Pathological Characterization and Molecular Analysis of *Elsinoe* Isolates Causing Scab Diseases of Citrus in Jeju Island in Korea

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ABSTRACT

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Two scab diseases are recognized currently on citrus: (i) citrus scab caused by *Elsinoe fawcettii*, which has several pathotypes; and (ii) sweet orange scab caused by *E. australis*. Pathogenicity and cultural characteristics among 36 isolates collected from Jeju Island were investigated. Of 30 isolates from satsuma mandarin, yuzu, and kinkoji, all were *E. fawcettii*; 27 were similar to the Florida broad host range pathotype and 3 were similar to the Florida narrow host range pathotype by inoculation of differential hosts. Six isolates from natsudaikai were nonpathogenic to satsuma mandarin, rough lemon, sour orange, grapefruit, cleopatra mandarin, and natsudaikai leaves, and were only pathogenic to natsudaikai fruit. Isolates from natsudaikai usually produced unique tomentose colonies on potato dextrose agar compared with isolates from other citrus species. The colonies were relatively fast growing, radially sulcate, larger, and more expansive than the gummy, mucoid colonies of other isolates. Isolates from Florida, Australia, Argentina, and Jeju Island (Korea) were genetically differentiated using random amplified polymorphic DNA markers. *E. fawcettii* from Korea, Florida, and Australia, *E. australis* from Argentina, and natsudaikai isolates clustered closely within groups, but were clearly distinguishable among groups.

Scab diseases of citrus produce fruit blemishes that reduce the value of the fruit for the fresh market and are among the most important diseases reducing farmers' income in Jeju Island, Korea. Three scab diseases were described originally on citrus: (i) citrus scab caused by *Elsinoe fawcettii* Bitancourt & Jenk. (anamorph *Sphaeloma fawcettii* Jenk.); (ii) Tryon's scab caused by *S. fawcettii* var. *scabiosa* Jenk. (teleomorph unknown); and (iii) sweet orange scab caused by *E. australis* Bitancourt & Jenk. (anamorph *S. australis* Bitancourt & Jenk.) (10). Subsequently, Tryon's scab was demonstrated to be caused by two pathotypes of *E. fawcettii*, and *S. fawcettii* var. *scabiosa* was not morphologically distinguishable from *E. fawcettii* (9,11). Citrus scab is the most widely distributed disease, occurring in almost every area where rainfall conditions are conducive to infection. Sweet orange scab has been reported only in South America.

Whiteside (12,13) differentiated two biotypes of *E. fawcettii* causing citrus scab

based on host range in Florida, which were subsequently designated as the Florida narrow host range (FNHR) and Florida broad host range (FBHR) pathotypes of citrus scab (11). The FBHR pathotype affects the leaves and fruit of lemon (*Citrus limon* (L.) Burm. f.), rough lemon (*C. jambhiri* Lush), grapefruit (*C. paradisi* Macf.), sour orange (*C. aurantium* L.), Temple and Murcott tangors (*C. sinensis* (L.) Osbeck × *C. reticulata* Blanco); and the fruit of sweet orange (*C. sinensis*). The FNHR type attacks all of the above except sour orange, Temple tangor, and sweet orange fruit. Timmer et al. (11) used five differential hosts for determination of pathotypes of *E. fawcettii* and recognized four pathotypes: FBHR, FNHR, Tryon's, and Lemon, as well as sweet orange scab caused by *E. australis*. Studies have indicated that random amplified polymorphic DNA (RAPD) markers are useful for the identification and differentiation of races or pathotypes of many fungi (2,3,5). Tan et al. (9) readily differentiated *E. australis* from the Florida and Australian biotypes of *E. fawcettii* by restriction analysis of the amplified internal transcribed spacers (ITS) of ribosomal DNA with several endonucleases, by sequencing of the ITS region, and by RAPD analysis.

Satsuma mandarin (*C. unshiu* (Macf.) Marc.) and yuzu (*C. junos* Sieb ex Tanaka) are commercially cultivated on Jeju Island.

Satsuma mandarin, which occupies over 95% of 26,000 ha devoted to citrus, is severely affected by scab. Kinkoji (*C. obovoidea* Hort ex Tak.) and natsudaikai (*C. natsudaikai* Hayata), which are not commercially cultivated but are used for garden trees, are affected by scab, and disease is often severe on kinkoji. Determination of the taxonomic relationships and host ranges of the described species and pathotypes of citrus scab is important for regulatory purposes, breeding of disease-resistant cultivars, and in the selection of cultivars for use in various citrus areas. However, the distribution and host range of the species and pathotypes of citrus scab pathogens on Jeju Island in Korea have not been determined. Therefore, this study was carried out to compare the cultural and pathological characteristics among isolates of citrus scab pathogens in Jeju Island, and to determine the level of genetic variability among the isolates, pathotypes, and species by RAPD analysis.

