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SHORT COMMUNICATION

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Differentiation of pathogenic and nonpathogenic isolates of *Geotrichum candidum* sensu Suprapta et al. (1995) on citrus fruit based on PCR-RFLP analysis of rDNA ITS and PCR using specific primers designed in polygalacturonase genes

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Abstract Pathogenic and nonpathogenic isolates of *Geotrichum candidum* sensu Suprapta et al. (1995) that affect citrus fruit are indistinguishable morphologically. In this work, differentiation of the two pathogenicity types based on the internal transcribed spacer regions of ribosomal DNA (rDNA-ITS) and polygalacturonase (PG) genes was attempted. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of rDNA ITS and PCR using specific primers to PG genes from each type could clearly differentiate the two pathogenicity types, and the profiles by PCR-RFLP of rDNA ITS genes corresponded with those by type-specific PCR of PG genes. These results indicate that the two pathogenic types can be differentiated at a molecular level and that PG genes are alternatively useful for distinguishing between the two types.

Key words Differentiation · *Geotrichum candidum* citrus race · *Geotrichum citri-aurantii* noncitrus race · Polygalacturonase gene · rDNA ITS region

Geotrichum candidum Link: Fr. is a yeast-like fungus that causes diseases in humans, animals, and plants (Carmichael 1957; Butler 1960; Butler et al. 1965; Suprapta et al. 1996a). As a plant pathogen, the fungus causes sour rot, which is an important cause of postharvest loss of citrus fruits and has been reported from many areas in the world where citrus is grown (Smith 1917; El-Tobshy and Sinclair 1965; Eckert 1978; Brown and Eckert 1988; Harshenhorn et al. 1992). Sour rot of lemon fruit was first described by Smith (1917), and the pathogen was designated as *Oospora citri-aurantii* (Ferr.) Sacc. & Syd. Since then, several different names have been used to designate the pathogen. In 1965, Butler et al. designated *G. candidum* as the causal agent of citrus sour rot and they gave the term of citrus race to this pathogen to differentiate it from the noncitrus isolates based on pathogenicity on citrus fruit and physiological characteristics, although citrus race and noncitrus isolates are indistinguishable morphologically (Butler et al. 1965). Suprapta et al. (1995) referred to the noncitrus isolates as noncitrus race. Later on, Butler et al. (1988) also designated the causal agent of citrus sour rot as *Geotrichum citri-aurantii* (Ferr.) Butler (anamorph) and *Galactomyces (Gal.) citri-aurantii* Butler (teleomorph). They also reported that only *Gal. citri-aurantii* causes sour rot of citrus and *Gal. geotrichum* (teleomorph of *Geotrichum candidum*) does not (Butler et al. 1988), and further that the taxonomic difference between the two fungi is the type of colony on medium containing only D-glucose or sorbose with amino acids and the pattern of utilization of carbon sources, with only one difference, ribitol. It follows from what has been stated that the citrus race is *Gal. citri-aurantii* and the noncitrus race is *Gal. geotrichum*. In Japan, however, the sexual stage has not yet been found, and development of the stage also has not been demonstrated. In recent work on citrus sour rot, *G. candidum* citrus race is commonly cited to indicate the citrus sour rot pathogen (Barash et al. 1984; Mor et al. 1984; Baudoin and Eckert 1985; Chalutz and Wilson 1990; Wild 1992; Suprapta et al. 1995, 1996a,b; Nakamura et al. 2001, 2003); therefore, in this article, we adopt the name of *G. candidum* sensu Suprapta et al. (1995). In addition, because, in plant pathology, the term race is used to express strains that show different pathogenicity on different cultivars, the terms citrus race and noncitrus race are not appropriate. Thus, in this report, to express citrus race and noncitrus race, the terms citrus type and noncitrus type, respectively, as also used by Butler et al. (1965) in their report, are used for the sake of simplicity.

