Reactive oxygen species involved in regulating fruit senescence and fungal pathogenicity

Shiping Tian · Guozheng Qin · Boqiang Li

Abstract Senescence is a vital aspect of fruit life cycles, and directly affects fruit quality and resistance to pathogens. Reactive oxygen species (ROS), as the primary mediators of oxidative damage in plants, are involved in senescence. Mitochondria are the main ROS and free radical source. Oxidative damage to mitochondrial proteins caused by ROS is implicated in the process of senescence, and a number of senescence-related disorders in a variety of organisms. However, the specific sites of ROS generation in mitochondria remain largely unknown. Recent discoveries have ascertained that fruit senescence is greatly related to ROS and incidental oxidative damage of mitochondrial protein. Special mitochondrial proteins involved in fruit senescence have been identified as the targets of ROS. We focus in discussion on our recent advances in exploring the mechanisms of how ROS regulate fruit senescence and fungal pathogenicity.

Keywords Fruit senescence · Fungal pathogenicity · Mitochondrial protein · Oxidative damage · Molecular target

Introduction

Fruit senescence is a developmentally programmed degeneration process and regulated by the various internal and external factors such as genetic factors, developmental signals, hormones, light and temperature (Adams-Phillips et al.

2004; Giovannoni 2004; Vrebalov et al. 2009; Karlova et al. 2011; Klee and Giovannoni 2011; Qin et al. 2012). Senescence greatly impacts fruit postharvest quality and resistance to pathogen attack and environmental stress (Tian et al. 2004). In general, senescent fruit are easily attacked by fungal pathogens and the diseases caused by fungal pathogens can greatly accelerate fruit senescence after harvest (Tian et al. 2007). It has been well known that ethylene plays a crucial role in regulating climacteric fruit ripening and senescence (Picton et al. 1993; Alba et al. 2005; Lee et al. 2012), whereas non-climacteric fruits do not require increased ethylene biosynthesis in the ripening and senescence process (Alexander and Grierson 2002; Causier et al. 2002). Certain common mechanism shared by all types of fruit may exist as the regulator of fruit senescence.

ROS contribute to aging and diseases causing lipid oxidation, protein oxidation, DNA strand break and base modification, and modulation of gene expression (Ames et al. 1993; Simon et al. 2000; Stadtman 2000; Spiteller 2001). Since mitochondria are the primary site of generation of ROS such as superoxide anion ($O_2{}^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals, and singlet oxygen (Turrens 2003), ROS accumulation can cause oxidative damage to mitochondrial proteins, resulting in dysfunction of various mitochondrial components and finally accelerating aging (Chan 2006). The important role of ROS in fruit senescence and fungal pathogenic ability has been recognized (Brennan and Frenkel 1977; Lacan and Baccou 1998; Rogiers et al. 1998; Jimenez et al. 2002; Tian et al. 2006; Chan et al. 2007; Qin et al. 2009b). Characterization of ROS roles that regulate fruit senescence is also crucial to understand the biology underlying the senescence phenomenon itself and the plant life cycle, and exploring the molecular and biochemical basis of fruit senescence and defense response to pathogen attack.

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The role of ROS in regulating fruit senescence

Previous studies indicate that the steady-state amounts of the products of oxygen free radicals that attack macromolecules tend to increase with age, especially in long-lived, post-mitotic cells (Yan and Sohal 1998). Oxidative damage caused by intracellular ROS to biological macromolecules, including proteins, DNA, and lipids, contributes to the irreversible, deleterious changes of biological systems (Genova et al. 2004; Kujoth et al. 2005). The generation of ROS and the degree to which they cause oxidative damage play important roles in the progression of senescence and various senescence-associated disorders (Stadtman 1992). A variety of studies have shown that ROS, as highly reactive molecules, are primarily generated by mitochondria, the complex organelles within the eukaryotic cell that play critical roles in multiple cellular processes, such as adenosine triphosphate (ATP) synthesis, β-oxidation, calcium homeostasis, apoptosis and cell signaling (Brown 1992; Møller 2001; Turrens 2003). Fruit ripening, as an oxidative phenomenon, requires the removal of ROS such as H_2O_2, and there is a balance between the production of ROS and their removal by antioxidant systems (Jimenez et al. 2002). In general, during ripening or senescence, antioxidant enzymes eliminate O_2^- and H_2O_2 and thus inhibit the rate of postharvest senescence (Lacan and Baccou 1998; Lester 2003).

