REVIEW ARTICLE

Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*

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Abstract

Host-selective toxins (HSTs) produced by fungal plant pathogens are generally low-molecular-weight secondary metabolites with a diverse range of structures that function as effectors controlling pathogenicity or virulence in certain plant–pathogen interactions. There are now seven known diseases caused by *Alternaria alternata* in which HSTs are responsible for fungal pathogenesis. The pathogens have been defined as pathotypes of *A. alternata* because of morphological similarity but pathological differences. Chemical structures of HSTs from six pathotypes have been determined. The role of *A. alternata* HSTs in pathogenesis has been studied extensively, and discovery of the release of HSTs from germinating conidia prior to penetration aids in understanding the early participation of HSTs to induce susceptibility of host cells by suppressing their defence reactions. Many attempts have been made to find the target sites of *A. alternata* HSTs, and four cellular components, plasma membrane, mitochondrion, chloroplast and a metabolically important enzyme, have been identified as the primary sites of each HST action, leading to elucidation of the molecular mechanisms of HST sensitivity in host plants. Studies of the molecular genetics of HST production have identified supernumerary chromosomes encoding HST gene clusters and have provided new insights into the evolution of *A. alternata* pathotypes.

Introduction

A number of plant pathogenic fungi produce toxins that can damage plant tissues. Toxins are often classified as host selective (host specific) or nonspecific. Host-selective toxins (HSTs) are toxic only to host plants of the fungus that produces the toxin. In contrast, nonspecific toxins can affect many plants regardless of whether they are a host or non-host of the producing pathogen. Yoder (1980) classified toxins of plant pathogens as a pathogenicity factor or a virulence factor by considering the possible involvement of toxins in pathogenesis: pathogenicity is the ability to cause disease (a qualitative term), whereas virulence refers to the extent or severity of the disease caused (a quantitative term). Most HSTs are considered to be pathogenicity factors, which the fungi producing them require to invade tissue and induce disease (Yoder, 1980; Nishimura & Kohmoto, 1983; Scheffer & Livingston, 1984; Markham & Hille, 2001; Wolpert *et al.*, 2002; Howlett, 2006). All isolates of the pathogen that produce an HST are pathogenic to the specific host; all isolates that fail to produce HSTs lose pathogenicity to the host plants. Plants that are susceptible to the pathogen are sensitive to the toxin. Such correlations between HST production and pathogenicity in the pathogens and between toxin sensitivity and disease susceptibility in plants provide persuasive evidence that HSTs can be responsible for host-selective infection and disease development. On the other hand, the exact roles of nonspecific toxins in pathogenesis are largely unknown, but some are thought to contribute to features of virulence, such as symptom development and *in planta* pathogen propagation.

The HSTs known to date are limited to fewer than 20 pathogenic fungi. Most are low-molecular-weight secondary metabolites. However, two wheat pathogens, *Pyrenophora tritici-repentis* and *Stagonospora nodorum*, produce a proteinaceous HST, ToxA, which is encoded by orthologous genes of the two pathogens (Friesen *et al.*, 2006,

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2008). Friesen *et al.* (2006) showed that the *ToxA* gene originated and evolved in *S. nodorum* and was transferred recently from *S. nodorum* to *P. tritici-repentis*. Among low-molecular-weight HSTs, seven are from the genus *Alternaria* and four from *Cochliobolus* (anamorph *Bipolaris*), which are often considered saprophytic pathogens. In this review, we focus on HSTs produced by *Alternaria* species. We provide an overview of the HST-producing *Alternaria* pathogens and chemical characteristics of HSTs, followed by a summary of the role of HSTs in pathogenesis and the mode of toxin action. We also examine recent advances in the molecular genetics of toxin production and discuss the evolution of HST-producing pathogens.

Pathotypes of *Alternaria alternata*

The genus *Alternaria*, the *Hyphomycetes* in the *Fungi Imperfecti*, includes saprophytes on organic substrates and parasites on living plants (Rotem, 1994; Thomma, 2003). The involvement of an HST in plant disease was first suggested in the black spot disease of Japanese pear in 1933 (Tanaka, 1933). The disease appeared after the new cultivar Nijisseiki was introduced. This cultivar was discovered in 1888 as a spontaneous mutant seedling at a damp yard in Chiba Prefecture, Japan, and the economic cultivation began after 20 years of its discovery. Since then, black spot disease has become a serious threat to farmers growing Nijisseiki pears. Nijisseiki was extraordinarily susceptible to the pathogen, while older cultivars were immune. The causal organism was identified and named *Alternaria kikuchiana* (Tanaka, 1933). Tanaka (1933) reported that the fungus-free culture filtrates showed toxicity to fruits of Nijisseiki, but not to those of resistant cultivars. This work was the first confirmed example of selective toxicity by culture filtrates and suggested the presence of a host-selective fungal metabolite, which was later isolated and named AK-toxin. Studies on the toxin were resumed by several groups in Japan in the 1950s (Nishimura & Kohmoto, 1983). Beginning in the 1970s, HSTs were identified from other *Alternaria* pathogens, and there are now seven diseases caused by *Alternaria* in which HSTs are responsible for fungal pathogenicity (Table 1) (Nishimura & Kohmoto, 1983; Kohmoto & Otani, 1991; Otani *et al.*, 1995; Markham & Hille, 2001; Thomma, 2003).

Previously, species names were adopted for five of the seven *Alternaria* fungi known to produce HSTs (Table 1): *A. kikuchiana*, causing black spot of Japanese pear (Tanaka, 1933; Nakashima *et al.*, 1985); *Alternaria mali*, causing Alternaria blotch of apple (Sawamura, 1962; Okuno *et al.*, 1974); *Alternaria longipes*, causing brown spot of tobacco (Lucas, 1975; Kodama *et al.*, 1990); and two biotypes of *Alternaria citri*, causing leaf spot of rough lemon (Doidge, 1929; Gardner *et al.*, 1985a) and brown spot of tangerine (Pegg, 1966; Kohmoto *et al.*, 1993; Timmer *et al.*, 2000) (Table 1). The two other toxin-producing fungi were identified as pathogenic variants within *A. alternata*. Grogan *et al.* (1975) reported the occurrence of a stem canker of tomato and characterized the causal agent as *Alternaria alternata* f. sp. *lycopersici* (Table 1). This pathogen was later found to produce an HST, AAL-toxin (Bottini & Gilchrist, 1981). The causal agent of the Alternaria black spot of strawberry was designated as the strawberry pathotype of *A. alternata* and appeared to produce an HST, AF-toxin (Table 1) (Maekawa *et al.*, 1984; Nakatsuka *et al.*, 1986a).

None of the HST-producing *Alternaria* pathogens has a reported sexual stage, but all are morphologically similar enough in their asexual stages to be classified as *A. alternata* (Fries) Keissler (Neergaard, 1945; Ellis, 1971; Nishimura *et al.*, 1978; Nishimura, 1980). *Alternaria alternata* is

Table 1. *Alternaria alternata* host-selective toxins known to date

	Pathogen		Host	
Disease	Pathotype (previous name)	Toxin	Host range (susceptible cultivar)	Genetic background (dominance)
Alternaria blotch of apple	Apple pathotype (*A. mali*)	AM-toxin I, II and III	Apple (Red Gold, Starking)	Single (susceptible)
Black spot of strawberry	Strawberry pathotype	AF-toxin I, II and III	Strawberry (Morioka-16)	Single (susceptible)
Black spot of Japanese pear	Japanese pear pathotype (*A. kikuchiana*)	AK-toxin I and II	Japanese pear (Nijisseiki)	Single (susceptible)
Brown spot of tangerine	Tangerine pathotype (*A. citri*)	ACT-toxin I and II	Mandarins and Tangerines (Dancy, Emperor, Minneola)	Unknown
Leaf spot of rough lemon	Rough lemon pathotype (*A. citri*)	ACR-toxin I	Citrus rootstocks (Rough lemon, Rangpur)	Unknown
Alternaria stem canker of tomato	Tomato pathotype (*A. alternata* f. sp. *lycopersici*)	AAL-toxin Ta and Tb	Tomato (Earlypak 7, First)	Single (resistance)
Brown spot of tobacco	Tobacco pathotype (*A. longipes*)	AT-toxin	Tobacco	Unknown

fundamentally a ubiquitous, saprophytic fungus isolated from various dead plant materials and is also familiar as a weak pathogen causing indefinite or opportunistic disease on a number of crops (Rotem, 1994; Thomma, 2003). Regardless of similarity in conidial morphology of the HST-producing pathogens, they have limited host range, by which they are distinguished. All isolates belonging to *A. alternata* possess a potential general aggressiveness, recognizable as the ability to penetrate artificial membranes, such as cellulose and poly(vinyl formal) membranes, through appressoria of germinated conidia (Nishimura *et al.*, 1978; Nishimura, 1980). Thus, it is likely that the pathogenicity of *A. alternata* pathogens consists of potential aggressiveness, common to all isolates belonging to *A. alternata*, and HSTs, which are essential for host-selective infection and disease development. Based on these features, Nishimura (1980) proposed that each pathogen should be considered a distinct pathotype of *A. alternata* (Table 1). This classification, however, has not been accepted widely because of difficulties in unambiguous discrimination of small-spored *Alternaria* species with few morphological characteristics, which are strongly influenced by not only intrinsic factors, but also environmental conditions.

Simmons (1992) reported that in addition to the range in size of conidia, three-dimensional conidiation patterns are critical for differentiating similar species in the small-spored group. For example, *A. alternata* conidia are produced in a loose, three-dimensional tuft of branching chains under specific growth conditions. *Alternaria* fungi, the conidia of which are produced in a single linear chain under the same conditions, were considered as a separate taxon even if their conidia showed the same size range. For example, Simmons & Roberts (1993) sorted a number of small-spored, chain-forming *Alternaria* isolates from black spot lesions of Japanese pear into six conidiation groups (morphospecies) on the basis of three-dimensional conidiation patterns, although almost all isolates fell in the same conidial size group of typical *A. alternata*. They also reported no correlation between toxigenicity (AK-toxin production) and conidiation group among the isolates from Japanese pear and concluded that AK-toxin production might not be species-restricted within the genus *Alternaria*.