MATERIALS AND METHODS

Isolates. Isolates from Jeju Island were collected from leaves or fruits of four citrus species: satsuma mandarin, yuzu, kinkoji, and natsudaikai in Jeju Island (Table 1). Isolations were made by disinfesting fruit for 1 min in 75% ethanol and then 1 min in 1% sodium hypochlorite followed by rinsing in sterile water. After drying, scab pustules were scraped with a scalpel to deposit flakes of diseased tissue onto half-strength PDA (12.0 g of potato dextrose broth and 15 g of agar in 1 liter of distilled water). Colonies recovered from the plates were derived from a small piece of a single pustule which theoretically is produced by a single conidium; therefore, cultures were not single spored.

Isolates of *E. fawcettii* were obtained from Florida and from Australia (M. Priest, New South Wales Agriculture, Orange, NSW). Isolates of *E. australis* were obtained directly from Argentina (S. M. Garrán, Instituto Nacional de Tecnología Agropecuaria, Concordia, Entre Ríos) or from the collection of M. Priest in Australia. All isolates were maintained on PDA slants.

Production of conidia. Conidia were produced by the method of Whiteside (13). Small pieces of mycelium (5 to 10 mm³) were removed from a 2- to 3-week-old

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culture on PDA, crushed in a plastic petri plate with a spatula, and resulting fragments stirred into liquid-modified Fries medium (12). Plates were incubated for 1 or 2 days at 27°C. The plates were then washed three times with sterile, distilled water, and incubated overnight in the dark.

Pathogenicity. Pathogenicity was determined on leaves of the differential hosts previously described (11): rough lemon, grapefruit, sour orange, cleopatra mandarin (*C. reshni* Hort. ex Tan), plus satsuma mandarin and natsudaikai. Seedlings of these citrus species were grown in a greenhouse maintained at 18 to 33°C. Plants were pruned to stimulate production of uniform flushes of new leaves and inoculated when leaves were one-quarter mature size by spraying with a suspension of 10^6 conidia/ml. The plants were covered with plastic bags for 48 h for infection, and symptom severity was recorded when the leaves were mature. A plant was considered positive for infection if a clearly distinguishable scab lesion developed.

Pathogenicity tests on fruit were conducted by spraying the fruit 6 weeks after petal fall with a conidial suspension at 10^6 /ml. In all, 5 to 10 fruit were used for each isolate. Fruit were covered with plastic bags for 7 days after inoculation and symptoms rated 30 days later. Pathogenic-

ity tests were repeated at least once in almost all cases. All tests giving negative results were conducted at least three times.

RAPD assay. A total of 10 isolates of the *E. fawcettii* composed of FNHR and FBHR pathotypes from Florida and Jeju Island, 3 *E. fawcettii* isolates from Australia, 5 natsudaikai isolates, and 6 isolates of *E. australis* from Argentina were compared using RAPD analysis. Total genomic DNA of each fungal isolate was extracted from colonies grown on PDA according to the procedure described in Hyun et al. (6), which basically followed the method of Yoon (15), and amplified with 30 random 10-mer primers from Operon Primer Kit P and X (Operon Technologies, Alameda, CA) and 12 random 20-mer primers from SRILS UniPrimer Kit I (Seoul Scientific Co., Seoul, Korea) designed from repetitive sequences of Korean red rice. Amplification reactions were done in a 25- μ l reaction mixture containing 2.5 μ l of 10 $\times$ *Taq* DNA polymerase reaction buffer (Promega Corp., Madison, WI), 2.5 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.2 μ M of each primer, with approximately 5 ng of template DNA and 1 unit of *Taq* DNA polymerase (Promega Corp.). Each reaction was overlaid with two drops of sterile mineral oil. The polymerase chain reaction (PCR) was performed in a programmable thermocycler (Perkin-Elmer

Cetus thermal cycler, model 480), using an initial denaturation step of 95°C for 2 min, followed by 40 amplification cycles of denaturation at 94°C for 30 sec, annealing at 40°C for the Operon Primer Kit or 50°C for the SRILS UniPrimer Kit for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. Primers that produced strong, reproducible PCR products (bands) were finally selected. After PCR, 12.0 μ l of the product were electrophoresed in a 1.4% agarose gel in Tris-acetate-EDTA (TAE) buffer for 2 h at 3 V/cm and visualized by ethidium bromide staining. A 1-kb DNA Ladder (GeneRuler, MBI, Fermentas) was used for molecular weight markers. All RAPD PCR reactions were run at least twice with similar results.