The accumulation of ROS accelerating fruit senescence

Fruit senescence is an oxidative phenomenon accompanied by a pronounced increase in ROS, particularly H_2O_2 and O_2^- accumulation (Brennan and Frenkel 1977; Frenkel and Eskin 1977; Warm and Laties 1982). Recent results indicate that H_2O_2 contents are greatly enhanced in senescing peach fruit (Qin et al. 2009b), muskmelon (Lacan and Baccou 1998) and tomato (Jimenez et al. 2002). Since manganese superoxide dismutase (MnSOD) scavenges O_2^- produced in the mitochondria, decreasing catalytic activity of MnSOD usually results in the accumulation of O_2^-. MnSOD activity in senescing apple fruit was higher than that of young fruit, leading to higher levels of O_2^- (Qin et al. 2009a). However, lower ROS content by lowering oxygen concentration (2–5 %) in storage environment can effectively retard fruit senescence, whereas treatment of fruit with H_2O_2 enhances senescence (Tian et al. 2004; Qin et al. 2007). High oxygen treatment can increase the production of H_2O_2 in mitochondria (Turrens et al. 1982) and affect the activity of antioxidant enzymes in fruit and accelerate fruit senescence (Wang et al. 2005). These results demonstrate that oxidative stress causes ROS accumulation and is implicated in the process of fruit senescence.

Mitochondrial proteins in response to fruit senescence

Mitochondria are ubiquitous organelles within eukaryotic cells that perform a variety of biochemical functions (Chan 2006). As the center of energy metabolism in cell, almost all oxidative and reductive reactions are carried out in mitochondria (Brown 1992). The primary biochemical functions of mitochondria are the oxidation of organic acids through the tricarboxylic acid (TCA) cycle and the synthesis of ATP to meet cellular energy demands (Heazlewood et al. 2004). Mitochondria consist of an outer membrane, inner membrane and inter-membrane space. The inner mitochondrial membrane is the major intracellular site of generation of ROS (Yan and Sohal 1998). Oxidative modification of mitochondria components have been implicated in various pathophysiological states associated with oxidative stress and senescent changes (Kraytsberg et al. 2006; Toroser et al. 2007).

Proteomics has been a powerful tool for screening the specific proteins that are differentially expressed in response to various stresses in the last few years (Ali and Komatsu 2006; Qin et al. 2007; Aghaei et al. 2008). Changes in proteins in response to fruit ripening and resistance have been identified based on redox proteomics, including antioxidant proteins and pathogenesis related-proteins (Chan et al. 2007), heat shock proteins and dehydrogenases (Chan et al. 2008), as well as 1-aminocyclopropane-1-carboxylic acid synthase, CBS domain-containing protein and alcohol dehydrogenase 1 (Wang et al. 2009). Moreover, some mitochondrial proteins, which are responsible for oxidative stress, are differentially expressed in the process of fruit senescence (Fig. 1a, b). The sites of specific proteins in mitochondria have also been defined, including two porin proteins located in outer membrane, six proteins related to electron transport chain and carbon metabolism in inner membrane, three proteins belong to citric acid cycle, and other four proteins (Mn-SOD, heat shock protein, Beta-cyanoalanine synthase 1 and formate dehydrogenase) in inner space (Fig. 1c). These results provide evidence that ROS may regulate fruit senescence by changing expression profiles of specific mitochondrial proteins and impairing the biological function of these proteins.

Oxidative damage resulting in mitochondria dysfunction

Mitochondria, as a primary generator of endogenous ROS, are particularly vulnerable to oxidative damage (Sweetlove et al. 2002). Mitochondrial DNA is highly susceptible to oxidative damage because it is located close to the inner mitochondrial membrane, where the ROS are generated (Kujoth et al. 2005). Accumulating evidence suggests that mitochondrial protein oxidation is directly related to their

biochemical characteristics such as enzyme activities, structural functions, and susceptibility to proteolysis (Bulteau et al. 2006). Oxidative damage to mitochondria caused by ROS has been implicated in the process of senescence, as well as a number of senescence-related disorders in a variety of organisms (Balaban et al. 2005; Nyström 2005; Scheckhuber et al. 2007). The impairments of mitochondrial function caused by ROS include oxidative damage of respiratory chain, mtDNA deletion and lipid peroxidation (Yan et al. 1997; Das et al. 2001), which cause redox signaling, mitochondrial dysfunction and apoptosis and result in diseases and aging (Balaban et al. 2005; Kujoth et al. 2005). The oxidative modification of specific mitochondrial proteins was reported to be related to senescence process in plants (Møller and Kristensen 2006).