Molecular phylogenetic studies based on ribosomal DNA, several protein-coding genes and noncoding, anonymous regions of the genome have frequently failed to resolve morphospecies and host association within the small-spored *Alternaria* complex (Kusaba & Tsuge, 1995; Pryor & Gilbertson, 2000; Peever *et al.*, 2004; Andrew *et al.*, 2009). In the phylogenetic studies, *Alternaria* species, the conidia of which are morphologically distinguishable from *A. alternata*, were clearly differentiated from *A. alternata* (Kusaba & Tsuge, 1995; Pryor & Bigelow, 2003; Peever *et al.*, 2004). In contrast, the HST-producing pathogens were not associated with any specific clade corresponding to the host plant for any loci within the small-spored *Alternaria* (Kusaba & Tsuge, 1995; Peever *et al.*, 2004; Andrew *et al.*, 2009). Rotem (1994) pointed to the high variability of morphological and physiological characters in *A. alternata* and concluded that morphological variation should not be used to assign different species within the genus *Alternaria*. We now recommend that morphological differences reported within HST-producing fungi be interpreted as intraspecific variability.

Chemical structures of *Alternaria* HSTs

Chemical structures of six *A. alternata* HSTs, excluding that of AT-toxin of the tobacco pathotype, have been determined (Fig. 1). The *Alternaria* HSTs involve a diverse group of low-molecular-weight substances, and most were found in culture filtrates as families of closely related compounds. The major form of each HST is shown in Fig. 1. The *Alternaria* HSTs cause necrosis on leaves of susceptible cultivars at concentrations as low as 10^{-8} to 10^{-9} M and no necrosis on leaves of resistant cultivars even at higher concentrations (Fig. 2) (Nishimura & Kohmoto, 1983; Kohmoto & Otani, 1991; Otani *et al.*, 1995).

Epoxy-decatrienoic acid (EDA) family: AK-toxin, AF-toxin and ACT-toxin

Toxins of the Japanese pear, strawberry and tangerine pathotypes were found to be structurally analogous metabolites that are esters of 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA) (Fig. 1) (Nakashima *et al.*, 1985; Nakatsuka *et al.*, 1986a; Kohmoto *et al.*, 1993). Free EDA is not toxic to any of the plants tested. The Japanese pear pathotype produces two related molecular species, AK-toxins I and II, with toxin I being the more abundant and biologically active species (Fig. 1) (Nakashima *et al.*, 1985; Otani *et al.*, 1985; Hayashi *et al.*, 1990). Both toxins exhibit toxicity only on susceptible pear cultivars (Fig. 2) (Otani *et al.*, 1985). AK-toxin I is esterified at the 8-hydroxy group of EDA with a phenylalanine derivative, *N*-acetyl-β-methyl-phenylalanine (Fig. 1).

The strawberry pathotype affects Japanese strawberry cultivar Morioka-16 in the field. Interestingly, this pathotype was also found to be pathogenic to Japanese pear cultivars susceptible to the Japanese pear pathotype in laboratory tests (Maekawa *et al.*, 1984). Such host range can be completely reproduced by toxin preparations from culture filtrates of the pathogen. The pathogen produces three related molecular species, AF-toxins I, II and III,

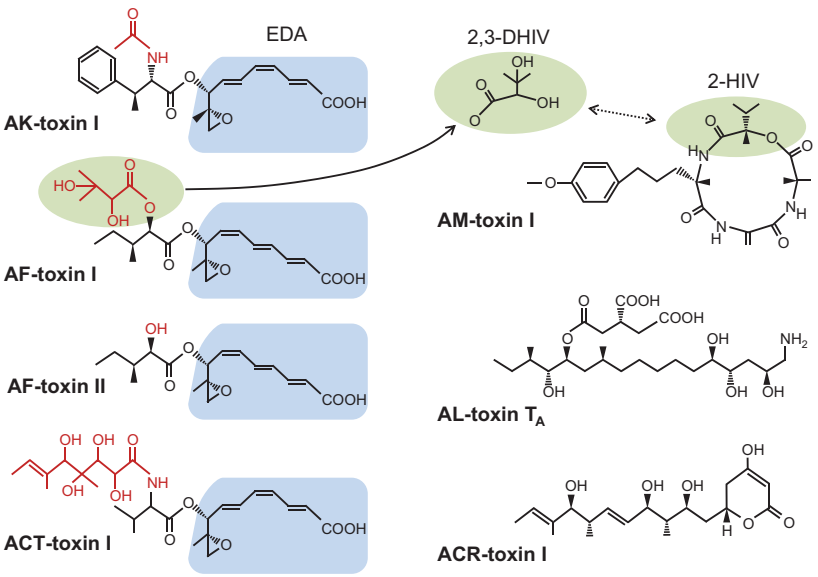

Fig. 1. Chemical structures of HSTs produced by *Alternaria alternata*. EDA, 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid; 2,3-DHIV, 2,3-dihydroxy-isovaleric acid; 2-HIV, 2-hydroxy-isovaleric acid.

with toxin I being the most abundant (Nakatsuka *et al.*, 1986a; Nishimura & Nakatsuka, 1989; Hayashi *et al.*, 1990). AF-toxin I is toxic to both strawberry and pear, toxin II is toxic only to pear, and toxin III is highly toxic to strawberry and slightly to pear (Maekawa *et al.*, 1984). The structure of AF-toxin II was the first determined by Nakatsuka *et al.* (1986a). Intriguingly, AF-toxin II is esterified at the 8-hydroxy group of EDA with an isoleucine derivative, 2-hydroxyvaleric acid, instead of the phenylalanine derivative of AK-toxin I (Fig. 1). Structures of AF-toxins I and III have also been determined as

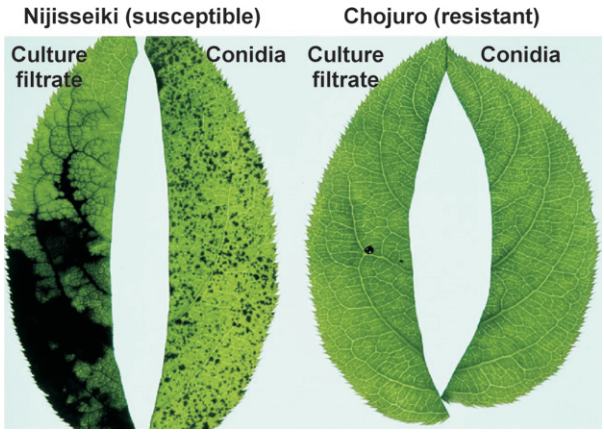

Fig. 2. Toxicity of AK-toxin produced by the Japanese pear pathotype of *Alternaria alternata*. The culture filtrate of the Japanese pear pathotype was dropped on slightly wounded points of left-half leaves. Right-half leaves were spray-inoculated with a conidial suspension. Leaves were incubated for 24 h.

2′-*O*-acyl derivatives of AF-toxin II (Fig. 1) (Nishimura & Nakatsuka, 1989). These two toxins have valine derivatives at the 2′-position: 2,3-dihydroxy-isovaleric acid in toxin I and 2-hydroxy isovaleric acid in toxin III. Interestingly, several 2′-*O*-acyl derivatives (acetyl, propionyl and isovaleryl) artificially synthesized from AF-toxin II exhibit toxicity on leaves of both strawberry and pear, just as toxin I and toxin III (Nishimura & Nakatsuka, 1989). It is likely that the structure at the 2′-*O*-acyl position serves as an essential part of the molecule determining toxicity to strawberry and pear.

The tangerine pathotype affects tangerines and mandarins (Timmer *et al.*, 2000; Akimitsu *et al.*, 2003). This pathotype was also found to be pathogenic to Japanese pear cultivars susceptible to the Japanese pear pathotype in laboratory tests, although no field occurrence of the disease has been recorded in Japan; the pathogen perhaps has never encountered susceptible pear cultivars (Kohmoto *et al.*, 1993). The tangerine pathotype produces ACT-toxins
I and II, with toxin I being the more abundant (Fig. 1) (Kohmoto *et al.*, 1993). ACT-toxin I is toxic to both citrus and pear; toxin II, the 5″-deoxy derivative of ACT-toxin I, is highly toxic to pear and slightly toxic to citrus. ACT-toxins consist of three moieties, EDA, valine and a polyketide (Fig. 1) (Kohmoto *et al.*, 1993).

AK-toxins and ACT-toxins have a (*2E*,*4Z*,*6E*)EDA moiety, whereas AF-toxins have a (*2E*,*4E*,*6Z*)EDA moiety (Fig. 1). Minor stereoisomers (*2E*,*4Z*,*6E* and *2E*,*4E*,*6E* at EDA) of AF-toxins have been isolated from culture filtrates (Nishimura & Nakatsuka, 1989). Differences in ste-

reostructure of EDA do not affect host selectivity of the toxins. However, AF-toxin II with the (*2E*,*4Z*,*6E*) form at EDA, like AK-toxins, showed stronger toxicity to pear than the major AF-toxin II with (*2E*,*4E*,*6Z*)EDA (Nishimura & Nakatsuka, 1989).

Cyclic depsipeptide (cyclic tetrapeptide): AM-toxin (syn. Alternariolide)

The chemical structure of AM-toxin I from the apple pathotype was elucidated as a cyclic depsipeptide, cyclo (-L-α-amino-*p*-methoxyphenylvaleryl-dehydroalanyl-L-alanyl-L-α-hydroxyisovaleryl-lactone) (Fig. 1) in the pioneering work on structural elucidation of HSTs (Okuno *et al.*, 1974; Ueno *et al.*, 1975, 1977). Depsipeptides with cyclic structures are also found in other toxins of plant pathogens, such as host-selective HC-toxin from *Cochliobolus carbonum* race 1 (Gross *et al.*, 1982; Walton *et al.*, 1982) and tentoxin from *Alternaria tenius* (Meyer *et al.*, 1975). These toxins are also referred to as nonribosomal peptides (NRPs) in consideration of their synthetic pathways. Although AM-toxin I is a unique NRP among *Alternaria* HSTs, NRPs are abundant fungal metabolites as well as polyketides (Keller *et al.*, 2005; Hoffmeister & Keller, 2007).

Aminopentol/polyketide (sphinganine analog): AAL-toxin

The tomato pathotype produces aminopentol ester toxins called AAL-toxins (Gilchrist & Grogan, 1976). The chemical structures of AAL-toxin T_A and T_B (minor) were determined (Fig. 1) (Bottini & Gilchrist, 1981; Bottini *et al.*, 1981). AAL-toxins contain propane-1,2,3-tricarboxylic acid (PTCA) side chains in common, and PTCA is esterified to 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol, which is structurally analogous to sphingosine and sphinganine. Bezuidenhout *et al.* (1988) isolated mycotoxin fumonisins, which are structurally related metabolites produced by the unrelated fungus *Fusarium verticillioides* (syn. *Fusarium moniliforme*). Fumonisins have a distinct structural similarity to AAL-toxin: AAL-toxins have one PTCA and fumonisins two PTCA side chains esterified to aminopentol backbones. AAL-toxins and fumonisins show similar toxicity to plants and mammalian cells and inhibitory activity to ceramide synthase, which is involved in sphingolipid biosynthesis (Gilchrist *et al.*, 1994; Gilchrist, 1998). Because of structural and functional similarity, AAL-toxins and fumonisins are collectively referred to as sphinganine-analogue mycotoxins (SAMs) (Gilchrist *et al.*, 1994). AAL-toxins affect organisms other than host plants, so their toxicity is host selective but not host specific. Molecular characterization of the biosynthetic genes of AAL-toxins and fumonisins revealed that the aminopentol backbone of each toxin is produced by the activity of a polyketide synthase (PKS) and then modified by other enzymes (Proctor *et al.*, 1999; Seo *et al.*, 2001; Akamatsu *et al.*, 2003).

