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Alternaria Pathosystems for Study of Citrus Diseases

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ABSTRACT

Leaf spot disease of rough lemon and brown spot disease of tangerines caused by distinct pathotypes of *Alternaria alternata*, and postharvest fruit black rot disease caused by *A. citri*, are the three major fungal diseases of citrus. The pathogenicity and specificity for the pathogens of Alternaria leaf spot and brown spot diseases are associated with the production of different host-specific toxins designated as ACR- and ACT-toxins. Production of endopolygalacturonase plays a role in causing disease symptoms of Black rot pathogen of *A. citri*. This review summarizes the recent progress on these citrus pathomodels.

Keywords: citrus defense, endopolygalacturonase, host-selective toxin, rough lemon
Abbreviations: endoPG, endopolygalacturonase; **GFP**, green fluorescent protein; **HR**, hypersensitive reaction; **HST**, host-selective toxin; **ORF**, open reading frame; **PR-protein**, pathogenesis-related protein

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INTRODUCTION

Although *Alternaria alternata* (Fries) Keissler is generally known as a saprophytic fungus, several types of *A. alternata* can be pathogenic to leaves and/or fruit of many citrus cultivars. There are three major *Alternaria* species known to be citrus pathogens, and the diseases caused by these pathogens include Alternaria leaf spot of rough lemon, Alternaria brown spot of tangerines, and Alternaria black rot of many citrus fruits, respectively. Since *Alternaria* species are difficult to distinguish morphologically, biological, biochemical and genetic aspects of determinations including pathogenicity tests, toxin assays or use of genetic markers, are required to distinguish between species of this fungus.

Alternaria leaf spot of rough lemon was first reported from South Africa (Doidge 1929). Alternaria black rot was reported as early as the 1900s in California (Pierce 1902; Roger and Earle 1917; Bartholomew 1926; Bliss and Fawcett 1944) and the disease is also called stem-end rot (e.g. Roger and Earle 1917; Bartholomew 1926; Brown and McCornack 1972). Alternaria brown spot disease was first reported on 'Emperor' mandarin in Australia in 1903 (Cobb 1903) and the pathogen was identified as *Alternaria* species (Kiely 1964; Pegg 1966). The disease later appeared in the USA (Whiteside 1976), and now occurs in many places in the world, including Israel (Solel 1991), South Africa (Schutte *et al*. 1992), Turkey (Canihos *et al*. 1997), Spain (Vicent *et al*. 2000), Brazil and Argentina (Peres *et al*. 2003). Due to the morphological similarities between the pathogens of brown spot and black rot, the pathogen of brown spot was once identified as *A. citri* Ellis & Pierce (Pegg 1966; Whiteside 1976), the same fungus that causes citrus black rot. More recently, the pathogen which affects tangerines has been classified as *A. alternata* (Kohmoto *et al*. 1979) following designation of the agent as *A. alternata* pv. *citri* (Solel 1991). Ten species of *Alternaria* were defined based on the branching patterns of conidial chains (Simmons 1999). Phylogenetic studies based on sequence data from genes encoding several different genes did not find molecular genetic evidences for differentiation of these 10 morphospecies (Peever *et al*. 2004). Despite the morphological and genetic similarity of citrus-associated *Alternaria* isolates, these pathogens were designated all as *A. alternata* by examination of different host-selective toxins (HSTs) production (Kohmoto *et al*. 1979, 1991) which will be referred to subsequently as the tangerine and rough lemon pathotypes (**Fig. 1**). HSTs can be defined as a group of chemically diverse and complex metabolites produced by plant pathogenic strains of certain fungal species, and they are essential determinants of pathogenicity or virulence (Yoder 1980; Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996).

In this review we will provide a brief overview of the biology of the diseases caused by these fungi and finally a discussion of the molecular biology and biochemistry of the interactions between *A. alternata* and citrus. The direct delivery of HST produced by *A. alternata* into the toxin-sensitive citrus causes mitochondrial dysfunction following cell

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Invited Review

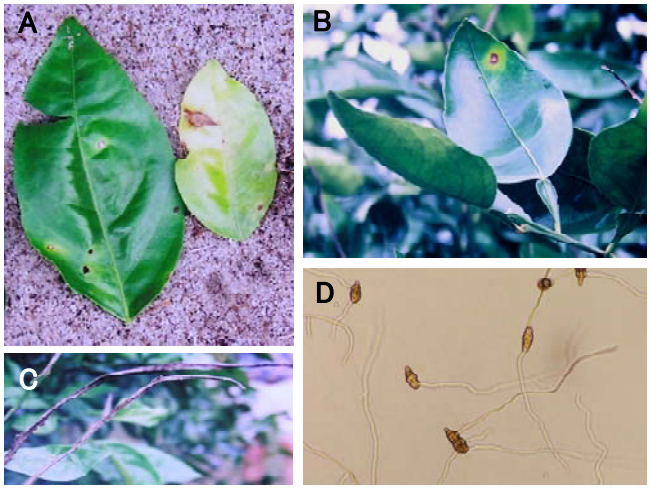

Fig. 1 Typical symptoms of Alternaria leaf spot and brown spot diseases in citrus. Brown spot appears as distinct brown lesions surrounded by a yellow halo for both Alternaria leaf spot (**A**) and Alternaria brown spot (**B**) diseases. Affected leaves often abscise and infected twigs die back, especially if the leaves have fallen (**C**). Pathogens causing these diseases are the different pathotypes of *A. alternata* (**D**: *A. alternata* rough lemon pathotype) and morphologically difficult to distinguish without examination of respective host ranges and HST produced by each pathogen.

death, but the same molecule could also act as an elicitor to the toxin-insensitive citrus by transcriptional induction of genes related to plant defense responses.

