Disruption of *ku70* involved in non-homologous end-joining facilitates homologous recombination but increases temperature sensitivity in the phytopatogenic fungus *Penicillium digitatum*

Mónica GANDÍA *, **Shaomei XU, Cristina FONT, Jose F. MARCOS**

Food Science Department, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), Avda Agustín Escardino 7, 46980 Paterna, Valencia, Spain

*Corresponding author: Dr. Mónica Gandía. Instituto de Agroquímica y Tecnología de Alimentos (IATA) - CSIC, Avenida Agustín Escardino 7, Paterna 46980, Valencia, Spain. e-mail: mgandia@iata.csic.es. Phone: (34)963.900.022. Fax: (34)963.636.301

E-mail addresses:
MG: mgandia@iata.csic.es
SX: Shao_mei93@hotmail.com
CF: cris_font_ma@hotmail.com
JFM: jmarcos@iata.csic.es

Highlights

- Disruption of *ku70* enhanced homologous recombination frequency in *P. digitatum.*
- The mutation of *ku70* did not affect *P. digitatum* phenotypes at optimal temperature.
- Δ*ku70* strains showed defects in growth and conidia production at high temperatures.
- Fungal Δ*ku70* recipient strains should be used with caution.

ABSTRACT

The dominant mechanism to repair double-stranded DNA breaks in filamentous fungi is the non-homologous end joining (NHEJ) pathway, and not the homologous recombination (HR) pathway that operates in the mutation of genes by replacement of target DNA for selection cassettes. The key to improve HR frequency is the inactivation of the NHEJ pathway by eliminating components of its Ku70/80 heterodimeric complex. We have obtained *ku70* mutants of *Penicillium digitatum*, the main citrus postharvest pathogen. The increased efficiency of HR in Δ*ku70* strains was demonstrated by the generation of mutants in two different chitin synthase genes (*PdchsII* and *PdchsV*). *P. digitatum* Δ*ku70* strains showed no differences from the parental strain in vegetative growth, asexual development or virulence to citrus fruit, when experiments were conducted at the optimal temperature of 24 ºC. However, growth of Δ*ku70* strains at temperatures higher than 24 ºC demonstrated a detrimental effect in axenic growth and conidia production. These observations are in agreement with previous studies describing differences between *ku70* mutants and their parental strains in some fungal species, and must be taken into account for future applications of the Δ*ku* approach to increase HR efficiency in fungi.

Keywords: Gene targeting, *ku70*, *Penicillium digitatum,* citrus fruit.

1. Introduction

Gene disruption based on DNA homologous recombination (HR) is a common approach in fungal genetics to replace genes and study their functional roles. The mechanism to generate knockout mutants by HR involves integration at the target site of a gene replacement cassette, which contains a selectable marker. However, exogenous DNA integration can also be produced at non-target locations by the non-homologous end-joining pathway (NHEJ), which depends on the Ku70/Ku80 protein complex (Dudasova et al. 2004; van Attikum et al. 2001). These proteins form a heterodimer that binds DNA ends and is also involved in telomere maintenance (Downs and Jackson 2004; Gravel and Wellinger 2002).

As opposed to *Saccharomyces cerevisiae*, the dominant pathway to repair DNA double-strand breaks in filamentous fungi seems to be NHEJ and thus HR efficiency is very low (Meyer et al. 2007). An approach to improve and enhance HR frequency has been to change this balance by inactivating the NHEJ pathway through the deletion of *ku70/ku80* genes. This strategy has been used in many different fungi, such as *Neurospora crassa* (Ninomiya et al. 2004), *Aspergillus* (Meyer et al. 2007; Takahashi et al. 2006; Zhang et al. 2011), *Botrytis cinerea* (Choquer et al. 2008) and *Penicillium chrysogenum* (Snoek et al. 2009). The resulting strains have not been reported to demonstrate noticeable phenotypic differences with the parental strains and they have been used as new parental strains to delete target genes of interest.

