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ORIGINAL ARTICLE

PCR–RFLP analysis for detecting potential QoI and CAA fungicide resistance in onion and lettuce downy mildews

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Abstract

Downy mildew of onion and lettuce, caused by *Peronospora destructor* and *Bremia lactucae*, respectively, is distributed worldwide. Control of these serious diseases largely relies on oomycete fungicides including quinone-outside inhibitors (QoIs) and carboxylic acid amides (CAAs), both of which bear the risk of resistance development in pathogens. Resistance to QoI and CAA fungicides has not been confirmed in field isolates of onion and lettuce downy mildews to date, and monitoring using biological methods is challenging as both pathogens are obligate biotrophs and cannot be grown in agar culture. In this study, the RFLP-method using PCR-amplified gene fragments encoding the fungicide targets cytochrome b (*cytb*) and cellulose synthase 3 (*CesA3*) has been developed for detecting potential QoI and CAA resistance in *P. destructor* and *B. lactucae*, as this is likely to develop in the future. This method will make it possible to diagnose resistance prior to efficacy reduction of fungicides contributing to sustainable disease control.

Keywords *Bremia lactucae* · CAA fungicides · Fungicide resistance · *Peronospora destructor* · QoI fungicides

Introduction

Downy mildew of onion and lettuce, caused by *Peronospora destructor* (Berk.) Casp. ex Berk. and *Bremia lactucae* Regel, respectively, is widespread in Africa, Asia, Europe, North America, Oceania, and South America (https://www.cabi.org/isc/datasheet/39701 and https://www.cabi.org/isc/datasheet/9937 Accessed July 31, 2022) and is one of the most devastating diseases in these crops (Schwartz 2008; van Bruggen and Scherm 1997). Control of these diseases largely relies on oomycete fungicides, although breeding of resistant cultivars has made some progress (Parra et al. 2021; Scholten et al. 2007). The phenylamide mefenoxam (= metalaxyl-M) has been used for a long time, but resistance to this fungicide developed in *P. destructor* (O'Brien 1992; Syobu and Watanabe 2022) and *B. lactucae* (Crute et al. 1987; Schettini et al. 1991) resulting in control failure. Quinone-outside inhibitor (QoI) and carboxylic acid amide (CAA) fungicides have been subsequently registered for these two pathogens (Cohen et al. 2008). However, QoI and CAA fungicides possess high and medium risk of resistance, respectively [Fungicide Resistance Action Committee (FRAC), http://www.frac.info/ Accessed July 31, 2022]. For downy mildews, QoI resistance has been reported for field isolates of *Pseudoperonospora cubensis* in cucumber (Ishii et al. 2001) and *Plasmopara viticola* in grapevine (Sierotzki et al. 2005). Furthermore, the presence of QoI-resistant strains has been suspected in onion downy mildew in inoculation tests (Wright and Beresford 2019). With respect to CAA fungicides, no resistant isolates have been reported for *P. destructor* and *B. lactucae* to date. However, field resistance has been found in several other oomycete pathogens such as *P. viticola* (Toffolatti et al. 2018), *P. cubensis* (Zhu et al. 2007), and *Pseudoperonospora humuli* (Higgins et al. 2021), and isolates carrying double resistance to QoI and CAA fungicides have been found in field populations of *P. viticola* (Santos et al. 2020).

A point mutation in the fungicide target mitochondrial cytochrome *b* (*cytb*) genes leading to the substitution of glycine to alanine at codon 143 (G143A) is closely associated

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with high QoI resistance (Gisi et al. 2000), and a mutation causing the change of phenylalanine to leucine at codon 129 (F129L) results in moderate levels of resistance (Sierotzki 2015). For CAA fungicides, the major G1105S mutation, in addition to the G1105V mutation was found in the *CesA3* gene in resistant isolates of *P. viticola* encoding the target cellulose synthase 3 enzyme (Blum et al. 2010, 2012). Similarly, G1105V/W mutations were associated with cross-resistance among the three CAA fungicides dimethomorph, benthiavalicarb, and mandipropamid in *P. cubensis* isolates (Blum et al. 2011). Thus, it is possible that field isolates of *P. destructor* and *B. lactucae* mutate at codon 143 of cytb and/or codon 1105 of CesA3 proteins responsible for high QoI and CAA fungicide resistance, respectively.