ALTERNARIA LEAF SPOT OF ROUGH LEMON

Alternaria leaf spot affects only rough lemon (*Citrus jambhiri*) and rangpur lime (*C. limonia*, a hybrid of acid mandarin and rough lemon), two common rootstocks in some citrus-growing areas. Thus, this disease is only important in nurseries and seed production blocks. Symptoms on leaves are very similar to those produced on tangerines by the pathogen of Alternaria brown spot disease (Timmer *et al*. 2000). The HST produced by this pathotype is different in chemical structure from the HST produced by the tangerine pathotype (Kohmoto *et al*. 1979), and is called ACR-toxin or ACRL-toxin (Gardner *et al*. 1985; Nakatsuka *et al*. 1986a). Symptoms do not appear on rough lemon leaves for about 2 to 3 days following inoculation compared to within 24 hr for the brown spot disease. Symptoms on fruit are merely small brown specks (Timmer *et al*. 2000) and are quite reduced relative to the tangerine pathotype.

MECHANISMS OF SPECIFICITY TO ACR-TOXIN IN CITRUS CELLS

The fungal genera of *Alternaria* and *Cochliobolus* have been known to produce HSTs of low molecular compounds (Yoder 1980; Nishimura and Kohmoto 1983; Scheffer and Livingston 1984; Walton 1996). HSTs have high specificity and toxicity, and induce cell death at 10^{-9} to 10^{-8} M in susceptible plants while even 10^{-12} M of toxin does not cause cell death to resistant plants.

We have identified the mechanism of ACR-toxin sensitivity in rough lemon (Akimitsu *et al*. 1989; Ohtani *et al*. 2002). ACR-toxin is an HST produced by the *A. alternata* rough lemon pathotype, and citrus cultivar rough lemon and rangpur lime are known to be sensitive to the toxin and susceptible to its producer (Kohmoto *et al*. 1991; Akimitsu *et al*. 2003). The target site of ACR-toxin was identified as the mitochondrion by electron microscopic examination (Kohmoto *et al*. 1984) and the monitoring of an oxidative phosphorylation using isolated physiologically active mitochondria (Akimitsu *et al*. 1989). ACR-toxin caused uncoupling of the mitochondrial oxidative-phosphorylation similar to classic protonophores, such as 2,4-Dinitrophenol (DNP) or Carbonyl Cyanide M-Chloro Phenyl Hydrazone (CCCP), with a loss of membrane potential, but the effects differed slightly from other uncouplers because the toxin also causes leakage of co-factor, NAD^+ from the TCA cycle (Akimitsu *et al*. 1989). These effects of ACR-toxin are specific to mitochondria isolated from susceptible cultivars. Mitochondria from 'Dancy' tangerine, 'Emperor' mandarin, and grapefruit, which are susceptible to ACT-toxin, are completely insensitive to ACR-toxin (Akimitsu *et al*. 1989). These responses of citrus mitochondria to ACR-toxin are very similar to those of mitochondria from Texas cytoplasm male-sterile (T-cms) maize to HMT-toxin, a HST from *C. heterostrophus* race T. Both ACR and HMT-toxins are structurally similar, consisting of long-chain fatty acid-like polyketides, and having similar polyol moieties (Kono and Daly 1979; Gardner *et al*. 1985; Nakatsuka *et al*. 1986a). The mode of action of these toxins is very similar to that of the respective host mitochondria. However, both toxins are highly selective for the respective hosts, and HMT-toxin has no toxic or protective effects on leaf tissue of or mitochondria from rough lemon (Akimitsu *et al*. 1989). Conversely, ACR-toxin has no effect on T-cms maize (Akimitsu *et al*. 1989).

The source of the gene that confers sensitivity of *Citrus* species to ACR-toxin has recently been identified from the mitochondrial genome of rough lemon (*C. jambhiri*) (Ohtani *et al*. 2002). The gene confers ACR-toxin sensitivity to *Escherichia coli*, and the mechanism of specificity in plants is alternative transcript processing of the gene conferring ACR-toxin sensitivity (**Fig. 2**) (Ohtani *et al*. 2002). Mitochondrial DNA was isolated from rough lemon, and random *Bam*HI fragments were expressed in *E. coli*, which is normally resistant to ACR-toxin. Addition of the toxin at minimum concentration of 50 nM completely abolished oxygen uptake by toxin-sensitive *E. coli* strain J104 (Ohtani *et al*. 2002). The plasmid in toxin-sensitive *E. coli* strain J104 that conferred sensitivity to ACR-toxin contained a 355-bp insert, named *ACRS* (ACR-toxin Sensitivity gene), and a search of the non-redundant databases identified that the region is located within the intron of the tRNA-Ala gene. This intron, known as a self-splicing group II intron, catalyzes its own splicing (e.g., Leaver and Gray 1982), and many of these introns have been reported to contain open reading frames (ORFs) encoding polypeptides (e.g., Fassbender *et al*. 1994).

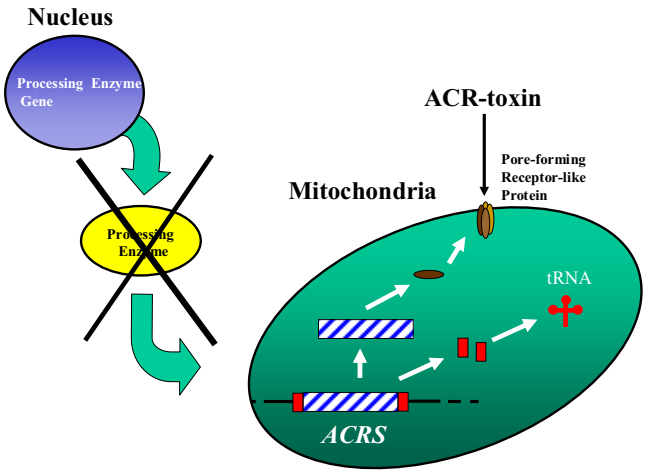

Fig. 2 The mechanism of specificity of ACR-toxin in citrus is alternative transcript processing of the gene (*ACRS*) conferring ACR-toxin sensitivity. *ACRS* (ACR-toxin Sensitivity gene) is located within the intron of the tRNA-Ala gene. Sensitivity to the toxin was not controlled by the presence or absence of the *ACRS* but rather by post-transcriptional modification (= processing) of the *ACRS* RNA (Ohtani *et al*. 2002). *ACRS* transcripts were translated to the oligomeric pore-forming transmembrane protein controlling ACR-toxin sensitivity, and lack of the translation leads insensitivity to the toxin. Based on Ohtani *et al*. (2002).