Carbonylated proteins can be formed by direct oxidation of amino acid side chains or via indirect reactions with lipid peroxidation products (Nyström 2005). The advent of proteomics and mass spectrometry make it possible to identify the specific proteins that are susceptible to oxidative modifications (England et al. 2004). Among a variety of methods for assessing protein oxidative damage, protein carbonylation has been used extensively. Using two-dimensional gel electrophoresis coupled with immuno-blotting to determine protein carbonylation (damaged proteins) in mitochondria of peach fruit during the senescence process, 28 proteins that ranged in molecular mass from 30 to 60 kDa and exhibited obvious carbonylation were identified by Qin et al. (2009b). Damaged proteins included outer mitochondrial membrane transporter, TCA cycle enzymes and antioxidant proteins (Fig. 2). Based on the analysis of the mitochondrial permeability transition, Qin et al. (2009a) suggested that oxidative modification is attributed to the alteration of mitochondrial function and permeability transition. Mitochondrial dysfunction is a
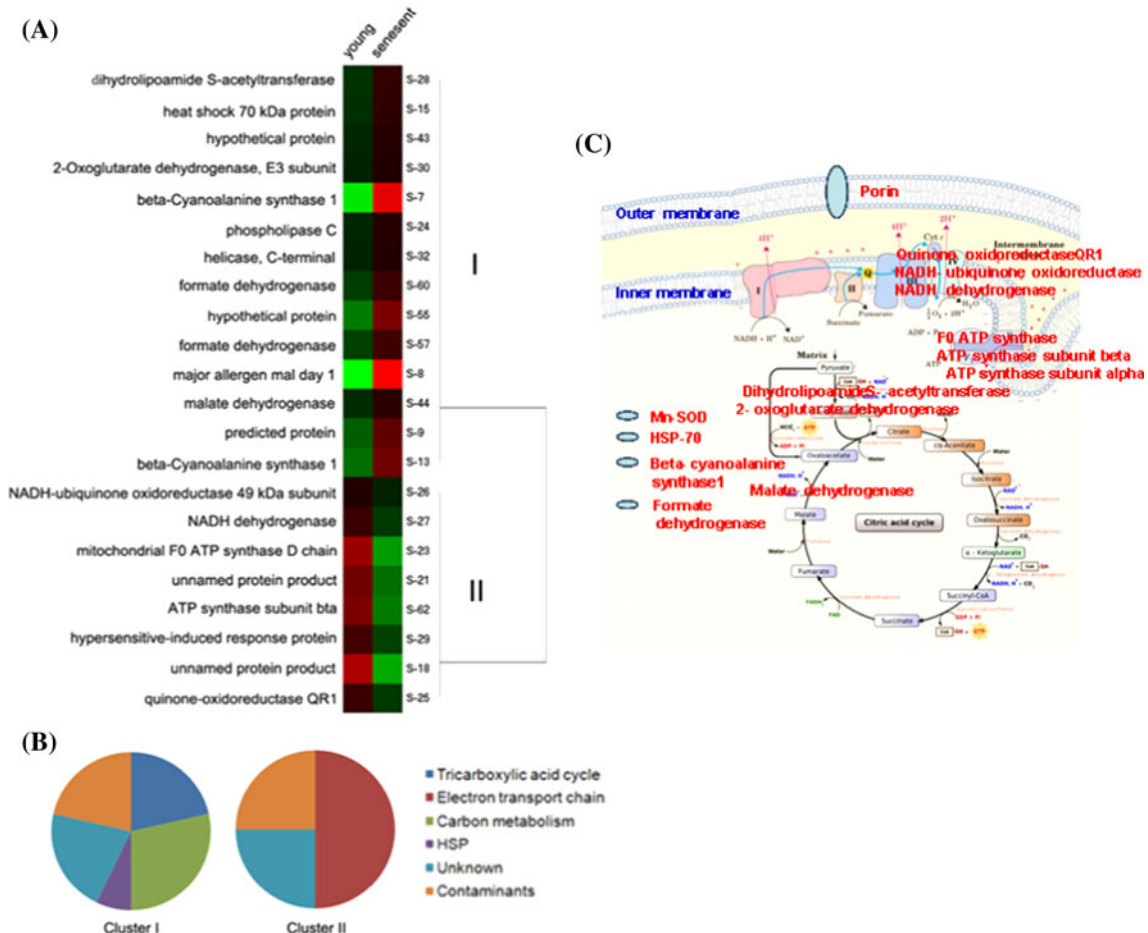

Fig. 1 Hierarchical clustering analysis of the changes in mitochondrial protein expression between young and senescent fruit (derived from Qin et al. 2009a). **a** Spots were clustered into two clusters (I and II) according to their percentage of volume using the Pearson clustering algorithm. *Each row* in the *color* heat map indicates a single protein, and each *column represents* proteins from young and senescent fruit. A *bright red color* indicates a high protein expression value for a specific protein spot, and a *bright green color* represents a low protein expression value. For each protein, the spot number and the functional annotation are shown. **b** Functional classification of proteins in each cluster. **c** Localization of identified proteins in the mitochondria

major cause of senescence in a variety of organisms (Nyström 2005; Toroser et al. 2007). The undesirable accumulation of ROS causes oxidative damage of mitochondrial proteins, resulting in the collapse of mitochondrial membrane potential and cellular dysfunctions or cell death (Genova et al. 2004). Oxidative damage of specific mitochondrial proteins would result in impairment of mitochondrial function, thereby, leading to fruit senescence.