Penicillium digitatum is the main citrus postharvest pathogen that is responsible for the green postharvest decay that causes important economic losses. Recent sequencing of the genome of different strains of *P. digitatum* (Marcet-Houben et al. 2012; Sun et al. 2013) and the development of genetic transformation protocols (Wang and Li 2008) have paved the way for molecular and genetic studies on this fungus (González-Candelas et al. 2010; Sun et al. 2011) that include the generation of mutants to study gene function (Gandía et al. 2014; Harries et al. 2015; López-Pérez et al. 2015; Wang et al. 2014; Wang et al. 2015; Zhang et al. 2013a; Zhang et al. 2013b; Zhang et al. 2013c; Zhu et al. 2015). However, the generation of knockout mutants of *P. digitatum* is a difficult task due to the very low frequency of HR. For example, in our two recent publications the highest HR frequency was 0.6% in the case of the protein *O*–mannosyltransferase Pmt2 mutant (Harries et al. 2015). A

recent work has demonstrated that the deletion of *ku80* increased gene targeting efficiency in *P. digitatum* and did not affect growth, sporulation, tolerance to salt, or virulence (Xu et al. 2014). In this study we explored a similar approach by mutating the alternative *ku70* gene in *P. digitatum*, and discovered unexpected results that must be taken into account for the future use of *ku70/80* mutants in filamentous fungi.

2. Materials and Methods

2.1. Strains, media and culture conditions

The *P. digitatum* reference strain used was CECT20796 (PHI26) (Marcet-Houben et al. 2012). All *P. digitatum* strains were cultured on potato dextrose agar (PDA) plates for 7 days at 24 ºC. Growth of strains was evaluated by depositing 5 µL of a conidial suspension (5 x 10^4 conidia mL^{-1}) on PDA plates, and the growth diameter was measured daily. To assess conidia production at 7 days, the assay was performed as described previously (Gandía et al. 2014). Statistical analyses were conducted using the Statgraphics Centurion XVI software (Statpoint Technologies, VA, USA). The *Agrobacterium tumefaciens* AGL-1 was used for fungal transformation (Gandía et al. 2014).

2.2. Generation of disruption strains

Primers used in this work are described in Table 1. To produce the *ku70* disruption construct, the nourseothricin resistance cassette (*nat1*) flanked by the FLP recombinase recognition targets (FRT) (FRT-*nat1*) was used as positive selection marker and amplified from the pDNAT-FRT1,2 vector (Kopke et al. 2010) with the universal primers T7 and SP6. This FRT-*nat1* was fused to two flanking fungal DNA fragments of ~1200 bp (primers OJM383-OJM384 for the 5´ fragment and OJM385-OJM386 for the 3´ fragment, respectively, amplified from *P. digitatum* CECT20796 genomic DNA) (Fig 1A) by fusion PCR, purified and cloned into the *Spe* I-*Sal* I restriction sites of the binary vector pGKO2 as described (Harries et al. 2015; Khang et al. 2006). The T-DNA of pGKO2 also contains the Herpes Virus thiamine kinase gene (HSV*tk*), which was used as negative selection marker for 5-fluoro-2-deoxyuridine (F2dU). The resulting vector pGKO2_Δ*ku70* was transformed into *A. tumefaciens* AGL-1. Fungal transformation of the parental CECT20796 strain was

performed by *Agrobacterium tumefaciens*-mediated transformation (ATMT) as previously described (Harries et al. 2015). Fungal colonies were selected first positively for nourseothricin resistance and then negatively for F2dU. Transformants were monitored and confirmed by PCR amplification of genomic DNA obtained from fungal mycelium (Harries et al. 2015; Khang et al. 2006); different primers used were located at different positions on the target locus and the disruption constructs (Fig 1A).

2.3. Fruit infection assays

Fruit infection assays with *P. digitatum* strains on mature freshly harvested orange fruits (*Citrus sinensis* L. Osbeck cv Navelina) were conducted as previously described (González-Candelas et al. 2010). Three replicates of five fruits were inoculated with 5 µL of conidial suspensions (10^5 conidia mL^{-1}) at four wounds around the equator. Data presented are the mean value ± SD of the percentage of infected wounds per replica at different days post-inoculation (dpi).

3. Results

3.1. Disruption of the P. digitatum ku70 gene

The *P. digitatum ku70* gene (PDIG_32990, *Pdku70*) was identified in the genome of *P. digitatum* (Marcet-Houben et al. 2012). To obtain marker-free *ku70* disruption transformants, we designed a two-steps strategy based on the recyclable FRT-*nat1* cassette (Kopke et al. 2010) and adapted to the ATMT protocol that we follow with *P. digitatum* (Gandía et al. 2014; Harries et al. 2015). First, a fragment of the *Pdku70* ORF was replaced by the FRT-*nat1* cassette that confers resistance to nourseothricin (Fig 1A), and second, transient expression of FLP recombinase would recombine and excise the flanking FRT sequences, also removing the *nat1* sequence.

