Technical advance

Establishment of a selection marker recycling system for sequential transformation of the plant-pathogenic fungus *Colletotrichum orbiculare*

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SUMMARY

Genome sequencing of pathogenic fungi has revealed the presence of various effectors that aid pathogen invasion by the manipulation of plant immunity. Effectors are often individually dispensable because of duplication and functional redundancy as a result of the arms race between host plants and pathogens. To study effectors that have functional redundancy, multiple gene disruption is often required. However, the number of selection markers that can be used for gene targeting is limited. Here, we established a marker recycling system that allows the use of the same selection marker in successive transformations in the model fungal pathogen *Colletotrichum orbiculare*, a causal agent of anthracnose disease in plants belonging to the Cucurbitaceae. We identified two *C. orbiculare* homologues of yeast URA3/ pyrG, designated as URA3A and URA3B, which can be used as selection markers on medium with no uridine. The gene can then be removed from the genome via homologous recombination when the fungus is grown in the presence of 5-fluoroorotic acid (5-FOA), a chemical that is converted into a toxin by URA3 activation when the fungus is grown in the presence of 5-fluoroorotic acid. The establishment of a URA3A-based marker recycling system in plant-pathogenic fungi enables the genetic analysis of multiple genes that have redundant functions, including effector genes.

Keywords: *Colletotrichum orbiculare*, Cucurbitaceae plants, plant-pathogenic fungus, selection marker recycling, URA3/ pyrG

INTRODUCTION

Phytopathogens have evolved various strategies to overcome plant immunity, including the use of effectors that facilitate a parasitic lifestyle by regulating their host’s immune system. For example, effectors that target pattern-triggered immunity, the first layer of plant immunity, have been reported in various pathogens. In turn, plants have developed so-called resistance proteins to detect effectors, inducing a strong defence response against pathogens, called effector-triggered immunity. During the process of evolution, phytopathogens and plants have developed mutual attack and defence systems that have resulted in functional redundancy and the duplication of pathogen effectors and plant immunity-related proteins (Asai and Shirasu, 2015; Hogenhout et al., 2009; Jones and Dangl, 2006).

The *Colletotrichum* genus comprises over 600 species, including hemibiotrophic fungi that cause anthracnose disease in various plants, e.g. economically important crops, vegetables and fruits (Cannon et al., 2012). Therefore, the *Colletotrichum* genus is recognized by researchers in the plant–microbe interaction community as one of the 10 most important phytopathogenic fungi (Dean et al., 2012). Within the genus, *Colletotrichum higginsianum* and *Colletotrichum orbiculare* have been recognized as model pathosystems, as they can infect the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana*, respectively (O’Connell et al., 2004; Perfect et al., 1999; Shen et al., 2001; Takano et al., 2006). The functions of several effectors have been reported in *C. higginsianum* and *C. orbiculare*. For example, the lysin motif domain (LysM) contains effectors ELP1 and ELP2 of *C. higginsianum*, which play dual roles in appressorial function and suppression of chitin-triggered plant immunity (Takahara et al., 2016). In *C. orbiculare*, the effector NIS1 has a cell death-inducing effect on *N. benthamiana* in an *SGT1*- and HSP90-dependent manner (Yoshino et al., 2012). Furthermore, transcriptome analysis has revealed a characteristic expression pattern of effector-encoding genes during the infection stage transition of *C. higginsianum* and *C. orbiculare*, implying that...
the coordinate expression of different sets of effectors is orchestrated for successful infection (Gan et al., 2013; Kleemann et al., 2012; O’Connell et al., 2012). Although the functions of several effectors have been elucidated, the vast majority of Colletotrichum effectors are still obscure. One reason for this lack of knowledge is the limited availability of selection markers for transformation. There are only four reported combinations of antibiotics (bialaphos, geneticin/G418, hygromycin and sulfonylurea) and corresponding resistance genes that can be used for the transformation of C. higginsianum and C. orbiculare, making it difficult to analyse redundant effectors (Chung et al., 2002; Dallery et al., 2017; Irieda et al., 2014).