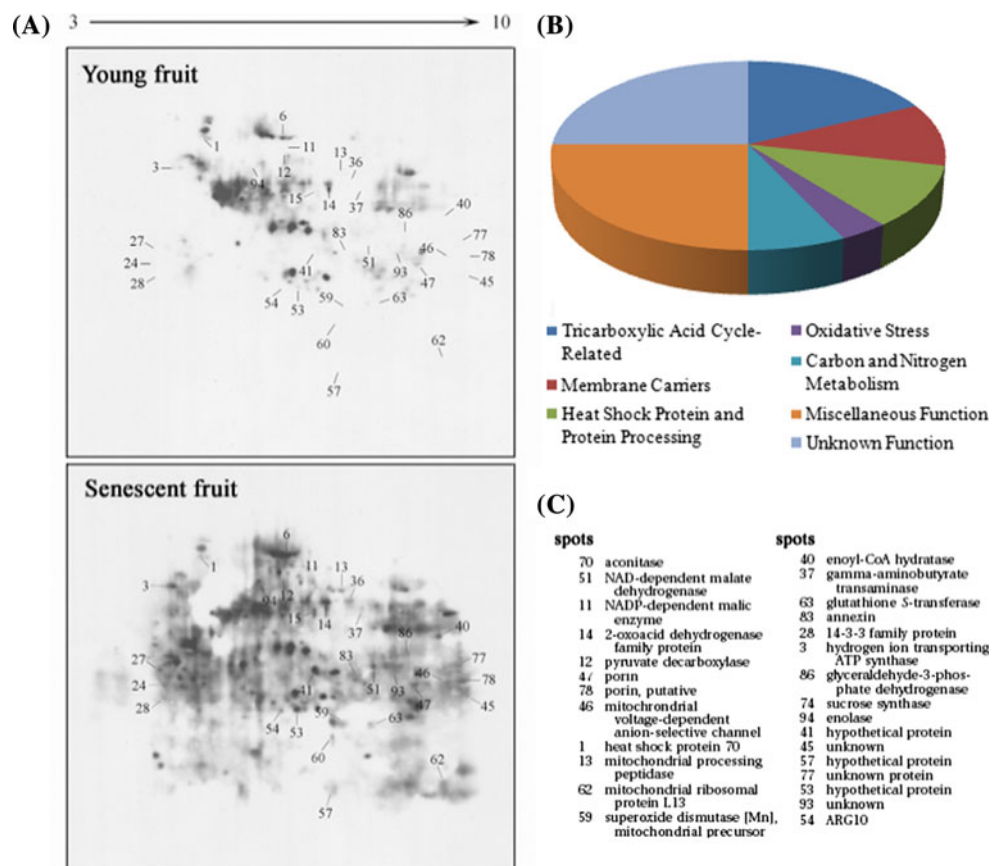## The function of ROS in regulating fungal pathogenicity

Plants have intra- and intercellular signaling mechanisms to generate both local and systemic responses to pathogen infection (Dietrich et al. 1994). Responses to pathogens are triggered by recognition of pathogen-encoded molecules, subsequent signal transduction, and biosynthesis or molecules acting to halt pathogen growth (Dixon and Lamb 1990). When fungal pathogens attack fruits, they often encounter the defense strategies of the host, including the accumulation of barrier-forming substances and the production of antimicrobial compounds that act directly to prevent pathogen invasion (Tian et al. 2006). Cellular environmental factors within the host, such as constitutive and induced toxic molecules, represent a challenge to an invading fungus. An oxidative burst, during which large

quantities of ROS are generated by different host enzyme systems of a plant, is one of the earliest host responses after pathogen attack (Mellersh et al. 2002). In general, pathogens can develop several defense responses, including increased activity of antioxidant enzymes and nonenzymatic protective molecules to protect their cells from ROS damage (Moradas-Ferreira et al. 1996).