Polyketide: ACR-toxin (syn. ACRL-toxin)

The rough lemon pathotype produces ACR-toxins. The major toxin, ACR-toxin I, is a C19 polyalcohol with an α-dihydropyrone ring (Fig. 1) (Gardner *et al.*, 1985a, b; Nakatsuka *et al.*, 1986b). Minor toxins were characterized as having a pyrone ring with polyalcohol side chains different in length and showing weaker toxicity (Kono *et al.*, 1985). ACR-toxins have structural features typical of polyketides. Recently, the gene encoding a PKS essential for ACR-toxin biosynthesis was identified from the rough lemon pathotype, indicating that ACR-toxins are polyketide secondary metabolites (Izumi *et al.*, 2012).

Role of HSTs in pathogenesis

The definition of HST has been postulated as compounds possessing the following characteristics: (1) host-selective toxicity, (2) selective toxicity matching the specificity of the HST-producing pathogen, (3) plants insensitive to the HST must be resistant to the pathogen producing the compound, (4) the compound must be released on germination of the pathogen's conidia at the site of infection, (5) the compound can reproduce the initial physiological changes in host cells caused by the HST-producing pathogen and (6) the initial physiological changes caused by HST in host cells lead to penetration or initial colonization by the HST-producing pathogen (Yoder, 1980; Nishimura & Kohmoto, 1983; Scheffer & Livingston, 1984; Kohmoto & Otani, 1991; Otani *et al.*, 1995). The role of *Alternaria* HSTs has been considered to be the effector determining pathogenicity because simultaneous treatment with HSTs and conidia of non-pathogenic *A. alternata* strains leads to induction of infection (Otani *et al.*, 1975; Yamamoto *et al.*, 1984; Akimitsu *et al.*, 1989; Yamagishi *et al.*, 2006), and conversely, a loss of HST production by target gene disruption or silencing of the biosynthetic genes leads to disappearance of pathogenicity (Tanaka *et al.*, 1999; Johnson *et al.*, 2000; Ruswandi *et al.*, 2005; Harimoto *et al.*, 2008; Miyamoto *et al.*, 2008; Akagi *et al.*, 2009a).

A basic concept of pathogenesis for an HST-producing pathogen consists of three hypothetical processes: (1) HST released before penetration is a signal molecule for host recognition; (2) the released HST binds selectively to the host receptor site; and (3) the HST signal suppresses the host defence against fungal penetration or initial

colonization prior to cell death (Nishimura & Kohmoto, 1983; Scheffer & Livingston, 1984; Kohmoto & Otani, 1991; Otani *et al.*, 1995). Examples have proved this hypothesis initially proposed by Pringle & Scheffer (1964) based on the HST-receptor model.

The mitochondrial gene T-*urf13* from Texas cytoplasmic maize encodes the receptor of the polyketide HST, T-toxin (HMT-toxin), produced by *Cochliobolus heterostrophus* race T, the causal agent of southern corn leaf blight (Dewey *et al.*, 1988). T-*urf13* is responsible for the sensitivity to T-toxin as well as to the structurally related HST, PM-toxin, produced by *Mycosphaerella zeae-maydis*, the causal agent of yellow leaf blight of corn (Dewey *et al.*, 1988). T-*urf13* encodes a 13-kDa protein, and the URF13 protein specifically binds to radiolabelled PM-toxin in maize mitochondria (Braun *et al.*, 1990). The gene also conferred sensitivity to T-toxin in *Escherichia coli* cells (Dewey *et al.*, 1988). Braun *et al.* (1990) verified the binding of the toxin to URF13 in *E. coli* cells. A gene, *ACRS* (ACR-toxin sensitivity gene), encoding a protein with similar features was also identified in the mitochondrial genome from rough lemon, which is sensitive to ACR-toxin produced by the rough lemon pathotype of *A. alternata* (Ohtani *et al.*, 2002). The gene confers ACR-toxin sensitivity to *E. coli*, and the mechanism of specificity in plants is alternative processing of the gene transcript, which confers sensitivity to ACR-toxin (Ohtani *et al.*, 2002). Details for *ACRS* are described in a later section. *Cochliobolus victoriae* produces the cyclic peptide HST victorin (HV-toxin) and causes victoria blight of oats. The ^{125}I-labelled victorin and an immunochemical approach using anti-victorin antibodies both identified a 100-kDa binding protein, a candidate for the victorin receptor (Wolpert & Macko, 1989; Akimitsu *et al.*, 1992).

Although HSTs are highly toxic to host plant cells and cause cell death, the establishment of infection by the HST-producing pathogen is probably caused by a genotype-specific suppression of defence responses rather than the induction of cell death. Interestingly, several lines of evidence indicate that the HST itself has the property of suppression of defences in addition to toxicity, and the roles of the HST in toxicity and suppression of defences can be separated. For example, development of leaf necrosis on apple caused by AM-toxin of the apple pathotype is suppressed by light irradiation, with wavelengths near 602 nm being the most active (Tabira *et al.*, 1989). At least 5 h of darkness is necessary for necrosis development; however, light does not affect AM-toxin-induced electrolyte leakage and photosynthetic CO_2 fixation inhibition (Tabira *et al.*, 1989). When the leaves were inoculated with conidia of the pathogen, light had no protective effect against their invasion, suggesting that host cell death is not necessary for fungal invasion. Observation of infection behaviour of *C. victoriae* conidia on susceptible and resistant oats suggested that cell death may not be necessary for pathogenesis (Nishimura & Scheffer, 1965; Yoder & Scheffer, 1969). HC-toxin produced by *C. carbonum* race 1, the causal agent of northern corn leaf spot, is essential for pathogenesis, but is thought to be cytostatic rather than toxic (Walton, 1996; Wolpert *et al.*, 2002). Furthermore, delay or suppression of induction of defence-related genes was observed in rough lemon leaves inoculated with the rough lemon pathotype producing ACR-toxin (Gomi *et al.*, 2002a, b, 2003b). The suppressive effects have been observed only in the case of AM-toxin and ACR-toxin and their respective host plants. One putative target site of other *Alternaria* HSTs, such as AK-toxin, AF-toxin and ACT-toxin, is the plasma membrane, and the damage to host cells caused by these toxins is extremely rapid, and the time required for cell death is extremely short (Maekawa *et al.*, 1984; Otani, *et al.*, 1985; Kohmoto *et al.*, 1993). Thus, it will probably be difficult to identify the suppressive effects of any defensive function by these toxins because cell death is induced by these toxins faster than suppression of defensive responses in the toxin-sensitive cells.

For most HSTs, the mechanism of cell death has not been characterized at the molecular level. However, AAL-toxin from the tomato pathotype of *A. alternata* and victorin from *C. victoriae* appear to have a common mechanism causing programmed cell death. AAL-toxin induces an apoptotic-like response in toxin-sensitive tomato, as demonstrated by TUNEL-positive cells, DNA laddering and the formation of apoptotic-like bodies (Wang *et al.*, 1996; Gilchrist, 1998). Victorin also induces a form of cell death in toxin-sensitive oats that shares many of the biochemical and molecular characteristics of apoptosis, such as DNA laddering, heterochromatin condensation, cell shrinkage and protease activation (Navarre & Wolpert, 1999; Yao *et al.*, 2001; Wolpert *et al.*, 2002; Curtis & Wolpert, 2004). These responses are similar to a type of programmed cell death observed in the defence response of plants, suggesting that these toxins may elicit the defence response (Gilchrist, 1998; Richael & Gilchrist, 1999; Jones, 2001; Markham & Hille, 2001; Wolpert *et al.*, 2002). The functions of these two toxins seem different from that of ACR-toxin, which suppresses induction of defence-related genes in toxin-sensitive plants. Although it is unknown whether programmed cell death is a common feature for many HSTs, which affect different plants by different modes of action, HST-producing fungi can indeed survive in, derive nutrients from and form conidia on dead tissue. Previous comprehensive reviews have dealt with this subject (Gilchrist, 1998; Richael & Gilchrist, 1999; Markham & Hille, 2001; Wolpert *et al.*, 2002).

It has also been reported that HST induces expression of defence-related genes in toxin-insensitive plants. Defence-related genes are highly inducible in rough lemon leaves by inoculation of the tangerine pathotype producing ACT-toxin, which is not toxic on rough lemon (Kohmoto *et al.*, 1979, 1991). All defense-related genes tested, including lipoxygenase (Gomi *et al.*, 2002b), chalcone synthase (Gotoh *et al.*, 2002; Nalumpang *et al.*, 2002a), polygalacturonase-inhibiting protein (Gotoh *et al.*, 2002; Nalumpang *et al.*, 2002a, b), chitinases (Gomi *et al.*, 2002a), hydroperoxide lyase (Gomi *et al.*, 2003b), epoxide hydrolase (Gomi *et al.*, 2003a), miraculin-like protein (Tsukuda *et al.*, 2006), thaumatin-like protein (Kim *et al.*, 2009) and nonspecific lipid transfer protein (Nishimura *et al.*, 2008), were highly inducible in rough lemon leaves by inoculation with the tangerine pathotype conidia, which could not invade rough lemon leaf cells. Although nonpathogenic strain O-94 of *A. alternata*, which is not pathogenic to any citrus tested and produces no HST (Akimitsu *et al.*, 1989; Kohmoto *et al.*, 1991), also induced expression of these genes, the timing and intensity of the induction were not as fast or strong as those induced by the tangerine pathotype. Because there are no apparent morphological or biochemical differences between the tangerine pathotype and the nonpathogenic strain except that the former produces ACT-toxin, the toxin is expected to have a role as an elicitor in faster and greater accumulation of transcripts of defence-related genes. Recently, the pattern of induction of these defence-related genes in rough lemon following inoculation with an ACT-toxin-minus mutant of the tangerine pathotype, which was made by silencing the ACT-toxin biosynthetic gene *ACTT2* (Miyamoto *et al.*, 2008), was examined. This experiment showed that the intensity and timing of the induction pattern were similar to those induced by O-94 (H. Shishido *et al.*, unpublished results). These results indicated that ACT-toxin is an effector with functions of necrotrophic pathogenicity in toxin-sensitive plants and also in defence elicitation of toxin-insensitive plants (Fig. 3).