Because citrus and noncitrus types are morphologically indistinguishable (Butler et al. 1965), a pathogenicity test on citrus fruit or a physiological approach is commonly applied to distinguish between the pathogenicity types of the fungus. Butler et al. (1965) reported that the pyridoxine

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Table 1. Isolates of *Geotrichum candidum* used in this study

Isolates[a]	Locality	Origin/cultivated plants	Pyridoxine requirement
Citrus type			
S7 (+)[b]	Kagoshima	Soil/*Citrus unshiu* Marc.	+
S31 (+)	Ehime	Soil/*C. unshiu*	+
S148 (+)	Saga	Soil/*C. unshiu*	+
S181 (+)	Wakayama	Soil/*C. unshiu*	+
M13 (+)	Oita	Soil/*Pinus thunbergii* Parl.	+
Te2 (+)	Shizuoka	Soil/*Thea sinensis* L.	+
Pt3 (+)	Saga	Soil/*Solanum tuberosum* L.	+
R2 (+)	Miyazaki	Soil/*Oryza sativa* L.	+
D2 (+)	Oita	Soil/*Raphanus sativus* L.	+
Kk10 (+)	Hiroshima	Soil/*Diospyros kaki* Thunb.	+
Noncitrus type			
S63 (–)[b]	Kumamoto	Soil/*C. unshiu*	–
R9 (–)	Saga	Soil/*O. sativa*	–
R10 (–)	Saga	Soil/*O. sativa*	–
R21 (–)	Hiroshima	Soil/*O. sativa*	–
R22 (–)	Hiroshima	Soil/*O. sativa*	–
Tm5 (–)	Kumamoto	Soil/*Lycopersicon esculentum* Mill.	+
Ig1 (–)	Kumamoto	Soil/*Juncus effuses* L.	–
W4 (–)	Oita	Soil/*Triticum aestivum* L.	–
W6 (–)	Fukuoka	Soil/*T. aestivum*	–
Mm 2(–)	Kumamoto	Soil/*Prunus persica* Batsch	–

[a] All isolates were isolated from soils by Suprapta et al. (1995, 1996a,b)
[b] (+), pathogenic to citrus fruit; (–), nonpathogenic to citrus fruit

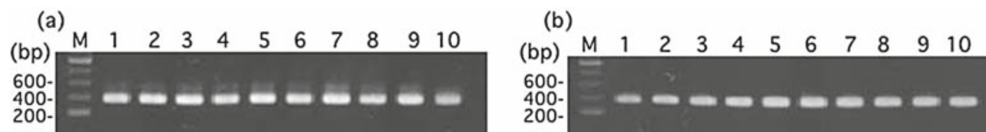

Fig. 1. Polymerase chain reaction (PCR) amplification products of the internal transcribed spacer region of the ribosomal DNA of *Geotrichum candidum* isolates. **a** Citrus type isolates: *lane M*, 200-bp ladder marker; *lane 1*, S31; *lane 2*, S7; *lane 3*, S148; *lane 4*, S181; *lane 5*, M13; *lane 6*, Te2; *lane 7*, Pt3; *lane 8*, R2; *lane 9*, D2; *lane 10*, Kk10. **b** Noncitrus type isolates: *lane* M, 200-bp ladder marker; *lane 1*, S63; *lane 2*, R9; *lane 3*, R10; *lane 4*, R21; *lane 5*, R22; *lane 6*, m5; *lane 7*, Ig1; *lane 8*, W4; *lane 9*, W6; *lane 10*, Mm2

requirement for growth on asparagine-glucose agar and the growth in autoclaved lemon juice at pH 2.2 or 2.7 could be used to distinguish between the two types. Suprapta et al. (1995, 1996a,b) collected 369 isolates of citrus and noncitrus types from citrus groves and noncitrus fields in Japan and investigated their pathogenicity and physiological properties. The result of the growth in lemon juice agreed with the report of Butler et al. (1965). However, they reported that the use of pyridoxine requirement was not effective in differentiating the two types (Suprapta et al. 1996b). To date, classification based only on physiological methods has been conducted, but none based on molecular methods has been carried out. In this work, to distinguish between the two types, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the rDNA-internal transcribed spacer (ITS) and type-specific PCR of PG genes were conducted.