Antioxidant enzymes related to fruit defense response

Antioxidant enzymes play an important role in oxidative stress resistance of fungal pathogens. Catalase (CAT), the enzyme that catalyzes the degradation of H_2O_2 into water and oxygen, is considered to be one of the major H_2O_2 scavenging enzymes in all aerobic organisms (Yang and Poovaiah 2002). The enzyme is present from lower to higher organisms and its activity is associated with lower rates of cancer and diabetes, and slower aging in mammalian systems (Melov et al. 2000; Preston et al. 2001). Similarly, glutathione S-transferase (GST), the enzyme that detoxifies hazardous compounds such as fatty acid peroxides by conjugating glutathione to these toxic compounds, is also important in protecting cells from oxidative stress (Veal et al. 2002). Recent reports indicate that antioxidant proteins (CAT, GST) are up-regulated in peach and sweet cherry fruit treated by salicylic acid (SA), suggesting that

Fig. 2 Identification of oxidatively damaged protein from peach mitochondria during fruit senescence (derived from Qin et al. 2009b). **a** Two-dimensional (2D) immunoblots of carbonylated mitochondrial proteins during fruit senescence. Mitochondrial proteins isolated from young and senescent peach fruit were separated by 2D gel electrophoresis using 13 cm Immobiline Drystrip with a pH 3–10 nonlinear gradient. After electrophoresis, proteins were transferred to PVDF membrane for immunoblot analysis with anti-dinitrophenyl-group antibodies. **b** Protein classification of identified proteins. **c** Proteins that have been identified by mass spectrometry

spots		spots	
70	aconitase	40	enoyl-CoA hydratase
51	NAD-dependent malate dehydrogenase	37	gamma-aminobutyrate transaminase
11	NADP-dependent malic enzyme	63	glutathione S-transferase
14	2-oxoacid dehydrogenase family protein	83	annexin
12	pyruvate decarboxylase	28	14-3-3 family protein
4	porin	3	hydrogen ion transporting ATP synthase
78	porin, putative	86	glyceraldehyde-3-phosphate dehydrogenase
46	mitochrondrial voltage-dependent anion-selective channel	74	sucrose synthase
1	heat shock protein 70	94	enolase
13	mitochondrial processing peptidase	41	hypothetical protein
62	mitochondrial ribosomal protein L13	45	unknown
59	superoxide dismutase [Mn], mitochondrial precursor	57	hypothetical protein
		77	unknown protein
		53	hypothetical protein
		93	unknown
		54	ARG10

the antioxidant enzymes are involved in defense responses of fruit (Chan et al. 2007, 2008).

ROS level affecting fungal pathogenic ability

Many exogenous factors that inhibit oxidative enzyme activity result in higher ROS level of fugal pathogens and suppression of fungal growth and pathogenicity (Qin et al. 2007). Borate treatment induced higher ROS levels in cells of *Penicillium expansum* (Fig. 3b), resulting in stronger inhibitory effects on spore germination of the pathogen in vitro (Fig. 3a) and on its pathogenic ability in the apple fruit (Fig. 3c). CAT and GST exhibited reduced expression levels under the borate stress, suggesting that the two antioxidant enzymes act as ROS scavenger in *P. expansum* (Qin et al. 2007). Borate also stimulated ROS generation in *Colletotrichum gloeosporioides* causing anthracnose in mango fruits, and leading to mitochondria degradation of the fungal pathogen (Shi et al. 2012). Borate-treated pathogen showed slower spore germination rate in vitro and lower pathogenic ability in mango fruit (Shi et al. 2012).

Also nitric oxide (NO) in high concentration has been reported to induce the generation of intracellular ROS of *P. expansum*, which subsequently causes severe oxidative damage to proteins crucial to the process of spore germination, leads to suppression of spore germinability (Lai et al. 2011). Application of exogenous superoxide dismutase (SOD) and ascorbic acid (Vc) to maintain ROS at basal level can repair cellular damage caused by ROS and reduce the detrimental effects of NO on *P. expansum* (Lai et al. 2011). Oxidative stress caused by NO may be attributed to: (1) the increase of ROS production and the decrease of ROS detoxifying ability (Mur et al. 2006), because NO has high-affinity binding activity to Cu^{2+}-B center of cytochrome oxidases (complex I II, IV), the final electron acceptor, which limits the flow of electrons from NADH to the ubiquinol pool and markedly increased the O_2^- yield (Carreras et al. 2004; Giulivi et al. 2006); and (2) NO can bind to the heme moiety of CAT to form a ferric nitrosyl and Fe–NO adduct, preventing the binding of H_2O_2 to the metal ion, thus inhibiting CAT activity (Moradas-Ferreira et al. 1996). Therefore, antioxidant defense systems are important to mediate ROS levels. The suppression of expression of antioxidant proteins and genes in fungal spores under the exogenous factor stresses can impair the ability of scavenging ROS, leading to the accumulation of ROS, greater growth and pathogenic ability of fungal pathogens.