Mode of action and mechanism of specificity

Many attempts have been made to find the primary site of action of HSTs, as knowledge of the mechanism underlying the initial physiological event in toxin-treated host cells is fundamental to understanding the mutual recognition between hosts and pathogens. Four cellular components have been proposed as inhibitory sites of toxin action: plasma membrane, mitochondrion, chloroplast and a metabolically important enzyme (Fig. 4).

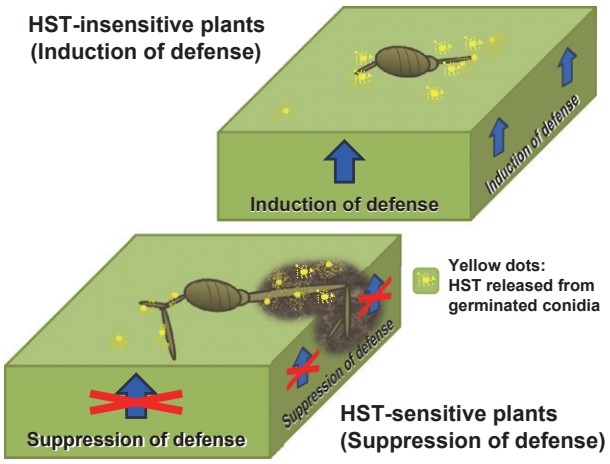

Fig. 3. HST works as an effector, having a suppressor function to HST-sensitive plants as well as an elicitor function to HST-insensitive plants.

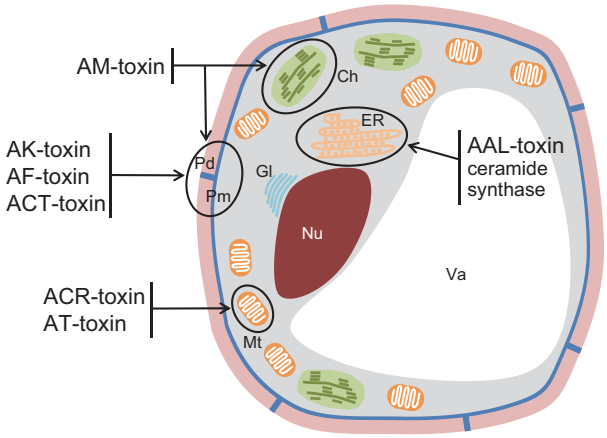

Fig. 4. Schematic presentation of target sites of HSTs produced by *Alternaria alternata*. Ch, chloroplast; ER, endoplasmic reticulum; Gl, Golgi apparatus; Mt, mitochondrion; Nu, nucleus; Pd, plasmodesma; Pm, plasma membrane; Va, vacuole.

AK-toxin, AF-toxin and ACT-toxin

All toxins in this family show toxicity to host plants at a minimum concentration ranging from 1 to 10 nM in a leaf-necrosis bioassay using susceptible plant leaves (Fig. 2) (Otani *et al.*, 1985; Namiki *et al.*, 1986a; Kohmoto *et al.*, 1993). No necrosis, however, is induced on resistant cultivars or non-host plants at practically applicable concentrations. When an aqueous solution of these toxins is dropped on leaves, a slightly wounded, necrotic appearance usually becomes visible within 12 h after treatment and develops along the leaf vein, resulting in the appearance of veinal necrosis (Fig. 2). Yamamoto *et al.* (1985) reported that roots of strawberry are more sensitive to AF-toxin than

leaves. The minimum concentration causing root growth inhibition is 10- to 100-fold lower than that causing leaf necrosis. The sensitivity of Japanese pear to AK-toxin and strawberry to AF-toxin was controlled by a semidominant locus at a pair of alleles (Kozaki, 1973; Yamamoto *et al.*, 1985; Sanada, 1988). Homozygotes and heterozygotes of the dominant locus are sensitive to toxin, but the homozygotes are more sensitive than the heterozygotes. Cultivars lacking this dominant locus are insensitive to toxin. Such toxin sensitivity is completely consistent with the susceptibility to the pathogen. Japanese pear cultivar Nijisseiki and strawberry cultivar Morioka-16 were identified as heterozygotes.

These toxins exert primary effects on the plasma membrane of susceptible cells (Fig. 4) (Maekawa *et al.*, 1984; Otani *et al.*, 1985; Kohmoto *et al.*, 1993; Park & Ikeda, 2008). They cause an instantaneous, marked increase in loss of K^+: the increase in K^+ efflux is detectable within a few minutes after toxin treatment, with a linear relationship between the amount of efflux and time after treatment. The toxin-induced dysfunction of the plasma membrane was confirmed by electron micrographs showing many invaginations in the membranes, along with much fragmentation and vesiculation, extension of desmotubules from plasmodesmata and fusion of Golgi vesicles to the damaged plasma membrane occurring within 1–3 h after toxin treatment (Park & Ikeda, 2008). No adverse modifications were observed for intracellular organelles other than the plasma membrane of the host cell. Electrophysiological studies showed that AK-toxin and AF-toxin irreversibly depolarized the plasma membrane of susceptible genotypes (Namiki *et al.*, 1986b; Otani *et al.*, 1989). The membrane potential gradient of toxin-treated susceptible cells decreased, but did not disappear completely. A decrease in polarization occurred mostly in the respiration-dependent component of the membrane potential, which is sustained by an H^+ pump; there was little effect on the diffusion potential component. This indicated the possibility that these toxins directly affect the plasma membrane H^+-ATPase. However, AK-toxin and AF-toxin had no direct effect on ATPase activity in an isolated plasma membrane fraction of susceptible host cells (Otani *et al.*, 1991, 1995).

There are several lines of indirect evidence suggesting the presence of toxin receptors in susceptible genotypes. For example, a study on the structure–selectivity relationships of AK-, AF- and ACT-toxins suggested that the initial interaction between the toxin and its putative receptor site on the plasma membrane of susceptible cells probably involves a biological reaction, such as ligand binding to a receptor, rather than a simple physicochemical reaction (Nishimura & Nakatsuka, 1989). An agonist–antagonist relationship was found between AF-toxin I and inert AF-toxin II in susceptible strawberry tissue (Namiki *et al.*, 1986a; Lee *et al.*, 1992). When AF-toxin II, which is toxic to pear but not strawberry, was applied to leaves or suspension-cultured cells of strawberry before AF-toxin I treatment, the damage caused by toxin I was markedly reduced. Preincubation of strawberry leaves with toxin II also reduced the number of lesions when sprayed with conidial suspension of an AF-toxin producer.

AM-toxin

AM-toxins have two target sites for affecting susceptible apple cells: one on the plasma membrane and another on chloroplasts (Fig. 4) (Park *et al.*, 1981). AM-toxin causes invagination of the plasma membrane and electrolyte loss. However, the response of the plasma membrane in apple to AM-toxin is not as drastic as that of Japanese pear to AK-toxin. In the chloroplasts of susceptible apple leaves, membrane fragments and vesicles appear to be derived from grana lamellae within 3 h after toxin treatment. The chloroplast disorganization is compatible with the reduction in chlorophyll content and inhibition of photosynthetic CO_2 assimilation in the toxin-treated susceptible leaves (Kohmoto *et al.*, 1982). AM-toxin does not cause dysfunction of plasma membranes or chloroplasts in resistant apple tissue. A marked tissue specificity is expressed by susceptible apple cultivars in response to AM-toxin. Reactions are detected only in green tissues such as leaves, and the photosynthetic activity of chloroplasts isolated from leaves is inhibited (Otani *et al.*, 1991), suggesting that the chloroplast is a primary target site of AM-toxin.

Saito and Takeda (1984) reported that susceptibility of apple to *Alternaria* blotch is basically controlled by a single dominant gene and that the susceptible cultivars are heterozygous, whereas the resistant cultivars are recessive homozygous. Tabira *et al.* (1998) isolated a mutant resistant to *Alternaria* blotch from gamma-ray-irradiated meristem cultures of susceptible cultivar Indo. The resistant mutant showed about 10 000-fold increased tolerance to AM-toxin. The mutant not only is of horticultural value due to having a good agronomic trait, but also is expected to be useful for characterization of the molecular mechanisms responsible for AM-toxin sensitivity in apple.

AAL-toxin

In the tomato pathotype–tomato interactions, a major factor in pathogenicity is the production of AAL-toxin, which is capable of inducing cell death only in susceptible cultivars (Akamatsu *et al.*, 1997; Brandwagt *et al.*, 2000; Yamagishi, *et al.*, 2006). AAL-toxin and the mycotoxin fumonisin are SAMs (Gilchrist *et al.*, 1994; Gilchrist,

1998). Fumonisin is as selectively toxic to the AAL-toxin-sensitive tomato genotypes as AAL-toxin (Gilchrist *et al.*, 1994; Gilchrist, 1998). As described above, SAMs induce programmed cell death in susceptible tomato cells and mammalian cells by inhibiting ceramide biosynthesis (Fig. 4) (Wang *et al.*, 1996; Spassieva *et al.*, 2002, 2006). When AAL-toxin-sensitive tomato tissue was treated with SAMs, sphinganine and phytosphingosine accumulated in the tissue (Abbas *et al.*, 1994). AAL-toxin-induced cell death could be avoided in sensitive tomato leaves by feeding ceramide, indicating that a ceramide imbalance is critical in causing cell death (Brandwagt *et al.*, 2000; Markham and Hille, 2001).

In tomato and other plants, insensitivity to AAL-toxin and fumonisin and resistance to the tomato pathotype are conferred by the *Asc1* (*Alternaria* stem canker resistance gene 1) gene, a homologue of the yeast longevity assurance gene *Lag1*, which mediates resistance to SAM-induced apoptosis (Brandwagt *et al.*, 2000; Spassieva *et al.*, 2002, 2006). Yeast *Lag1* and *Lac1* (longevity assurance gene cognate 1) are thought to encode ceramide synthase, the target of SAMs, and each gene can compensate for the absence of the other: deletion of either gene will not affect ceramide synthase activity, but deletion of both genes markedly impairs activity (D'Mello *et al.*, 1994; Schorling *et al.*, 2001). *Asc1* partially compensated for the growth defect in the *Lag1*/*Lac1*-deleted yeast strain, indicating a common function of these plant and yeast ceramide synthase genes (Spassieva *et al.*, 2002; Mullen *et al.*, 2011). It has also been shown that sensitivity to AAL-toxin is governed by *Asc1*/*Lag1* homologues in other plants such as *Arabidopsis thaliana* and *Orobanche cumana* (Gechev *et al.*, 2004, 2008; de Zelicourt *et al.*, 2009).