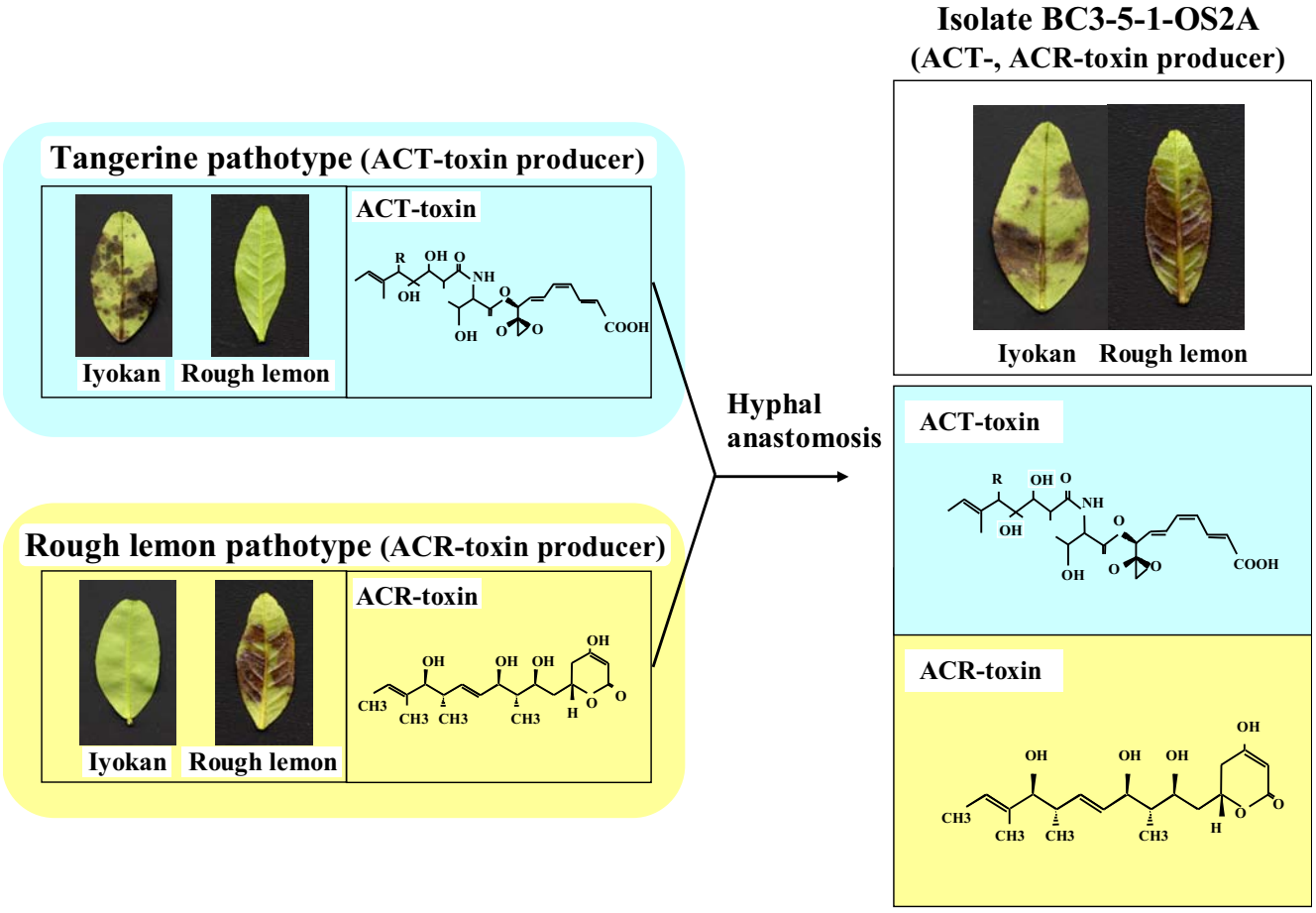

Fig. 3 A field isolate producing two HSTs, ACT-and ACR-toxins, has both gene clusters responsible for the biosynthesis of the two HSTs. A dispensable chromosome carrying the gene cluster controlling biosynthesis of one of the HSTs might be transferred horizontally by hyphal anastomosis and rearranged by duplication and/or translocation in another isolate of the fungus carrying genes for biosynthesis of the other HST. Based on Masunaka *et al.* (2005).

To investigate the relationship of *ACRS* to sensitivity to ACR-toxin and hence susceptibility to *A. alternata* rough lemon pathotype, we sequenced this DNA region of the mitochondrial genome from resistant cultivars and species of citrus, including citron (*C. limonimedica*), grapefruit (*C. paradisi*), Iyokan (*C. iyo*), lemon (*C. limon*), lime (*C. latifolia*), mexican lime (*C. aurantifolia*), navel orange (*C. sinensis*), satsuma mandarin (*C. unshiu*), trifoliate orange (*Poncirus trifoliate*), trovita orange (*C. sinensis*), rangpur lime (*C. limonia*), yuzu (*C. junos*) and volkamer lemon (*C. volkameriana*), and found that the regions in the resistant citrus were identical to that of rough lemon (*C. jambhiri*) (Ohtani *et al*. 2002). However, examination of transcripts of *ACRS* demonstrated that the sensitivity to the toxin was not controlled by the presence or absence of the *ACRS* but rather by post-transcriptional modification (= processing) of the *ACRS* RNA transcript (Ohtani *et al*. 2002). The peptide encoded by the *ACRS* was detected by immunoblotting only in rough lemon mitochondria, but not in other toxin-insensitive citrus mitochondria, and the peptide appeared to consist of SDS-resistant oligomers that have been reported for many pore-forming transmembrane proteins (Ohtani *et al*. 2002). The known physiological effects of ACR-toxin are consistent with it forming pores in membranes, because ACR-toxin-treated mitochondria show not only increased permeability to protons, but also to NAD+ (Akimitsu *et al*. 1989). Several series of deletion experiments and examination of *ACRS* transcripts revealed that the putative ORF region required for the translation to the oligomeric pore-forming transmembrane protein controlling ACR-toxin sensitivity is predicted as 171-bp, and the calculated molecular weight of the product from the ORF is 6683 (Ohtani *et al*. 2002). Since the *ACRS* antibodies detected three proteins with molecular weights of 14, 21 and 28 kDa in extracts from rough lemon mitochondria, the proteins detected by the immunoblotting could be the dimer, trimer, and tetramer that are not fully dissociated during SDS-PAGE.

