ATP-regulation of antioxidant properties and phenolics in litchi fruit during browning and pathogen infection process

Chun Yi a, b, Yueming Jia ng a, John Shi b, *, Hongxia Qu a, * c, Sophia Xue b, Xuewu Duan a, Jingyu Shi a, Nagendra K. Prasad a

a South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, PR China
b Food Research Center, Agriculture and Agri-Food Canada, Guelph, Ont., Canada N1G 5C9

c* Corresponding authors. Tel.: +1 519 780 8035; fax: +1 519 829 2600 (J. Shi).
E-mail addresses: shij@agri.gc.ca (J. Shi), q-hxia@scib.ac.cn (H. Qu).

1. Introduction

Adenosine triphosphate (ATP) serves as the ‘energy currency of the cell’ and is determinant of cell function and viability (Pradet & Raymond, 1983). Plant energy metabolism is considered as a mature field, but several important observations in plants initiated a research renaissance in ATP (both cellular and extracellular) in the last decade (Jiang, Jiang, Qu, Duan, & Jiang, 2007). This includes: (a) an ATP threshold existing for membrane lipid synthesis (Rawyler, Pavelic, Gianinazzi, Oberson, & Braendle, 1999); (b) effective preservative ways of horticultural crops closely associated with elevated ATP levels (Duan et al., 2004; Saquet, Streif, & Bangerth, 2001), energy deficiency, fruit browning and decay (Saquet, Streif, & Bangerth, 2001; Yi et al., 2008); and (c) extracellular nucleotides (eATP, eADP and hydrolyzable analogues of them) that function as regulatory agents in plant signal transduction (Roux, Song, & Jeter, 2006).

Mitochondrial oxidative phosphorylation is not only the main pathway of ATP production for eukaryotic cell, but also the major target of oxidative stress, mediated by reactive oxygen species (ROS), including superoxide anion (O2•−), hydrogen peroxide (H2O2), hydroxyl (HO•), peroxyl (ROO•) and alkoxyl (RO•) (Blokmina, Virolainen, & Fagerstedt, 2003; Fleury, Mignotte, & Vayssiere, 2002). Such ROS, also termed active oxygen species, can disrupt cellular membranes, induce oxidative changes in DNA, and disturb cellular metabolism (Yu, 1994).

The accumulation of ROS could be prevented by two defence systems: non-enzymatic antioxidant system containing low molecular weight antioxidants (ascorbic acid, glutathione, tocopherols) and ROS-interacting enzymatic system comprising enzymes such as superoxide dismutases (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX). Oxidative stress occurs due to the imbalance between ROS and ROS-scavenging compounds and enzymes. During senescence and stress conditions, plants cells could sense lethal dose of ROS and subsequently activate several defence enzymes and synthesise compounds as adaptional responses to scavenge ROS amount within a safe level. The processes for enzyme activation and synthesis of low molecular weight antioxidants require energy. Veltman, Lenthéric, Van der Plas, and Peppelenbos (2003) showed that energy production and ATP levels in tissues of pears play a role in regulating/inducing antioxidant enzymes, including SOD, CAT, and APX. Saquet et al. (2000) demonstrated that ATP played a vital role in the synthesis of ROS scavenging enzymes and non-enzyme scavengers in the ascorbate-gluthatione cycle. Energy depletion may result in a more dramatic oxidative stress, and consequently, damage the cell. Franck et al. (2007) suggested that membrane dysfunction, an early
response to senescence and stresses, is characterised by shortage of ATP and an increase in ROS production.

Browning is a common symptom during senescence or injury in fruit, resulting from breakdown of cellular compartments and consequently reaction between polyphenol oxidase (PPO) and its substrates (phenolic compounds) (Jiang, 2000). During storage, excess ROS generation accelerates the oxidation of phenolic compounds, resulting in decreased contents of phenolics and pericarp browning (Underhill & Critchley, 1994; Zhang, Quantick, & Grigor, 2000).

The infection by pathogens increases the amount of ROS produced during fruit storage, the so-called “oxidative burst” in response to pathogen attack (Bolwell & Wojtaszek, 1997). The oxidative burst is crucial for the disease resistance in higher plants, whilst it may result in peroxidative destruction of cellular constituents if they are not timely averted. Thus, the intracellular ROS level has to be kept under tight control to prevent cell damage (Bartoli, Simontacchi, Montaldi, & Puntarulo, 1996).

Litchi (Litchi chinensis Sonn.) is a subtropical/tropical fruit of high commercial value in the international trade. Harvested litchi fruits are highly perishable due to water loss, pericarp browning and disease development. The objectives of present work were to measure antioxidant enzymes activities, scavenging activities and phenolic compounds contents to test whether exogenous ATP supply will regulate the balance between the ROS and its defence system in litchi fruit during browning and pathogen infection process.