Data analysis. Each profile was compared on the basis of the presence versus absence, indicated by 1 and 0, respectively, of RAPD products of the same electrophoretic mobility. Relative relatedness among isolates was determined by multivariate analysis of similarity coefficients, and a matrix of similarity coefficients for each pair of isolates was constructed. A cluster analysis of the Jaccard similarity coefficients was performed with the unweighted pair-group method algorithm (UPGMA). All calculations were conducted using the computer program NTSYS-pc (8).

Table 1. Host, geographic origin, pathotypes, and pathogenicity of scab isolates from citrus on various citrus species in the greenhouse

Location, isolate ^b	Host ^c	Pathogenicity ^a						Pathotype ^d
		SM	RL	SO	GF	CM	NDF	
Jeju								
SM 3-1, 24-1, Sang 2-1	SM	+	+	+	+	+	–	FBHR
SM 12-1, 16-8, X2-5	SM	+	+	–	+	+	–	FNHR
SM 12-2, 16-1, 16-4, 16-7, 24-4, 24-5, SJIN, MUD, Sang 1-1, Yeon-3	SM	+	+	+	+	+	NT	FBHR
Yuzu-3	YZ	+	+	+	+	+	–	FBHR
Yuzu-1, 2, 2a, Ryuzu-2, 5, SM 16-3, SM 12-5	YZ	+	+	+	+	+	NT	FBHR
X1-5	KK	+	+	+	+	+	–	FBHR
Ryuzu-1, Maru-1, 2, 3, 4	KK	+	+	+	+	+	NT	FBHR
Na-1, -2, -3, -4, -5, -6	NDF	–	–	–	–	–	+	
Florida								
Scott	Tem	+	+	–	+	+	NT	FNHR
S38162 (70254), CC-1	GF	+	+	–	+	+	NT	FNHR
70033	Volk	+	+	–	+	+	NT	FNHR
70255, 70257, Russ-15, 5 Moak-1	Tem	+	+	+	+	+	NT	FBHR
SCSK	SCSK	+	+	+	+	+	NT	FBHR
Australia								
70027 ^e	Lemon	NT	+	NT	NT	+	NT	Tryon's
70217 ^e	Lemon	NT	+	NT	NT	–	NT	Lemon
70298 ^e	Emp	NT	+	NT	NT	+	NT	Tryon's
Argentina								
70041 ^e	Val	NT	–	–	–	NT	NT	<i>Elsinoe australis</i>
70212 ^e	SM	NT	–	–	–	NT	NT	<i>E. australis</i>
Ea-2, 3	Val	NT	NT	NT	NT	NT	NT	<i>E. australis</i>
Ea-1, 4	SM	NT	NT	NT	NT	NT	NT	<i>E. australis</i>

^a Symbols: + = pathogenic, – = nonpathogenic, and NT = not tested.

^b Underlined isolate numbers were those used in the random amplified polymorphic DNA assays. All five-digit isolate members beginning with 7 are from the collection of M. Priest (New South Wales Agriculture, Orange, Australia).

^c SM = satsuma mandarin, RL = rough lemon, SO = sour orange, GF = grapefruit, CM = cleopatra mandarin, NDF = natsudaikai fruit, YZ = yuzu, KK = kinkoji, Val = Valencia sweet orange, Emp = Emperor mandarin, Tem = Temple tangor, Volk = *Citrus volkameriana* Ten. and Pasq., and SCSK = Sun chu sha kat mandarin.

^d FNHR = Florida narrow host range and FBHR = Florida broad host range.

^e Data from reference 10.

RESULTS

Observations and cultural characteristics. Thirty-six isolates were recovered from four citrus species on Jeju Island. Scab was observed on only six fruit in about 400 trees of natsudaikai in eight surveyed locations. Scab pustules on infected natsudaikai fruit were similar in size, shape, and degree of elevation to those on satsuma mandarin, but were more reddish. Conidia of many isolates were examined for possible distinguishing characteristics. All were hyaline and variable in shape and produced buds. No size differences were apparent.

