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Interference of Oxidative Metabolism in Citrus by *Xanthomonas citri* pv *citri*

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1. Introduction

Citrus are one of the most important fruit crops grown worldwide. Among the pathogens that cause disease of *Citrus sp.* and closely related genera, *Xanthomonas citri* pv *citri* (*Xcc*) causes citrus canker, a devastating disease that is found in 30 countries worldwide and has caused significant economic loss (Del Campo et al., 2009; Rigano et al., 2010). The principle mode of transmission of *Xcc* is through heavy rain and high wind events and thus the disease is most severe in regions that experience occasional tropical storms and hurricanes (Graham *et al.,* 2004). Citrus canker outbreaks in Florida, for example, have contributed to a decline in acreage of grapefruit to 61 % by 2009 compared to the acreage in 1994 (Anonymous, 2009). Severe canker can cause fruit drop and even tree death (Graham et al., 2004). Further economic losses can be incurred through restricted movement of infected fruits especially to citrus growing regions where canker is not found (Schubert *et al.,* 2001).

The commercial and dietary importance of citrus and the severity of canker have led to extensive research to identify resistant genotypes that would serve as models of study as well as germplasm for crop improvement. Most commercial citrus are within the *Citrus* genus, however closely related genera are capable of hybridizing with *Citrus sp.* and thus have been included in studies to evaluate variation in plant defense to canker. Citrus genotypes can be classified into four broad classes based on susceptibility to canker (Gottwald, 2002). The most highly-susceptible commercial genotypes are 'Key' lime [*C. aurantifolia* (Christm.) Swingle], grapefruit (*C. paradisi* Macfad.), lemon (*C. limon*), and pointed-leaf Hystrix (*C. hystrix*). Susceptible genotypes include limes (*C. latifolia*), sweet oranges (*C. sinensis*), trifoliate orange (*P. trifoliata*) citranges and citrumelos (*P. trifoliata* hybrids), and bitter oranges (*C. aurantium*). Resistant genotypes include citron (*C. medica* L.) and mandarins (*C. reticulata* Blanco). Highly resistant genotypes include Calamondin [*Citrus margarita* (Lour.)] and kumquat [*Fortunella margarita* (Lour.) Swingle]. The high degree of resistance to Asiatic citrus canker by calamondin, kumquat, and Ichang papeda (*C. ichangenesis*) has been noted in the field (Reddy, 1997; Viloria *et al.,* 2004).

Although *Xcc* can cause disease in kumquat, the cankers are normally much smaller than in *Citrus* species indicating greater resistance (Viloria et al., 2004). Kumquat resistance to *Xcc* has been utilized in breeding programs to produce intergeneric hybrids with *Citrus* species that are more canker resistant than the *Citrus sp.* parent (Viloria et al., 2004). Kumquat is also

being used as a model system in research programs to determine the underlying resistance mechanism (eg., Khalaf et al., 2007) with the long term goal of identifying specific genes that could be inserted into commercial *Citrus* species and avoid the much greater genetic variability in yield and fruit quality typically introduced through crosses in traditional breeding programs.

Although development of resistant genotypes is a long-term research goal, commercial industries have been forced to implement a variety of management practices to reduce the impact of this devastating disease including the use of resistant species and cultivars, applications of bactericides especially copper, and in extreme cases removal of infected trees in an attempt to eradicate the disease from a particular region. Resistance alone is insufficient for commercial production, eradication in high wind and rain-prone areas have largely proven ineffective and copper sprays are often unreliable, in part because of increased resistance by the pathogen (Graham et al., 2004). Multiple management approaches will be required to maintain commercial production. One approach that has received limited attention is the application of biotic and abiotic agents that would promote systemic acquired resistance and induced systemic resistance (Valad and Goodman, 2008). Advances in the use of systemic acquired resistance and induced systemic resistance will require a working hypothesis of how *Xcc* interferes with citrus defense. The comparison of resistant and susceptible genotypes has revealed new information regarding the deficiencies in susceptible genotypes that can be developed into a working hypothesis as to how *Xcc* interferes with citrus defense, and from that knowledge strategies can be developed to restore the defense mechanism.

2. Pathogenesis of canker in citrus

Metabolic changes in plants to pathogens coincide with the plant parts affected and the development of the disease. Canker affects all above ground parts of the plant including the leaves, stems and fruit (Graham et al., 2004). Only one bacterium is required to cause canker formation, which enters the plant through stomatal apertures or wounds using its flagella (Gottwald and Graham, 1992; Koizumi and Kuhara, 1982; Stall et al., 1982). Once inside, the bacterium multiplies to reach a population density of 1×10^3 to 1×10^4 bacteria per canker lesion, which is sufficient to act as source of inoculum and under specific conditions promote dispersal (Graham *et al*., 2004).

Cankers are a localized phenomenon such that plant response in an infected area differs from uninfected areas, and thus bulk sampling of tissues would include both areas. To facilitate sampling of only infected tissues, studies have utilized injection of *Xcc* suspensions into leaf tissues (Khalif et al., 2007). Upon injection, an initial water soaked area is observed and subsequent disease symptoms develop in this region. Thus, sampling the original water soaked area allows sampling of only diseased tissues. The advantage of this approach has been demonstrated by changes in H_2O_2 concentrations in *Xcc* infected areas induced through injection (Kumar et al, 2011a), whereas whole leaf sampling of trees sprayed with *Xcc* suspensions demonstrated inconsistent or no differences in H_2O_2 concentrations (Kumar, data unpublished).