Following the first step, eighteen *P. digitatum* transformants resistant to nourseothricin and F2dU were obtained by ATMT with AGL-1 harboring the binary vector pGKO2_Δ*ku70*. These transformants were monitored using a set of PCR amplifications with primers designed outside or inside the replacement construction

to discriminate positive disruption transformants from negative ectopic or parental strains (Fig 1B).

Second, in an attempt to excise the *nat1* marker, we tried to use protoplast transformation with the self-replicating plasmid pPTRII_PcFLP containing the FLP recombinase (Kopke et al. 2010). After succeeding in stablishing protocols for the isolation, selection and regeneration of protoplasts with our *P. digitatum* strains, we were unable to demonstrate protoplast transformation (data not shown). This negative result is consistent with the reported difficulty of transforming *P. digitatum* protoplasts (Xu et al. 2014). As an alternative, we tried to select spontaneous FRT recombination events that would excise the *nat1* marker. We conducted five rounds of sub-culturing on PDA plates in the absence of selection pressure (nourseothricin), and randomly selected 100 colonies after each round of culture. However, we could not identify any colony that would have lost the heterologous *nat1* cassette. Therefore, we did not excise *nat1* and the study continued with the strains shown in Fig 1B. Indirectly, this result confirms the high genetic stability of the disruption transformants.

The six positive Δ*ku70* strains shown in Fig 1B (for instance, PDMG314) were selected for further analyses. At the optimal temperature (24-25 ºC), the inactivation of *ku70* did not affect the vegetative fungal growth or conidia development (see below), hyphal morphology (data not shown), or the virulence to citrus fruit (Fig 2). The sensitivity of Δ*ku70* strains to a battery of different compounds such as H_2O_2, calcofluor white (CFW), sodium dodecyl sulfate (SDS), sodium chloride (NaCl), sorbitol or fungicides was determined as described previously (Gandía et al. 2014), and no differences were found compared with the CECT20796 strain (data not shown). At this point, all data confirm that the *P. digitatum ku70* mutants are phenotypically undistinguishable from the parental wild-type strain.

3.2. Inactivation of ku70 improved HR and decreased ectopic integration

The effect of *ku70* disruption on HR frequency was studied by disrupting the class II (*chsII*) or class V (*chsV*) chitin synthase genes by ATMT of PDMG314 (Δ*ku70*) and CECT20796 as parental strains. Detailed characterization of the *PdchsII* and *PdchsV* mutants will be described elsewhere. The strategy was similar to that used for *ku70* and previously in other fungal genes, with derivatives of pGKO2 and

hygromycin (Hyg) as a positive selection marker (Gandía et al. 2014; Harries et al. 2015). Disruption transformants of *chsII* were identified with both parental strains. Up to 300 hygromycin resistant transformants were obtained from CECT20796, two of which were F2dU resistant and were confirmed by PCR as Δ*chsII* positives (HR frequency of 0.6 %) (Table 2). When we used PDMG314 as the parental strain, 70 hygromycin resistant transformants were obtained, and 29 of them were F2dU resistant; twelve of these were analysed by PCR and eight were confirmed to be disruption strains, demonstrating an increase of HR frequency from 0.6 % to ~11.4 %. In another set of experiments, we obtained only two Δ*chsV* positives using PDMG314 as the parental strain, but none with CECT20796 (Table 2). Therefore, our data confirmed an improvement of HR frequencies when *ku70* was inactivated.

Another experiment was designed to assess the function of NHEJ in CECT20796 and PDMG314 by determining the random ectopic integration of the hygromycin resistance cassette (*hph*) from the pBHt2 vector (Harries et al. 2015; Khang et al. 2006). A large number (>100) of hygromycin-resistant colonies were obtained with CECT20796 in each of the two Hyg-PDA plates used in the experiment. When PDMG314 was used as the parental strain, we obtained only 34 colonies in total in the two plates, confirming a lower frequency of NHEJ in Δ*ku70* strains.

3.3. The P. digitatum ku70 mutants showed altered growth and asexual development at increasing temperatures

P. digitatum is a fungus sensitive to high temperature and does not grow at 30 ºC, with the optimal growth temperature being 24-25 ºC. As part of the phenotypic characterization of Δ*ku70* strains, we investigated their growth at different temperatures (Fig 3). The results showed no differences with the parental strain at 24 or 26 ºC. At 28 ºC, the parental CECT20796 strain showed a small reduction of growth, but surprisingly growth of Δ*ku70* strains at 28 ºC was slower and much more reduced (Fig 3A and 3B). We measured the conidia production in each strain at each temperature. Parental CECT20796 did not show significant differences in conidia production per surface of colony at the three different temperatures including 28 ºC (Fig 3C). However, the Δ*ku70* strains showed a significant reduction in conidia

production even at 26 ºC (where no growth differences were observed) and a reduction of more than 80 % at 28 ºC (Fig 3C).