The characteristics of scab colonies on PDA were extremely variable even within the same isolate. All isolates typically formed very slow-growing, pulvinate, or raised and deeply fissured, gummy to mucoid colonies on PDA medium, but formed tomentose colonies in some cases. Tomentose colonies were found in cultures from all species but were more common among natsudaikai isolates. Gummy or mucoid colonies of isolates from natsudaikai did not differ from those of isolates from other citrus species, but tomentose colonies of isolates from natsudaikai were distinguishable from those from other citrus species. Tomentose colonies of isolates from natsudaikai produced relatively fast-growing, radially sulcate colonies covered with whitish to grayish mycelium. Com-

pared with colonies of isolates from other citrus species, those isolates from natsudaikai were larger and more expansive (Fig. 1A and B). When isolates producing tomentose colonies were grown in liquid Fries medium, no cloudiness or other evidence of bacterial or yeast contamination was observed. No bacteria or yeast were found in microscopic examination of these isolates. Single-spore isolates of cultures Na-1 and Na-2 also produced tomentose colonies.

Whiteside (14) developed a semiselective medium for the isolation of *E. fawcettii* from citrus scab pustules by using dodine, and this medium has been usually used for isolation from host tissues. However, in our experiment, isolates of all pathotypes of *E. fawcettii*, as well as isolates of *E. australis*, grew well on PDA containing dodine at 400 µg a.i./ml, but natsudaikai isolates did not grow on this medium.

Pathogenicity and pathotype. In pathogenicity tests with 30 isolates from

satsuma mandarin, yuzu, and kinkoji on four citrus species, 27 isolates were pathogenic to satsuma mandarin, rough lemon, sour orange, grapefruit, and cleopatra mandarin, and thus were considered to be the FBHR pathotype of *E. fawcettii*. Three isolates were pathogenic to satsuma mandarin, rough lemon, grapefruit, and cleopatra mandarin, but produced no symptoms on sour orange (Table 1) and were considered to be the FNHR pathotype. Six isolates from natsudaikai were not pathogenic to leaves and fruits of satsuma mandarin, leaves of rough lemon, sour orange, grapefruit, cleopatra mandarin, or natsudaikai, but were only pathogenic to fruit of natsudaikai (Table 1).

RAPD analysis. Of the 10, 20, and 12 primers in Operon kit P, X, and SRILS UniPrimer kit I, 1, 10, and 5, respectively, led to amplification of PCR products, including at least one clear polymorphic product per primer. The number of polymorphic fragments produced with each primer varied from 1 to 10 for each isolate.

Fig. 1. Colony morphology of **A**, colonies of *Elsinoe fawcettii* and **B**, colonies of isolates from natsudaikai, cultured for 30 days on potato dextrose agar.

Fig. 2. **A**, Electrophoretic patterns for amplified fragments generated from isolates of citrus scab pathogens with random primer OPX-7 on a 1.4% agarose gel. Lane M, marker DNA (1-kb ladder); lanes 1 to 5, *Elsinoe fawcettii* Florida narrow host range isolates SM 12-1, SM 16-8, and X2-5 from Korea and isolates Scott and CC-1 from Florida, respectively; lanes 6 to 10, *E. fawcettii* Florida broad host range isolates Maru-1, MUD, and Yuzu 3 from Korea and isolates Russ-15 and 70255 from Florida; lanes 11 to 13, *E. fawcettii* isolates of the Tryon's, lemon, and Tryon's pathotypes (70027, 70217, and 70298, respectively) from Australia; lanes 14 to 18, isolates from natsudaikai Na-5, -6, -1, -2, and -3, respectively, from Korea; lanes 19 to 24, *E. australis* isolates 70041, 70212, Ea-1, -2, -3, and -4, respectively, from Argentina. **B**, Electrophoretic patterns for amplified fragments generated from isolates of citrus scab pathogens with random primer OPX-13 on a 1.4% agarose gel. Lanes as described above.

Two hundred twenty-six polymorphic fragments were used for cluster analysis. Based upon the presence or absence of amplification products ranging in size from 250 to 3,000 bp, all primers selected revealed polymorphisms useful for classifying *E. fawcettii*, natsudaïdai isolates, and *E. australis*. Primers OPX-7 and 13 had especially distinct polymorphisms among pathotypes of *E. fawcettii*, *E. australis*, and natsudaïdai isolates (Fig. 2A and B). However, no primers revealed distinct polymorphisms for classifying FNHR and FBHR pathotypes. The similarity coefficients among the isolates ranged from 0.42 to 1.00, where 1.00 is identical. Ten isolates of the *E. fawcettii* composed of FNHR and FBHR pathotypes were clustered into one genetically distinct group, with similarity among isolates in the group ranging from 0.97 to 1.00. However, Korean and Floridian isolates of *E. fawcettii* were differentiated by one marker with primer OPX-8 in the RAPD assay (*data not shown*). Two of three isolates of *E. fawcettii* from Australia were very close to each other, and the similarity value between the two isolates (70027 and 70298, Tryon's pathotype) and other pathotypes of *E. fawcettii* was 0.88. The other Australian isolate (70217, lemon pathotype) was more distant, with a similarity value of 0.76.