Injection of known concentrations of a specific strain of *Xcc* and maintaining plants under consistent environmental conditions allows repetition of a specific sequence of disease events to which plant response can be correlated. Using this approach, a specific sequence of

events in the pathogenesis of *Xcc* in citrus has been described (Burnings and Gabriel, 2003). Following artificial inoculation, the bacterial cells occupy intercellular spaces and begin to divide by the end of the first day after inoculation. Once a critical population threshold is reached, which is about 1 x 10^3 to 1 x 10^4 bacteria per canker lesion, a quorum sensing mechanism (da Silva et al., 2002) is likely the impetus that turns on pathogenicity factors (Bassler, 1999) that includes Rpf encoding genes (Slater et al., 2000). Within 2 days after inoculation, *Xcc* attaches to plant cell walls via specialized proteins called "adhesins" (Lee and Schneewind, 2001) by hrp (hypersensitivity response and pathogenicity) pili or by type IV pili as observed during *xanthomonas* pv. *malvaceraum- Gossypium hirsutum* interaction (Burnings and Gabriel, 2003). Once attached, *Xcc* uses it T3S system to turn on additional pathogenicity genes (Pettersson et al., 1996) and inject pathogenicity factors into the cell including Avr, Pop and Pth proteins such as PthA (Brunings and Gabriel, 2003). PthA presumably stimulates plant cell division and enlargement within 3 days after inoculation that reaches a maximum by 7 days after inoculation (Lawson et al., 1989). Cell enlargement, presence of the bacteria in the apoplast, and its production of hydrophilic polymers causes watersoaking symptoms starting 4 days after inoculation (Duan et al., 1999). The maximum bacterial populations occur at 7 days after inoculation (Khalaf et al., 2007) and about 8 days after inoculation the epidermis ruptures allowing bacteria to egress to the surface (Brunings and Gabriel, 2003). By 10-14 days after inoculation, necrosis develops in the infected areas (Duan et al., 1999) and by 21 days after inoculation leaves abscise (Khalaf et al., 2007).

3. Oxidative response of plants to pathogens

The hypersensitive response (HR) involves a rapid, widespread change in plant cell metabolism intended to alter the chemistry of the region within and surrounding the infected area in order to impact the pathogen by deterring its metabolism, isolating it within the infected region, and even directly killing it (Lamb and Dixon, 1997). As part of the response, programmed cell death (PCD) of plant cells within and adjacent to the infected region is often elicited (Lamb and Dixon, 1997). The HR includes alteration of oxidative metabolism to produce reactive oxygen species (ROS) that promote PCD, sicken pathogen metabolism, and promote changes in cell wall chemistry that isolate the pathogen (Azvedo *et al.,* 2008; Kuzniak and Urbanek, 2000; Lamb and Dixon, 1997). In the case of citrus canker, PCD is evident around infection sites by chlorosis, with the chlorotic rings widening as the canker spreads radially from the infection point and along the plane of the leaf blade (Burnings and Gabriel, 2003).

Reactive oxygen species produced during HR and PCD in response to pathogens include superoxide radicals ($O2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) (Chen et *al.,* 2008; Lamb and Dixon, 1997; Wojtaszek, 1997). Production of ROS occur during normal metabolism of uninfected plants and maintained at low concentrations by several enzymatic and non-enzymatic pathways. In response to infection by pathogens, concentrations of ROS are increased and compartmentalized during HR and PCD via several pathways mediated by signals including salicylic acid, nitrous oxide, and the MAP kinase cascade mechanism (Durrant and Dong, 2004; Vlot et al., 2009) to alter the chemistry within and surrounding the infection site (Mittler, 2002).

One important ROS is H_2O_2, the concentration of which has been correlated with disease resistance (Lamb and Dixon, 1997; Mittler *et al.,* 1999). H_2O_2 concentrations can increase very rapidly from 0 to 6 days after inoculation during plant-bacterial pathogen interactions (Wojtaszek, 1997). Early after infection, elevated concentrations of H_2O_2 serve as diffusible signals to induce defense genes in adjoining cells with the later elevated concentrations serving in the direct inhibition of pathogens (Alverez *et al.,* 1998; Dat et al., 2000; Lamb and Dixon, 1997). The role of H_2O_2 in promoting disease resistance has been confirmed in transgenic potato plants that over-expressed a fungal glucose oxidase gene and accumulated sub-lethal concentrations of H_2O_2 (Wu *et al.,* 1997).

A major source of H_2O_2 is by dismutation of $O_2^{\cdot-}$ via the activity of superoxide dismutase (SOD) (Alscher *et al.,* 2002; Voludakis et al., 2006). SODs are regarded as a first step in reducing oxidative stress by converting $O_2^{\cdot-}$ to H_2O_2 during normal metabolism (Babhita et al., 2002). In response to biotic stress, SOD genes and enzyme concentrations are often up-regulated as part of the resistance mechanism against viral, bacterial and fungal diseases (Barna *et al.,* 2003; Bolwell and Wojtaszek, 1997; Buonaurio *et al.,* 1987; Delledonne *et al*., 2001; Montalbini and Buonaurio, 1986; Tertivanidis *et al*., 2004; Voludakis *et al.,* 2006). The importance of SOD in the production of H_2O_2 has been demonstrated in rose cells treated with the Cu-Zn-SOD inhibitor N,N-diethyldithiocarbamate and exposed to phytophthora (Auh and Murphy, 1995). Furthermore, pearl millet (*Pennisetum glaucum*) demonstrated higher SOD activity in resistant genotypes compared to susceptible genotypes when challenged with *Sclerospora graminicola* (Babhita et al., 2002). Similarly, SOD activity was higher in *Xanthomonas campestris* pv. *campestris* resistant cabbage (*Brassica oleracea*) varieties (Gay and Tuzun, 2000).

Based on their metal co-factor, SODs can be classified into three categories: iron SOD (Fe-SOD), manganese-SOD (Mn-SOD), and copper-zinc SOD (Cu-Zn-SOD), each of which is specifically compartmentalized in the cell (Alscher *et al.,* 2002). Fe-SOD is located in the chloroplasts, Mn-SODs in the mitochondria and peroxisomes, and Cu-Zn-SOD in the chloroplast, cytosol, and possibly in the apoplast (Alscher *et al.,* 2002). The various SODs play important roles in plant/pathogen interactions. Fe-SOD, for example, appears to be involved in the early signaling with H_2O_2 by plant cells after infection (Mur *et al.,* 2008; Zurbriggen *et al*, 2009). Mn-SOD has been reported to play an important role in early apoptotic events during PCD in *Gossypium hirsutum-Xanthomonas campestris* pv. *malvecearum* interaction (Voludakis et al., 2006). However, Kukavica et al. (2009) showed the existence of a cell wall bound Mn-SOD that generated $OH^{\cdot}$ in pea roots and probably facilitates cell elongation.