Although the roles of toxins as effectors of disease susceptibility have been well characterized in many plant–pathogen interactions, there is little knowledge about the mechanisms of general, basal or nonhost resistance in plants against toxigenic and necrotrophic pathogens that depend on toxins for their pathogenesis. Among several signalling molecules shown to be involved in plant defences, the plant hormone jasmonic acid (JA) has a crucial role in defence signalling pathways against necrotrophic pathogens, such as *Botrytis cinerea* and *Alternaria brassicicola* (Thomma *et al.*, 1998, 2001; Penninckx *et al.*, 2003). A possible role of JA in basal defence responses of plants against toxigenic, necrotrophic pathogens was analysed using the tomato pathotype–tomato interaction as a model system (Egusa *et al.*, 2009a, b). The results suggested that the JA-dependent signalling pathway is not involved in host defence against the toxigenic *A. alternata* pathogen, but might affect pathogen accessibility by mediating a susceptible response.

The mechanisms of nonhost resistance to *A. alternata* were further investigated using *Arabidopsis* mutants. Wild-type *Arabidopsis* showed either no symptoms or a hypersensitive reaction (HR) when inoculated with conidia of AAL-toxin-producing and nonproducing strains of *A. alternata*. Yet, when *Arabidopsis penetration* (*pen*) mutants were challenged with the strains, fungal penetration was possible, and HR-like cell death occurred. However, further development and conidiation of both strains were limited on the *pen* mutants. In contrast, only an AAL-toxin-producing strain could invade *loh* mutants, which have a defect in a homologue of the AAL-toxin resistance *Asc1* gene (Gechev *et al.*, 2004), subsequently allowing the fungus to complete its life cycle. These results show that a compatible interaction with *Arabidopsis* could be established as a consequence of interference with ceramide biosynthesis due to AAL-toxin production (M. Egusa *et al.*, unpublished results). However, in at least some cases, AAL-toxin alone is not sufficient for infection of the toxin-sensitive plants by the toxin-producing isolates. Brandwagt *et al.* (2001) identified many *Nicotiana* species sensitive to AAL-toxin and found that the majority were resistant to the tomato pathotype. Although many *Fusarium* species produce fumonisin (Rheeder *et al.*, 2002; Stępień *et al.*, 2011), they cannot infect SAM-sensitive tomato. The nonhost resistance consists of multilayered defence systems that include preinvasion resistance and postinvasion resistance. Fungi must therefore overcome multiple defence mechanisms to establish compatibility on host plants.

ACR-toxin

Alternaria leaf spot of rough lemon was first reported in South Africa (Doidge, 1929). The causal pathogen, the rough lemon pathotype of *A. alternata*, affects only rough lemon (*Citrus jambhiri*) and rangpur lime (*Citrus limonia*) (a hybrid of acid mandarin and rough lemon), two common rootstocks in some citrus-growing areas.

The target site of ACR-toxin was first expected to be the mitochondrion based on electron microscopic examination (Kohmoto *et al.*, 1984), and monitoring of oxidative phosphorylation using isolated physiologically active mitochondria confirmed a specific mode of action of ACR-toxin on rough lemon mitochondria (Fig. 4) (Akimitsu *et al.*, 1989). ACR-toxin caused uncoupling of mitochondrial oxidative-phosphorylation similar to that of classic protonophores, such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenyl hydrazone, with a loss of membrane potential, but the effects differed slightly from other uncouplers because the toxin also causes leakage of the cofactor NAD^+ from the tricarboxylic acid cycle (Akimitsu *et al.*, 1989). These effects of ACR-toxin

are specific to mitochondria isolated from susceptible cultivars. Mitochondria from resistant citrus are 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 T-toxin produced by *C. heterostrophus* race T. Both ACR- and T-toxins are structurally similar, consisting of long-chain fatty acid-like polyketides, and having similar polyol moieties (Kono & Daly, 1979; Gardner *et al.*, 1985a, b; Nakatsuka *et al.*, 1986b). Although there are similarities found in their modes of action as well as their structures, both toxins are highly selective for the respective hosts, and T-toxin has no toxic or protective effects on leaf tissue or mitochondria from rough lemon, and conversely, ACR-toxin has no effect on T-cms maize (Akimitsu *et al.*, 1989).

The *ACRS* gene that confers sensitivity of *Citrus* species to ACR-toxin has been identified from the mitochondrial genome of rough lemon (Ohtani *et al.*, 2002). The gene confers ACR-toxin sensitivity to *E. coli*, and the mechanism of specificity in plants is alternative processing of the transcript of the gene conferring ACR-toxin sensitivity (Fig. 5) (Ohtani *et al.*, 2002). For initial screening of the gene, mitochondrial DNA was isolated from rough lemon, and random BamHI fragments were expressed in *E. coli*, which is normally resistant to ACR-toxin. Addition of the toxin at a minimum concentration of 50 nM abolished oxygen uptake by a toxin-sensitive *E. coli* strain. The plasmid in the toxin-sensitive *E. coli* strain that conferred sensitivity to ACR-toxin contained a 355-bp insert containing *ACRS*, and a search of the nonredundant databases in NCBI identified the region as located within the intron of the tRNA-Ala gene. This intron, known as a self-splicing group II intron, catalyses its own splicing (Leaver & Gray, 1982), and many of these introns have been reported to contain ORFs encoding polypeptides (Fassbender *et al.*, 1994).

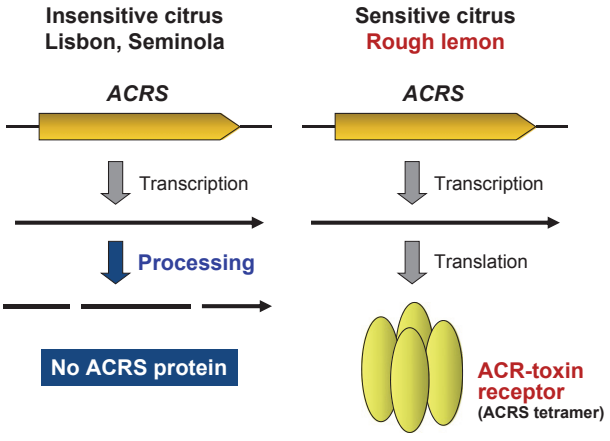

Fig. 5. Mechanism of ACR-toxin sensitivity controlled by receptor transcript processing in mitochondria.

To investigate the relationship of ACRS to sensitivity to ACR-toxin and hence susceptibility to *Alternaria* leaf spot pathogen, we sequenced this region of the mitochondrial genome from resistant cultivars and 13 species of citrus and found that the regions in the resistant citrus are identical to that of rough lemon (Ohtani *et al.*, 2002). However, examination of *ACRS* transcripts demonstrated that sensitivity to the toxin is not controlled by the presence or absence of *ACRS* but rather by post-transcriptional modification of the *ACRS* transcripts (Fig. 5) (Ohtani *et al.*, 2002). The peptide encoded by *ACRS* was detected by immunoblotting only in rough lemon mitochondria, but not in toxin-insensitive citrus mitochondria, and the peptide appeared to consist of sodium dodecyl sulphate (SDS)-resistant oligomers that have been reported for many pore-forming transmembrane proteins (Fig. 5) (Ohtani *et al.*, 2002). The known physiological effects of ACR-toxin are consistent with its forming pores in membranes, because ACR-toxin-treated mitochondria show increased permeability not only to protons but also to NAD^+ (Akimitsu *et al.*, 1989). Several series of deletion experiments and examination of *ACRS* transcripts revealed that the putative protein-coding region required for the translation to the oligomeric pore-forming transmembrane protein controlling ACR-toxin sensitivity is predicted to be 171 bp, and the calculated molecular weight of the product is 6683 kDa (Ohtani *et al.*, 2002). As ACRS antibodies detected three proteins with molecular weights of 14, 21 and 28 kDa in extracts from rough lemon mitochondria, the proteins detected by immunoblotting could be the dimer, trimer and tetramer that are not fully dissociated during SDS polyacrylamide gel electrophoresis (Ohtani *et al.*, 2002).

Genetics of HST production

Alternaria alternata pathogens producing HSTs are good models for studying the development of pathogenic specialization in plant pathogenic fungi, because their HSTs have been well characterized as effectors of host-specific pathogenesis. To assess the molecular basis of the pathogenic specialization of these pathogens, the gene clusters required for HST production have been identified from six pathotypes (Japanese pear, strawberry, tangerine, apple, tomato and rough lemon) (Table 2).

AK-toxin, AF-toxin and ACT-toxin

HSTs produced by the Japanese pear, strawberry and tangerine pathotypes have a common moiety, EDA, in their

Table 2. HST biosynthetic genes identified from *Alternaria alternata* pathotypes

Pathogen	Gene	Gene product	Chromosome (Mb)
Apple pathotype	*AMT* genes	AM-toxin cyclic depsipeptide	< 1.8
Strawberry pathotype	*AFT* genes	AF-toxin EDA ester	1.0
Japanese pear pathotype	*AKT* genes	AK-toxin EDA ester	< 2.0*
Tangerine pathotype	*ACTT* genes	ACT-toxin EDA ester	< 2.0
Rough lemon pathotype	*ACRT* genes	ACR-toxin polyketide	< 1.5
Tomato pathotype	*ALT* genes	AAL-toxin polyketide ester	1.0

*Exceptional strain 15A was found to have a 4.1-Mb chromosome encoding *AKT* genes (Tanaka & Tsuge, 2000).

structures (Fig. 1) (Nakashima *et al.*, 1985; Nakatsuka *et al.*, 1986a; Kohmoto *et al.*, 1993). Free EDA is detected in culture filtrates of these pathotypes (Feng *et al.*, 1990; Nakatsuka *et al.*, 1990; Nishimura & Nakatsuka, 1989; Miyamoto *et al.*, 2009). When the ^{3}H-labelled EDA was added to a growing liquid culture of the Japanese pear pathotype strain, it was efficiently converted to AK-toxin (Feng *et al.*, 1990), showing that EDA is an intermediate for the toxin pathways. Thus, it is easy to presume that these three pathotypes share genes for EDA biosynthesis.