Twenty isolates of *G. candidum* citrus type and noncitrus type (10 in each type) were collected from Japan, mainly from Kyushu (Table 1). Fungal genomic DNA was extracted and purified by the method of Wu et al. (1997), with minor modifications. The rDNA-ITS was amplified with primers ITS1 and ITS4 (White et al. 1990). PCR was performed in a final volume of 50 µl containing 1× Ex Taq buffer (Takara Bio, Shiga, Japan), 1 µM each primer, 0.2 mM dNTPs, 20 ng genomic DNA, and 2.5U Ex Taq polymerase (Takara Bio). Cycling conditions were 95°C for 2 min for predenaturation, followed by 32 cycles at 95°C for 1 min, at 65°C for 30 s, and at 72°C for 30 s. Reactions were held at 4°C after a final extension at 72°C for 5 min. Using the ITS1/ITS4 primer set, we obtained PCR products of about 370 bp, including ITS1, 5.8S rDNA, and ITS2, for the *G. candidum* citrus type and noncitrus type isolates listed in Table 1 (Fig. 1). The products were digested with several restriction enzymes such as *Alu*I, *Hha*I, *Msp*I, and *Hin*fI to find which enzyme could differentiate the two types (data not shown). Digested fragments were run on 7.5% polyacrylamide gels (Nacalai Tesque, Kyoto, Japan) in 1× TBE buffer [89 mM Tris base, 89 mM boric acid, 2 mM ethylenediaminetetraacetic acid (EDTA)) at 100 V for 1–2 h. Only *Hin*fI could cleave the products, resulting in two bands (about 190/180 bp) for citrus type isolates and three bands (about 185/100/85 bp) for noncitrus type isolates (Fig. 2). The findings of this experiment showed that the citrus and noncitrus types were clearly differentiated into two groups corresponding to their pathogenicity.

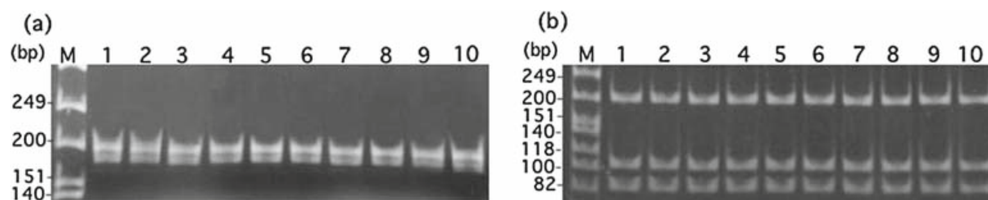

Fig. 2. Restriction fragments of amplified internal transcribed spacer products of *Geotrichum candidum* isolates digested with *Hin*fI. **a** Citrus type isolates: *lane M*, øX174/*Hin*fI digest; *lane 1*, S31; *lane 2*, S7; lane 3, S148; *lane 4*, S181; *lane 5*, M13; *lane 6*, Te2; *lane 7*, Pt3; *lane 8*, R2; *lane 9*, D2; *lane 10*, Kk10. **b** Noncitrus type isolates: *lane M*, øX174/*Hin*fI digest; *lane 1*, S63; *lane 2*, R9; *lane 3*, R10; *lane 4*, R21; *lane 5*, R22; *lane 6*, Tm5; *lane 7*, Ig1; *lane 8*, W4; *lane 9*, W6; *lane 10*, Mm2