2. Materials and methods

2.1. Sample preparation

Litchi fruits were harvested when commercially matured from a local orchard (Guangzhou, China). They were washed in water containing 0.1% thiram/dazol, and then infiltrated under vacuum with sterile distilled water (SDW) as control, 1.0 mM ATP and 0.5 mM DNP solution under 75 kPa for 3 min, separately. Peronophythora litchii was isolated from infected litchi fruits and cultured on potato dextrose agar medium at 28 °C for 4 days. Half of the litchi fruits treated above were inoculated as previously reported by Yi et al. (2008). The fruits were kept in covered plastic boxes (5 × 15 × 4 cm) and stored at 25 °C (85–90% RH). Twenty fruits from different treatments were randomly sampled every 2 days of storage until the end of storage time (0, 2, 4, 6 days for inoculated fruits; 0, 2, 4, 6, 7 days for non-inoculated fruits). Pericarp tissue were immediately frozen in liquid nitrogen and stored at −70 °C until analysis (less than 3 months).

2.2. Determination of SOD, CAT, APX activities

Litchi pericarp tissue (2 g) was homogenised in 10 mL 0.05 M potassium phosphate buffer (pH 7.8) for SOD activity, 0.1 M potassium phosphate buffer (pH 7.0) for CAT and APX activities. The homogenate was filtered through two layers of miracloth and centrifuged at 20,000 g for 15 min at 4 °C. The resulting supernatants were collected for the enzyme assays described below. Protein content was determined according to the Bradford (1976) method with bovine serum albumin as standard.

SOD activity was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) using the method of Beyer and Fridovich (1987). One unit of SOD activity was defined as the amount of enzyme required to effect 50% inhibition of the reduction of NBT per mg protein as monitored at 560 nm.

CAT activity was assayed by measuring the disappearance of H₂O₂ according to Aebi (1984). The assay mixture (3 mL) contained 2.95 mL phosphate buffer (0.05 M, pH 7.0), 15 mM H₂O₂ and 50 μL of extract. CAT activity was usually calculated by a decrease in absorbance at 240 nm and was expressed as U per mg protein.

APX activity was measured following the oxidation of ascorbic acid at 290 nm (extinction coefficient 2.8 mM⁻¹ cm⁻¹) according to Nakano and Asada (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM sodium ascorbate, 0.1 mM H₂O₂, 0.5 mM ethylene diamine tetraacetic acid and 1 mL of extract. APX activity was calculated by the change of absorbance per minute per mg protein.

2.3. Determination of Scavenging activities of DPPH radicals and reducing power

Litchi pericarp tissue (2 g) was ground in liquid nitrogen with a mortar and pestle and homogenised in 10 mL methanol by stirring for 30 min at room temperature. The homogenate was filtered through two layers of miracloth and extracted again. Combined extract were stored at −20 °C until analysis.

DPPH radical scavenging activity was measured according to Shimada, Fujikawa, Yahara, and Nakamura (1992) with slight modification. The reaction mixture contained 0.1 mL of sample extract and 2.9 mL of 0.1 mM DPPH methanol solution. The absorbance at 517 nm was measured after 30 min of incubation at 25 °C. The inhibition of DPPH radicals by samples was calculated according to the following equation: DPPH scavenging activity (%)(1 – absorbance of sample/absorbance of control) × 100.

Reducing power was measured at 700 nm according to Duan, Jiang, Su, Zhang, and Shi (2007). A control, devoid of any hydrolysates and a blank, containing only hydrolysates samples, was used because proteins also absorb at the same wavelength. Increased absorbance of the reaction mixture indicated increased reducing power.

2.4. Determination of total phenolic compounds

Litchi pericarp tissue (5 g) from 12 fruits was extracted three times in 100 mL methanol containing 0.1 M HCl at 25 °C for 2 h. The extract solutions were filtered and combined for total phenolic determination. Phenolic content of the extract was determined by a modification of the Prussian blue assay of Price and Buttlerr (1977). The extracts (50 μL) was diluted with 3 mL distilled water, and then 100 μL of 50 mM FeCl₃ in 0.1 M HCl plus 100 μL of 8 mM K₃Fe(CN)₆ added to the solution, which was incubated for 20 min. Total phenolic compounds were determined by measuring the absorbance at 720 nm on a spectrophotometer with gallic acid as standard.

2.5. Statistical analysis

Significant differences were tested by one-way analysis of variance (ANOVA) (SPSS, version 10.0). Each experiment was repeated 2–4 times.