Molecular targets for the ROS attack in mitochondria

Plant hosts release high levels of ROS, mostly O_2^- and H_2O_2 upon recognizing a pathogen (Bolwell et al. 1995).
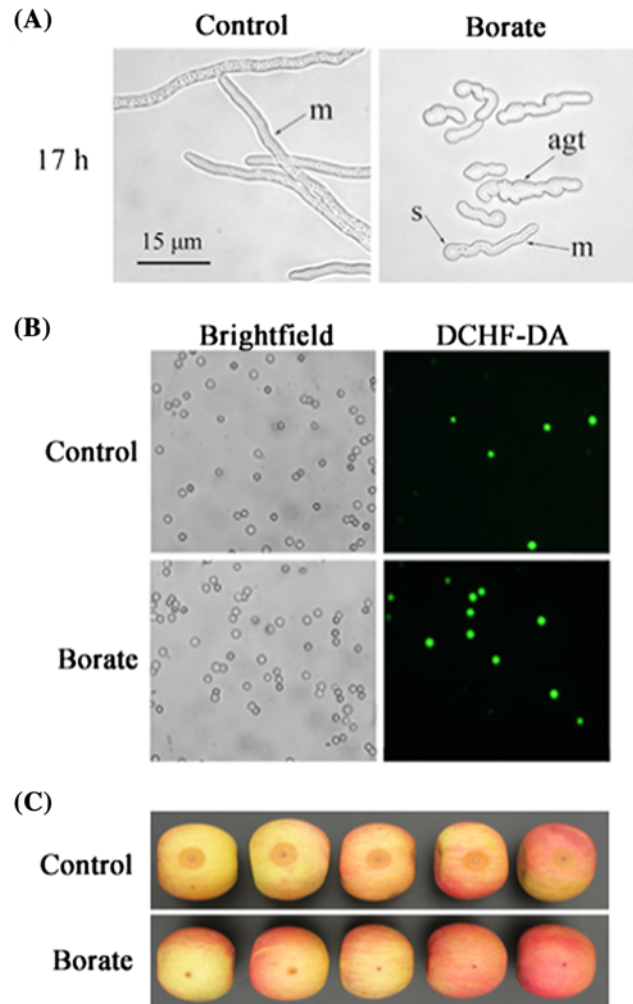

Fig. 3 Effects of borate on growth, ROS production, and pathogenicity of *Penicillium expansum*. **a** Microscopic observation of pathogen growth after 17 h of incubation at 25 °C. s, m, and agt represent spore, mycelium, and abnormal germ tube, respectively (Qin et al. 2007). **b** Generation of ROS in spores of *P. expansum*. Spores were cultured at 25 °C in potato dextrose broth medium supplemented with 0 and 0.1 % potassium tetraborate. After incubation for 4 h, spores were stained with the oxidant-sensitive probe DCHF-DA and observed with a fluorescence microscope (Qin et al. 2007). **c** Effects of borate on decay caused *P. expansum* in apple fruit

This rapid production of ROS, called the oxidative burst, provides an extremely hostile environment for pathogen (Hamann et al. 2008). Mitochondria are important organelles for infection process of fungal pathogen and play a crucial role in the survival of fungal pathogen under oxidative stress of H_2O_2 (Ingavale et al. 2008). Being adjacent to the site of ROS generation, mitochondrial components such as proteins are particularly vulnerable to oxidative damage (Yan and Sohal 1998). Exogenous H_2O_2 can cause the accumulation of ROS within the cell of fungal pathogens (Das et al. 2001). H_2O_2 exposure causes a concentration-dependent loss of cell viability in *P. expansum*, and

cell death of the pathogen is companied by the severe decrease of $\Delta\Psi_m$, a crucial parameter of mitochondrial function (Qin et al. 2011). The results suggest that mitochondria serve as the major intracellular target of exogenous H_2O_2.

Mitochondrial membrane proteins serving as ROS targets

The integrity of the plasma membrane is related to whether oxygen radicals can lead to rapid disintegration of biological membranes. Damage to plasma membrane can result in loss of osmotic balance and influx of fluids and ions, as well as loss of proteins and ribonucleic acids, eventually leading to the onset of cell death (Qin et al. 2010). Sources of cellular ROS include leakage from the mitochondrial electron transport chain as well as a number of ROS-generating plasma membrane and cytosolic enzymes (Thannickal and Fanburg 2000; Yagoda et al. 2007). Upon exposure to half lethal dose of H_2O_2, *P. expansum* showed a limited loss of plasma membrane integrity, indicating that membrane damage was not the main reason for H_2O_2-induced cell death and that an intracellular target might be involved in this process (Qin et al. 2011). Two-dimensional gel electrophoresis coupled with immunoblotting has revealed that mitochondrial proteins including outer membrane transporters (porin), antioxidant proteins (MnSOD) and TCA cycle enzymes (malate dehydrogenase and aconitase) of peach fruit experienced an oxidative damage during the senescence process (Qin et al. 2009b).