The effect of ACR-toxin is specific to the disruption of mitochondrial functions of rough lemon, following a shortage of ATP supply from mitochondria, and ends up with cell death. This host-selective cell death by the toxin has a similarity to programmed cell death caused by the hypersensitive reaction (HR). However, we think the cell death caused by ACR-toxin is different from that caused by HR, because HR is usually accompanied by several reactions of defense for plant resistance, while ACR-toxin causes suppression of defense-related genes such as lipoxygenase (*RlemLOX*), chalcone synthase, chitinase (*RlemAchi*) hydroperoxide lyase (*RlemHPL*), polygalacturonase-inhibiting protein (*RlemPGIP*) and allene oxide synthase (Gomi *et al*. 2002a, 2002b; Gotoh *et al*. 2002 Gomi *et al*. 2003b) to establish susceptibility to the toxin-producing *A. alternate* (**Fig. 3**). It is still not clear at this point whether the suppression of defense is through the mitochondrial disruption by ACR-toxin or whether ACR-toxin has some other mode(s) of action to regulate the transcription of such genes. However, since the only known action of ACR-toxin so far is on the mitochondria, the development of a study on communications between the organelle and nuclei may solve, in the near future, this cross talk function to seal the fate of cells attacked by a pathogen secreting ACR-toxin.

ALTERNARIA BROWN SPOT OF TANGERINES

The tangerine pathotype of *A. alternata* affects many tangerines and hybrids (Timmer *et al*. 2000). The disease produces minute brown to black spots on young leaves and fruit. Symptoms can appear in as little as 24 hr after infec-

tion. Lesions usually continue to expand and large areas of the leaf may be killed by the host-selective ACT-toxin (Kohmoto *et al*. 1993), even without tissue colonization. Chlorosis and necrosis can extend along the veins as toxin is translocated upward. On mature leaves, brown spot appears as distinct brown lesions surrounded by a yellow halo (**Fig. 1**). Affected leaves often abscise and infected twigs die back, especially if the leaves have fallen. On fruit, lesions can vary from minute spots to large crater-like lesions (**Fig. 1**). Corky eruptions sometimes form and can be dislodged forming a pockmark on the surface. Severely affected fruit abscise reducing yield and blemishes on the remaining fruit diminishes marketability greatly (Timmer *et al*. 2000; Akimitsu *et al*. 2003).

ACT-TOXIN BIOSYNTHESIS GENE CLUSTER ANALYSIS

The tangerine pathotype of *A. alternata* causes Alternaria brown spot disease, which affects many tangerines and their hybrids due to its ability to produce the host-selective ACT-toxin (Kohmoto *et al*. 1979; Kohmoto and Otani 1991; Akimitsu *et al*. 2003). The mode of action of ACT-toxin appears to be complex, but ultrastructural changes of cells treated with the toxin have indicated that the primary site of action is likely the plasma membrane. Toxin at a concentration of 2×10^{-8} M causes veinal necrosis on leaves with a rapid loss of electrolytes from host cells (Kohmoto and Otani 1991; Kohmoto *et al*. 1993).

The structure of ACT-toxin I (Kohmoto *et al.* 1993) was identified in culture filtrates of *A. alternata* tangerine pathotype. Another selective toxin, named ACTG-toxin, was also identified by Kono *et al.* (1986). However, ACT-toxin is considered as the major HST because it is highly toxic and the only major product detected in conidium-germinating fluid (Kohmoto *et al.* 1993). The structure of ACT-toxin is closely related to AK- and AF-toxins, which are the HSTs produced by Japanese pear and strawberry pathotypes of *A. alternata*, respectively (Nakashima *et al.* 1985; Nakatsuka *et al.* 1986a, 1986b; Kohmoto *et al.* 1993). A cluster of genes controlling the biosynthesis of ACT-toxin was identified by using heterologous probes of *AKT* sequences that control biosynthesis of a 9, 10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety in AK-toxin from the Japanese pear pathotype of *A. alternata* (Tanaka *et al.* 1999). Portions of these genes used as heterologous probes detected homologs in several isolates of *A. alternata* tangerine pathotype, but did not detect homologs in isolates that do not produce ACT-toxin, such as the rough lemon pathotype, non-pathogens, or black rot isolates (Masunaka *et al.* 2000). Homologs of two genes responsible for biosynthesis of the decatrienoic acid moiety in the Japanese pear pathotype are also found in the tangerine and strawberry pathotypes (Tanaka *et al*. 1999). The entire sets of genes required for biosynthesis of the decatrienoic acid moiety are homologous among these three fungi. Genes involved in the production of fungal secondary metabolites, e.g. penicillin in *Aspergillus nidulans*, *Penicillium chrysogenum* and various actinomycetes (Aharonowitz *et al*. 1992), trichothecene in *Fusarium* spp. (Hohn *et al*. 1993), lovastatin (Kennedy *et al*. 1999), gibberellins (Tudzynski and Holter 1998), and the ergot alkaloids (Tudzynski *et al*. 1999) are often clustered in the genome (Walton 2000).