Some of the major enzymes involved in H_2O_2 dismutation and that have been shown to change during pathogenesis include catalase (CAT), ascorbate peroxidase (APOD) and class III peroxidase (POD) (Able et al., 2000; Dat et al., 2003; De Pinto et al., 2006; Gonzalez et al., 2010). Catalase and APOD are the most important enzymes involved in maintaining H_2O_2 at low concentrations in the symplast of healthy plants (Mittler, 2002). Catalase is a tetrameric iron porphyrin that converts millions of H_2O_2 to water and oxygen per second and is generally limited to the peroxisomes where H_2O_2 forms rapidly as a by-product of photorespiration (Willekens et al., 1997). The importance of CAT in disease resistance has been shown in transgenic tobacco (*Nicotiana tabacum* cv AS1) that had reduced CAT1 mRNA and protein (AS1) which demonstrated a HR leading to necrotic lesions upon challenge with *Pseudomonas syringae* pv. *tabaci* (Mittler *et al*., 1999).

Ascorbate peroxidases contain a heme cofactor and use ascorbate as a substrate as part of the glutathione-ascorbate cycle (Foyer et al., 2009). Ascorbate peroxidase is ubiquitous throughout the cell and thus is important in catalyzing H_2O_2 that is produced as a waste product of different metabolic pathways (Mittler, 2002). The importance of APOD in disease resistance has been shown in transgenic tobacco transformed with antisense cAPX (*Nicotiana tabacum* cv Bel W3) that exhibited PCD accompanied by fragmentation of nuclear DNA after being challenged with *Pseudomonas syringae* pv. *tabaci, Pseudomonas syringae* pv. *phaseolicola* NPS3121 and *Pseudomonas syringae* pv. *syringae* (Mittler *et al*., 1999; Polidoros et al., 2001).

The use of guaiacol as a substrate to test peroxidase activity is limited to the Class III peroxidases (POD) that are characterized by secretion into the apoplast and utilize phenolic compounds as substrates to cross-link cell walls during cell maturation (De Gara, 2004; Liszkay et al., 2003; Sasaki et al., 2004). During infection, the class III PODs promote lignification, suberization, cross-linking of cell wall proteins, and phytoalexin synthesis to sicken metabolism and isolate the pathogen (Sasaki *et al*., 2004; Quiroga et al., 2000). The peroxidative cycle of POD uses H_2O_2 as an oxidant to convert phenolic compounds to phenoxy radicals that spontaneously combine to form lignin responsible for cell wall stiffening (Liszkay et al., 2003; Martinez *et al.,* 1998).

4. Comparative analysis of oxidative metabolism in *Xcc* resistant and susceptible genotypes

Recent studies on various *Citrus sp.* and closely related genera have increased our understanding of deficiencies in oxidative metabolism in susceptible genotypes. The most commonly studied resistant genotype is kumquat (*Fortunella margarita* (Lour.) Swingle). The kumquats have been characterized as canker resistant based on fewer canker lesions per leaf and reduced internal bacterial populations per lesion compared to susceptible genotypes (Khalaf *et al*., 2007; Viloria *et al*., 2004). Resistance of kumquat has been exhibited in hybrids with *Citrus sp.* such as 'Lakeland' limequat, a cross between the highly *Xcc*-susceptible 'Key' lime and kumquat, which has demonstrated greater canker resistance than 'Key' lime alone under field conditions (Viloria *et al.,* 2004). Furthermore, the Asiatic strain of canker (Canker A) has been shown to reach populations densities consistent with a compatible reaction (Stall *et al*., 1980) and the lower concentrations of *Xcc* in kumquat indicates a disease resistance mechanism (Viloria *et al*., 2004). Although oxidative metabolism is complex, recent research has focused on comparing kumquat resistant and susceptible *Citrus* genotypes on their H_2O_2 metabolism in part due to its importance in cell signaling and its involvement in cell wall chemistry during growth and plant defense.

The basal antioxidant metabolism has been shown to vary in different citrus genotypes (Kumar et al., 2001a) which relate to their fundamental differences in resistance. Kumquat, for example, was shown to have higher total SOD activity in kumquat than grapefruit and sweet orange, yet H_2O_2 was lower in kumquat in part because of higher CAT activity. These fundamental differences in basal metabolism are the starting point for changes in oxidative metabolism when challenged with *Xcc*.

5. Oxidative metabolism in canker-resistant kumquat

Using an Asiatic strain of canker (Canker A) and infiltration of kumquat leaves, Kumar et al., (2011c) showed that the *Xcc* populations peaked 4 days after inoculation and declined

thereafter. Chlorosis was evident the first day after inoculation and persisted throughout the infection process (Fig. 1). Water soaking was delayed until 4 days after inoculation. H_2O_2 concentrations increased rapidly 1 day after inoculation to almost 2x the controls, about 10 ml, from 6 to 8 days after inoculation and declined slightly thereafter but remained above the controls throughout the infection process (Figs. 1 and 2). The pattern of *Xcc* population and H_2O_2 concentrations is consistent with the latter's role in impeding bacterial growth and promoting PCD, which occurred from 10 to 12 days after inoculation. The rapid necrosis in the localized region of the infected kumquat tissue by *Xcc* has been suggested to be consistent with a hypersensitive response (HR) and induced PCD (Khalaf et al., 2007). Lipid peroxidation was shown to increase rapidly and remain several times higher than the controls in kumquat-*Xcc* interaction (Kumar et al., 2011e). Lipid peroxidation generates free radicals, which in turn are toxic to plant and bacterial cells and is consistent with PCD as part of the HR to pathogens (Gobel *et al.,* 2003; Kumar et al., 2011e; Rusterucci et al., 1996). It is interesting that using the injection method, kumquat did not display much swelling of the epidermis, which is required for egress of Xcc to the leaf surface. Kumar et al., (2011c,e) concluded that the retention of bacteria in the leaf coupled with early leaf abscission, which occurred from days 10 through 12, is consistent with a disease avoidance mechanism.