The gene cluster involved in HST biosynthesis of *A. alternata* pathogens was first isolated from the Japanese pear pathotype (Tanaka *et al.*, 1999). Restriction enzyme-mediated integration transformation was used to isolate AK-toxin-minus mutants (Tanaka *et al.*, 1999). Molecular analysis of one of the mutants identified the affected gene, which is essential for AK-toxin biosynthesis. Structural and functional analysis of a cosmid clone containing the tagged site demonstrated that six AK-toxin biosynthetic genes (*AKT1*, *AKT2*, *AKT3*, *AKT4*, *AKTR* and *AKTS1*) (Tanaka *et al.*, 1999; Tanaka & Tsuge, 2000; R. Nishikawa *et al.*, unpublished results). *AKTR* encodes a putative transcription regulator containing a zinc binuclear cluster DNA-binding domain typical of the fungal Zn(II)2Cys6 family of proteins (Tanaka & Tsuge, 2000). The other genes encode proteins with similarity to many enzymes reported from fungi (Tanaka *et al.*, 1999; Tanaka & Tsuge, 2000; R. Nishikawa *et al.*, unpublished results). Thus, it is likely that *AKTR* is a regulatory gene for expression of the other genes encoding enzymes for AK-toxin biosynthesis. It also appeared that the Japanese pear pathotype strains have multiple copies of functional or nonfunctional homologues of the *AKT* genes (Tanaka *et al.*, 1999; Tanaka & Tsuge, 2000).

DNA gel blot analysis was used to assess the distribution of the *AKT* homologues in *A. alternata* pathogens. *AKTS1* was unique to the Japanese pear pathotype, and the others were also present in the strawberry and tangerine pathotypes (Tanaka *et al.*, 1999; Tanaka & Tsuge, 2000; R. Nishikawa *et al.*, unpublished results). This result supported that these three pathotypes share common genes required for EDA biosynthesis.

To isolate AF-toxin and ACT-toxin biosynthetic genes from the strawberry and tangerine pathotypes, genomic cosmid libraries of the two pathotypes were screened with the *AKT* gene probes. Structural analysis of a clone of the strawberry pathotype found *AFT1*, *AFT3* and *AFTR* having strong similarity to *AKT1*, *AKT3* and *AKTR*, respectively, of the Japanese pear pathotype (Hatta *et al.*, 2002). A clone of the tangerine pathotype was found to contain *ACTT2*, *ACTT3* and *ACTTR*, with strong similarity to *AKT2*, *AKT3* and *AKTR*, respectively (Masunaka *et al.*, 2000; Miyamoto *et al.*, 2008). The respective gene pairs of these three pathotypes share > 90% nucleotide identity. However, the arrangement of genes in the clusters differs among the three pathotypes. The strawberry pathotype clone analysed lacks the *AKT2* and *AKT4* homologues and contains two new genes, *AFT8* and *AFTS1* (Hatta *et al.*, 2002; Ito *et al.*, 2004). *AFT8* homologues are also present in the Japanese pear and tangerine pathotypes, and *AFTS1* is unique to the strawberry pathotype. The tangerine pathotype clone analysed lacks *AKT1* and *AKT4* homologues and has two new genes, *ACTT5* and *ACTT6*, which are also present in the Japanese pear and strawberry pathotypes (Miyamoto *et al.*, 2009). Analysis of bacterial artificial chromosome (BAC) clones containing part of the *ACTT* cluster identified *ACTTS* genes, which are present only in the tangerine pathotype (Ajiro *et al.*, 2010; Miyamoto *et al.*, 2010). Thus, the *AKT*, *AFT* and *ACTT* clusters consist of pathotype-specific genes as well as genes common to the three pathotypes. Strains of the strawberry and tangerine pathotypes also have multiple copies of functional or nonfunctional homologues of the toxin biosynthetic genes in their genomes, as do the Japanese pear pathotype strains (Hatta *et al.*, 2002; Ruswandi *et al.*, 2005; Miyamoto *et al.*, 2008, 2009, 2010; Ajiro *et al.*, 2010). These results imply structural and functional complexity of the genomic regions controlling HST biosynthesis in these three pathotypes.

AK-toxin biosynthetic enzymes Akt1, Akt2 and Akt3 of the Japanese pear pathotype and their orthologues in the strawberry and tangerine pathotypes have peroxisomal targeting signal type 1 (PTS1)-like tripeptides at their C-terminal ends and are localized in peroxisomes (Imazaki *et al.*, 2010). Mutation of *AaPEX6*, which encodes a peroxin protein essential for peroxisome biogenesis, in the Japanese pear pathotype resulted in lack of functional

peroxisomes and the complete loss of AK-toxin production and pathogenicity (Imazaki *et al.*, 2010). As peroxisomes have the function of β-oxidation of fatty acids (van Roermund *et al.*, 2003), the involvement of peroxisomes in EDA biosynthesis is an evolutionarily interesting characteristic of this type of toxin production.

Disruption of *AFTS1*, which encodes a protein with similarity to an aldo-keto reductase, in the strawberry pathotype provided a persuasive example for the critical role of HSTs in host-specific pathogenicity of *A. alternata* pathogens (Ito *et al.*, 2004). The strawberry pathotype produces AF-toxins I and II and is pathogenic not only to strawberry, but also to Japanese pear susceptible to the Japanese pear pathotype (Maekawa *et al.*, 1984). When *AFTS1* was disrupted, the mutants lacked AF-toxin I, toxic to both strawberry and pear, and produced only AF-toxin II, toxic only to pear. The mutants were pathogenic to pear, but not strawberry, resembling the Japanese pear pathotype (Ito *et al.*, 2004). This experiment represented a novel example of the host range of a plant pathogenic fungus being restricted by modification of secondary metabolism.

Toxin biosynthetic genes appear to be clustered on small chromosomes of < 2.0 Mb in most strains of the three pathotypes tested (Table 2) (Hatta *et al.*, 2002; Ito *et al.*, 2004; Masunaka *et al.*, 2005; Miyamoto *et al.*, 2008). During the course of targeting the *AFT* genes in the strawberry pathotype strain NAF8, mutants that lack the 1.0-Mb chromosome encoding *AFT* genes were isolated (Hatta *et al.*, 2002). The mutants lost the ability to produce AF-toxin and to cause disease on leaves of strawberry and Japanese pear. However, they grew normally and formed conidia on culture media. Thus, this chromosome appears to be a conditionally dispensable (CD) chromosome, which is not required for growth, but does confer an advantage for colonizing certain ecological niches (Covert, 1998; Hatta *et al.*, 2002). Recently, we determined the structure of the 1.0-Mb chromosome of strain NAF8 and sequenced the entire *AFT* region of about 390 kb in the chromosome (R. Hatta *et al.*, unpublished results). We found two to seven copies of more than 20 putative *AFT* genes in this region. Interestingly, this region also contains many transposon-like sequences, most of which are inactive transposon fossils (Hatta *et al.*, 2006).

AM-toxin

The apple pathotype produces cyclic peptide AM-toxins (Okuno *et al.*, 1974; Ueno *et al.*, 1975, 1977). Cyclic peptides are generally synthesized via non-ribosomal pathways by large multifunctional enzymes called nonribosomal peptide synthetases (NRPS), which have highly conserved domains (Keller *et al.*, 2005). Johnson *et al.* (2000) isolated *AMT1* (previously named *AMT*), which encodes an NRPS required for AM-toxin biosynthesis, by PCR-based cloning with primers designed to conserved domains of fungal NRPS genes. *AMT1* encodes a 479-kDa NRPS containing four catalytic domains responsible for the activation of each residue in AM-toxin (Fig. 1).

The apple pathotype strains were found to have a homologue, *AMT2*, of *AFTS1* of the strawberry pathotype (Ito *et al.*, 2004). Although AF-toxin and AM-toxin are classified into different chemical groups, a similarity in structure of AF-toxin I and AM-toxins was found (Fig. 1). AM-toxins consist of four components, and one of the components is 2-hydroxy-isovaleric acid. AF-toxin I contains 2,3-dihydroxy-isovaleric acid, which is synthesized by addition of a 3-hydroxy group to 2-hydroxy-isovaleric acid. Thus, 2-hydroxy-isovaleric acid is probably a common precursor of AF-toxin I and AM-toxins. Genetic complementation of the *AFTS1* mutant with *AMT2* showed that these two genes have a conserved function (Ito *et al.*, 2004). Involvement of *AMT2* in AM-toxin biosynthesis was confirmed by disruption of *AMT2* in the apple pathotype (Harimoto *et al.*, 2008). AftS1 and Amt2 both have significant similarity to enzymes of the aldo-keto reductase superfamily, suggesting that these enzymes catalyse the conversion of 2-keto-isovaleric acid, the last intermediate in the biosynthetic pathway of L-valine, to 2-hydroxy-isovaleric acid.

Johnson *et al.* (2001) found an AM-toxin-minus, nonpathogenic mutant from laboratory stocks of the apple pathotype strains. The original strain of this mutant had a 1.1-Mb chromosome encoding the *AMT* genes and was pathogenic to susceptible apple cultivars, producing AM-toxin. The AM-toxin-minus mutant, however, lacked the 1.1-Mb chromosome. This mutant grew normally and formed conidia on media, strongly suggesting that *AMT* is located on CD chromosomes in the apple pathotype strains (Johnson *et al.*, 2001). Harimoto *et al.* (2007) examined the chromosomal distribution of the *AMT* genes in apple pathotype strains collected from different prefectures in Japan and found that the genes are encoded by single small chromosomes of < 1.8 Mb in the strains (Table 2).

Harimoto *et al.* (2007) performed expressed sequence tag analysis of a 1.4-Mb chromosome encoding *AMT* genes in strain IFO8984. A cDNA library from AM-toxin-producing culture was screened with the 1.4-Mb chromosome probe, and 196 expressed sequence tags (80 unigenes) were selected from 40 980 clones. Comparison of the transcription levels of the genes in toxin-producing and nonproducing cultures identified 21 genes, including *AMT1* and *AMT2*, that were upregulated (> 10-fold) in toxin-producing cultures. Sequence analysis suggested that the upregulated genes include candidates for novel

AM-toxin biosynthetic genes. Disruption of three genes, *AMT2*, *AMT3* and *AMT4*, upregulated in toxin-producing cultures demonstrated that IFO8984 has multiple copies of these genes in the genome, and all have a similar structure. However, single- or double-copy mutants of the genes produced a smaller amount of AM-toxin than the wild type and showed reduced pathogenicity. Thus, these genes were verified to be required for AM-toxin biosynthesis and hence pathogenicity (Harimoto *et al.*, 2007, 2008). These results indicate that multiple copies of *AMT* genes are required for production of enough AM-toxin to express full pathogenicity in this pathogen.

Recently, we determined the structure of the 1.4-Mb chromosome of strain IFO8984 and identified putative *AMT* clusters in the chromosome (Y. Harimoto *et al.*, unpublished results). IFO8984 seems to have a cluster containing 15 putative *AMT* genes and additional sets of part of the cluster on one side of the chromosome. The 15 putative genes are present in one to four copies in the chromosome. This region also contains many transposon fossils, resembling those of the *AFT* region of the strawberry pathotype (Hatta *et al.*, 2006).