4. Discussion

The *ku70* disruption in *P. digitatum* resulted in a significant HR increase and NHEJ frequency decrease, therefore enhancing the efficiency of targeted knockout mutant generation but reducing ectopic non-targeted integration of foreign DNA. This genetic modification is neutral with regard to all tested phenotypic properties assayed at the optimal growth temperature for this fungus. These observations and conclusions are similar to those reported for *ku70*/*ku80* mutations in many other filamentous fungi including the previous Δ*ku80* strains in *P. digitatum* (Xu et al. 2014).

The Ku70/80 heterodimeric complex has important functions in the maintenance of genome integrity with implications in eukaryotic cell cycle control, response to DNA damage and stress, and senescence (Downs and Jackson 2004; Fell and Schild-Poulter 2015). The relevance of these functions is often overlooked with the extended use of *ku70/80* mutants in fungal genetics. We found a significant reduction of growth and conidia production of *P. digitatum* Δ*ku70* strains at higher than optimal temperatures. To our knowledge, very few previous studies report similar conclusions in filamentous fungi. A similar temperature dependent phenotype has been described only in the unicellular yeast *S. cerevisiae*, in which strains lacking *ku70* could grow at 23 ºC but were unable to develop at elevated temperatures that are optimal for this microorganism, due to defects in telomeric repeat maintenance (Gravel and Wellinger 2002). In filamentous fungi, it has been demonstrated that inactivation of *ku70* in the citrus leaf pathogen *Alternaria alternata* reduced conidial formation and production of pigments (Wang et al. 2011). These phenotypes were observed at the optimal growth temperature. In our study, the *P. digitatum* Δ*ku70* strains appeared as the wild type at the optimal temperature with very similar growth, conidia production, coloration or pigment content. Both *A. alternata* and *P. digitatum* Δ*ku70* strains were not affected in their pathogenicity to citrus, as is the case of the *P. digitatum* Δ*ku80* (Xu et al. 2014). Interestingly, the *A. alternata ku70* mutation had unexpectedly no effect on homologous integration and

therefore did not enhance gene disruption efficiency. It is important to note that Ku complex is essential for cell viability in the basidiomycete *Ustilago maydis*, where inactivation of Ku cause DNA and telomere defects (de Sena-Tomas et al. 2015). In another work, deletion of *ku70* affected fruiting body development in the agaricomycete *Coprinopsis cinerea* (Nakazawa et al. 2011). *P. digitatum* and *P. chrysogenum* are closely related fungal species (Marcet-Houben et al. 2012). *P. chrysogenum ku70* knockout strains generated by protoplast transformation using the same *nat1* marker used in our experiments were not reported to be different from the parental in relation to growth, conidia production, response to mutagens or oxidative stress; however, they exhibited higher sensitivity to osmotic stress and induction of stress-related genes (Hoff et al. 2010). This is not the case of our strains that were able to growth as the parental under continued osmotic stress (high concentration of NaCl) (data not shown). All these observations confirm important roles of the Ku complex in fungal development, maintenance of genomic integrity, and response to stress, which may present differences depending on the fungal species under study, and whose mechanistic causes will need further study.

The significance of our conclusions is illustrated by the yet unpublished characterization of our *P. digitatum chsV* mutants. In a previous work, we demonstrated that the disruption of the *chsVII* gene resulted in defects in cell wall, reduced growth and conidia production, alterations in hyphae morphology, reduced virulence and higher sensitivity to elevated temperatures (Gandía et al. 2014). Both ChsV and ChsVII chitin synthases contain a myosin motor-like domain (Gandía et al. 2012), and their absence in other fungi results in growth and morphology defects, altered cell wall and temperature sensitivity too (Kim et al. 2009; Larson et al. 2011; Liu et al. 2004; Muszkieta et al. 2014). The mutation of *chsV* on a *P. digitatum* Δ*ku70* genetic background (PDMG314) resulted in extreme temperature sensitivity (data not shown) that must be taken with caution because is likely a synergistic effect from the absence of both Ku70 and ChsV proteins.