In general, the marker recycling method is used to resolve the limitation of selection markers (Kopke et al., 2010; Zhang et al., 2017). For example, in Aspergillus fungi, the pyrG (a homologue of URA3)-based marker recycling system has been used and developed (d’Enfert, 1996; Nielsen et al., 2006; Oakley et al., 1987). The URA3/pyrG gene encodes an orotidine-5′-phosphate decarboxylase involved in uridine/uracil synthesis (Weld et al., 2006). In Saccharomyces cerevisiae, mutants of URA3 show growth defects on medium lacking uridine. The uridine auxotrophy of ura3 enables URA3 expression cassettes to work as a selection marker for transformation in the ura3 mutant background. In addition, URA3/pyrG can be applied to negative selection (Boeke et al., 1984). Orotidine-5′-phosphate decarboxylases encoded by pyrG or URA3 orthologues convert 5-fluoroorotic acid (5-FOA), an analogue of the uracil precursor, to 5-fluorouracil, a toxic compound that inhibits DNA and RNA synthesis (Flynn and Reece, 1999). Therefore, when URA3/pyrG is positioned between homologous sequences, excision of the genomic URA3/pyrG sequence by homologous recombination can be selected by 5-FOA treatment. By utilizing this strategy, the URA3/pyrG expression cassette can be removed from the fungal genome. Indeed, the URA3/pyrG-based marker recycling system has been utilized in S. cerevisiae (Alani et al., 1987), Aspergillus nidulans (Nielsen et al., 2006; Oakley et al., 1987), Aspergillus fumigatus (d’Enfert, 1996), Neurospora crassa (Turner et al., 1997), Candida albicans (Fonzi and Irwin, 1993) and Mucor circinelloides (Garcia et al., 2017). However, this system has never been applied to phytopathogenic fungi.

Here, we report the establishment of a URA3-based marker recycling method in C. orbiculare 104-1. As a proof of concept, we knocked out PKS1, a gene encoding a polyketide synthase that is required for melanin synthesis involved in virulence (Takano et al., 1995), using the URA3B (one of two pyrG homologues in C. orbiculare) expression cassette as a selection marker. The PKS1 mutants showed reduced virulence on plants, consistent with previous studies demonstrating that the URA3B selection marker can be applied to study virulence-related genes. In the pks1 mutant background, DMAT3, a secondary metabolism key enzyme encoding gene, was disrupted using the URA3 selection marker, demonstrating that the marker recycling system can be applied to sequential transformation and gene deletion. The establishment of a URA3-based marker recycling system enables genes that have redundant functions, such as effectors in phytopathogenic fungi, to be studied.

RESULTS

The C. orbiculare genome encodes two URA3/pyrG homologues, URA3A and URA3B

To check whether URA3 homologues are present in plant-pathogenic fungi, including C. orbiculare, BLAST (blastp; default setting) search was performed using S. cerevisiae Ura3p as a query. Figure 1a shows that each of the 10 different plant fungal pathogens, which were selected as the 10 most important fungal pathogens based on scientific/economic importance (Dean et al., 2012), has at least one URA3/pyrG homologue, except for Melampsora lini. In particular, C. orbiculare has two putative URA3/pyrG genes, named URA3A (Cob_06825) and URA3B (Cob_03887). As shown in Fig. S1 (see Supporting Information), URA3A is more similar than URA3B to S. cerevisiae Ura3p. However, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis revealed that URA3B is the major URA3 gene, because of its constitutive expression in all tested developmental stages (Fig. 1b). This notion is supported by the fact that Fusarium oxysporum URA3, which is the only predicted URA3 homologue in the genome, is more similar to URA3B than URA3A (Fig. S1).

URA3A and URA3B double knock-out mutants exhibit uridine auxotrophy and 5-FOA insensitivity

