 2007). The production rate of superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) content were measured following the method of Wang and Luo (1990) and Patterson *et al*. (1984), respectively. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured following the method of Beyer and Fridovich (1987). Catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7) activity were measured according to the method described by Aebi (1984) and Hammerschmidt *et al*. (1982), respectively. Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined as described by Nakano and Asada (1987). Monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) activity was measured according to Hossain and Asada (1984). Dehydroascorbate reductase (DHAR; EC 1.8.5.1) activity was determined as described by Nakano and Asada (1981). Glutathione reductase (GR; EC 1.6.4.2) activity was determined according to Foyer and Halliwell (1976). Protein content was determined following the method of Bradford (1976). The ascorbate (ASC) content, ASC/oxidised ascorbate (DHA) ratio and glutathione (GSH) content, GSH/oxidized glutathione (GSSG) ratio were measured as described by Hernandez *et al*. (2010) and Wang *et al*. (2009), respectively.

Gene-specific primers were designed using *Vector NTI Suite 5.5* software to detect the *cytSOD, CAT, POX, APX*, *MDHAR, DHAR* and *GR* gene relative transcription during development and senescence of cucumber fruit. Primer sequences are shown in Table 1. 18S ribosomal RNA (18S rRNA) gene of *C. sativus* was used as a housekeeping gene (Zhao *et al*. 2010), and immature cucumber fruit was set as control. All operations were followed the method of Zhao *et al*. (2010).

The experimental design was completely randomized. The data were expressed as mean ± standard deviation (SD) for triplicate determinations. Data were subjected to *ANOVA*, and the differences between means were determined using Tukey's test calculated at $P < 0.05$. Statistical procedures were carried out with *SPSS 16.0* for *Windows* (*SPSS Inc*., Chicago, IL, USA).

Both electrolyte leakage and MDA content increased during fruit development and no significant difference ($P < 0.05$) was found during later periods (mature, breaker, and yellow). The production rate of $O_2^{\bullet-}$ and H_2O_2 content both increased significantly ($P < 0.05$) in yellow fruit (Table 2).

The SOD and POX activities consistently increased with the development of cucumber fruit, whereas CAT activity decreased significantly after achieving a peak in mature fruit (Table 2). Both APX and GR activities

Table 1. Primers of genes used for real time (RT)-PCR assay. [a] - GenBank accession number according to the National Center for Biotechnology Information (NCBI) database; [b] - according to reference Zhao *et al*. (2010).

GenBank ID[a]	Gene	Primer	Sequences 5' - 3'
EU407180.1	*Cyt-SOD*	sense	AAGCCCGGGCTTCATGGATTCC
		antisense	TGAACCACAACAGCCCTTCCGA
AY274258.1	*CAT*	sense	TCCACTGGAAACCAACTTGCGG
		antisense	ACCAATCTCCCCACCGGCTGTA
M91373.1	*POX*	sense	GCTGCTGCTAAGCTCATTCGCC
		antisense	CCCAAATTGGTCCTCCCGACAA
FJ890985.1	*APX*	sense	ACAGGACAAACCCGAGCCACCA
		antisense	CGTTCCTTGTGTGCCCTACCCA
DQ641068.1	*MDHAR*	sense	ATGGCAGATGAGACCTTCAAAT
		antisense	ACCAATCAGGAAGCAACCTCTC
EF468515.1	*DHAR*	sense	AATGGATCCCGGATTCTGATG
		antisense	TTTTGAGCCCACAGAGGCA
GU248528.1	*GR*	sense	ACACGTCGTCGTTCAATCCAA
		antisense	TGCGCCAAGGACCTTTTGT
AF206894	18S ribosomal RNA[b]	sense	GGCGGATGTTGCTTTAAGGA
		antisense	GTGGTGCCCTTCCGTCAAT

declined gradually before the fruit turned yellow, after which dramatic increases in activities were observed (Table 2). MDHAR activity declined with fruit development. However, DHAR activity increased rapidly up to the breaker stage (Table 2).

The ASC content increased with the development of the fruit. The highest content was found in yellow fruit (34.11, 15.45, and 2.29 times the corresponding values in immature, mature, and breaker fruits, respectively). The ASC/DHA ratio dramatically increased during the development of cucumber fruit. The GSH content and the GSH/GSSG ratio declined continuously with cucumber fruit development (Table 2).