Biosynthetic genes controlling the production of HSTs have shown a similar clustering (Ahn and Walton 1996; Tanaka *et al*. 1999; Hatta *et al*. 2002). Using *AKTR* as a heterologous probe (Tanaka and Tsuge 2000), we have identified a contiguous 33,974 bp region of chromosomal DNA from a cosmid clone of the tangerine pathotype SH20 responsible for toxin production. The 33,974 bp region contained five ORFs identified by sequencing both genomic DNA and corresponding cDNA, and we reported the sequence, structure and function of ORFs that encode a putative acyl-CoA synthetase (*ACTT5*), enoyl-CoA hydratase (*ACTT6*) and hydrolase (*ACTT2*) for the purpose of identification of their role in ACT-toxin production and pathogenicity (Miyamoto *et al.* 2008, 2009). Genomic Southern blots demonstrated that these genes were present in tangerine pathotype isolates producing ACT-toxin and also in Japanese pear pathotype isolates producing AK-toxin and strawberry pathotype isolates producing AF-toxin (Masunaka *et al*. 2000; Miyamoto *et al.* 2008, 2009). ACT-, AK-, and AF-toxins from these three pathotypes share a common 9, 10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety. Targeted gene disruption of two copies of *ACTT5* significantly reduced ACT-toxin production and virulence (Miyamoto *et al.* 2009). Targeted gene disruption of two copies of *ACTT6* led to complete loss of ACT-toxin production and pathogenicity and a putative decatrienoic acid intermediate in ACT-toxin biosynthesis accumulated in mycelial mats (Miyamoto *et al.* 2009). These results indicate that *ACTT5* and *ACTT6* are essential genes in ACT-toxin biosynthesis in the tangerine pathotype of *A. alternata* and both are required for full virulence of this fungus (Miyamoto *et al.* 2009).

Including *ACTT5* and *ACTT6*, several genes responsible for ACT-toxin biosynthesis (*ACTT* genes) have often many paralogs that are clustered on a small, conditionally dispensable chromosome making it difficult to disrupt entire functional copies of *ACTT* genes using homologous recombination-mediated gene disruption. To overcome this problem, we attempted to use RNA silencing, which has never been employed in *Alternaria* spp., to knock down the functional copies of one of *ACTT* genes with a single silencing event. *ACTT2*, which encodes a putative hydrolase and is present in multiple copies in the genome, was successfully silenced by transforming the fungus with a plasmid construct expressing hairpin *ACTT2* RNAs (Miyamoto *et al*. 2008). The *ACTT2* RNA-silenced transformant (S-7-24-2) completely lost *ACTT2* transcripts, ACT-toxin production as well as pathogenicity (Miyamoto *et al*. 2008). These results indicated also that RNA silencing may be a useful technique for studying the role of *ACTT* genes with multiple copies responsible for HST biosynthesis in *A. alternata*. Further, this technique may be broadly applicable to the analysis of many genes present in multiple copies in fungal genomes which are difficult to analyze using recombination-mediated knockdown.

Alternaria species are ubiquitous and cosmopolitan saprophytic and pathogenic fungi, and seven distinct pathotypes (pathogenic variants) of *A. alternata* are known that produce HSTs (Kohmoto *et al*. 1979, 1991). Despite the morphological similarity of these pathotypes, it is possible to distinguish them based on host range and this host specificity is due to the production of a particular HST (Kohmoto *et al*. 1979, 1991). A strain of *A. alternata* that is pathogenic to two different hosts and that produces two distinct HSTs, ACT-toxin and ACR-toxin was discovered (Masunaka *et al.* 2005). The quantity of each HST produced by BC3-5-1-OS2A in culture filtrates appears similar to that produced by each pathotype independently. Spray inoculation of BC3-5-1-OS2A on leaves of both ACT-toxin-sensitive and ACR-toxin-sensitive citrus cultivars resulted in necrosis on both cultivars. The dual host specificity and toxin production by BC3-5-1-OS2A is not common in populations of *A. alternata* on citrus. BC3-5-1-OS2A is the only isolate with pathogenicity to two different citrus hosts that has been found among hundreds of isolates examined to date. The expansion of host range may increase the fitness of pathogens because it increases their capacity for infection of and reproduction on a larger number of plant species. However, there may be a fitness cost to this expanded host range that limits the frequency of broad host range isolates like BC3-5-1-OS2A. One hypothesis is that one of the HSTs produced by BC3-5-1-OS2A could act as an elicitor of plant defense responses in toxin-insensitive plants (Masunaka *et al.* 2005). For example, when BC3-5-1-OS2A infects rough lemon leaves, ACR-toxin functions as a pathogenicity factor while at the same time, ACT-toxin might act as an elicitor of defense responses in rough lemon.