To summarize, our study reinforces the perception that if Δ*ku70*/*ku80* are used as recipient strains for gene-targeting approaches, the involvement of target genes in temperature, conidia production and other stress-related and developmental functions must be characterized with care.

Acknowledgements: This work was funded by the following grants: BIO2012-34381 from the “Ministerio de Economía y Competitividad” (MINECO, Spain) and PROMETEOII/2014/027 from “Conselleria d’Educaciò” (Generalitat Valenciana, Comunitat Valenciana, Spain). We acknowledge Dr. Ulrich Kück (Ruhr-Universität Bochum, Bochum, Germany) for providing the pDNAT-FRT1,2 and pPTRII_PcFLP plasmids.

5. References

Choquer M, Robin G, Le Pêcheur P, Giraud C, Levis C, Viaud M, 2008. *Ku70* or *Ku80* deficiencies in the fungus *Botrytis cinerea* facilitate targeting of genes that are hard to knock out in a wild-type context. *FEMS Microbiology Letters* **289**: 225-232.

de Sena-Tomas C, Yu EY, Calzada A, Holloman WK, Lue NF, Perez-Martin J, 2015. Fungal Ku prevents permanent cell cycle arrest by suppressing DNA damage signaling at telomeres. *Nucleic Acids Research* **43**: 2138-2151.

Downs JA, Jackson SP, 2004. A means to a DNA end: the many roles of Ku. *Nature Reviews Molecular Cell Biology* **5**: 367-378.

Dudasova Z, Dudas A, Chovanec M, 2004. Non-homologous end-joining factors of *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* **28**: 581-601.

Fell VL, Schild-Poulter C, 2015. The Ku heterodimer: Function in DNA repair and beyond. *Mutation Research/Reviews in Mutation Research* **763**: 15-29.

Gandía M, Harries E, Marcos JF, 2012. Identification and characterization of chitin synthase genes in the postharvest citrus fruit pathogen *Penicillium digitatum*. *Fungal Biology* **116**: 654-664.

Gandía M, Harries E, Marcos JF, 2014. The myosin motor domain-containing chitin synthase PdChsVII is required for development, cell wall integrity and virulence in the citrus postharvest pathogen *Penicillium digitatum*. *Fungal Genetics and Biology* **67**: 58-70.

González-Candelas L, Alamar S, Sánchez-Torres P, Zacarías L, Marcos JF, 2010. A transcriptomic approach highlights induction of secondary metabolism in citrus fruit in response to *Penicillium digitatum* infection. *BMC Plant Biology* **10**: 194.

Gravel S, Wellinger RJ, 2002. Maintenance of double-stranded telomeric repeats as the critical determinant for cell viability in yeast cells lacking Ku. *Molecular and Cellular Biology* **22**: 2182-2193.

Harries E, Gandía M, Carmona L, Marcos JF, 2015. The *Penicillium digitatum* protein *O*–Mannosyltransferase Pmt2 is required for cell wall integrity, conidiogenesis,

virulence and sensitivity to the antifungal peptide PAF26. *Molecular Plant Pathology* **16**: 748-761.

Hoff B, Kamerewerd J, Sigl C, Zadra I, Kuck U, 2010. Homologous recombination in the antibiotic producer *Penicillium chrysogenum*: strain Δ*Pcku70* shows up-regulation of genes from the HOG pathway. *Applied Microbiology and Biotechnology* **85**: 1081-1094.

Khang CH, Park SY, Rho HS, Lee YH, Kang S, 2006. Filamentous Fungi (*Magnaporthe grisea* and *Fusarium oxysporum*). *Methods in Molecular Biology* **344**: 403-420.

Kim JE, Lee HJ, Lee JK, Kim KW, Yun SH, Shim WB, Lee YW, 2009. *Gibberella zeae* chitin synthase genes, GzCHS5 and GzCHS7, are required for hyphal growth, perithecia formation, and pathogenicity. *Current Genetics* **55**: 449-459.

Kopke K, Hoff B, Kück U, 2010. Application of the *Saccharomyces cerevisiae* FLP/FRT Recombination System in Filamentous Fungi for Marker Recycling and Construction of Knockout Strains Devoid of Heterologous Genes. *Applied and Environmental Microbiology* **76**: 4664-4674.

Larson TM, Kendra DF, Busman M, Brown DW, 2011. *Fusarium verticillioides* chitin synthases *CHS5* and *CHS7* are required for normal growth and pathogenicity. *Current Genetics* **57**: 177-189.