Because URA3B is constitutively expressed, we first knocked out URA3B by homologous recombination using the pNK028 plasmid harbouring neomycin phosphotransferase II (NPTII), a geneticin/G418 resistance gene (Fig. 2a). At least four ura3b knock-out strains were obtained and the gene disruption was confirmed by genomic PCR using the primer sets Po1/Po2, Po3/Po4 and Po5/Po6 (Fig. 2a,b). As predicted, all ura3b mutants showed no growth on potato dextrose agar (PDA), but were able to grow on PDA supplemented with 10 μM uridine, suggesting that URA3B is indispensable for uridine synthesis in vivo (Fig. 2c). The growth of wild-type C. orbiculare was inhibited by the addition of 1 mg/mL 5-FOA to PDA, implying that URA3B also synthesizes a toxic compound from 5-FOA. However, the 5-FOA sensitivity varied among the ura3b mutants (Fig. 2c), suggesting that residual URA3 enzyme activity was contributed by the other URA3 homologue URA3A. Therefore, we decided to knock out URA3A in ura3b and established four double knock-out lines by homologous recombination using pNK032 (Fig. 2d). As shown in Fig. 2e, genomic PCR confirmed that the URA3A gene was disrupted in
The URA3 expression cassette functions as a selection marker in ura3a/b mutants

To test whether the uridine auxotrophy selection marker is usable in C. orbiculare, PKS1, which encodes a polyketide synthase involved in melanin synthesis (Takano et al., 1995, 1997), was targeted for disruption by the pNK059 plasmid. The plasmid has the URA3B expression cassette as a selection marker, in which URA3B is driven by the Tef (TRANSLATION ELONGATION FACTOR) promoter of Aureobasidium pullulans (Wymelenberg et al., 1997) (Fig. 3a). Successful knock out of PKS1 was confirmed by genomic PCR (Fig. 3b). Consistently, pks1/ura3a/b-Tef::URA3B#1 and pks1/ura3a/b-Tef::URA3B#2 showed the albino phenotype, characteristic of pks1 mutants and caused by a lack of melanin (Takano et al., 1995) (Fig. 3c).

The excision of the URA3B expression cassette can be selected by 5-FOA treatment, demonstrating establishment of the marker recycling system in C. orbiculare

In the pks1/ura3a/b-Tef::URA3B strains, the Tef::URA3B cassette is designed to be located between 500 bp of completely homologous sequences (Fig. 4a, green boxes). If pks1/ura3a/b-Tef::URA3B is incubated on PDA plates containing 5-FOA, the strains that lose the Tef::URA3B cassette by homologous recombination should be selected. As predicted, pks1/ura3a/b strains without the Tef::URA3B cassette could be isolated from pks1/ura3a/b-Tef::URA3B strains after growth on PDA containing 1 mg/mL 5-FOA and 10 mM uridine. Then, using eight randomly selected colonies per strain, the removal of the Tef::URA3B cassette was checked by genomic DNA PCR. No bands were observed from genomic DNA of all pks1/ura3a/b strains (#1–4 are shown as representatives in Fig. 4b) using the primer set Po13/Po14, which generates 1200-bp amplicons in the presence of the Tef::URA3B cassette. The pks1/ura3a/b strains were unable to grow on PDA, but grew on PDA supplemented with 10 mM uridine (Fig. 4c). These strains also show low sensitivity to 5-FOA treatment, suggesting the absence of the Tef::URA3B cassette. Together, we conclude that the removal of the Tef::URA3B marker from pks1/ura3a/b-Tef::URA3B can be selected by 5-FOA treatment.

URA3B marker knock in enables in planta virulence assay in C. orbiculare

We tested whether the marker recycling system could be applied to study virulence in planta. As shown in Fig. 5d, ura3a/b mutants did not trigger disease symptoms on cucumber cotyledons, most probably because uridine was not acquired from the host plant. To check whether externally added uridine complements the phenotype of ura3a/b, wild-type, ura3a/b and ura3a/b supplemented with 10 mM uridine were inoculated onto cucumber
cotyledons. Externally added uridine partially complemented the disease phenotype of ura3a/b (Fig. S2c,d, see Supporting Information), showing that uridine is required for the virulence of C. orbiculare. Then, to determine at which stage the pathogenicity of ura3a/b mutants was arrested, the rates of conidial penetration, an early event of infection, were assessed. Although appressoria were formed as in the wild-type, the ura3a/b mutants could not penetrate into the cucumber cells at all (Fig. S2a,b).

This deficiency in the penetration rate of ura3a/b was partially complemented by externally added uridine (Fig. S2d). These results suggest that infection of ura3a/b is arrested at the penetration stage and uridine is required for successful penetration.