The production of H_2O_2 occurs mainly through SOD activity. Kumar et al. (2011e) showed that total SOD activity demonstrated two peaks during the course of *Xcc* infection of kumquat with peaks at 1-2 days after inoculation and 6-8 days after inoculation, although the total SOD activity was always higher than the uninfected controls. Analysis of the activity and isoforms of the various SODs were shown to be altered indicating compartmentalization of H_2O_2 production (Kumar et al, 2011c,e). The first peak in total SOD activity was associated with a rapid increase in Fe-SOD activity to 2x the controls by 1 day after inoculation, but the activity dropped rapidly near or below the controls thereafter. Fe-SOD is compartmentalized in chloroplasts and studies on other plant-pathogen interactions have shown that chloroplasts are an important source of ROS signals that initiate changes in oxidative metabolism in other cellular compartments (Mur *et al.,* 2008; Zurbriggen *et al*, 2009). Cu-Zn-SOD is also found in the chloroplasts (Alscher *et al.,* 2002), but Kumar et al. (2011e) found no activity of this SOD isoform during the kumquat-*Xcc* interaction. Mitogen-activated protein kinase (MAPK), which respond to external stimuli, are activated in plant-pathogen interactions and promote ROS generation in chloroplasts by inhibiting CO_2 assimilation that serves as a sink for ROS generated by light (Liu *et al*., 2007; Zurbriggen *et al,* 2009). Evidence that this mechanism functions during kumquat-*Xcc* interaction is supported by differential expression of related genes (Khalaf *et al*., 2007). Although Fe-SOD activity initially surged, high concentrations of H_2O_2 have been shown to deactivate Fe-SOD (Giannopolitis and Ries, 1977), which is consistent with suppression of Fe-SOD activity after the first day (Kumar et al., 2011e).

Keeping in mind that total SOD activity in kumquat-*Xcc* interaction increased and remained high throughout pathogenesis, the decline in Fe-SOD activity beyond the first day after inoculation had to be replaced by a different form of SOD that would dominate during the second peak of total SOD activity. Kumar et al., (2011e) found that Mn-SOD activity increased from 2x to 3x that of the control starting 2 days after inoculation and reached a maximum during the second peak of total SOD activity from 6 to 8 days after inoculation. The prolonged, elevated Mn-SOD activity indicated that this class of SOD was responsible for the majority of total SOD activity throughout the entire pathogenesis process. Mn-SOD is

generally considered to be limited to mitochondria and peroxisomes (Alscher *et al*, 2002) and recent evidence indicates the importance of mitochondria in generating ROS to promote PCD (Mur *et al*, 2008; Yao *et al.,* 2002). Thus, the elevated H_2O_2 concentration during kumquat-*Xcc* interaction is promoted by SOD activity, first in the chloroplast and thereafter in the peroxisome and mitochondria. Thus, the sustained production of H_2O_2 in peroxisomes and mitochondria indicates that these organelles serve as important generators of H_2O_2 during kumquat-*Xcc* interactions.

The fate of H_2O_2 in kumquat-*Xcc* interaction is determined, in part, by enzymes involved in its dismutation. Catalase is considered the major H_2O_2 scavenging enzyme and is located in peroxisomes of plant cells (Kamada et al., 2003; Hu et al., 2010). During kumquat-*Xcc* interaction, total CAT activity remained similar to the controls up to 5 days after inoculation but declined starting 6 days after inoculation to almost half of the controls (Kumar et al., 2011c). Interestingly, CAT demonstrated qualitative and temporal changes in isoforms (Kumar et al., 2011c). Plants have been shown to contain three CAT genes that code for three subunits and generate at least six isoforms that are classified into three classes (Hu et al., 2010). Class I CATs are abundant in tissues that contain chloroplasts, Class II CATs are mainly expressed in vascular tissues, and Class III CATs are generally found in young and senescent tissues. In uninfected kumquat leaves, Kumar et al. (2011c) identified 4 CAT isoforms (CAT 1-4) that appeared to be constitutive and therefore belong in Class I and II. CAT-3 disappeared, CAT-2 declined starting at 4 days after inoculation, and CAT-4 declined starting at 10 days after inoculation, probably due to termination of all metabolic activity because of necrosis. A novel CAT isoform, CAT-5, was expressed 4 days after inoculation, and appears to belong to Class III since senescence as indicated by chlorosis rapidly developed at this time. There was no evidence of CAT-6.

The decline in CAT activity coincided with the highest concentrations of H_2O_2 but during the stationary phase of *Xcc* population growth (Kumar et al., 2011e). *Xcc* during the log phase of growth in kumquats is highly susceptible to H_2O_2 with almost no survival upon exposure to 1 mM H_2O_2 in comparison to stationary phase populations that can resist up to 30 mM of H_2O_2 (Tondo et al., 2010). H_2O_2 increased to almost 10 mM (Kumar et al., 2011c,e), which was high enough to restrict *Xcc* during the log phase but not enough to impact bacterial populations during the stationary phase of growth (Tondo et al., 2010). The *Xcc* stationary phase populations were able to resist higher external H_2O_2 concentrations due to high bacteria CAT activity via the expression of four CAT genes (*katE, catB, srpA, and katG*) (Tondo et al., 2010). Thus, it appears that the reduced plant CAT activity, which occurred during the stationary phase of *Xcc* population growth, was too late to directly impact the pathogen. Perhaps molecular modification that increasing CAT activity earlier in kumquat would suppress *Xcc* concentrations further by allowing H_2O_2 concentrations to increase during the log phase of *Xcc* growth (Chaouch et al., 2010).