Liu H, Kauffman S, Becker JM, Szaniszlo PJ, 2004. *Wangiella (Exophiala) dermatitidis* WdChs5p, a Class V Chitin Synthase, Is Essential for Sustained Cell Growth at Temperature of Infection. *Eukaryotic Cell* **3**: 40-51.

López-Pérez M, Ballester AR, González-Candelas L, 2015. Identification and functional analysis of *Penicillium digitatum* genes putatively involved in virulence towards citrus fruit. *Molecular Plant Pathology* **16**: 262-275.

Marcet-Houben M, Ballester AR, de la Fuente B, Harries E, Marcos JF, González-Candelas L, Gabaldón T, 2012. Genome sequence of the necrotrophic fungus *Penicillium digitatum*, the main postharvest pathogen of citrus. *BMC Genomics* **13**: 646.

Meyer V, Arentshorst M, El-Ghezal A, Drews AC, Kooistra R, van den Hondel CAMJJ, Ram AFJ, 2007. Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. *Journal of Biotechnology* **128**: 770-775.

Muszkieta L, Aimanianda V, Mellado E, Gribaldo S, Alcàzar-Fuoli L, Szewczyk E, Prevost MC, Latgé JP, 2014. Deciphering the role of the chitin synthase families 1 and 2 in the in vivo and in vitro growth of *Aspergillus fumigatus* by multiple gene targeting deletion. *Cellular Microbiology* **16**: 1784-1805.

Nakazawa T, Ando Y, Kitaaki K, Nakahori K, Kamada T, 2011. Efficient gene targeting in Δ*Cc.ku70* or Δ*Cc.lig4* mutants of the agaricomycete *Coprinopsis cinerea*. *Fungal Genetics and Biology* **48**: 939-946.

Ninomiya Y, Suzuki K, Ishii C, Inoue H, 2004. Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. *Proceedings of the National Academy of Sciences of the USA* **101**: 12248-12253.

Snoek ISI, van der Krogt ZA, Touw H, Kerkman R, Pronk JT, Bovenberg RAL, van den Berg MA, Daran JM, 2009. Construction of an *hdfA Penicillium chrysogenum* strain impaired in non-homologous end-joining and analysis of its potential for functional analysis studies. *Fungal Genetics and Biology* **46**: 418-426.

Sun X, Ruan R, Lin L, Zhu C, Zhang T, Wang M, Li H, Yu D, 2013. Genomewide investigation into DNA elements and ABC transporters involved in imazalil resistance in *Penicillium digitatum*. *FEMS Microbiology Letters* **348**: 11-18.

Sun X, Wang J, Feng D, Ma Z, Li H, 2011. *PdCYP51B*, a new putative sterol 14α-demethylase gene of *Penicillium digitatum* involved in resistance to imazalil and other fungicides inhibiting ergosterol synthesis. *Applied Microbiology and Biotechnology* **91**: 1107-1119.

Takahashi T, Masuda T, Koyama Y, 2006. Enhanced gene targeting frequency in *ku70* and *ku80* disruption mutants of *Aspergillus sojae* and *Aspergillus oryzae*. *Molecular Genetics and Genomics* **275**: 460-470.

van Attikum H, Bundock P, Hooykaas PJJ, 2001. Non-homologous end-joining proteins are required for *Agrobacterium* T-DNA integration. *Embo Journal* **20**: 6550-6558.

Wang JY, Li HY, 2008. *Agrobacterium tumefaciens*-mediated genetic transformation of the phytopathogenic fungus *Penicillium digitatum*. *Journal of Zhejiang University-Science B* **9**: 823-828.

Wang M, Chen C, Zhu C, Sun X, Ruan R, Li H, 2014. *Os2* MAP kinase-mediated osmostress tolerance in *Penicillium digitatum* is associated with its positive regulation on glycerol synthesis and negative regulation on ergosterol synthesis. *Microbiological Research* **169**: 511-521.

Wang M, Sun X, Zhu C, Xu Q, Ruan R, Yu D, Li H, 2015. *PdbrlA*, *PdabaA* and *PdwetA* control distinct stages of conidiogenesis in *Penicillium digitatum*. *Research in Microbiology* **166**: 56-65.

Wang NY, Yang SL, Lin CH, Chung KR, 2011. Gene inactivation in the citrus pathogenic fungus *Alternaria alternata* defect at the *Ku70* locus associated with non-homologous end joining. *World Journal of Microbiology and Biotechnology* **27**: 1817-1826.

