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The *Podosphaera fusca TUB2* gene, a molecular "Swiss Army knife" with multiple applications in powdery mildew research

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ABSTRACT

The powdery mildew fungus *Podosphaera fusca* (synonym *Podosphaera xanthii*) is the main causal agent of cucurbit powdery mildew and one of the most important limiting factors for cucurbit production worldwide. Despite the fungus' economic importance, very little is known about the physiological and molecular processes involved in *P. fusca* biology and pathogenesis. In this study, we isolated and characterised the β-tubulin-encoding gene of *P. fusca* (*PfTUB2*) to develop molecular tools with different applications in powdery mildew research. *PfTUB2* is predicted to encode a protein of 447 amino acid residues. The coding region is interrupted by six introns that occur at approximately the same positions as the introns present in other fungal *TUB2*-like genes. Once cloned, the *PfTUB2* sequence information was used in different applications. Our results showed that the *TUB2* gene is a good marker for molecular phylogenetics in powdery mildew fungi but it is unsuitable for the analysis of intraspecific diversity in *P. fusca*. The expression of *PfTUB2* was proven to be stable in different temperature conditions, supporting its use as a reference gene in quantitative gene expression studies. Furthermore, an allele-specific PCR assay for the detection of resistance to methyl-2-benzimidazole carbamate (MBC) fungicides in *P. fusca* was developed based on the correlation between the single amino acid change E198A in β-tubulin and the MBC resistance phenotype. Lastly, *PfTUB2* was used as a target gene in the development of a high-throughput method to quantify fungal growth in plant tissues.

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Introduction

Powdery mildew diseases remain among the most important plant pathological problems worldwide. The diseases' causal agents, powdery mildew fungi (*Erysiphales*), are likely the largest group of plant pathogens that remains largely uncharacterised from genetic and molecular perspectives. Their nature as obligate biotrophic parasites and consequent inability to grow on culture media have significantly hampered research into these parasites (Fernández-Ortuño *et al.* 2007). Fortunately, the advent of the new 'omic' technologies is progressively palliating this situation by contributing to the exponential increase in our current knowledge of model species such as *Blumeria graminis*, a powdery mildew that affects cereals (Zhang *et al.* 2005).

Numerous vegetable crops are susceptible to powdery mildew, but cucurbits are arguably the group that is most severely affected (Pérez-García *et al.* 2009). In Spain, as in many other countries around the world, *Podosphaera fusca* (synonym *Podosphaera xanthii*) is considered to be the main causal agent of powdery mildew in cucurbits and one of the most important limiting factors in cucurbit production (Del Pino *et al.* 2002; Fernández-Ortuño *et al.* 2006). However, despite the fungus' economic importance, very little is known about the physiological and molecular processes involved in *P. fusca* biology and pathogenesis (Pérez-García *et al.* 2009).

As elementary subunits of the microtubules, tubulin proteins represent major components of the cytoskeleton and eukaryotic flagella. In association with other proteins such as kinesins and dyneins, tubulins assemble the dynamic polymers of the microtubule which provide the molecular basis for chromosome segregation, cell division, the generation and maintenance of cell shape, intracellular transport and cell motility by ciliary/flagellar movement (Einax & Voigt 2003). Tubulins constitute a small family of globular proteins involving seven members (McKean *et al.* 2001). In a eukaryotic cell, the most abundant members of the tubulin family are α-tubulins and β-tubulins, the proteins that are the primary constituents of microtubules (Einax & Voigt 2003).

Mitosis and cell division are important targets of fungicidal action. In fact, the first systemic fungicides launched into the market were the mitosis and cell division inhibitors thiabendazole and benomyl. These fungicides are members of the benzimidazole family, which also includes carbendazim, fuberidazole, and thiophanate-methyl (Quaranta 2012). Benzimidazoles in their original state are not toxic to fungi in their original state, but rather must be converted into ester metabolites to become toxic. The active moiety of these compounds is methyl-2-benzimidazole carbamate (MBC), also known as carbendazim, which is why this class of compounds is also known as MBC fungicides (Ware & Whitacre 2004). These metabolites are thought to disrupt cell division by inhibiting microtubule assembly, as single-point mutations in the β-tubulin gene have been associated with benzimidazole resistance in several fungi (Albertini *et al.* 1999).

The tubulin genes, and especially the β-tubulin gene, have been particularly useful in several research applications. The β-tubulin gene is fairly conserved, with at least 60 % amino acid similarity between the most distantly related lineages (Juuti *et al.* 2005). One of those applications has been the clarification of evolutionary relationships at the kingdom, complex species groups and intraspecific levels by phylogenetic analyses (Baldhauf *et al.* 2000; Ayliffe *et al.* 2001; Edgcomb *et al.* 2001). Other applications of the β-tubulin genes have been their use as molecular targets in real-time PCR technologies for the accurate and reliable quantification of fungal DNA in environmental samples, including host tissues (Schena *et al.* 2004), or as reference genes in quantitative gene expression analysis in fungi (Hao-Zhi & Ruey-Fen 2006).