Outer membrane transporters are channel-forming proteins that form the major pathway in mitochondria and control metabolite flux across the membrane (Lemasters and Holmuhamedov 2006). Small metabolites up to 5,000 daltons can be permitted to transported across these membrane-spanning channels in its open configuration (Harris and Thompson 2000). The change in the specific modification state of outer membrane in mitochondria was considered to be involved in mitochondrial dysfunction (Fukada et al. 2004). Outer membrane transporters are linked to the mitochondrial permeability transition pore (PTP), which consists of several proteins, including the adenine nucleotide translocator, the cyclophilin-D, and sites between the mitochondrial inner and outer membranes (Shimizu et al. 2001). Opening of PTP would result in the swelling of the mitochondrial matrix space and rupture of the outer mitochondrial membrane (Madesh and Hajnóczky 2001). ROS regulate fruit senescence via changing expression profiles of specific mitochondrial proteins, particularly outer membrane transporters, and impairing their biological function, which serve as target attacked by ROS in mitochondria.

Mitochondrial complex III is responsible for ROS production

There are subunits in mitochondrial respiratory chain. The mitochondrial complex I has a central role in the regulation of longevity and regulates aging through at least two mechanisms: (1) an ROS-dependent mechanism that leads to mitochondrial DNA damage; and (2) an ROS-independent mechanism through the control of the NAD^+ to NADH ratio (Stefanatos and Sanz 2011). The mitochondrial complex II of the electron transport chain contributes to localized mROS that regulates plant stress and defense responses (Gleason et al. 2011). The mitochondrial complex III is thought to contribute to hypoxia-induced ROS production in animals and yeasts (Guzy et al. 2005, 2007).

Mitochondrial complexes I, III and V have been identified in *P. expansum* by our lab (Qin et al. 2011). We monitored the change in intracellular ROS levels by using DCHF-DA, a cell-permeable ROS indicator that penetrates live cells but does not fluoresce unless oxidized by ROS, and observed that more cells were stained with DCHF-DA under H_2O_2 stress, implying that more ROS were generated (Fig. 4a). Based on the analysis showing that fluorescent signals of DCHF-DA can be co-localized with those of Mitotracker (a fluorescent dye that stains mitochondria), the mitochondria are proven to be responsible for H_2O_2-induced ROS production (Fig. 4b). After myxothiazol was applied, the mitochondrial complex III showed a depletion of DCHF-DA signal, which represented the reduction in the production of ROS (Qin et al. 2011). These data suggest that exogenous H_2O_2 can cause the accumulation of ROS in cell of the fungi by targeting mitochondrial complex III. Also the mitochondrial complex III is the major site for ROS production in *P. expansum* under oxidative stress of H_2O_2.

Concluding remarks

A model describing the involvement of ROS in regulation of fruit senescence and fungal pathogenicity is presented (Fig. 5). When fruit age, ROS accumulation in cells causes oxidative damage and changes biochemical characteristics of the mitochondrial proteins, including outer membrane transporter, TCA cycle enzymes, and some antioxidant proteins, which serve as the targets attacked by ROS in mitochondria. The oxidative damage of specific mitochondrial proteins caused by ROS is responsible for impairment of protein targets, which in turn facilitates further release of ROS and enhances oxidative damage to mitochondrial protein, eventually leading to the mitochondrial dysfunction and fruit senescence. Moreover, H_2O_2 stress can induce ROS accumulation in mitochondria
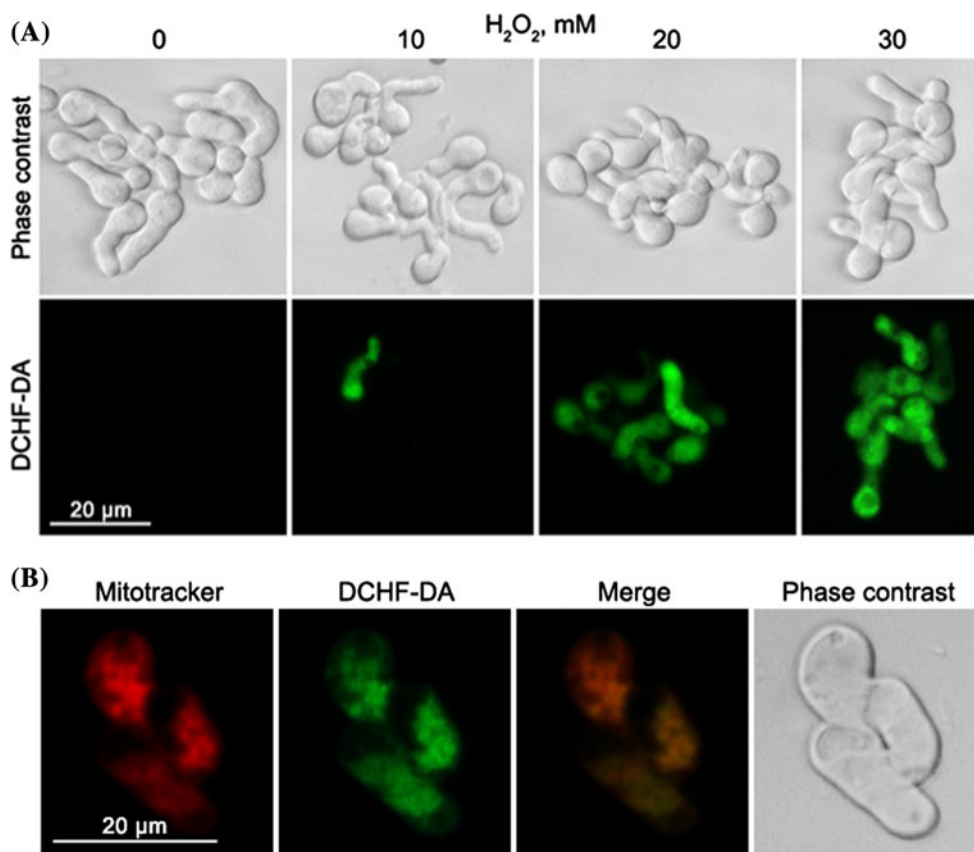
Fig. 4 Determination of ROS production and cellular location of ROS formation in *Penicillium expansum* under oxidative stress (Qin et al. 2011). **a** Production of ROS in *P. expansum* after H$_2$O$_2$ treatment assessed by the oxidant-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA). **b** Co-localization of the Mitotracker orange (a fluorescent dye that stains mitochondria) and DCHF-DA staining inside the germlings. Before fluorescent staining, the fungal spores were cultured in potato dextrose broth medium until germination, and then treated with indicated concentrations of H$_2$O$_2$ for 60 min at 25 °C. *Scale bar*, 20 μm