Early detection of resistant isolates is crucial to prevent loss of fungicide efficacy, and molecular diagnosis of resistance is particularly useful for obligate pathogens such as the downy mildews. Therefore, in this study, we examined partial sequences of the *cytb* and *CesA3* genes in onion and lettuce downy mildews and introduced restriction-fragment length polymorphism (RFLP) analyses to develop methods to detect potential QoI and CAA resistance.

Materials and methods

Sample collection and DNA extraction

Downy mildew-infected symptomatic leaves of onion and lettuce were sampled from commercial and experimental fields in Hyogo, Saga, and Ibaraki prefectures, Japan from 2016 to 2020 (Table S1). Zoosporangia and hyphae of 16 *P. destructor* and five *B. lactucae* isolates from fresh lesions were collected into 5 µl of distilled water per 1 cm^2 leaf segment. Total DNA was extracted using the Sigma REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer's instructions (Furuya et al. 2010).

Identification of *P. destructor* by polymerase chain reaction (PCR)

To confirm that the total DNA extracted from the isolates was derived from *P. destructor*, their rDNA-ITS regions were amplified by PCR using the species-specific primers Pd ITS 614F: 5′–CTTTTCACGTTGCCCCTTCC–3′ and Pd ITS 614R: 5′–CCCTTAGACGAGGCAAGCTC–3′ (Fujiwara et al. 2021). PCR was performed in 25 µL volumes containing 0.5 µL of total DNA, 12.5 µL of Go Taq Green Master Mix (Promega, Madison, WI, USA) and primers at 0.2 µM each. The temperature conditions were an initial preheat for 1 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 64 °C for 1 min and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis and stained.

Characterization of *cytb* and *CesA3* genes by PCR and sequence analysis

To amplify the *cytb* gene of both pathogens, primers RSCBF1: 5′–TATTATGAGAGATGTAAATAATGG–3′ and RSCBR2: 5′–AACAATATCTTGTCCAATTCATGG–3′ were used for PCR according to Ishii et al. (2001). To amplify the *CesA3* gene of *P. destructor*, primers KES2089: 5′–GCTGATTCGATTCTTTGAGTATGA–3′ and KES2090: 5′–AGCTGCACGAATACCACAATG–3′ and for the *CesA3* gene of *B. lactucae*, primers BlCesA3F: 5′–AGGATAAGTCGTGGGCGAAC–3′ and BlCesA3R: 5′–GCTGTACGAACACGACAACG–3′ were used. PCR was carried out with the following temperature conditions: an initial preheat for 2 min at 94 °C, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, and terminated with a final extension at 72 °C for 7 min.

The PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced (Macrogen Japan Corp., Kyoto and Tokyo, Japan) using the same primers employed for PCR. After sequencing, the nucleotide and deduced amino acid sequences were analyzed using the National Center for Biotechnology Information (NCBI)/GenBank database using basic local alignment search tools (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Restriction-fragment length polymorphism (RFLP) analysis of *cytb* and *CesA3* genes

RFLP analyses were conducted using PCR products of the *cytb* and *CesA3* genes. PCR fragments of the *cytb* genes were *Fnu*4HI-digested (New England BioLabs Japan, Tokyo, Japan). Similarly, *CesA3* gene fragments were *Alu*I-digested (New England BioLabs Japan). Because no resistant isolates were available in downy mildew of onion and lettuce yet, PCR amplicons of the *cytb* genes from the QoI-resistant isolate Beto R (G143A) and -sensitive isolate Beto FS (wild-type G143) of cucumber downy mildew (Ishii et al. 2001) were used as a positive and negative control, respectively. For the *CesA3* genes, the amplicons from CAA-resistant [PV 884, bearing G1105S (nucleotide: AGC) mutation] and sensitive wild-type [PV1357, possessing G1105 (nucleotide: GGC)] isolates of grapevine downy mildew (Stammler et al. unpublished) were used.

Results

Identification of *P. destructor* by PCR

It was reported previously that the primer set Pd ITS 614F and Pd ITS 614R was specific to *P. destructor* among the tested species of the Peronosporaceae, and the specificity was unlikely to be affected by contaminants or substances derived from onion seedlings (Fujiwara et al. 2021). The species-specific primer set yielded PCR products of 204 bp in this study (Fig. S1) confirming that all DNA samples were from *P. destructor*.