AAL-toxin

AAL-toxins are polyketide-derived compounds similar to fumonisins. Polyketides are synthesized by PKSs, which are large multifunctional enzymes. Akamatsu *et al.* (2003) identified *ALT1*, which encodes a type I PKS required for AAL-toxin biosynthesis, by PCR-based cloning with primers designed to conserved domains of fungal PKS genes. *ALT1* has similarity to *FUM1*, encoding a PKS required for fumonisin biosynthesis in *F. verticillioides* (Proctor *et al.*, 1999; Seo *et al.*, 2001; Akamatsu *et al.*, 2003; Yamagishi *et al.*, 2006).

A genomic BAC library of strain As-27 of the tomato pathotype was screened with the *ALT1* probe, and a 120-kb genomic region of a positive clone was sequenced. As the fumonisin biosynthetic gene (*FUM*) cluster of *F. verticillioides* contains 17 genes within a 45.5-kb region (Proctor *et al.*, 2003; Waalwijk *et al.*, 2004), the 120-kb sequence was compared with the *FUM* cluster sequence. The 120-kb region was found to include at least 13 genes with significant similarity to the genes in the *FUM* cluster, suggesting that the region is involved in AAL-toxin biosynthesis (Y. Akagi *et al.*, unpublished results). Disruption of some of the cluster genes confirmed their involvement in AAL-toxin biosynthesis by the tomato pathotype: *ALT1* (*FUM1* homologue), encoding type I PKS; *ALT2* (*FUM6* homologue), encoding cytochrome P450 monooxygenase; *ALT3* (*FUM7* homologue), encoding aminotransferase; *ALT6* (*FUM13* homologue), encoding short-chain dehydrogenase/reductase; and *ALT13* (*FUM21* homologue), encoding Zn(II)2Cys6 transcription factor (Proctor *et al.*, 1999; Seo *et al.*, 2001; Akamatsu *et al.*, 2003; Proctor *et al.*, 2003; Akagi *et al.*, 2009a; Y. Akagi *et al.*, unpublished results). Although the respective gene pairs in the *ALT* and *FUM* clusters share significant similarity, the arrangement of genes in the clusters differs between the two clusters.

Akagi *et al.* (2009a) found that the *ALT* genes are encoded by single small chromosomes of ~ 1.0 Mb in all strains of the tomato pathotype, including those from different countries (Table 2). They also isolated a mutant lacking the 1.0-Mb chromosome from strain As-27 and identified the chromosome as a CD chromosome. Recently, the structure of the 1.0-Mb chromosome of As-24 was determined (Y. Akagi *et al.*, unpublished results). As-27 has two sets of the AAL-toxin biosynthetic gene cluster, consisting of 13 putative *ALT* genes, on the chromosome.

ACR-toxin

The rough lemon pathotype produces polyketide ACR-toxin (Gardner *et al.*, 1985a, b; Nakatsuka *et al.*, 1986b). As described above, HST biosynthetic genes of the other pathotypes are clustered on small chromosomes (Akamatsu *et al.*, 1999; Johnson *et al.*, 2001; Hatta *et al.*, 2002; Masunaka *et al.*, 2005; Harimoto *et al.*, 2007; Miyamoto *et al.*, 2008, 2010; Akagi *et al.*, 2009a). Masunaka *et al.* (2005) found that the rough lemon pathotype strains have small chromosomes of 1.2–1.5 Mb. Mass sequence analysis of the 1.5-Mb chromosome of strain HC1 identified multiple genes, which are possibly involved in ACR-toxin biosynthesis and are designated *ACRT* genes (Izumi *et al.*, 2012).

The candidate genes often have multiple paralogues, making it difficult to disrupt entire functional copies of the genes using homologous recombination-mediated gene disruption. To overcome this problem, RNA silencing was used to knock down the functional copies, just like the example of the first trial of RNA silencing for the *Alternaria* HST biosynthesis gene of *ACTT2*, which encodes a putative hydrolase and is present in multiple copies in the genome of the tangerine pathotype (Miyamoto *et al.*, 2008). One of the candidates, *ACRT2*, which encodes a PKS of 2514 amino acids and is unique to the rough lemon pathotype, was silenced by transforming the rough lemon pathotype strain with a plasmid construct expressing hairpin *ACRT2* RNA. The *ACRT2*-silenced transformants, in which *ACRT2* transcripts were not detectable, lost ACR-toxin production and pathogenicity, indicating that this gene encodes a PKS essential for ACR-toxin biosynthesis and hence pathogenicity (Izumi *et al.*, 2012).

Evolution of *A. alternata* pathogens producing HSTs

HST biosynthetic gene clusters

In filamentous fungi, genes involved in the same secondary metabolite biosynthetic pathway are often located at the same locus in the genome and coexpressed, defining gene clusters (Rosewich & Kistler, 2000; Walton, 2000; Keller *et al.*, 2005; Hoffmeister & Keller, 2007). The involvement of horizontal gene transfer (HGT) in the evolution of fungal secondary metabolite gene clusters has been frequently discussed, because the clustering of the genes for a pathway raises the possibility of transfer of the pathway in a single event by HGT (Walton, 2000; Oliver & Solomon, 2008; Mehrabi *et al.*, 2011). Recently, a plausible example for interspecific HGT of a fungal secondary metabolite gene cluster has been provided by Slot & Rokas (2011). They discovered that a complete sterigmatocystin gene cluster in *Podospora anserina* had been horizontally transferred from *Aspergillus*. Several *Aspergillus* species produce the highly toxic mycotoxins, aflatoxin and sterigmatocystin (the precursor of aflatoxin), and have biosynthetic gene clusters in a 50- to 70-kb region encoding at least 23 genes (Brown *et al.*, 1996; Yu *et al.*, 2004; Carbone *et al.*, 2007). The *P. anserina* cluster is highly conserved in content, sequence and microsynteny with the sterigmatocystin cluster of *Aspergillus nidulans*, and the fungus was verified to produce sterigmatocystin (Slot & Rokas, 2011). The pine needle pathogen *Dothistroma septosporum* was also found to have ten orthologues of aflatoxin biosynthetic genes, which are required for biosynthesis of the aflatoxin-related compound dothistromin (Zhang *et al.*, 2007). Phylogenetic analysis suggested that these genes were also obtained from *Aspergillus* by HGT (Zhang *et al.*, 2007; Slot & Rokas, 2011). These observations suggest that HGT events have been frequent in the evolution of aflatoxin cluster-related genes.

The tomato pathotype of *A. alternata* produces a SAM, AAL-toxin, which is similar in structure to the fumonisin produced by *Fusarium* species, and the AAL-toxin gene cluster includes at least 13 orthologues of the *FUM* genes of *F. verticillioides* (Y. Akagi *et al.*, unpublished results). To date, 15 *Fusarium* species have been reported to produce fumonisins, and some have been verified to have *FUM* genes (Rheeder *et al.*, 2002; Proctor *et al.*, 2008; Stępień *et al.*, 2011). For example, the *Fusarium oxysporum* cluster has the same order and orientation of the 17 *FUM* genes as the *F. verticillioides* cluster (Proctor *et al.*, 2008). In contrast, no species in the genus *Alternaria* other than the tomato pathotype of *A. alternata* has been identified to produce SAMs, suggesting that the SAM biosynthetic gene cluster originated and evolved in *Fusarium* and was transferred from *Fusarium* to *A. alternata*.

The *AFT* cluster region on the 1.0-Mb CD chromosome of the strawberry pathotype contains many DNA transposon-like sequences (Hatta *et al.*, 2006). Most of the sequences, however, have incomplete ORFs for transposases owing to deletions, termination codons and/or frameshifts, indicating that they are inactivated elements (fossils). They are unique to the AF-toxin gene cluster region in the genome. Accumulation of transposon fossils was also found in the *AMT* cluster region on the 1.4-Mb chromosome of the apple pathotype (Y. Harimoto *et al.*, unpublished results). The presence of unique transposon fossils in the HST gene cluster regions suggests the involvement of HGT for establishment of the *AFT* and *AMT* gene clusters in the CD chromosomes, although candidate donor fungi having homologous clusters in their genomes have not been identified.

The *A. alternata* pathotypes have multiple copies of the toxin biosynthetic genes, and the genomic regions controlling biosynthesis of HSTs are not simple genetic loci, but instead are large, complex regions of DNA resulting from extraordinary duplication and recombination events (Tanaka *et al.*, 1999; Johnson *et al.*, 2000; Masunaka *et al.*, 2000; Tanaka & Tsuge, 2000; Hatta *et al.*, 2002; Ruswandi *et al.*, 2005; Harimoto *et al.*, 2007; Miyamoto *et al.*, 2008; Akagi *et al.*, 2009a). In disruption experiments of toxin biosynthetic genes in the apple, strawberry and tangerine pathotypes, it was shown that multiple copies of the genes are a prerequisite for the pathotypes to produce enough toxin to express full pathogenicity (Ruswandi *et al.*, 2005; Harimoto *et al.*, 2007, 2008; Miyamoto *et al.*, 2008, 2009, 2010). This suggests that the duplication of toxin biosynthetic gene clusters in the genomes was the critical event for the pathogens to gain considerable virulence, increasing fitness by efficiently infecting host plants. As mentioned above, many transposon fossils are distributed within HST gene cluster regions of the strawberry and apple pathotypes. It is possible that transposon-like elements were involved in the duplication events of HST gene clusters, enabling the pathogens to gain stable pathogenicity.

Structural complexity of HST biosynthetic gene loci has also been reported in two *Cochliobolus* pathogens, *C. heterostrophus* race T, producing T-toxin, and *C. carbonum* race 1, producing HC-toxin. The *Tox1* locus controlling T-toxin production is composed of two large segments, *Tox1A* and *Tox1B*, of DNA (totalling approximately 1.2 Mb) that map to two chromosomes in *C. heterostrophus* race T. To date, nine genes have been identified at the *Tox1* locus, including two PKSs, one decarboxylase, five dehydrogenases and a protein with unknown function (Yang *et al.*, 1996; Rose *et al.*, 2002;

Baker *et al.*, 2006; Inderbitzin *et al.*, 2010). The *TOX2* locus controlling HC-toxin production extends over more than 540 kb and contains multiple copies of multiple genes (Ahn & Walton, 1996; Walton, 2006). During the past two decades, various researchers have shown that the genes for production of a broad range of fungal secondary metabolites are clustered (Walton, 2000; Keller *et al.*, 2005; Hoffmeister & Keller, 2007). Gene clusters, other than for HSTs, generally reside as a single set in the fungal genome. The presence of multiple copies of genes for biosynthesis of a single secondary metabolite is characteristic of the molecular evolution of HST biosynthesis and has pathological significance.