Fig. 5 Model for ROS regulating fruit senescence and fungal pathogenicity
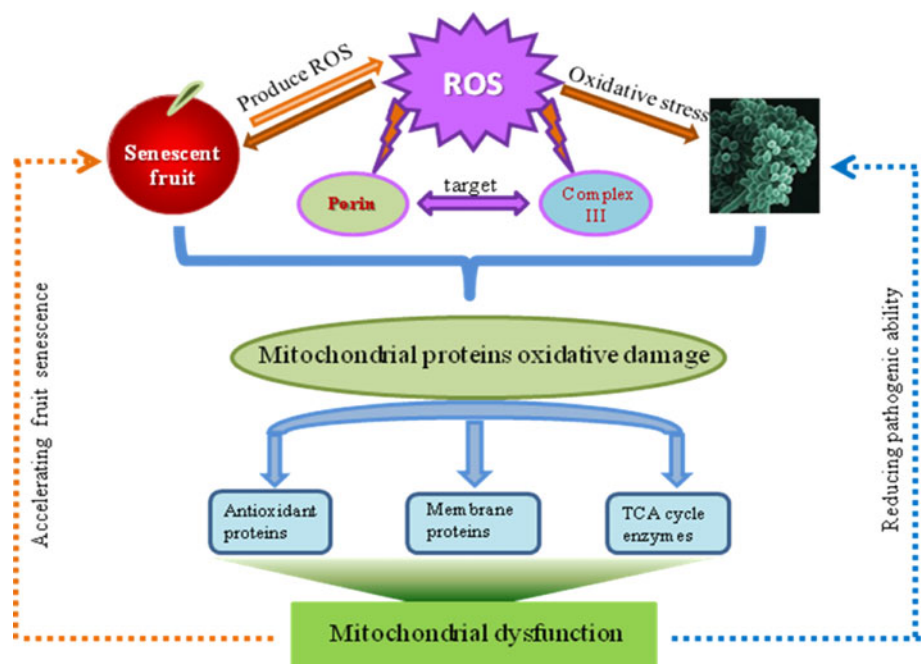
of fungal pathogens, resulting in oxidative damage of specific mitochondrial proteins, such as respiratory chain complexes I and III, F1F0 ATP synthase and mitochondrial phosphate carrier protein. Among of them, the mitochondrial complex III is proven to be molecular target attacked by ROS. The oxidative damage of mitochondrial proteins can destroy mitochondrial structure and cause mitochondrial dysfunction, finally leading to reduce fungal pathogenic ability to fruit host. Therefore, exploring the site of ROS generation and their molecular targets is not only beneficial for understanding the mechanisms that ROS regulate fruit senescence and fungal pathogenicity, but also for providing a basis for future development of novel control technologies of harvested fruit quality and anti-fungal agents.

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