Next, we knocked in the URA3B gene in ura3a/b to its original locus using pNK062 (Fig. 5a). The successful knock in of the URA3B gene was confirmed by genomic DNA PCR (Fig. 5b) and phenotypic analysis (Fig. 5c). No significant difference in disease...
symptoms between wild-type and URA3B knocked-in strains was observed, demonstrating that URA3B alone was sufficient to complement the reduced virulence phenotype of ura3a/b (Fig. 5d,e). Then, we knocked in URA3B in pks1/ura3a/b mutants to produce pks1/ura3a mutants, and infected cucumber (Cucumis sativus) cotyledons. As shown in Fig. 5d,e, pks1/ura3a showed significantly less virulence than ura3a, indicating the involvement of PKS1 in virulence, as described previously (Takano et al., 1995). These data demonstrate that the marker recycling system can be applied to the in planta assays to test genes involved in virulence.

**DMAT3 knock-out in the pks1/ura3a/b mutant using the marker recycling system**

To check whether the marker recycling system can be repeatedly used for transformation and gene targeting, we performed one round of additional gene disruption in the pks1/ura3a/b

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**Fig. 4** The Tef::URA3B cassette was removed by homologous recombination and selected by 5-fluoroorotic acid (5-FOA) treatment. (a) Schematic diagrams of the removal of the Tef::URA3B cassette (Cob_09513). Green arrows show the homologous 500-bp sequences for recombination. (b) Genomic DNA polymerase chain reaction (PCR) showed that Tef::URA3B was removed. The primer set Po13/Po14 generates the 1200-bp amplicon if the Tef::URA3B cassette is present. The 266-bp bands corresponding to CHITIN SYNTHASE (CHS) were amplified using the primer set CHS_79F and CHS_345R (Carbone and Kohn, 1999) to show the presence of genomic DNA. The primers used are listed in Table S3 (see Supporting Information). (c) ura3a/b#1, pks1/ura3a/b-Tef::URA3B #1 and pks1/ura3a/b#1-4 strains were cultured on potato dextrose agar (PDA), PDA with 10 mM uridine and PDA with 10 mM uridine plus 1 mg/mL 5-FOA for 6 days at 25 °C in the dark. All pks1/ura3a/b#1-4 strains showed uridine auxotrophy and 5-FOA insensitivity similar to ura3a/b#1, demonstrating successful removal of the Tef::URA3B cassette. The details of each strain are listed in Table S1 (see Supporting Information). [Colour figure can be viewed at wileyonlinelibrary.com]
As a target, DMAT3 (Cob_04983), which encodes a predicted secondary metabolite (SM) key gene, dimethylallyl transferase, was selected for gene knock-out by the pNK098 plasmid (Fig. 6a). First, the Tef::URA3B expression cassette was inserted into the upstream (Up) and downstream (Down) sequences of the DMAT3 coding sequence (CDS) in the Colletotrichum orbiculare 104-T genome. The complete DMAT3 CDS, including intron, is located between the Up and Down sequences.

Mutant. As a target, DMAT3 (Cob_04983), which encodes a predicted secondary metabolite (SM) key gene, dimethylallyl transferase, was selected for gene knock-out by the pNK098 plasmid.

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was confirmed by genomic DNA PCR (Fig. 6b), resulting in three independent strains, named pNK098HR pkst1/ura3a/b. In these strains, the Tef::URA3B cassette and the DMAT3 CDS were located between 500 bp of completely homologous sequences (Fig. 6a, green boxes) for the marker and CDS removal. Second, removal of the Tef::URA3B cassette and DMAT3 CDS was selected for by incubating pNK098HR pkst1/ura3a/b on PDA plates containing 5-FOA and uridine. The successful removal of the Tef::URA3B cassette and DMAT3 CDS was confirmed by genomic DNA PCR (Fig. 6c), resulting in three independent dmat3/pks1/ura3a/b mutant strains. These results prove the concept of sequential transformation and gene targeting using the marker recycling system reported here.