Xu Q, Zhu CY, Wang MS, Sun XP, Li HY, 2014. Improvement of a gene targeting system for genetic manipulation in *Penicillium digitatum*. *Journal of Zhejiang University-Science B* **15**: 116-124.

Zhang J, Mao Z, Xue W, Li Y, Tang G, Wang A, Zhang Y, Wang H, 2011. *Ku80* gene is related to non-homologous end-joining and genome stability in *Aspergillus niger*. *Current Microbiology* **62**: 1342-1346.

Zhang T, Sun X, Xu Q, Candelas LG, Li H, 2013a. The pH signaling transcription factor PacC is required for full virulence in *Penicillium digitatum*. *Applied Microbiology and Biotechnology* **97**: 9087-9098.

Zhang T, Sun X, Xu Q, Zhu C, Li Q, Li H, 2013b. *PdSNF1,* a sucrose non-fermenting protein kinase gene, is required for *Penicillium digitatum* conidiation and virulence. *Applied Microbiology and Biotechnology* **97**: 5433-5445.

Zhang TY, Xu Q, Sun XP, Li HY, 2013c. The calcineurin-responsive transcription factor Crz1 is required for conidation, full virulence and DMI resistance in *Penicillium digitatum*. *Microbiological Research* **168**: 211-222.

Zhu C, Wang W, Wang M, Ruan R, Sun X, He M, Mao C, Li H, 2015. Deletion of *PdMit1*, a homolog of yeast *Csg1*, affects growth and Ca2+ sensitivity of the

fungus *Penicillium digitatum*, but does not alter virulence. *Research in Microbiology* **166**: 143-152.

Figure Captions

Fig 1 - Construction of *P. digitatum* Δ*ku70* disruption strains and confirmation by PCR analysis. (A) Schematic representation of *ku70* in the parental strain CECT20796 and the Δ*Pdku70* disrupted gene. All primers used for PCR analysis are located in the figure. **(B)** PCR amplification of genomic DNA of the different *P. digitatum* strains with different primer pairs as indicated. Primers 395/396 (top panel) amplified a 1.8 kb fragment from the 5´region of *ku70* in the parental (CECT20796) and ectopic (PDMG324) strains but not in the positive Δ*ku70* mutants (PDMG301 to PDMG338). Conversely, these positive Δ*ku70* mutants showed a 2.0 kb amplicon with primers 395/399 (bottom panel). The PCR amplifications shown are representatives of all primer combinations tested, and all of them confirmed the structure of the disrupted Δ*ku70* locus.

Fig 2 - Infection assays of *P. digitatum* strains on orange fruits. The bar graph shows the incidence of infection caused by parental CECT20796, ectopic PDGM324, and disruption strains PDMG301, PDMG314 and PDMG338, determined as the percentage of infected wounds (mean value ± SD) from 2 to 5 days post-inoculation (dpi). No significant differences at any dpi were found among the different strains tested (ANOVA test, $p<0.05$).

Fig 3 - Growth in solid medium and conidia production of *P. digitatum* strains. (A) Phenotypic morphology of parental CECT20796 and disruption strains PDMG301, PDMG314 and PDMG338 after 7 days of growth at 24, 26 or 28 ºC temperature as indicated. **(B)** Colony diameter from 3 to 7 days of growth at 26 ºC (continuous lines) or at 28 ºC (dashed lines) on PDA solid medium plates, of the same strains shown in (A) as indicated. Data are mean values of four biological replicates. Note that growth lines of CECT20796 and disruption strains at 26 ºC, are overlapped. **(C)** Conidia production per surface of colony at different temperatures of parental strain CECT20796 (black bars) and disruption mutants PDMG301 and PDMG314 (grey bars). Data are mean values ± SD of four biological replicates. The

different letters present in bars show statistically significant differences among samples (ANOVA test, $p<0.05$).

Tables

Table 1 – Primers used in this study

Name	Use	Sequence 5´-3´	Tm (ºC)	Rest. Sites	Gene
OJM383	F	CG**ACTAGT**CCACTTCCACAATTCATTGAGG	60	*Spe*I	*ku70*
OJM384	R	CCCTATAGTGAGTCGTATTACGGATCAGGC GATGGACGAGGC	60		*ku70*
OJM385	F	GTATTCTATAGTGTCACCTAAATGCCTGTG ATCCGTATCATTGGG	60		*ku70*
OJM386	R	CC**GTCGAC**GGAGTGCTACAGGTATTGTAC	60	*Sal* I	*ku70*
T7	F	GTAATACGACTCACTATAGGG	58		
SP6	R	CATTTAGGTGACACTATAGAATAC	58		
OJM395	F	ACGATTCCGCCACTTCCAC	60		*ku70*
OJM396	R	CCGTCGATGCATTTGTATCCG	60		*ku70*
OJM399	R	GGTCAGCCGGCGGTTCCAGC	60		*nat*

Complementary sequences are underlined and restriction sites are in bold.