Strain BC3-5-1-OS2A from a leaf spot on rough lemon

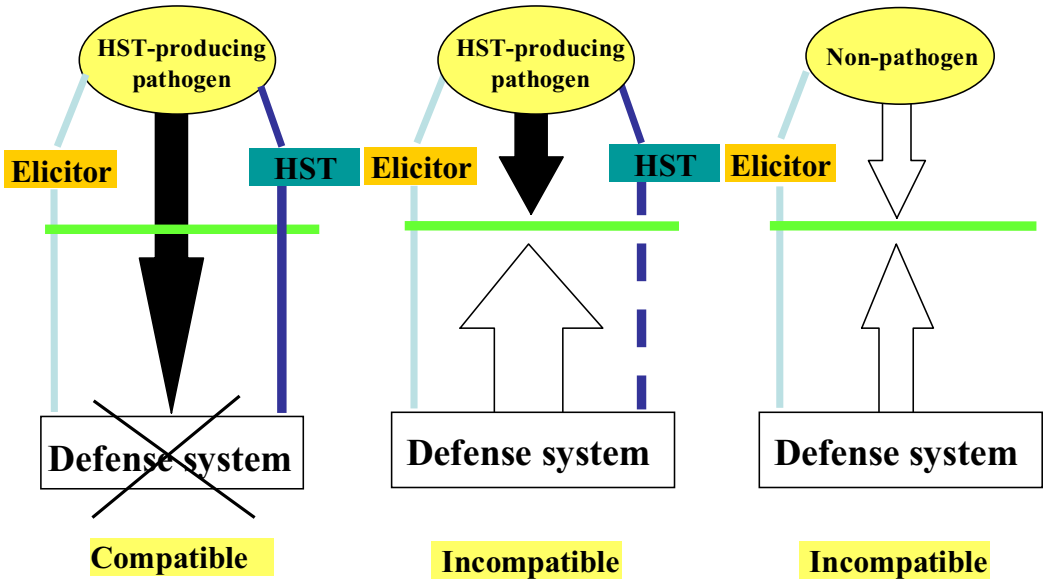

Fig. 4 HSTs have a PAMPs-like role acting as both elicitor and suppressor to plants. HST is a suppressor of the defense mechanisms of plants in a compatible relationship, while HST also works as an elicitation factor of the defense mechanism together with normal elicitors (e.g., chitin/pectin oligomers) in an incompatible relationship. The role of HST in the plant-microbe interactions can be considered as that of PAMPs (Pathogen-Associated Molecular Patterns) in this aspect.

in Florida, and this isolate is pathogenic on both Iyokan and rough lemon and also produces both ACT-toxin and ACR-toxin. BC3-5-1-OS2A carries both the genomic regions that exists one of which was known only to be present in ACT-toxin producers and the other genomic region that was known to exists only in ACR-toxin producers (**Fig. 3**) (Masunaka *et al.* 2005). Each of the genomic regions is present on distinct small chromosomes, one of 1.05 Mb and the other of 2.0 Mb. *Alternaria* species have no known sexual or parasexual cycle in nature and populations of *A. alternata* on citrus are clonal. Therefore, the ability to produce both toxins was likely not to have been acquired through meiotic or mitotic recombination. It was hypothesized that a dispensable chromosome carrying the gene cluster controlling biosynthesis of one of the HSTs was transferred horizontally and rearranged by duplication and/or translocation in another isolate of the fungus carrying genes for biosynthesis of the other HST (**Fig. 3**) (Masunaka *et al.* 2005).

INDUCTION OF CITRUS DEFENSE RESPONSES BY ALTERNARIA LEAF SPOT OF ROUGH LEMON

Defense responses of citrus inoculated with Alternaria leaf spot pathogens have not been examined in any detail to date. We have characterized the expression patterns of several defense-related genes including lipoxygenase (*RlemLOX*) (Gomi *et al*. 2002a), chalcone synthase (Moriguchi *et al*. 1999; Gotoh *et al*. 2002; Nalumpang *et al*. 2002a), polygalacturonase-inhibiting protein (*RlemPGIP1, 2, A* and *B*) (Gotoh *et al*. 2002; Nalumpang *et al*. 2002a, 2002b; Katoh *et al.* 2007a), chitinases (*RlemAchiI* and *RlemAchiII*) (Gomi *et al.* 2002b), hydroperoxide lyase (*RlemHPL*) (Gomi *et al*. 2003b), epoxide hydrolase (*RlemEH*) (Gomi *et al.* 2003a), miraculin-like protein (*RlemMLP1* and *RlemMLP2*) (Tsukuda *et al*. 2006), and non-specific lipid transfer protein (*RlemLTP*) (Nishimura *et al*. 2008) genes in rough lemon in response to the pathogenic and non-pathogenic *Alternaria*. All of these genes are highly inducible in rough lemon leaves by infection with nonpathogenic *A. alternata*, and expression of these genes was detected within 2 hr after wounding or inoculation of rough lemon leaves with conidia of the *A. alternata* tangerine pathotype (isolate SH20), which is pathogenic on tangerines and mandarins but not on rough lemon (Gomi *et al*. 2002a, 2002b; Gotoh *et al*. 2002; Nalumpang *et al*. 2002a, 2002b). Another nonpathogenic strain of *A. alternata* (isolate O-94), which is not pathogenic to any citrus tested (Akimitsu *et al*. 1989; Kohmoto *et al*. 1991), also induced expression of these genes within 2 hr, but the intensity of the bands was not as strong as that induced by SH20. Because there are no apparent morphological or biochemical differences between SH20 and O-94 except that SH20 produces ACT-toxin (Kohmoto *et al*. 1979, 1991, 1993; Masunaka *et al*. 2000), the toxin might have a role as elicitor in the greater accumulation of the transcripts (**Fig. 4**). In contrast, induction of these defense-related genes was delayed or suppressed when pathogenic *A. alternata* rough lemon pathotype (AC325) was inoculated on rough lemon leaves (Fig. 4) (Gomi *et al*. 2002a, 2002b; Gotoh *et al*. 2002; Nalumpang *et al*. 2002a, 2002b). Our system, using either an HST-producing or a non-producing *A. alternata* strain, which led to a clear susceptible or resistant response in rough lemon leaves, may be a good model for further evaluation of the role of other defense-related genes including pathogenesis-related (PR)-proteins. The major form of HST produced by the tangerine pathotype was designated ACT-toxin I (Kohmoto *et al*. 1993). ACT-toxin I and its derivatives are the only major products detected in fluids from germinating conidia (Kohmoto *et al*. 1993). The structure of ACT-toxin is related to AK- and AF-toxins, which are the HSTs produced by the Japanese pear and strawberry pathotypes of *A. alternata*, respectively (Kohmoto *et al*. 1993; Nakashima *et al*. 1985; Nakatsuka *et al*. 1986). These toxins share a common 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid moiety (Kohmoto *et al*. 1993; Nakashima *et al*. 1985; Nakatsuka *et al*. 1986).