Although the decline in CAT activity was too late to have a direct impact on *Xcc* populations, it may be part of the adaptive response of kumquat to promote necrosis and leaf abscission late in the infection process (Foyer et al., 2009). Recently, Yu et al, (2006) showed that selective degeneration of specific CATs in mouse cell lines subsequently caused an increase in ROS concentrations and induced PCD. Similarly, transgenic plants with reduced CAT expression exhibited necrotic lesions and displayed elevated concentrations of pathogenesis-related proteins in tobacco (*Nicotiana tabacum* cv. Bel w3; Mittler et al., 1999).

Because CATs are limited to peroxisomes, it appears that this organelle serves an important role in canker resistance by elevating H_2O_2 concentrations that diffuses to the rest of the cell and thus could become a promising site for resistance enhancement in susceptible citrus by genetic engineering of CAT gene expression or by post-translational modification of CAT proteins (Chaouch et al., 2010).

Ascorbate peroxidases are ubiquitous peroxidases that help maintain low H_2O_2 concentrations during normal metabolism (Mittler, 2002). During kumquat-*Xcc* interaction, APOD activity declined linearly after *Xcc* inoculation to less than half the activity of the controls by 12 days after inoculation (Kumar et al., 2011c). The immediate and increasing decline in APOD activity is an adaptive plant response to help promote elevated H_2O_2 concentrations throughout the sympast and is the principle enzyme that allowed H_2O_2 concentrations to increase in infected kumquat. There is evidence that higher H_2O_2 concentrations inactivate APODs at both the transcriptional and post-transcriptional levels (Zimmermann et al., 2006; Paradiso et al., 2005).

Higher H_2O_2 concentrations rather than $O_2^{\cdot-}$ in the symplast is interesting because it is a less reactive ROS, which may indicate another role for H_2O_2 than promoting senescence alone. *Xcc* are only found in the apoplast and any positive effect of higher H_2O_2 concentrations would require diffusion out of the symplast. H_2O_2 in the apoplast would allow it to serve as a substrate for the Class III PODs. During normal metabolism of uninfected plants, H_2O_2 is utilized by the Class III PODs to promote loosening of cell walls during cell enlargement and to cross-link cell wall polymers during cell maturation (de Gara, 2004). The Class III PODs are also an adaptive defense mechanism against pathogens since the cross linking of cell wall polymers diminishes their ability to enzymatically digest the cell wall and thus isolates the pathogen in a confined area (Bradley et al., 1992; Passardi et al., 2005). Kumquat POD activity tripled 1 day after inoculation with *Xcc* and continued to increase to 8 days after inoculation (Kumar et al., 2011c). No canker development occurred beyond the initial infection zone as evidenced by water soaking upon injection indicating isolation of the bacteria consistent with activity of the Class III PODs. No up-regulation of POD has been shown for kumquat, but transcriptional analysis has shown up-regulation of POD genes in sweet orange leaves 2 days after inoculation with *Xcc* (Cernadas et al., 2008).

In addition to cross linking cell walls using H_2O_2, Class III PODs are capable of catalyzing reactions utilizing other substrates (Passardi et al., 2005). PODs can convert $O_2^{\cdot-}$ and H_2O_2 to $OH^{\cdot}$ (Schweikert et al., 2000; Schopfer et al., 2002; Liszkay et al, 2003), however, apoplastic generation of $O_2^{\cdot-}$ has not been definitively determined in kumquat-*Xcc* interactions. A potential source of $O_2^{\cdot-}$ is by NADPH oxidase activity (Kasai *et al.,* 2006), which is generally regarded as a critical component of plant defense (Lamb and Dixon, 1997), but that enzyme has not been studied in kumquat exposed to *Xcc*. Any apoplastic SOD activity would de-activate $O_2^{\cdot-}$. One SOD reported to be located in plant apoplasts is Cu-Fe-SOD (Alscher et al., 2002) and in kumquat infected with *Xcc*, a putative Cu-Fe-SOD gene was up-regulated 2 to 7 days after inoculation (Khalaf et al., 2007), however activity of this SOD isoform was not detected (Kumar et al., 2011e). Mn-SOD was also suggested to be involved in cell elongation (Kukavica et al., 2009), which is one of the early events during canker development (Khalaf et al., 2007). Kukavica et al. (2009) proposed a novel role for cell wall bound Mn-SOD that assists in POD-mediated cell elongation by producing $OH^{\cdot}$ in the apoplast. Although the

formation of $OH^{\cdot}$ during kumquat-*Xcc* is not verified, its formation is consistent with plant defense considering its high toxicity to *Xanthomonas spp*. (Vattanaviboon and Mongkolsuk, 1998). Nevertheless, production of $O_2^{\cdot -}$ and conversion of it plus H_2O_2 to $OH^{\cdot}$ in kumquat-*Xcc* interactions needs to be determined.

In summary, kumquat respond to *Xcc* by promoting higher concentrations of H_2O_2 through temporal and qualitative changes in enzymes involved in its synthesis and dismutation. H_2O_2 is produced initially through increased chloroplastic SOD 1 day after inoculation and thereafter through increased mitochondrial and peroxisomal SOD activity. Elevated symplastic H_2O_2 concentrations are maintained by declining APOD and later CAT activity. We propose that the elevated concentration of H_2O_2 diffuses from the symplast to the apoplast where it directly inhibits bacterial metabolism and utilized by POD. The higher POD activity presumably utilizes H_2O_2 to cross-link cell walls and perhaps produce highly toxic $OH^{\cdot}$.

7. Oxidative metabolism in canker susceptible grapefruit and sweet orange

Using the same strain of Asiatic canker, infiltration method, and under the same growing conditions as in kumquat (Kumar et al., 2011c,e), the bacterial population in grapefruit and sweet orange leaves grew to 1 x 10^9 CFU/cm^2 (Kumar et al., 2011b,d), which was 10x that of kumquat (Kumar et al., 2011e). In general, the responses of grapefruit and sweet orange to *Xcc* were similar. Whereas the *Xcc* population peaked in kumquat 4 days after inoculation, the population peak occurred 8 days after inoculation in grapefruit (Figs. 1 and 3) and 14 days after inoculation in sweet orange. Chlorosis was evident in grapefruit and sweet orange by the first day after inoculation as in kumquat. However water soaking, which didn't occur until 4 days after inoculation in kumquat, occurred by the second day in grapefruit and sweet orange. Furthermore, swelling of the leaves in the inoculated region was evident starting 6 days after inoculation. Necrosis was evident from 16 to leaf abscission, which occurred a week later than kumquat.