**Fig. 6** DMAT3 knock-out in the pkst1/ura3a/b mutant. (a) Schematic diagrams of the DMAT3 coding sequence (CDS) knock-out experiments in the pkst1/ura3a/b background. The pNK098 plasmid includes about 2 kb of upstream (Up) and downstream (Down) sequences around the DMAT3 locus. The Down sequence contains the complete DMAT3 CDS shown by a black box. Green arrows show homologous 500-bp sequences for recombination originally from the downstream region of DMAT3 CDS. (b) Genomic DNA polymerase chain reaction (PCR) showed successful homologous recombination by pNK098, resulting in pNK098HR pkst1/ura3a/b strains. The primer sets Po17/Po19 and Po18/20 generate 2826- and 2674-bp bands, respectively, from pNK098HR pkst1/ura3a/b, but not from the genome of pkst1/ura3a/b. The 266-bp bands corresponding to CHITIN SYNTHASE (CHS) were amplified to show the presence of genomic DNA. (c) Genomic DNA PCR showed the successful removal of the Tef::URA3B expression cassette and the DMAT3 CDS. The primer sets Po13/Po14 and Po21/Po22 generate 1200- and 540-bp bands, respectively, from the genome of pNK098HR pkst1/ura3a/b, but not from that of dmat3/pks1/ura3a/b. The primers used are listed in Table S3 (see Supporting Information). [Colour figure can be viewed at wileyonlinelibrary.com]

**DISCUSSION**

The limited availability of selection markers represents a bottleneck for effector studies in many plant-pathogenic fungi. Here, we report the establishment of a marker recycling system using URA3/pyrG homologues in the phytopathogenic filamentous fungus, *C. orbiculare*. The URA3B cassette was successfully used as a selection marker in *C. orbiculare* ura3a/b double mutants to create PKST1 knock-out lines. Selection for loss of the URA3B cassette via homologous recombination can then easily be performed by growth on media supplemented with 5-FOA. In addition, we successfully knocked out DMAT3 in the pkst1 mutant background, proving the concept of sequential transformation by the marker recycling system. Importantly, the reintroduction...
of URA3B at its original locus is able to restore the growth of ura3a/b mutants to wild-type levels in planta. Thus, using this system, we should be able to assess the function of any gene of interest in planta. The avoidance of positional effects is critical, as shown in Candida albicans, an opportunistic fungal pathogen of animals, where the virulence phenotype of the fungus in mice varied as a function of the relocation of URA3 in the genome, as the expression of URA3 during infection is affected by its genomic locus (Staab and Sundstrom, 2003; Sundstrom et al., 2002). The reintroduction of URA3B is an additional step in the protocol, but the use of the same vector and the high efficiency of homologous recombination make this process straightforward. As the URA3B cassette can be recycled, knock-out analysis of multiple genes with functional redundancy is now possible in C. orbiculare. Similarly, the URA3-based marker recycling system can be applied to other pathogenic fungi that are transformable with a relatively high homologous recombination rate. The genome sequences of other transformable plant pathogens, Ustilago maydis, Zymoseptoria tritici, Botrytis cinerea, Magnaporthe oryzae, Fusarium graminearum, F. oxysporum and C. higginsianum, revealed that these organisms contain only one copy of URA3 (Fig. 1a). Thus, it is possible that setting up the ura3 knock-out system in these pathogens may be easier than that in C. orbiculare.

The URA3-based marker recycling system offers several advantages over other systems, such as Cre-loxP, Flp-FRT and β-recombinase-six, which have been used in prokaryotes and eukaryotes (Abuin and Bradley, 1996; Johansson and Hahn-Hägerdal, 2004; Lambert et al., 2007; Szewczyk et al., 2014; Yuliya et al., 2010; Zhang et al., 2017). Cre, Flp and β-recombinase catalyse the recombination between sites, named loxP, FRT and six, respectively. As these enzymes lead to the excision of DNA between the two recombination sites, one of the sites is left behind in the genome (Kilby et al., 1993; Wirth et al., 2007). Thus, unlike our homologous recombination-based URA3 system, foreign sequences accumulate in the genome if sequential transformation is performed, which potentially induces genome instability. Therefore, the URA3/pyrG-based marker recycling system should allow the creation of much more stable multiple knock-out lines. Further, the URA3B cassette has been demonstrated to function as both a positive and negative selection marker, avoiding the need for opposite markers as used in the β-recombinase-based system, which utilizes the bialaphos resistance gene as a positive selection marker for transformation and the thymidine kinase gene as a negative selection marker for excision (Szewczyk et al., 2014). Thus, the URA3/pyrG-based marker recycling system should allow the construction of smaller vectors.