Sequence analysis of *cytb* and *CesA3* genes

Approximately 300 bp fragments of *cytb* genes were PCR amplified from both pathogens. The nucleotide sequence of *P. destructor* matched 91% with that of *P. viticola* (NCBI accession DQ459461) and the sequence of *B. lactucae* was 98% identical to the accession FJ810099 for this pathogen. The isolates of both pathogens used in this study showed the wild-type *cytb* sequences and the deduced amino acid was phenylalanine (F), glycine (G), and glycine (G) at the position 129, 137, and 143, respectively (Fig. 1A). No substitutions were found in these deduced amino acids.

Approximately 250 bp fragments of *CesA3* gene were amplified from *P. destructor* isolates. In all isolates analyzed, the nucleotide sequence corresponding to the amino acid position 1105 was wild-type GGC (Fig. 2A). The deduced amino acid was glycine at this position and mutations responsible for CAA resistance were not found. From the lettuce isolates, fragments of the *CesA3* gene, 98–100% identical to the NCBI nucleotide accession JN561771, were amplified using the primer pair BlCesA3F and BlCesA3R, indicating that the samples were from *B. lactucae*. No mutations that led to substitutions of glycine were found at the amino acid position 1105 in the *CesA3* gene (Fig. 2A).

RFLP analysis of *cytb* and *CesA3* genes

The PCR fragments, derived from *P. destructor* and *B. lactucae* with the wild-type sequence GGAGCA at codon 143–144 of *cytb* (Fig. 1A), were not digested with the enzyme *Fnu*4HI which recognizes 5′–GC↓NGC–3′ (Fig. 1B (a)). A corresponding fragment from a QoI-sensitive isolate of cucumber downy mildew, used as a control, was also not digested (Fig. 1B (b)). In contrast, a fragment from a QoI-resistant isolate of cucumber downy mildew which carried the mutation GCTGCT at codon 143–144 (Ishii et al. 2001) was cut with the enzyme (Fig. 1B (b)).