CD chromosomes controlling HST production and pathogenicity

Pulsed-field gel electrophoresis analysis of chromosomes of *A. alternata* strains, including seven pathotypes and nonpathogenic strains, showed that the strains had nine to 11 chromosomal bands of 0.4–5.7 Mb, with genome sizes of ~ 30 Mb (Akamatsu *et al.*, 1999). The electrophoretic karyotypes could not be used to differentiate strains by correlation with pathotypes because of highly variable karyotypes among strains. This analysis distinguished an interesting difference in karyotypes between pathogenic and nonpathogenic strains. All strains from the pathotypes had small chromosomes of < 2.0 Mb, but nonpathogenic strains did not have these small chromosomes (Akamatsu *et al.*, 1999). Small chromosomes of several fungi have been identified as supernumerary (dispensable) chromosomes (Covert, 1998). The function of supernumerary chromosomes in most species is still cryptic. However, in *Nectria haematococca*, the causal agent of root rot of pea, the 1.6-Mb supernumerary chromosomes encode genes for phytoalexin detoxification as well as other virulence determinants (Miao *et al.*, 1991; Covert *et al.*, 1996; Wasmann & VanEtten, 1996; VanEtten *et al.*, 1998; Han *et al.*, 2001). The inherent instability of the chromosomes does not affect growth on media, but does affect the disease-causing capacity on host plants. Hence, such chromosomes have been termed CD chromosomes (Covert, 1998).

Molecular characterization of the HST biosynthetic gene clusters from six pathotypes of *A. alternata* demonstrated that the clusters reside on single small chromosomes of < 2.0 Mb in most strains tested (Table 2) (Akamatsu *et al.*, 1999; Johnson *et al.*, 2001; Hatta *et al.*, 2002; Masunaka *et al.*, 2005; Harimoto *et al.*, 2007; Miyamoto *et al.*, 2008, 2009; Akagi *et al.*, 2009a). Loss of the small chromosomes was observed in the strawberry, apple and tomato pathotypes, and the small chromosomes appeared to be CD chromosomes (Akamatsu *et al.*, 1997; Johnson *et al.*, 2001; Hatta *et al.*, 2002; Akagi *et al.*, 2009a). The fact that most strains of the Japanese pear, tangerine and rough lemon pathotypes also carry HST biosynthetic genes on small chromosomes suggests that host-specific pathogenicity of *A. alternata* pathotypes is controlled by these small CD chromosomes, encoding HST gene clusters.

Although the origin of none of the supernumerary chromosomes of fungi has been identified, the distribution of supernumerary chromosomes in certain strains within the same fungal species suggests that they are of a different origin from the essential chromosomes in the same genome, and that they may have been introduced into the genome by horizontal transfer from another species (Covert, 1998; Rosewich and Kistler, 2000; Walton, 2000). In *Colletotrichum gloeosporioides*, supernumerary chromosomes have been shown to have the capacity for transfer between otherwise genetically isolated strains (Masel *et al.*, 1996; He *et al.*, 1998). Recently, the occurrence of horizontal chromosome transfer (HCT) between pathogenic and nonpathogenic strains of *F. oxysporum* has been reported (Ma *et al.*, 2010). In cocultivation experiments using strains of the tomato wilt pathogen *Fusarium oxysporum* f. sp. *lycopersici* and nonpathogenic *F. oxysporum*, the 2.0-Mb chromosome of the tomato pathogen could be transferred into the nonpathogenic strain, which conferred pathogenicity to the nonpathogenic strain towards tomato (Ma *et al.*, 2010). This finding may explain the generation of new pathogenic genotypes within a fungal species and suggest that these events have also occurred in nature.

The CD chromosomal DNA encoding HST gene clusters of the strawberry, apple or tomato pathotype of *A. alternata* was found to strongly hybridize to only the CD chromosome itself in the same genome of each pathotype, suggesting that the CD chromosomes are structurally unrelated to the large, essential chromosomes in their genomes (Hatta *et al.*, 2002; Harimoto *et al.*, 2007; Akagi *et al.*, 2009a). In the tomato pathotype, the CD chromosomes of the strains collected worldwide had identical NotI-digestion patterns, and the sequences of some genes on the chromosomes were identical among the strains, suggesting that their structures are conserved (Akagi *et al.*, 2009a). In contrast, the sequences of other genes, such as β-tubulin, melanin biosynthetic PKS and mating-type genes, located on the essential chromosomes, showed considerable polymorphisms among the strains. These results indicated that the origin of the CD chromosomes might be different from that of the essential chromosomes in the tomato pathotype genome. Analysis of genetic relatedness among *A. alternata* pathotypes using DNA markers demonstrated that a single pathotype population does not form a monophyletic group (Adachi

et al., 1993; Kusaba & Tsuge, 1994, 1995). The fact that the *A. alternata* pathotypes have HST gene clusters on small CD chromosomes, which nonpathogenic strains do not have, suggests that the ability to produce HSTs in the pathotypes could be imparted by intraspecies transfer of CD chromosomes to nonpathogenic strains of *A. alternata*.

Protoplast fusion experiments provided evidence for intraspecies transfer of CD chromosomes in *A. alternata*. Hybrid strains between the tomato and apple pathotypes and between the tomato and strawberry pathotypes were made by protoplast fusion (Salamiah *et al.*, 2001; Akagi *et al.*, 2009a, b). The fusants produced two toxins produced by the parental strains and showed pathogenicity to both plants affected by the parents (Salamiah *et al.*, 2001; Akagi *et al.*, 2009a, b). The fusants carried two CD chromosomes, one derived from each of the parental strains. Thus, it appears that *A. alternata* is able to accept and maintain a small, exogenous chromosome in its genome.

If a cell fusion event between two different pathotypes occurred in nature, a fusion strain that harbours two CD chromosomes may have an expanded host range with an additional toxin. Masunaka *et al.* (2005) discovered an *A. alternata* isolate from a leaf spot on rough lemon in Florida that is pathogenic to both rough lemon and tangerine by producing two distinct HSTs, ACT-toxin and ACR-toxin. The quantity of each toxin produced by the isolate in cultures was similar to that produced by each pathotype independently. Inoculation of the isolate on leaves of both ACT-toxin-sensitive and ACR-toxin-sensitive citrus cultivars resulted in necrosis. The dual host specificity and toxin production by this isolate is not common in populations of *A. alternata* on citrus. This is the only isolate with pathogenicity to two different citrus hosts that has been found among hundreds of isolates examined to date. This isolate carries two small chromosomes of 1.05 and 2.0 Mb, which encode the *ACTT* and *ACRT* genes, respectively, indicating that the ability of the strain to produce two HSTs was conferred through the acquisition of two small chromosomes in the same genome (Masunaka *et al.*, 2005).

A possible mechanism of intraspecies transfer of CD chromosomes in *A. alternata* is a parasexual cycle through hyphal anastomosis. Hyphal anastomosis has been observed between different pathotype strains and between pathogenic and nonpathogenic strains of *A. alternata* in laboratory experiments (Huang *et al.*, 1996). Adachi and Tsuge (1994) observed the frequent occurrence of coinfection in single black spot lesions of Japanese pear leaves by two *A. alternata* isolates in the field and found cases of both nonpathogenic and pathogenic isolates of *A. alternata* coinfecting single lesions. Coinfection events may play a significant role in the parasexual cycle of *A. alternata* in the field because coinfected lesions enable different isolates to coexist and interact in a very small space.

Why have the HST gene clusters required for biosynthesis of chemically different metabolites toxic to different plants been retained on dispensable chromosomes in all *A. alternata* pathotypes? This phenomenon might be related to unique features of *A. alternata* pathogens. First, *A. alternata* is an asexual species that lacks sexual reproduction; hence, highly variable karyotypes are stably maintained. Extra chromosomes can survive through parasexual recombination because no homologous pair chromosomes exist. Second, the saprophytic life cycle dominates over the parasitic life cycle for *A. alternata*. HST production should increase the fitness of the producers in the presence of host plants because it increases their capacity for reproduction on host plants. However, there may be a fitness cost to HST production in the absence of host plants. In the saprophytic stage, the HST-producing isolates could become competitive by losing nonessential genes through loss of the CD chromosome, along with its HST genes. The CD chromosomes in the *A. alternata* pathotypes are regarded as pathogenicity chromosomes. The HCT hypothesis, in which intraspecies HCT of a CD chromosome is expected to confer the ability to produce an HST to a nonpathogenic *A. alternata* isolate, provides a possible mechanism whereby a new pathogenic genotype can arise in nature.

Concluding remarks

Plant infection appears to be a host-specific event. Participation of HSTs is one of the most clearly revealed mechanisms in host-selective pathogenesis in fungal plant diseases. There are now seven diseases identified as caused by *A. alternata* in which HSTs are responsible for fungal pathogenesis. As *A. alternata* is fundamentally a ubiquitous, saprophytic fungus, the pathotypes are a fascinating case for studying intraspecific variation and evolution of pathogenicity in plant pathogenic fungi. All isolates belonging to *A. alternata* possess appressorium-mediated, direct penetration ability. However, HST-producing isolates can directly penetrate epidermal cells only on toxin-sensitive plants. All *Alternaria* HSTs are produced by germinating conidia as well as by host-colonizing hyphae. HSTs affect regulation of metabolism, permeability and other key processes of toxin-sensitive plant tissue and suppress induction of defence responses. This potential of HST permits the producer to invade and colonize host cells by conditioning a toxin-sensitive plant as an appropriate host. Although the target molecules of toxins have been identified for the ACR-toxin–rough lemon and AAL-toxin–tomato systems, the targets of the other HSTs remain

to be explored. AAL-toxin causes programmed cell death on toxin-sensitive plants; however, it is unknown whether programmed cell death is a common feature for many HSTs. Defining how respective HSTs cause cell death will contribute to our understanding of the roles of cell death in establishment of a necrotrophic pathosystem.

The HST biosynthetic genes that have been isolated from six pathotypes of *A. alternata* and characterized are clustered, as are the genes responsible for production of other secondary metabolites in fungi. Detailed analysis of HST gene clusters has clarified their unique features, such as the presence of multiple sets on a single chromosome, high-density distribution of transposon-like sequences in the clusters and storage of the clusters in small CD chromosomes. Extensive phylogenetic analysis of the sequences of genes in the HST clusters and the CD chromosomes will shed light on how the clusters and their ability to produce HSTs evolved. Comparative genomics of CD chromosomes among multiple strains from different pathotypes is needed to understand the origin of the CD chromosomes controlling HST production and pathogenicity in *A. alternata* pathotypes, and may provide new insight into the evolution of fungal plant pathogens.

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