In Aspergillus nidulans, a model organism for fungal research, URA3/pyrG-based transformation and related technologies have been developed (Dohn et al., 2018; Oakley et al., 1987). For example, Szewczyk et al. (2006) were able to improve the speed of transformation experiments by utilizing PCR fragments (not plasmids) for gene targeting. Further, Nayak et al. (2006) identified and knocked out the A. nidulans homologue (nkuA) of the human KU70 gene, which is important for non-homologous end joining of DNA in double-strand breaks. A lack of nkuA reduces the frequency of non-homologous integration of DNA fragments for transformation, leading to higher gene targeting efficiency. The homologue of nkuA has also been knocked out in C. higginsianum (Ushimaru et al., 2010), the causal agent of anthracnose on Brassicaceae plants, leading to improved gene targeting efficiency. Thus, it is likely that the method may be applicable to other members of the Colletotrichum fungi. The application of these advanced technologies developed in A. nidulans and other fungi to C. orbiculare is theoretically possible and could make sequential gene targeting and transformation more rapid and easier in combination with the marker recycling system.

In recent years, the genomes of many phytopathogenic fungi, such as Colletotrichum species, M. oryzae, U. maydis and Z. tritici, have been sequenced and the presence of multiple effector proteins has been predicted (Dean et al., 2005; Gan et al., 2013; Ma et al., 2010; O’Connell et al., 2012; Spanu et al., 2010). In Colletotrichum, transcriptome analysis revealed that the expression of several SM synthesis-related genes is strongly induced during infection, especially at the biotrophic phase, in addition to effector proteins (Dallary et al., 2017; Gan et al., 2013). These findings suggest that SMs synthesized by fungi may have a virulence function. One SM synthesis-related gene, btcAco, whose expression is induced during infection, was knocked out, and its function was assessed (Gao et al., 2018). Although btcAco is involved in SM synthesis, virulence effects in btcAco disrupted mutants were not detected (Fig. S3, see Supporting Information). One possible reason for this lack of virulence phenotype is that SMs may also have functional redundancy in virulence. We anticipate that the URA3/pyrG marker recycling system will contribute to elucidate the functions of effectors, including proteins, and SMs.

**EXPERIMENTAL PROCEDURES**

**Fungal transformation**

Fungal transformation was performed using the polyethylene glycol-mediated protoplast transformation protocol described previously (Kubo et al., 1991). Colletotrichum orbiculare 104-T (MAFF240422) was used as the wild-type strain (Ishida and Akai, 1969). Derivative strains from C. orbiculare 104-T and plasmids used for transformation are listed in Tables S1 and S2 (see Supporting Information), respectively.
Selection of removal of the URA3B expression cassette after 5-FOA treatment

The strains pks1/ura3a/b:Tef::URA3B (CoNK0031) and pks1/ura3a/b:Tef::URA3B (CoNK0033) were cultured on PDA (Nissui Pharmaceutical Co., Ltd., Taito-ku, Tokyo, Japan) for 6 days and their conidia were collected. About 1 × 10^5 conidia of CoNK0031 and CoNK0033 were spread onto PDA with 1 mg/mL 5-FOA monohydrate (Wako Pure Chemical Industries, Ltd., Chuo-ku, Tokyo, Japan) and 10 μM uridine (Tokyo Chemical Industry Co., Ltd., Chuo-ku, Tokyo, Japan) in sterilized no. 2 square plates (Eiken Chemical Co., Ltd., Taito-ku, Tokyo, Japan). Then, the plates were incubated at 25 °C in the dark for 4 days. The surviving colonies were transferred to new PDA with 1 mg/mL 5-FOA and 10 μM uridine, and incubated under the same conditions for 4 days. The surviving colonies were selected and removal of the Tef::URA3B cassette was examined by fungal colony PCR. Selected transformants were designated as pks1/ura3a/b#1 (CoNK0041), pks1/ura3a/b#2 (CoNK0042), pks1/ura3a/b#3 (CoNK0043) and pks1/ura3a/b#4 (CoNK0044).