The similarity values among six isolates of *E. australis* from Argentina ranged from 0.89 to 1.00; of these, five isolates had very high similarity values (Fig. 3). The natsudaïdai isolates were clustered into one genetically distinct group, with similarity among isolates in the group ranging from 0.90 to 1.00. The similarity values between

the natsudaïdai isolates and *E. fawcettii* and *E. australis* were 0.41 and 0.58, respectively (Fig. 3).

DISCUSSION

The three scab diseases were originally differentiated primarily on the basis of morphological and cultural characteristics such as spore size and colony color (1,7). Tan et al. (9) readily differentiated three scab organisms using restriction analyses of the amplified ITS of ribosomal DNA with several endonucleases, RAPD analyses, and by sequence analysis of the ITS region. In the present study, the similarity coefficients between Australian and Floridian pathotypes of *E. fawcettii* were high, ranging from about 0.77 to 0.89. However, Australian isolates of *E. fawcettii* were readily differentiated from Florida isolates by some RAPD markers. Jenkins (7) mentioned the need for further taxonomic study to determine whether *S. fawcettii* var. *scabiosa* (Tyson's scab) should be accorded specific rank distinct from *S. fawcettii* var. *fawcettii*, the conidial state of *E. fawcettii*. Tan et al. (9) strongly suggested that *S. fawcettii* var. *scabiosa* is conspecific with *E. fawcettii* based on indistinguishable conidia and the formation of spindle-shaped conidia by both taxa. In the present study, the similarity coefficients between isolates of *S. fawcettii* var. *scabiosa* and *E. fawcettii* were relatively high compared with those between *E. fawcettii* and *E. australis*. This result supports classification of *E. fawcettii* and *S. fawcettii* var. *scabiosa* as different pathotypes of the same species.

Elsinoe spp. form two types of colonies: raised and gummy to mucoid colonies and tomentose colonies (16). *E. australis* and *E. fawcettii* have been differentiated on the basis of colony color (1,4) and length-to-width ratios of conidia (4). Natsudaïdai isolates produced two types of colonies, though it is not known under what conditions tomentose or gummy to mucoid colonies are formed. Natsudaïdai isolates commonly produced tomentose colonies in the original isolations from the host as well as after five to six transfers. In this study, natsudaïdai isolates were differentiated from other scab pathogens on the basis of colony characteristics and sensitivity to dodine.

Similarity of natsudaïdai isolates to isolates of *E. australis* and *E. fawcettii* based on RAPD markers was low (0.57 and 0.42, respectively). The five natsudaïdai isolates were clearly clustered into one genetically distinct group, with similarity among isolates in the group ranging from 0.91 to 1.00. The natsudaïdai isolates had distinct colony morphology and may represent a different pathotype or other variant of *E. australis* or may be a distinct species. Therefore, a taxonomic study including natsudaïdai isolates will be necessary to clarify the molecular, morphological, and cultural differences that exist among scab pathogens.

Even though isolates from both Korea and Florida were included in the RAPD analysis, the FBHR and FNHR isolates were clustered into one very close group, with similarity among isolates in the group ranging from 0.99 to 1.00. The two pathotypes of *E. fawcettii* were not distinguishable by RAPD assay, but isolates from Jeju

Fig. 3. Dendrogram of the random amplified polymorphic DNA data showing the relationships of *Elsinoe* isolates from citrus. E. f. = *Elsinoe fawcettii* isolates from Korea (K), Florida (FL), or Australia (Aus); FNHR = Florida narrow host range pathotype; FBHR = Florida broad host range pathotype; Nat = isolates from natsudaïdai in Korea; and E. a. = *E. australis* isolates from Argentina (Arg).

and Florida were distinguished by one marker (*data not presented*).

The FBHR pathotype is widespread on Jeju Island. With the broad host range of this pathotype, it will not be possible to choose other mandarin cultivars to avoid scab. The other known pathotypes, such as those from Australia, are mostly pathogenic to lemons and would not be a regulatory concern in cultivation of satsuma mandarin. The importance of isolates from natsudaikai for other cultivars is uncertain until the entire host range of these isolates has been determined.

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