Table 2 – Homologous recombination (HR) frequencies among CECT20796 and Δ*ku70* strains of*P. digitatum*

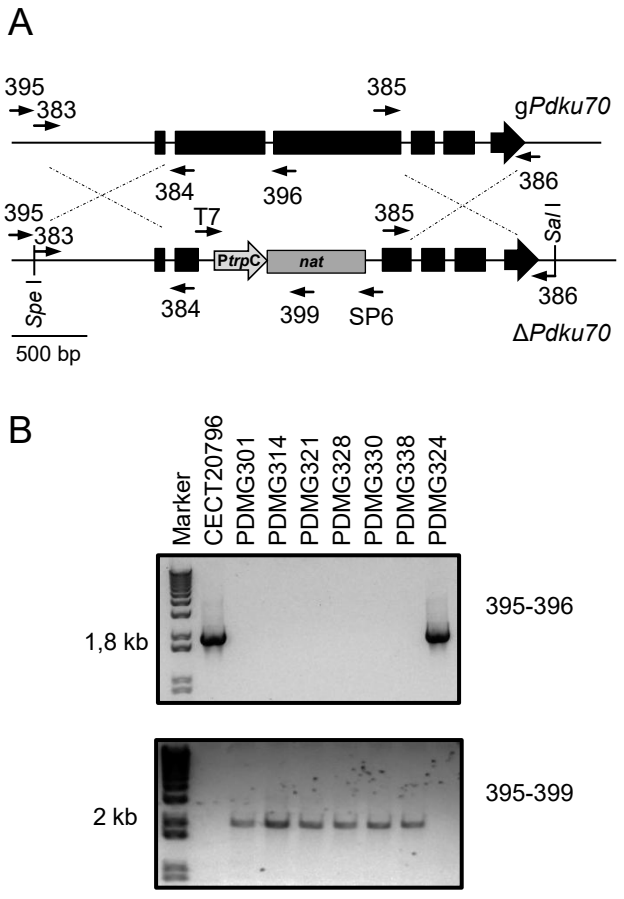
Parental strain	Gene deleted	No. of Hyg^Rcolonies	No. of $F2dU^R$colonies	No. positive transformants [a]	Frequency of HR (%) [b]
CECT20796	*PdchsII*	300	2	2 (2)	0.6
	PdchsV	110	1	0 (1)	-
PDMG314 (Δ*ku70*)	*PdchsII*	70	29	8 (12)	11.4
	PdchsV	30	4	2 (4)	6.6

[a] Number of positive HR transformants confirmed by molecular analyses. In parenthesis, the number of F2dU resistant transformants subjected to molecular analyses.

[b] HR frequency is the number of positive transformants divided by the number of Hyg^Rcolonies obtained.

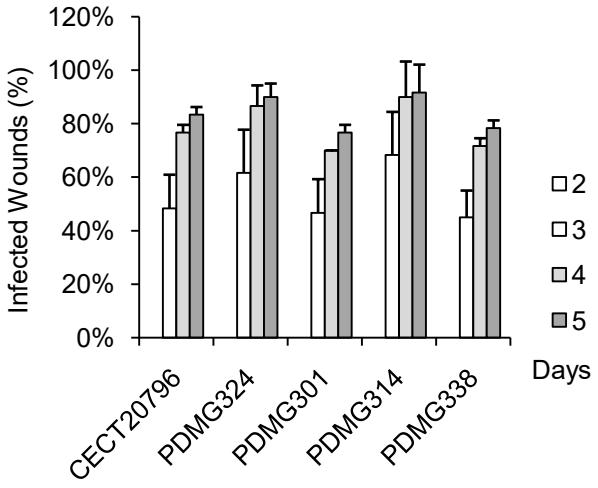
Figure(s)

Figure 1 (Gandía et al. 2015)



Figure(s)

Figure 2 (Gandía et al. 2015)



Figure(s)

Figure 3 (Gandía et al. 2015)