All the assayed genes showed relatively high mRNA content in immature fruit (Table 3). All the genes had low mRNA levels in mature fruit, but were up-regulated during the succeeding fruit maturity stages. A dramatic increase in *POX* gene expression was observed in breaker and yellow fruits (approximately 7- and 18-fold of that in immature fruit, respectively; Table 3).

Increased electrolyte leakage and MDA content showed progressive loss in membrane integrity during the development of cucumber fruit, which accompanied the increase of ROS production (Table 2). These results confirmed that fruit ripening and senescence are coupled with enhancement in oxidative stress, which is similar to findings in previous reports on tomato fruit (Jimenez *et al*. 2002). ROS, especially H_2O_2, is commonly believed to be

Table 2. Electrolyte leakage [%], MDA content [nmol g^{-1}(f.m.)], $O_2^{\bullet -}$ production rate [nmol g^{-1}(f.m.) min^{-1}], H_2O_2 content [µmol g^{-1}(f.m.)], SOD [U mg^{-1}(protein)], CAT [nmol(H_2O_2) mg^{-1}(protein) min^{-1}], POX [nmol(guaiacol) mg^{-1}(protein) min^{-1}], APX [nmol(ASC) mg^{-1}(protein) min^{-1}], MDHAR [nmol(NADH) mg^{-1}(protein) min^{-1}], DHAR [nmol(DHA) mg^{-1}(protein) min^{-1}], and GR [nmol(NADPH) mg^{-1}(protein) min^{-1}] activities, ASC content [nmol g^{-1}(f.m.)], ASC/DHA ratio, GSH content [nmol g^{-1}(f.m.)], GSH/GSSG ratio ($\times 10^{-3}$) in cucumber fruit at four developmental stages. The values represent mean ± SD of 3 replicates derived from composite samples from 5 fruits each. Values followed by different letters in same row are statistically different according to Tukey's multiple range test at $P < 0.05$.

Developmental stage	Immature	Mature	Breaker	Yellow
Electrolyte leakage	16.14 ± 1.92b	19.05 ± 1.35ab	20.03 ± 1.62a	20.37 ± 0.52a
MDA content	15.57 ± 0.27b	23.35 ± 1.90a	23.78 ± 3.15a	22.06 ± 0.13a
$O_2^{\bullet -}$ production rate	0.23 ± 0.08b	0.23 ± 0.05b	0.33 ± 0.13ab	0.52 ± 0.12a
H_2O_2 content	1.18 ± 0.79b	1.58 ± 0.64b	1.97 ± 0.15ab	3.16 ± 0.39a
SOD	0.78 ± 0.02b	0.79 ± 0.02b	0.99 ± 0.09ab	1.15 ± 0.10a
CAT	1.18 ± 0.01b	1.32 ± 0.04a	0.76 ± 0.07c	0.42 ± 0.07d
POX	49.51 ± 0.90d	66.19 ± 1.60c	78.87 ± 2.13b	142.57 ± 4.32a
APX	336.64 ± 11.0a	234.48 ± 6.25b	150.08 ± 8.41c	245.85 ± 1.49b
MDHAR	12.65 ± 0.93a	11.77 ± 0.32a	8.78 ± 0.40b	7.42 ± 0.40b
DHAR	1.15 ± 0.15d	2.18 ± 0.27b	4.61 ± 0.21a	1.64 ± 0.21c
GR	1.19 ± 0.03b	0.82 ± 0.03c	0.67 ± 0.22c	1.79 ± 0.01a
ASC content	1.49 ± 0.26c	3.29 ± 1.08c	22.17 ± 1.85b	50.83 ± 2.10a
ASC/DHA	0.02 ± 0.01b	0.20 ± 0.05b	1.02 ± 0.08b	7.66 ± 2.05a
GSH content	8.21 ± 0.18a	7.08 ± 0.26b	6.59 ± 0.40bc	6.35 ± 0.04c
GSH/GSSG	2.92 ± 0.10a	2.71 ± 0.09ab	2.63 ± 0.14b	2.19 ± 0.04c

Table 3. Relative transcription levels of genes coding antioxidative enzymes in cucumber fruit at four developmental stages. The gene transcription values of immature cucumber fruits are presented as 1. The values represent mean ± SD of 3 replicates derived from composite samples from 5 fruits each. The values followed by different letters in same row are statistically different according to Tukey's multiple range test at $P < 0.05$.