Plasmid construction

All primers used are listed in Table S3 (see Supporting Information). The genomic DNA of C. orbiculare 104-T used for PCR was isolated as described previously (Gan et al., 2013).

pNK028: PCR-1 and PCR-2 were amplified from C. orbiculare 104-T genomic DNA using the primer sets IF-pli99EcoRV+URA3BUP_F plus IF-pli99EcoRV+URA3BUP_R and pNK028-DW_F plus pNK028-DW_R. The pli99 plasmid harbouring NPTII, a geneticin/G418 resistance gene (Namiki et al., 2001), was digested with EcoRV (TaKaRa Bio, Inc., Kusatsu, Shiga, Japan) and the larger fragment was fused to PCR-1 using the In-Fusion HD Cloning Kit (TaKaRa Bio, Inc., Taito-ku, Tokyo, Japan). Then, the plates were incubated at 25 °C in the dark for 4 days. The surviving colonies were transferred to new PDA with 1 mg/mL 5-FOA and 10 μM uridine, and incubated under the same conditions for 4 days. The surviving colonies were selected and removal of the Tef::URA3B cassette was examined by fungal colony PCR. Selected transformants were designated as pks1/ura3a/b#1 (CoNK0041), pks1/ura3a/b#2 (CoNK0042), pks1/ura3a/b#3 (CoNK0043) and pks1/ura3a/b#4 (CoNK0044).

RT-qPCR

Total RNA isolation and DNA removal were carried out using an RNeasy Plant Mini Kit and RNase-Free DNase Set (Qiagen, Venlo, Limburg, Netherlands) following the manufacturer’s instructions. cDNAs were synthesized from isolated RNAs with the ReverTraAce qPCR RT Kit (Toyobo Co., Ltd., Kita-ku, Osaka, Japan) using the included primer mix and following the manufacturer’s instructions. All RT-qPCRs were performed with THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Kita-ku, Osaka, Japan) and an MX3000P Real-Time qPCR System (Stratagene, Santa Clara, California, USA). Primer sets qURA3A_F3 plus qURA3A_R3, qURA3B_F2 plus qURA3B_R2 and 8436qF_ref plus 8436qR_ref were used to detect transcripts of URA3A and URA3B, respectively. The primer sequences used in the experiments are listed in Table S3. Plasmids with the coding sequences of URA3A and URA3B were used as standards for the absolute quantification of URA3A and URA3B transcripts.

Fungal infection assay of cucumber

Fungal strains were cultured on PDA at 24 °C for 6 days in the dark. Then, their conidia were collected by centrifugation at 3000 g for 5 min and washed twice with sterilized water. Droplets (10 μL) of conidia at 5 × 10^5 conidia/mL in water were inoculated onto cucumber cotyledons at 10 days post-germination. Seeds of C. sativus, cucumber Suyo strain (Sakata Seed Corp., Yokohama, Kanagawa, Japan), were planted in a mix of equal amounts of Supermix A (Sakata Seed Corp.) and vermiculite. Then, cucumbers were grown at 24 °C under a 10-h light/14-h dark cycle.
Alignment of URA3 proteins

The amino acid sequence of *Saccharomyces cerevisiae* Ura3p and *C. orbiculare* URA3A and URA3B were aligned using MAFFT software (Katoh et al., 2002). Aligned sequences were formatted using CLC Genomics Workbench 8.0 (CLC bio, Aarhus, Midtjylland, Denmark).

**ACCESSION NUMBERS**

Ura3p (SGD:S000000747) from the Saccharomyces Genome Database (https://www.yeastgenome.org/). URA3A (ENH84876.1), URA3B (ENH87716.1), PKS1 (ENH81867.1), DMAT3 (ENH86929.1) and *F. oxysporum* f. sp. *cubense* URA3 (EMT68416.1) from the GenBank databases.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Amino acid sequence alignment of Saccharomyces cerevisiae Ura3p with Colletotrichum orbiculare URA3A and URA3B, and Fusarium oxysporum URA3.

Fig. S2 The lesser disease symptom phenotype of ura3a/b is partially complemented by externally added uridine.

Fig. S3 Colletotrichum orbiculare btcAco knock-out mutants do not show reduced virulence on cucumber leaves. The experiment was performed in the same conditions as in Fig. 5d.

Table S1 Fungal strain list.
Table S2 Plasmid list.
Table S3 Oligo list.
Data S1 DNA sequences of PCR-10 and PCR-11.