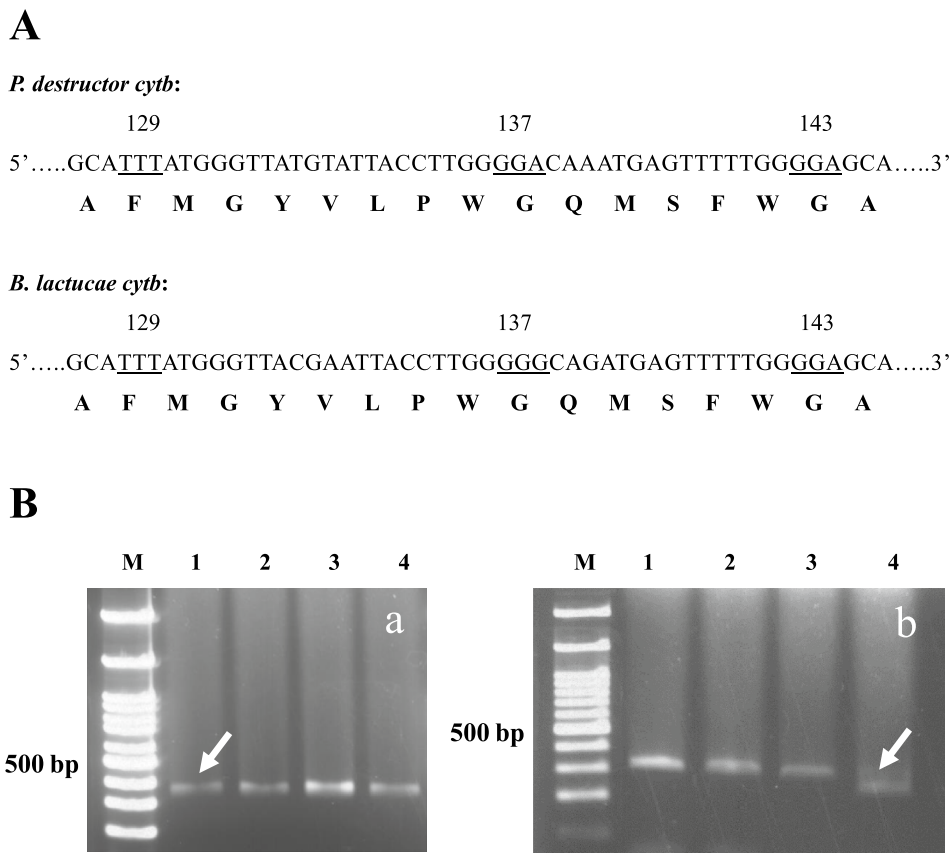

Fig. 1 **A** Partial nucleotide and deduced amino acid sequences (positions 128–144) of the cytochrome *b* (*cytb*) genes from the wild-type isolates of *Peronospora destructor* and *Bremia lactucae*. Sites of potential amino acid substitution are underlined. **B** *Fnu*4HI digestion pattern of *cytb* fragments of downy mildews: (a) M, 100-bp ladder; 1, untreated wild-type onion isolate H5-1; 2, treated H5-1; 3, untreated wild-type lettuce isolate Beto 4; and 4, treated Beto 4. An arrow indicates a fragment equivalent to ca. 400 bp in size. (b) M, 100-bp ladder; 1, untreated wild-type cucumber isolate Beto FS* (QoI-sensitive); 2, treated Beto FS; 3, untreated cucumber isolate Beto R* (QoI-resistant); and 4, treated Beto R. An arrow indicates a digested fragment. *Refer to Ishii et al. (2001)