BLACK ROT

Black rot affects the central columella of the fruit and can affect all species of citrus (Brown and McCornack 1972). External symptoms are not often apparent and, if present, appear as a small brown to black spots on the stylar end of the fruit (**Fig. 5**) (Brown and Eckert 2000). Affected fruit are more brightly colored than normal fruit due to ethylene generated in response to infection. A wound or a natural crack is required for penetration of the fungus. Although the black rot pathogen has been known to produce several toxins, such as tenuazonic acid, alternariol methyl ether, and alternariol (Logrieco *et al*. 2003), these toxins have no known role in pathogenesis. We have recently demonstrated that the pathogenicity of this pathogen depends upon pro-

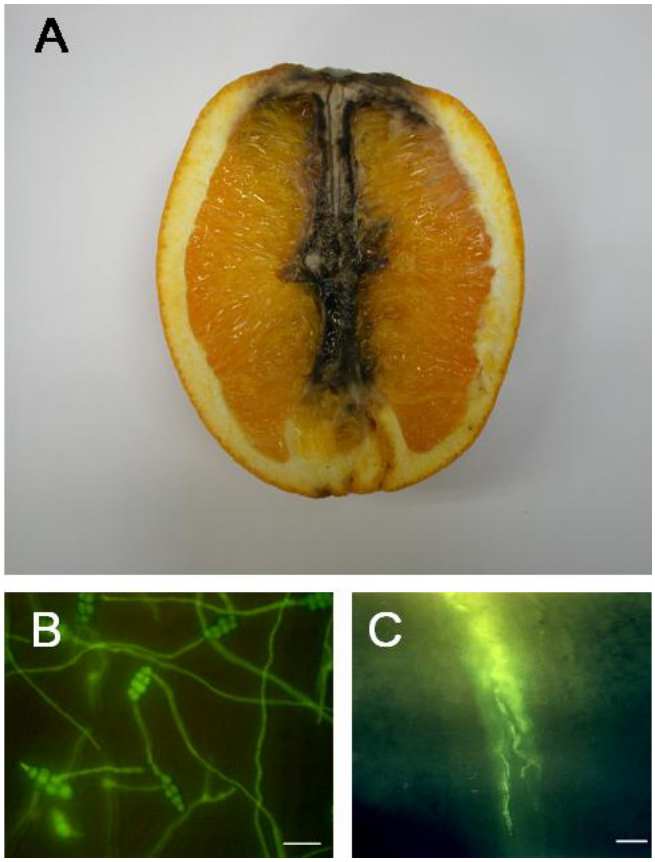

Fig. 5 Symptom of Alternaria black rot disease and observations of the infection through the peel using a GFP transformant of *A. citri*. Black rot affects the central columella of the fruit and can affect all species of citrus (**A**). A transformation plasmid pTEFEGFP carrying a GFP gene was introduced into wild type black rot pathogen, and green fluorescence was observed in spores, germ tubes, appressoria, and infection hyphae of the transformants (**B**) (Isshiki *et al*. 2003). Hyphae of the transformants penetrate the peel through central axis from the pedicel during infection (**C**) (Isshiki *et al*. 2003). **B** and **C** reprinted from **Isshiki A, Ohtani K, Kyo M, Yamamoto H, Akimitsu K** (2003) Green fluorescent detection of fungal colonization and endopolygalacturonase gene expression in the interaction of *Alternaria citri* with citrus. *Phytopathology* **93**, 768-773, ©2003, with kind permission of The American Phytopathological Society.

duction of an extracellular enzyme (endopolygalacturonase, endoPG (E.C.3.2.1.15)) that can degrade pectic polymers in cell walls during the infection stage (Isshiki *et al*. 2001). The plant cell wall is a potential barrier to the penetration and spread of phytopathogenic bacteria and fungi, and many plant pathogens make extracellular enzymes that can degrade cell wall polymers. Cell wall-degrading enzymes and their genes have been studied for their possible role in many aspects of pathogenicity, including penetration, maceration, nutrient acquisition, plant defense induction, and symptom expression (Cooper 1983, 1984; Walton 1994).

For our studies of endoPG from *A. citri*, we purified endoPGs and cloned their genes (*Acpg1* and *Aapg1*) from both a black rot pathogen and a rough lemon pathotype isolate (Isshiki *et al*. 1997, 2001). The sequences of these genes and biochemical characteristics of the enzymes they encode are highly similar. However, when we disrupted these genes in the respective pathogens by gene targeting, the phenotypes of the mutants were completely different (Isshiki *et al*. 1997, 2001). The ability of an endoPG mutant of the black rot pathogen to cause black rot symptoms and penetrate citrus fruits was significantly reduced as well as in the maceration of potato tissue. Moreover, the mutant could not colonize citrus peel segments (Isshiki *et al*. 2001). In contrast, an endoPG mutant of the rough lemon pathotype was unchanged in pathogenicity on rough lemon leaves (Isshiki *et al*. 2001). These results indicated that an endoPG was not required for pathogenicity of the rough lemon pathotype and that endoPG could play different roles in the pathogenicity of two closely related fungi.

To search for other essential factors causing symptoms in addition to endoPG in *A. citri*, a random mutation analysis of pathogenicity was also performed using restriction enzyme-mediated integration. Three isolates among 1694 transformants of *A. citri* showed a loss in pathogenicity in a citrus peel assay, and one of these three mutants was a histidine auxotroph. Gene *AcIGPD* that encodes imidazole glycerol phosphate dehydratase, the sixth enzyme in the histidine biosynthetic pathway, was cloned, and the mutant containing the disrupted target gene, *AcIGPD*, caused less black rot (Katoh *et al.* 2006).