3. Results

3.1. Effects of ATP and DNP on SOD, CAT and APX in litchi fruits

As shown in Fig. 1A and B, both the activities of SOD and CAT in control fruits slightly fluctuated in the first 4 days of storage and then continuously decreased to lower values as compared that of 0 day. Significant enhancements of both SOD and CAT activities were observed after fruits were treated with ATP (P < 0.05). Different with those in control fruits, the activities of SOD and CAT in ATP-treated fruits increased 23% and 45% in the first 4 days of storage, respectively. DNP-treated fruits showed much lower SOD and CAT activities than both control and ATP-treated fruits. SOD activity started to decrease after 2 days of storage and CAT activity began to decline from the beginning of the storage. Moreover, no signifi-
cant difference of CAT activity between the control and DNP-treated fruits was observed in the last 3 days of storage. APX activity showed distinct change pattern with activities of other two enzymes. The activity increase from 0.75 to 1.09 U mg\(^{-1}\) protein as storage time proceeded and declined at the end of storage (Fig. 1C). ATP treatment markedly enhanced the APX activity, whilst DNP treatment lowered the activity.

The levels of SOD, CAT and APX activities in \textit{P. litchi}-inoculated fruits were higher than those of non-inoculated fruits throughout the storage period (Fig. 1D, E, and F). Compared with non-inoculated fruits, inoculated fruits showed different trends in both SOD and CAT activities. SOD and CAT activities increased in the first 2 days of storage and then sharply decreased in the last 4 days of storage. The decrease in APX activity started from the 4 day of storage, which is 2 days earlier than that in the control and ATP-treated fruits. Likewise, higher activities of SOD, CAT and APX were found in ATP-treated fruit throughout the storage time, whilst lower activities were found in DNP-treated fruits.

### 3.2. Effect of ATP and DNP on DPPH scavenging ability and reducing power

Fig. 2 shows the effects of ATP and DNP treatment on DPPH radical scavenging ability and reducing power of litchi pericarp. In non-inoculated fruits, the scavenging activity of DPPH radicals gradually decreased as the storage time progressed. ATP treatment significantly reduced the activity of DPPH radicals (Fig. 2A). The DPPH scavenging ability decreased in DNP treated fruit. The change trends differed when the fruits were inoculated with \textit{P. litchii}. DPPH radical scavenging ability in \textit{P. litchii}-inoculated fruits was higher than those in inoculated fruits. It slightly increased from 100.1% to 105.2% in control fruits and significantly increased from 100.1% to 113.2% in the ATP-treated fruits in the first 2 days of storage, and then gradually declined (Fig. 2C). Also, remarkable enhancement of DPPH scavenging ability was observed in ATP-treated fruits and significantly lower scavenging ability was observed in DNP-treated fruits. The scavenging ability was in the order of ATP-treated \textit{P. litchii}-inoculated fruit > \textit{P. litchii}-inoculated fruit (control) > DNP-treated \textit{P. litchii}-inoculated fruit > \textit{P. litchii}-inoculated non-inoculated fruit > ATP-treated non-inoculated fruit > DNP-treated non-inoculated fruit, throughout the storage period.

For both the non-inoculated and \textit{P. litchii}-inoculated fruits, reducing powers of litchi pericarp extracts rapidly decreased from 1.6 to around 1.2 mg mL\(^{-1}\) (Fig. 2B and D). It was shown that the reducing power of ATP-treated fruits was higher than that in the control and DNP-treated fruits. After 2 day of storage, reducing powers in inoculated fruits were lower than those in non-inoculated fruits, suggesting stronger decline of antioxidant activity in infected tissues during storage.

Fig. 1. Effects of ATP and DNP on SOD (A, D), CAT (B, E), APX (C, F) activities in non-inoculated (A, B, C) and \textit{P. litchii}-inoculated (D, E, F) litchi fruits during storage. Each value is presented as mean ± standard error (\(n = 3\)). The vertical bars indicate standard errors where they exceeded the symbol size. (○, ATP; ●, Control; ▼, DNP).
3.3. Effect on the content of phenolic compounds

Contents of phenolic compounds of litchi fruit pericarp with different treatments are shown in Fig. 3. In the control fruits, the total phenolic contents decreased from 3.21 to 1.87 mg g\(^{-1}\) fresh weight (FW) in non-inoculated fruits and from 3.21 to 2.32 mg g\(^{-1}\) FW in \textit{P. litchii}-inoculated fruits, respectively. Higher contents of total phenolic compounds in inoculated fruits were detected than non-inoculated fruits, even at the end of storage. There is a slight increase in all fruits after 2 days of inoculation. Treatment with ATP elevated the phenolic compounds contents of litchi fruit pericarp. At the second day of storage, the contents in ATP-treated fruits were 29% higher in non-inoculated fruits and 32% higher in \textit{P. litchii}-inoculated fruits than those of DNP-treated fruits, respectively.