Unlike H_2O_2 concentrations in kumquat that increased and remained high until *Xcc* populations declined, H_2O_2 concentrations in grapefruit and sweet orange leaves demonstrated a biphasic pattern. There was an initial surge in H_2O_2 concentration in both susceptible genotypes to that found in kumquat except it was only to 1/3 the concentration and the surge only lasted until 4 days after inoculation (Kumar et al., 2011b,d). H_2O_2 concentrations declined to or below the controls and then surged a second time but only to the same concentrations or to concentrations slightly above the controls from 12-14 days after inoculation. The crash in H_2O_2 concentration occurred very late in the log phase of bacterial growth, the stage most susceptible to H_2O_2 (Tondo et al., 2010), which allowed extension of that phase resulting in the higher bacterial populations compared to kumquat.

The disturbance in H_2O_2 concentration was related to temporal and qualitative changes in enzyme activities related to H_2O_2 metabolism. Total SOD activity in grapefruit and sweet orange generally followed that of H_2O_2 concentration with a peak in activity occurring 4 days after inoculation followed by a rapid decline with concentrations similar to or less than the controls for the rest of the infection process (Kumar et al., 2011b,d). The initial increase in total SOD activity was due to a surge in Fe-SOD activity similar to that of kumquat. Three

Fe-SOD isoforms were detected in both infected and control leaves of grapefruit, but it was Fe-SOD 2 that contributed most of the Fe-SOD activity observed. Down regulation of *Fe-Sod1*transcription were observed in *Botrytis cinerea* infected cultured cells of *Pinus pinaster* (Azevedo et al., 2008), but whether this gene is involved in *Xcc*-susceptible citrus genotypes is unknown.

Manganese superoxide dismutase activity surged in a manner similar to kumquat but then crashed to concentrations similar to the controls by 4 days after inoculation (Kumar et al., 2011b,d). Thus the decline in H_2O_2 concentration in grapefruit and sweet orange was due in part to suppression of Mn-SOD activity. Four Mn-SOD isoforms were observed in grapefruit (Kumar et al., 2011d). Mn-SOD 3 was constitutively active however Mn-SOD 1 and 2 were higher from 2 and 4 days after inoculation but thereafter gradually disappeared. It appears then that the appearance of Mn-SOD 1 and 2 are originally promoted in response to *Xcc* infection, but response dissipates later in the infection process. A weakly stained Mn-SOD 4 was observed at 10 days after inoculation and appeared to be a last attempt by the host to generate more H_2O_2 to suppress *Xcc* or as part of PCD in the infected zone (Vattanaviboon and Mongkolsuk, 1998).

In addition to changes in activities of the various SODs, H_2O_2 degrading enzymes also demonstrated temporal and qualitative changes in activity (Kumar et al., 2011b,d). Catalase activity increased above the control in grapefruit starting 2 days after inoculation and remained up the control peaking 16 days after inoculation, which is opposite of kumquat where CAT activity was suppressed (Kumar et al., 2011b). Four CAT isoforms were detected in controls and six in *Xcc*-infected grapefruit, with CAT 4 and 5 novel in the latter plants and the intensity of the CAT 2 and 4 bands very high compared to the controls. Higher expression of CAT 2 mRNA in roots of potato was found during pathogenesis of *Corynebacterium sepedonicum* NCPPB 2137 and *Erwinia cartovora* spp. *cartovora* NCPPB 312 and provide the first evidence that class II CAT isoforms are also pathogen induced (Niebel et al., 1995). Thus the elevated CAT activity in grapefruit partially explains the decline in H_2O_2 concentrations in grapefruit.

Unlike kumquat where APOD activity was suppressed in *Xcc*-infected plants, APOD activity in grapefruit increased 4 days after inoculation and remained higher than the controls up to 16 days after inoculation (Kumar et al., 2011b). Like CAT, the higher APOD activity contributed to the lower H_2O_2 concentrations.

The class III POD activity levels were higher in *Xcc*-infected grapefruit and sweet orange leaves 1 days after inoculation (Kumar et al., 2011b,d), which was similar to that in kumquat. Three isoforms (POD 1, 2 and 3) were detected in control and infected leaves of both genotypes with higher intensity of all three bands in infected tissues. In a separate study of *Xcc* infected sweet orange, POD genes were shown to be up-regulated as early as 6 hours after inoculation (Cernadas *et al*., 2008). More than 70 isoforms of PODs have been identified in plants and it is currently difficult to assign a physiological function to each one due to gene redundancy (Sasaki et al., 2004). Nevertheless, it is interesting that unlike CAT and APOD where there was a differential response in susceptible (grapefruit and sweet orange) and resistant (kumquat) genotypes, POD activity in all three genotypes increased in response to *Xcc*.

8. Proposed model of citrus response to canker

A comparison of *Xcc* population, symptom development, H_2O_2, and activities of enzymes involved in H_2O_2 metabolism between the resistant genotype kumquat and a susceptible genotype such as grapefruit can reveal deficiencies in susceptible genotypes. Although similar concentrations of *Xcc* were injected in leaves of both genotypes, the population was 10x less in kumquat than grapefruit by 3 days after inoculation and remained substantially lower. Activity of chloroplastic Fe-SOD, an organelle that is presumed to be involved in pathogen sensing and signaling, increased 1 day after inoculation in kumquat but 2 days after inoculation in grapefruit, which indicates a delayed response in the latter genotype. The reduced *Xcc* population in kumquat compared to grapefruit was due, in part, to lower H_2O_2. Although H_2O_2 increased in both species upon infection, it was only 1/3 the concentration in grapefruit than kumquat at its peak 5 days after inoculation. The sustained H_2O_2 concentration in kumquat was due to higher and sustained Mn-SOD activity and lower CAT and APOD activities. In grapefruit, however, CAT increased 1 day after inoculation, APOD increased 3 days after inoculation, and Mn-SOD declined 5 days after inoculation. There are reports which showed that *Xanthomonas spp*. are naturally very resistant to $O_2^{\cdot-}$ but are susceptible to H_2O_2 (Loprasert et al., 1996; Tondo et al., 2010). Thus, although SOD activity was enhanced in grapefruit, the H_2O_2 was subsequently degraded by enhanced activities of CATs and APODs.