In order to investigate colonization of citrus fruit tissues by the black rot pathogen, a transformation plasmid (pTEFEGFP) carrying a green fluorescent protein (GFP) gene was introduced into wild type black rot pathogen and its endoPG-disrupted mutant M60 (**Fig. 5**) (Isshiki *et al*. 2003). Green fluorescence was observed in spores, germ tubes, appressoria, and infection hyphae of transformants G1 (derived from wild type) and GM4 (derived from M60), but not in the cell walls of spores from both transformants, indicating that that GFP accumulated in the cytoplasm of fungal cells. Hyphae of G1 but not GM4 penetrated the peel, but the hyphae of both G1 and GM4 spread equally in the juice sac area of citrus fruit (Isshiki *et al*. 2003). This result indicated that endoPG is essential for rotting (Isshiki *et al*. 2001) and penetration through the central axis from the pedicel during infection, but endoPG is not important for fungal colonization of the juice sac area (Isshiki *et al*. 2003). Hyphae of G1 did not grow on the surface of the fruit. Unlike other post-harvest pathogens of citrus such as *Penicillium digitatum* and *P. italicum* which colonize the surface of citrus fruit, the black rot pathogen only infects internal tissues of the fruit and causes black discoloration and maceration but never causes symptoms on the surface of fruit (Brown and McCornack 1972; Brown and Eckert 2000). It is currently unclear why this fungus is restricted to internal tissues and cannot colonize or cause symptoms on the surface of citrus fruits. However, the requirement for endoPG in the pathogenicity of this fungus might suggest that a lack of pectin in the cuticle layer of citrus surface leads to the absence of the fungal growth.

Green fluorescence of the transformant EPG7 carrying a GFP gene under control of the endoPG gene promoter was induced by pectin in the peel during the infection stage, but repressed completely in the juice sac area, likely due to carbon catabolite repression by sugars in the juice (Isshiki *et al*. 2003; Ohtani *et al*. 2003). Glucose, fructose and sucrose have been shown to be the major sugars found in citrus juice and their concentrations can reach 5 to 10% (McCready *et al*. 1950; Ting and Attaway 1971). We hypothesize that these sugars, which are present in the juice sac area, might be used preferentially as carbon sources over pectin for fungal growth. The concentrations of these sugars may be high enough to repress the endoPG gene expression in the juice sac area by carbon catabolite repression. In fact, our results showed that all of these sugars, at a concentration of 2%, repressed the endoPG gene expression on pectin plates, and the green fluorescence of EPG7 carrying a GFP gene under control of endoPG promoter was repressed completely in the juice sac area.

Expression of *Acpg1*, the gene cloned and characterized from the rough lemon pathotype, is regulated by substrate (pectic sugars) induction and carbon catabolite repression (Isshiki *et al*. 2003; Ohtani *et al*. 2003). A GFP gene was further employed as a reporter gene to define 813 bases upstream of the translation start site comprising the *Acpg1* promoter with use of several deletions (Ohtani *et al*. 2003). This upstream sequence contains five putative binding sequences of the catabolite repressive element A (CreA), a *cis*-acting zinc finger repressor involved in carbon catabolite repression. We constructed CreA-binding site-deleted

Acpg1 promoters with the GFP reporter gene and transformed them into the wild type black rot fungus. The construct (PGPDL4) which had a –401 to –813 deletion showed both substrate induction and catabolite repression, while PGPDL5 additionally deleted from –1 to –84, including one putative CreA-binding site, resulted in loss of catabolite repression function (Ohtani *et al*. 2003). Green fluorescence of PGPDL4 was induced by pectin in the peel, but repressed completely in the juice sac area of citrus fruit. However, green fluorescence of PGPDL5 was induced both in the peel and juice sac area, additionally indicating that the repression of *Acpg1* in the juice sac area is likely by carbon catabolite repression (Ohtani *et al*. 2003). EndoPG appears to be essential for the penetration of citrus peel by the black rot fungus and is essential for development of black rot symptoms. However, endoPG is not necessary for hyphal growth and colonization of the juice sac and expression of the endoPG gene is differentially regulated by carbon catabolite repression in different areas of citrus fruit.

Furthermore, differential regulation of a gene that encodes a cell wall-degrading enzyme during infection under different host environments has been examined, and host pH and sugars are involved in the regulation of the PG gene (Akimitsu *et al*. 2004; Katoh *et al*. 2007b). A gene (*AcCreA*) encoding a catabolite repression element (CreA) with two zinc fingers of the Cys_2His_2 type was isolated from a postharvest fungal pathogen *A. citri* (Katoh *et al*. 2007b). The *AcCreA* overexpression mutant AcOEC2 of *A. citri* showed normal growth on pectin medium and on segments of peel or the juice sac area from citrus fruits. Production of endoPG was similar in AcOEC2 and the wild type in pectin-containing media. However, addition of glucose to medium showed that carbon catabolite repression of endoPG gene (*Acpg1*) expression, as well as endoPG production, was lost in AcOEC2 (Katoh *et al*. 2007b). The wild type strain of *A. citri* causes rot mainly in the central axis of citrus fruits without development of rotting in the juice sac area, but AcOEC2 caused severe black rot symptoms in both the central axis and juice sac areas (Katoh *et al*. 2007b). These results also indicate that AcCreA-mediated catabolite repression controls the virulence and/or infection behaviors of this pathogen, and that the wild type *A. citri* does not cause symptoms in the juice sac area due to carbon catabolite repression by sugars in the juice of the juice sac area.

CONCLUSIONS AND FUTURE PERSPECTIVES

Alternaria diseases represent interesting pathomodels in citrus. Black rot pathogen of *A. citri* can affect all species of citrus, while the tangerine and rough lemon pathotypes of *A. alternata* produce respective HST of ACT-toxin and ACR-toxin and they can affect only citrus species which are sensitive to these toxins. ACT-toxin is closely related to other HSTs produced by similar fungi that affect Japanese pear and strawberry. ACR-toxin produced by the rough lemon pathotype differs chemically as well as its mode of action from ACT-toxin of the tangerine pathotype. The genes for these toxin productions are located on a small dispensable chromosome in each pathogen. The evolutionary history of these pathogens could be fascinating if it can be elucidated *A. alternata* is a common saprophyte on citrus leaves, but developed their own strategies to infect different citrus cultivars. The mechanism of specificity has been elucidating in the case of rough lemon and ACR-toxin. Surprisingly, the fail in degradation of tRNA intron in mitochondria is the cause of toxin sensitivity, and the protein complex responsible for the intron degradation is currently progressing. Further investigation of the specificity and pathogenicity of citrus *Alternaria* pathogens in citrus can provide a development of pathomodel explaining a unique evolutional traces of pathogenic *Alternaria* species.

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