Developmental stage	Immature	Mature	Breaker	Yellow
cytSOD	1.00 ± 0.08b	0.88 ± 0.02c	1.10 ± 0.01b	2.04 ± 0.01a
CAT	1.00 ± 0.19b	0.26 ± 0.02c	0.48 ± 0.03c	1.55 ± 0.05a
POX	1.00 ± 0.10c	0.62 ± 0.10c	7.22 ± 0.53b	18.35 ± 2.28a
APX	1.00 ± 0.03c	0.60 ± 0.01d	1.33 ± 0.04b	2.14 ± 0.08a
MDHAR	1.00 ± 0.02a	0.44 ± 0.08c	0.65 ± 0.02b	0.93 ± 0.06a
DHAR	1.00 ± 0.02a	0.26 ± 0.03b	0.20 ± 0.01b	0.80 ± 0.36a
GR	1.00 ± 0.07a	0.42 ± 0.04c	0.57 ± 0.01b	0.96 ± 0.05a

detrimental to plants, but recent evidence unequivocally points to the signaling function of ROS during plant development (Schopfer 2001). ROS production is believed to be programmed, and is an essential process in plant development (Schopfer 2001).

It has been reported that SOD, CAT, POX, and APX protect fruits from oxidative damage (Huang *et al*. 2007, Wang *et al*. 2009). In the present study, the increase in SOD (one H_2O_2-generating enzyme) activity and the decrease in the activities of CAT and APX (two main H_2O_2-scavenging enzymes) may cause the accumulation of H_2O_2 during cucumber development and senescence (Table 2). POX oxidize indolacetic acid causes the change in ratio of auxin/cytokinin, which is important for plant growth and development (Romano *et al*. 1991), participate in lignification during plant growth (Pomar *et al*. 2002), and have broad substrates for ROS detoxification (Kvaratskhelia *et al*. 1997). Increased POX activity and *POX* gene expression may be critical for the regulation of auxin and cell wall metabolism during the development and senescence of cucumber fruits.

During cucumber fruit development, MDHAR is less effective and DHAR is more efficient in ASC regeneration. The decline in GR activity reveals less GSH regeneration, and may cause decreases in GSH content during fruit growth (immature, mature and breaker stages; Table 2). The decline in GSH content, GSH/GSSG ratio and increase in ASC content, ASC/DHA ratio during fruit maturation suggest that GSH is important for cell division during the formation of cucumber fruits (Díaz-Vivancos *et al*. 2010), and ASC is critical for cell expansion during their development and senescence (Díaz-Vivancos *et al*. 2010).

The gene transcription levels of antioxidative enzymes could reflect genetic regulations toward oxidative stress. The relatively high mRNA levels of *cytSOD*, *CAT*, *POX,* and the enzymes in the ASC-GSH cycle in immature fruit could guarantee great potential for the enzymatic scavenging of ROS and may be the genetic control of ROS (Foyer and Noctor 2011). Decreased mRNA levels in mature fruit imply a decline in antioxidant capacity. All seven genes might be up-regulated by ROS accumulation during later developmental stages (breaker and yellow; Table 3). The obvious discrepancy between the mRNA levels and the activities of *CAT*, *APX*, *MDHAR* and *DHAR* (Tables 2 and 3) implies that the expressions of these genes are regulated by other factors. In view of these results, the increase in ROS content during cucumber fruit maturation appears to be programmed at the transcription level.

In conclusion, increased oxidative stress plays an important role in triggering the senescence of cucumber, and the change in antioxidative system regulates the balance between ROS biosynthesis and its removal during cucumber fruit maturation. Significant increases in *POX* gene expression and corresponding enzyme activity, and ASC content are conspicuous features that characterize the development and senescence of cucumber fruits, reflecting the critical roles of POX and ASC in the regulation of cucumber fruit maturation.

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