A correlation between the antioxidant activity in the DPPH assay and the contents of phenolic compounds were established (Fig. 4). As shown, there is a linear relationship between them with correlation coefficients of \(r^2 = 0.63\). The increase of phenolic compounds contents will result an increase in DPPH scavenging ability.

4. Discussion

The data confirm that the ability of defence system of ROS gradually decrease during browning and pathogen infection process. According to previous results (Yi et al., 2008), ATP-treated fruits clearly exhibit less browning and disease symptom. This study shows the effects of ATP and DNP on the anti-oxidation system during browning or loss of disease resistance process.

Senescence and/or browning were caused by overproduction of ROS. The enzymes are involved in scavenging superoxide radicals, thus they could protects cells from oxidative stress (Buchanan-Wollaston et al., 2003). SOD converts two superoxide anions into a molecule of hydrogen peroxide and one of oxygen, and CAT could convert hydrogen peroxide to water and oxygen (Boonsiri, Ketsa, & van Doorn, 2007). APX prevents the accumulation of ROS by the ascorbate-gluthatione cycle (Asada, 1994). The results showed that all enzymes activities were higher in ATP-treated fruits and lower in DNP-treated fruits throughout the storage. The increased activities of SOD, CAT, and APX in ATP-treated fruits could contribute to
activity (Bloknina et al., 2003). The reducing power might also be regulated by the balance between ROS generating and its enzymatic defence system, which could reduce the accumulation of ROS and prevent the abnormalities in cellular metabolism (Franck et al., 2007).

Free radical-scavenging is another known system for inhibiting lipid peroxidation by antioxidants. DPPH radical scavenging activity is one of the known methods used for evaluating antioxidant activity (Bloknina et al., 2003). The reducing power might also serve as a significant reflection of the antioxidant activity (Chang, Yen, Huang, & Duh, 2002). The noticeable elevated level of radical scavenging ability by ATP supply indicated that ATP might be involved in the synthesis of antioxidants, whilst decreased levels of radical scavenging ability in DNP-treated fruits corresponded to the accelerated browning process. Related to the energy levels, previous examined, energy levels are positively correlated with the free radical scavenging ability. The energy level in litchi pericarp tissue could regulate the non-enzymatic ROS defense system.

Phenolic compounds are effective antioxidants in plants (Jiang, 2000), which are demonstrated by the correlation between the antioxidant activity in the DPPH assay and the contents of total phenolic compounds. The results suggested that sufficient ATP level was essential for the synthesis of phenolic compounds, and then consequently, enhance the antioxidant activity of litchi pericarp.

The results showed higher enzyme activities, higher free radical scavenging ability, and higher phenolic compounds after pathogen attack. The increased protection by enzyme activities could contribute to responses of ROS to the pathogen stress and defence pathways (Desikin, Mackerness, Hancock, & Neill, 2001). The induction of APX activity in response to stress has been reported by Garcia-Limones, Hervas, Navas-Cortes, Jimenez-Diaz, and Tena (2002). A previous study suggested two-stage behaviours with respect to pathogen invasion in litchi fruits (Yi et al., 2008). The first stage was characterised by increased ATP levels and better preserved membrane (0–4 days for non-inoculated fruits and 0–2 days for inoculated fruits), whilst the second stage was characterised by reductions in ATP contents and accumulation of lipid peroxide product (Yi et al., 2008). The present results are consistent with the two-stage behaviours as the enzyme activities remained constant or increased during the first stage and decreased during the second stage.

Moreover, phenolic compounds played vital roles in pathogen resistance (Nicholson & Hammerschmidt, 1992). Obviously, higher content and slower decrease trend of total phenolic in inoculated fruits suggested the induction of phenolic compounds synthesis under pathogen attack. A higher level of ATP may provide an advantage for phenolic compounds synthesis of rapid disease resistance in response to pathogen attack.

5. Conclusions

A fine balance between the maintenance of ATP levels and the confinement of ROS production within the amount of signal requirement is essential for inhibiting browning and loss of disease resistance. Exogenous ATP supply provides sufficient available energy to maintain or enhance the antioxidant systems and the synthesis ability of phenolic compounds, and consequently, to inhibit the accumulation of ROS and maintain the integrity of membrane. In other words, energy levels could balance the ROS production and antioxidant system through enhancing both the enzymes activities and free radical scavenging ability.

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References