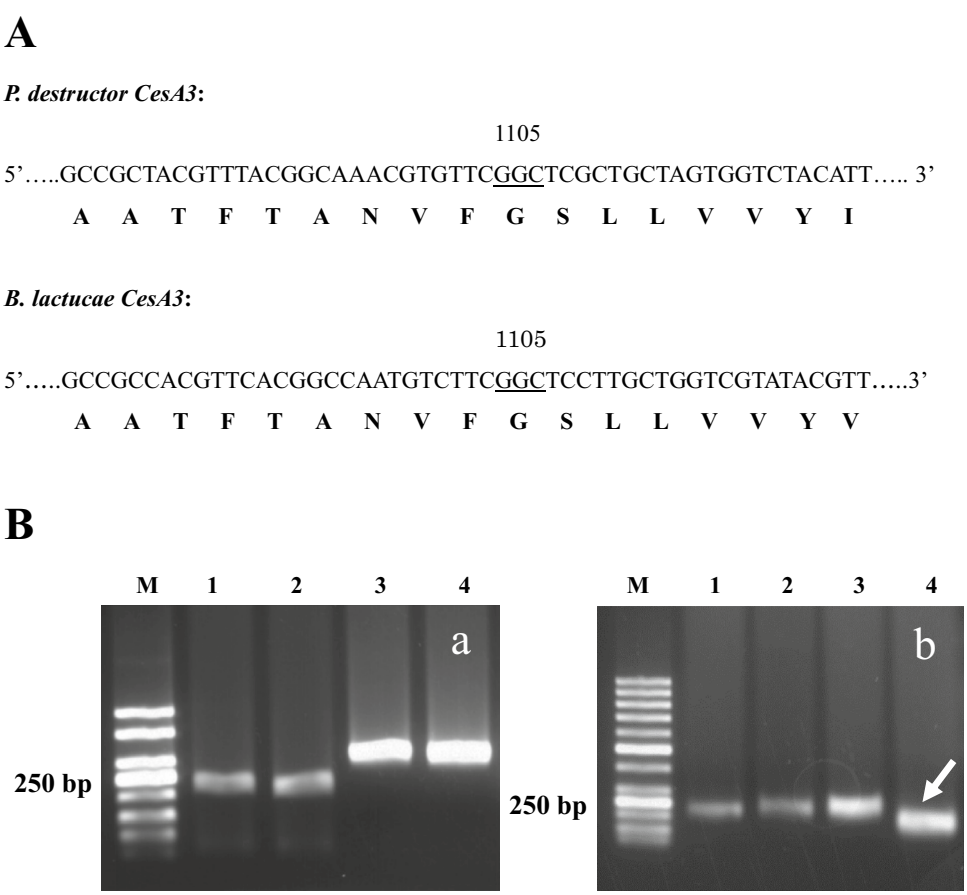

Fig. 2 **A** Partial nucleotide and deduced amino acid sequences (positions 1096–1112) of the cellulose synthase 3 (*CesA3*) genes from the wild-type isolates of *Peronospora destructor* and *Bremia lactucae*. Site of potential amino acid substitution is underlined. **B** *Alu*I digestion pattern of *CesA3* fragments of downy mildews: (a) M, 50-bp ladder; 1, untreated wild-type onion isolate H1-2; 2, treated H1-2; 3, untreated wild-type lettuce isolate Beto 1; treated Beto 1. (b) M, 50-bp ladder; 1, untreated wild-type grapevine isolate PV1357 (CAA-sensitive); 2, treated PV1357; 3, untreated grapevine isolate PV884 (CAA-resistant); and 4, treated PV884. An arrow indicates a digested fragment

The *CesA3* fragments from both *P. destructor* and *B. lactucae*, containing wild-type sequence GGCTCG and GGC TCC, respectively, at codon 1105–1106 were not digested with *Alu*I (Fig. 2B (a)), which recognizes the sequence 5′–AG↓CT–3′. A corresponding fragment from the sensitive wild-type isolate of *P. viticola*, used as a control, was also not digested with this enzyme (Fig. 2B (b)). In contrast, the fragment from G1105S resistant mutant isolate of this pathogen was digested by *Alu*I (Fig. 2B (b)).

Discussion

PCR–RFLP analysis of fungicide target protein gene is a useful approach to identify QoI and CAA fungicide resistance (Aoki et al. 2013; Ishii et al. 2007). *Cytb* genes encoding the QoI-target proteins are mitochondrially inherited and are present with variable hundred copies per cell. A heteroplasmic status of *cytb* genes could complicate a precise identification and quantification of QoI resistance in various pathogen species (Ishii et al. 2007; Lesemann et al. 2006). Fortunately, however, it seems that the *cytb* genes in the downy mildews investigated here are homoplasmic rather than heteroplasmic as no papers showing heteroplasmy have been published. Therefore, PCR–RFLP analysis was adopted in this study to develop a method for detecting potential resistance. The results from sequencing and those from the RFLP analysis coincided well, indicating that this method is reliable. All the isolates of *P. destructor* and *B. lactucae* randomly selected from commercial and experimental fields, where QoI and CAA fungicides had been used, were sensitive to these fungicides. However, further monitoring will be needed to test for the occurrence of mutations leading to fungicide resistance. It will also be needed to validate specificity and sensitivity of the method developed in this study when asymptomatic lesions contaminated with other microorganisms are used for DNA extraction.

Field resistance to QoI and CAA fungicides has not been clearly reported in downy mildews of onion and lettuce. However, the use of these fungicides is increasing, particularly in areas where metalaxyl-M resistance has evolved in *P. destructor* (Syobu and Watanabe 2022; Wright and Beresford 2019). Although control largely depends on multisite mancozeb and chlorothalonil, used as solo or in a mixture with metalaxyl-M in Japan (Furuta et al. unpublished; Ide et al. unpublished; Syobu and Watanabe 2022), regulation of these multisite inhibitors tightened due to health and environmental concerns (Irish Examiner 2021;

Lynxee consulting 2019) in the other countries. Currently, the Research Committee on Fungicide Resistance in the Phytopathological Society of Japan recommends limiting applications of QoI and CAA fungicides in onion to one to two times per season (http://www.taiseikin.jp/ Accessed 31 July 2022). High efficacy of mixed formulations, such as benthiavalicarb-isopropyl plus fluopicolide, mandipropamid plus oxathiapiprolin, and dimethomorph plus ametoctradin have now been registered for downy mildew control on onion and lettuce.

Rapid and accurate diagnosis of downy mildews (Crandall et al. 2018) and fungicide sensitivity is one of the most effective practices of resistance management. The PCR–RFLP analysis used in this study is cost-effective and allows simple discrimination of resistant and sensitive isolates and will contribute to delaying resistance evolution to QoI and CAA fungicides.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s41348-023-00732-w.

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Declarations

Conflict of interest Gerd Stammler is an employee of BASF.

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