Watersoaking developed earlier in grapefruit (2 days after inoculation) than kumquat (4 days after inoculation). Water soaking is a characteristic symptom of *Xcc* infection in citrus that is caused in part by increased uptake of water through capillary action as a consequence of loss of intercellular space between rapidly dividing and enlarging mesophyll cells (Khalaf et al., 2007; Popham et al., 1993). The earlier watersoaking of grapefruit and the higher raised epidermis is indicative of increased cell growth in this genotype, which was reflected in the observed raising of epidermis compared to kumquat. It is interesting that POD activity in both genotypes was elevated upon *Xcc* infection. Peroxidase serves a dual role of promoting cell enlargement by loosening the cell wall but is also involved in cross-linking of cell wall components during cell maturation, a process that inhibits cell enlargement (Passardi et al., 2004). Which process that occurs would be substrate dependent and would vary temporally and spatially. Such a temporal and spatial variation in POD activity has been shown to occur during cell growth of *Arabidopsis thaliana* leaves where cell enlargement was promoted early and cell wall stiffening occurred later (Abarca et al., 2001). The changes in CAT, APOD and Mn-SOD that lowered H_2O_2 concentrations in grapefruit preceded the raised epidermis and thus it is reasonable to assume that the concentrations of H_2O_2 were necessary to promote cell enlargement in this genotype, whereas the higher concentrations of H_2O_2 that occurred in kumquat were excessive and involved in suppression of *Xcc*. Thus, we propose that the lower H_2O_2 concentrations in grapefruit promoted plant cell growth whereas the higher H_2O_2 concentrations in kumquat were involved in cross linking of cell wall polymers and possibly the production of $OH^{\cdot}$. Solutions to solving *Xcc* in susceptible citrus genotypes such as grapefruit and sweet orange will need to include promoting earlier, higher, and sustained H_2O_2 concentrations.

The comparative studies of oxidative metabolism in susceptible and resistant genotypes to *Xcc* have identified deficiencies in susceptible genotypes. Altering their response either through exogenous applications of chemicals that evoke systemic acquired resistance and

induced systemic resistance or through genetic modification should be a focus of future research. In particular, stimulation of Mn-SOD activity, which is important for sustained production of H_2O_2, and suppression of CAT and APOD activity to maintain high concentrations of H_2O_2 in susceptible genotypes should improve resistance to *Xcc*. Strategies that improve H_2O_2 metabolism to enhance resistance should provide new cultural management approaches in commercial groves for reducing the economic impact of this disease.

	Xcc			H_2O_2		Enzyme activity[x]											
	Population[z]	Symptom[y]				Total-SOD		Fe-SOD		Mn-SOD		CAT		APOD		POD	
dai	K/G	K	G	K	G	K	G	K	G	K	G	K	G	K	G	K	G
0	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
1	↔	C	C	↑	↑	↑	↑	↑	↔	↔	↑	↔	↔	↓	↔	↑	↑
2	↔	C	C,W	↑	↑	↑	↑	↔	↑	↑	↑	↔	↑	↓	↔	↑	↑
3	↓	C	C,W	↑	↑	↑	↑	↔	↑	↑	↑	↔	↑	↓	↑	↑	↑
4	↓	C,W	C,W	↑	↑	↑	↑	↔	↑	↑	↑	↔	↑	↓	↑	↑	↑
5	↓	C,W	C,W	↑	↔	↑	↔	↓	↑	↑	↔	↔	↑	↓	↑	↑	↑
6	↓	C,W	C,W,E	↑	↓	↑	↔	↓	↔	↑	↓	↓	↑	↓	↑	↑	↑
7	↓	C,W	C,W,E	↑	↓	↑	↓	↓	↓	↑	↓	↓	↑	↓	↑	↑	↑
8	↓	C,W	C,W,E	↑	↓	↑	↓	↓	↓	↑	↓	↓	↑	↓	↑	↑	↑
9	↓	C,W	C,W,E	↑	↓	↑	↓	↓	↓	↑	↓	↓	↑	↓	↑	↑	↑
10	↓	C,W,N	C,W,E	↑	↓	↑	↓	↓	↓	↑	↓	↓	↑	↓	↑	↑	↑
11	↓	C,W,N	C,W,E	↑	↓	↑	↓	↓	↓	↑	↔	↓	↑	↓	↑	↑	↑
12	↓	C,W,N	C,W,E	↑	↔	↑	↔	↓	↔	↑	↔	↓	↑	↓	↑	↑	↑
13			C,E		↔		↔		↔		↔		↑		↑		↑
14			C,E		↔		↔		↔		↓		↑		↑		↑
15			C,E		↓		↔		↓		↓		↑		↑		↑
16			C,E,N		↓		↔		↔		↓		↑		↑		↑
17			C,E,N		↓		↔		↔		↓		↑		↑		↑
18			C,E,N		↔		↔		↔		↓		↔		↔		↑
19			E,N		↔		↔		↔		↓		↔		↔		↑
20							↓		↓		↓		↔		↔		↑

z Populatin concentrations are shown as the ratio of kumquat and grapefruit

y Symptom classification: C= chlorosis, W= watersoaking, E= raised epidermis, N= necrosis

x Enzyme classification: SOD= superoxide dismutase and their various forms as indicated by their metal cofactor, CAT= catalase, APOD= ascrobate peroxidase, and POD= the class III peroxidase

xThe arrows indicate the ratio in Xcc population between kumquat and grapefruit

Fig. 1. Comparison of *Xcc* population, canker symptoms, H_2O_2, and activities of enzymes involved in H_2O_2 metabolism for kumquat (K) and grapefruit (G) by days after inoculation (dai). Arrows for H_2O_2 and enzyme activities indicate a comparison of *Xcc*-infected to uninfected leaves. Data were taken from Kumar et al., 2011b,c,d,e.