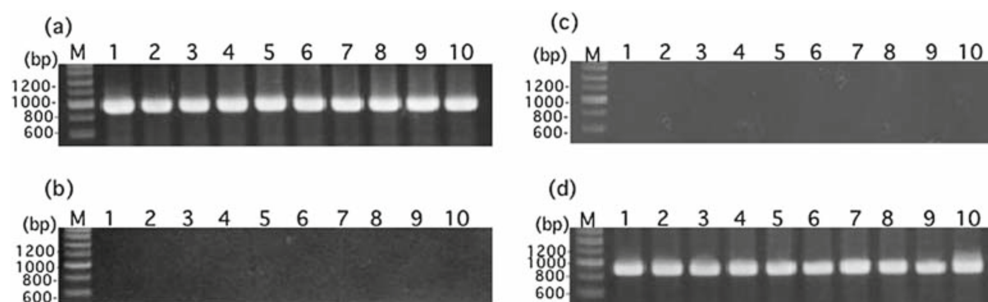

Fig. 3. Type-specific PCR to amplify polygalacturonase genes of *Geotrichum candidum* isolates. **a, b** Citrus type isolates: *lane M*, 200-bp ladder marker; *lane 1*, S31; *lane 2*, S7; *lane 3*, S148; *lane 4*, S181; *lane 5*, M13; *lane 6*, Te2; *lane 7*, Pt3; *lane 8*, R2; *lane 9*, D2; *lane 10*, Kk10. **c, d** Noncitrus type isolates: *lane M*, 200-bp ladder marker; *lane 1*, S63; *lane 2*, R9; *lane 3*, R10; *lane 4*, R21; *lane 5*, R22; *lane 6*, Tm5; *lane 7*, Ig1; *lane 8*, W4; *lane 9*, W6; *lane 10*, Mm2. **a, c** PCR specific to *S31pg1*. **b, d** PCR specific to *S63pg1*

Next, to find another useful gene to distinguish between the citrus and noncitrus types of *G. candidum*, a type-specific PCR was developed. *G. candidum* secretes PG, one of the pectic enzymes, during germination and growth (Barash 1968). We have reported that PGs from *G. candidum* citrus type were responsible for its pathogenicity to citrus fruit but that those from *G. candidum* noncitrus type had no pathogenicity (affinity to protopectin; protopectinase activity) (Nakamura et al. 2003). Since the PG genes, *S31pg1* and *S63pg1*, have been isolated from citrus type S31 and noncitrus type S63, respectively (Nakamura et al. 2001), primer sets specific to each PG gene were designed. Primers specific to *S31pg1* are S31-Sp2 (5′-ATCTTCAGGCCCGT GGCGGCGC-3′) and S31-Sp3 (5′-GCGTTGGTACCAGA GCTCTT-3′). Primers specific to *S63pg1* are S63-Sp1 (5′-ATCTTTATGCTCGTGATTCTTG-3′) and S63-Sp2 (5′-TAGTTAATACCCTTAGCAAG-3′). PCR was performed in the same reaction mixtures as already described. Cycling conditions were 94°C for 2 min for predenaturation, followed by 30 cycles at 94°C for 1 min, at 58°C for 45 s, and at 72°C for 1 min. Reactions were held at 4°C after a final extension at 72°C for 5 min. Amplified DNA was detected by electrophoresis in 1% agarose gel for ≥1-kbp fragments (Nacalai Tesque) in 1× TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 40 min. Specificity of those primers was determined by using *S31pg1* and *S63pg1* cloned into plasmids as a template (data not shown). Using the primer sets, we conducted PCR reciprocally with genomic DNA from all the isolates of each type listed in Table 1. As a result, the primer set (S31-Sp2/S31-Sp3) specific to *S31pg1* amplified a 919-bp fragment from all the isolates of the citrus type but none from the noncitrus type (Fig. 3a,b). On the other hand, the primer set (S63-Sp1/S63-Sp2) specific to *S63pg1* amplified a 913-bp fragment from all the isolates of the noncitrus type but none from the citrus type (Fig. 3c,d). These results indicate that because *S31pg1* is distributed only among the citrus type isolates and *S63pg1* is found only among the noncitrus type isolates, PG genes are useful for easily distinguishing between the two types by conducting type-specific PCR. Hirano and Arie (2006) also reported that PCR targeting PG genes in races of *Fusarium oxysporum* f. sp. *lycopersici* effectively differentiated the pathogenic types of the fungus.

Butler et al. (1965) reported that the pyridoxine requirement for growth on asparagine-glucose agar could be used to distinguish between the two types: the citrus type requires pyridoxine and the noncitrus type does not. However, Suprapta et al. (1996b) reported that this test was not effective. In fact, the isolate Tm5 is a noncitrus type, but it requires pyridoxine (Suprapta et al. 1996b). However, this isolate was distinguished from the citrus type by the molecular techniques just described here. Type-specific PCR is neither a difficult nor time-consuming task compared to the PCR-RFLP analysis of rDNA-ITS. Because in this study we conducted type-specific PCR of PG genes against citrus and noncitrus types isolated from soils but not directly against diseased samples or soils, we may need a further experiment to evaluate the usefulness of the technique in natural samples. However, to distinguish between the citrus and noncitrus types of *G. candidum*, so far, a pathogenicity test

(inoculating the fruit with the fungus) has been the most reliable technique, but type-specific PCR of PG genes is much easier and can be useful for epidemiological studies.

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