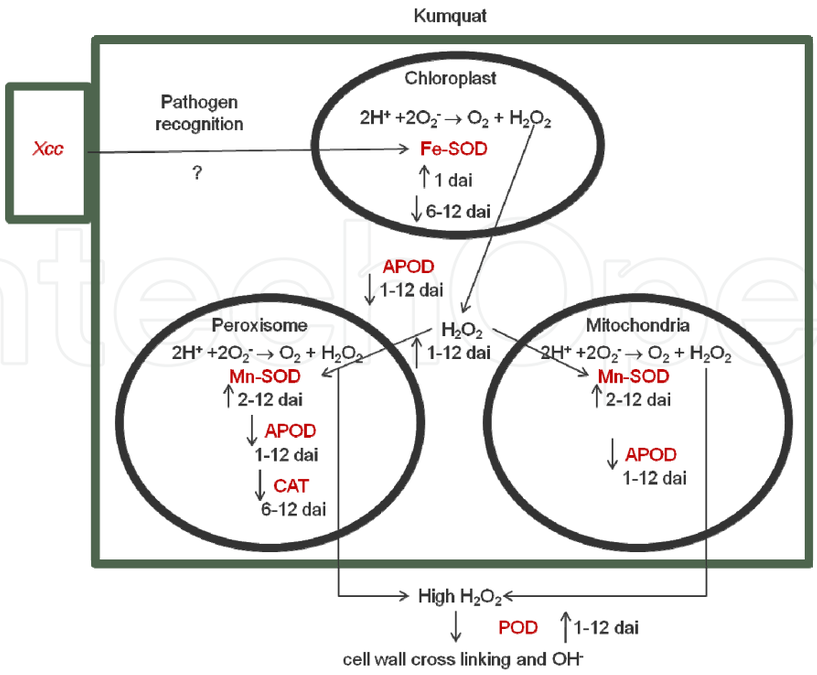

Fig. 2. Proposed mechanism of oxidative metabolism that promotes disease resistance in kumquat. Changes in enzyme activities and H_2O_2 concentration taken from Kumar et al. 2011c,e.

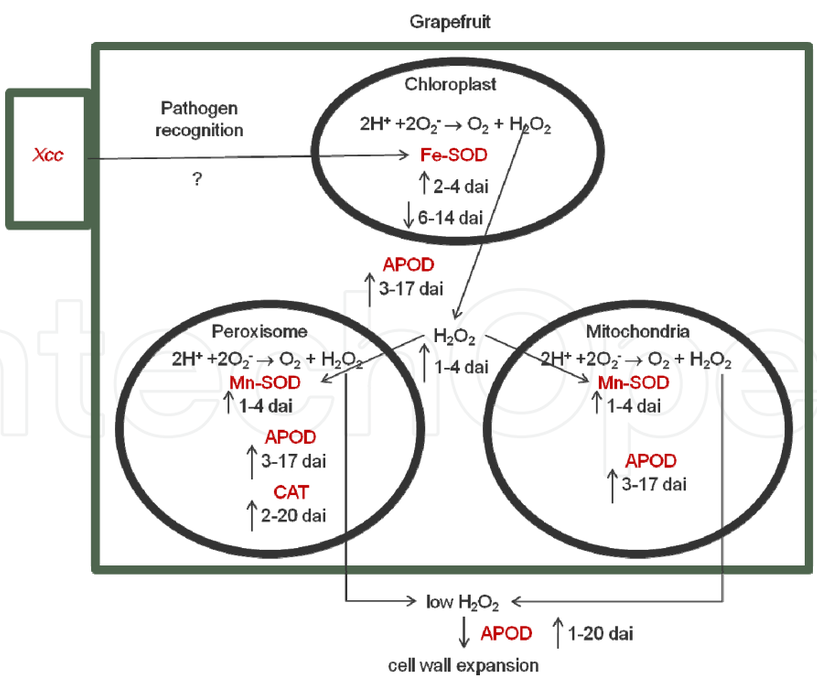

Fig. 3. Proposed mechanism of oxidative metabolism in grapefruit that promotes population growth of *Xcc*. Changes in enzyme activities and H_2O_2 concentration taken from Kumar et al. 2011b,d.

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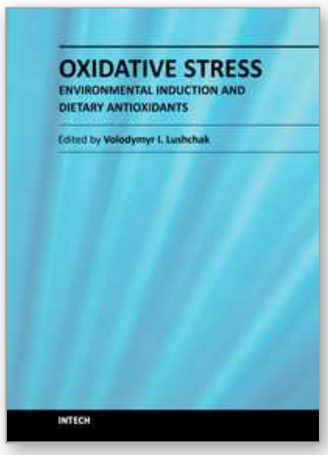

Oxidative Stress - Environmental Induction and Dietary Antioxidants

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This book focuses on the numerous applications of oxidative stress theory in effects of environmental factors on biological systems. The topics reviewed cover induction of oxidative stress by physical, chemical, and biological factors in humans, animals, plants and fungi. The physical factors include temperature, light and exercise. Chemical induction is related to metal ions and pesticides, whereas the biological one highlights host-pathogen interaction and stress effects on secretory systems. Antioxidants, represented by a large range of individual compounds and their mixtures of natural origin and those chemically synthesized to prevent or fix negative effects of reactive species are also described in the book. This volume will be a useful source of information on induction and effects of oxidative stress on living organisms for graduate and postgraduate students, researchers, physicians, and environmentalists.

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