As mentioned above, despite of its economic importance as a phytosanitary problem, very little is known about *P. fusca* at the genetic and molecular levels. To develop specific genetic and molecular tools to assist in *P. fusca* research, the aim of this study was to isolate and characterise of the *P. fusca* β-tubulin gene *TUB2*. The sequence information was the starting point that allowed us to clarify evolutionary and intraspecific relationships and to design primers to facilitate both quantitative gene expression studies and rapidly detect MBC resistance in *P. fusca* populations. Lastly, a high-throughput quantification method for the pathogen in host tissues was developed, using *TUB2* as a target DNA.

Materials and methods

Fungal isolates and plant material

The *Podosphaera fusca* isolates used in this study are listed in Table 1. Isolates were grown on zucchini (*Cucurbita pepo*) cotyledons cv. Negro Belleza (Semillas Fitó), a cultivar that is very susceptible to powdery mildew, and maintained *in vitro* as previously described (Álvarez & Torés 1997). Plants were grown from seeds in a growth chamber at 25 °C under a 16 h photoperiod. Single-spores isolates were kept at −80 °C until use (Pérez-García *et al.* 2006).

Isolation of nucleic acids

Nucleic acids were isolated from fungal tissues and from infected leaf material. Conidia and mycelia were collected from heavily powdery mildew-infected cotyledons. These fungal materials were collected by removing the entire epiphytic fungal biomass, which was frozen in liquid nitrogen and stored at −80 °C until use. Total DNA was extracted using the CTAB method (Stewart & Via 1993), as modified by Robinson *et al.* (2002). Total RNA was extracted using TRI Reagent (Sigma–Aldrich). Infected leaf material was frozen in liquid nitrogen and stored at −80 °C until use. Total DNA was isolated from frozen zucchini cotyledons inoculated with *Podosphaera fusca* using the DNeasy Plant Mini Kit (Qiagen).

PCR-strategy to isolate the Podosphaera fusca TUB2 *gene*

First, the ascomycete specific primer pair Bt2a/Bt2b (Glass & Donaldson 1995) was used to amplify an initial fragment of the *P. fusca* β-tubulin-encoding gene (Table 2). A PCR assay of genomic DNA resulted in a 402-bp fragment that was directly sequenced. The DNA sequence of this fragment revealed a high identity with other known fungal β-tubulin proteins.

Table 1 – Isolates of *Podosphaera fusca* used in this study.

Isolates	Year of isolation	Location	Host	EC_{50}/MIC[a] (μg ml^{-1})	Amino acid at position 198[b]
SF48	1996	Malaga	Melon	<10/10	E
2086	1997	Greece	Melon	<10/10	E
SF60	1999	Greece	Zucchini	>1000/>1000	A
SF61	1999	Greece	Cucumber	<10/10	E
SF220	1999	Malaga	Melon	>1000/>1000	A
3167	2002	Murcia	Melon	>1000/>1000	A
81216	2002	Badajoz	Melon	<10/10	E
31426	2003	Murcia	Melon	>1000/>1000	A
31435	2003	Murcia	Melon	>1000/>1000	A
31469	2003	Murcia	Melon	>1000/>1000	A
45631	2003	Valencia	Pumpkin	>1000/>1000	A
45636	2003	Valencia	Pumpkin	>1000/>1000	A
81695	2003	Badajoz	Melon	<10/10	E
23785	2004	Almeria	Melon	>1000/>1000	A
31856	2004	Murcia	Melon	>1000/>1000	A
711012	2004	Ciudad Real	Melon	>1000/>1000	A
311128	2008	Murcia	Melon	<10/10	E

a Sensitivity to thiophanate-methyl.
b Presence of the typical E198A substitution at β-tubulin conferring resistance to MBC fungicides.

To amplify the flanking sequences of the *P. fusca TUB2* gene, a PCR method for walking into unknown flanking genomic DNA was used (Zhang & Gurr 2000). Briefly, nested sets of gene-specific primers were used to amplify the upstream and downstream sequences of the gene in combination with adaptor specific primers. PCR reactions were developed using the Advantage 2 Polymerase Mix (Clontech) and the cycling conditions were: three cycles at 94 °C for 2 s and 72 °C for 3 min; three cycles at 94 °C for 2 s and 70 °C for 3 min; three cycles at 94 °C for 2 s and 68 °C for 3 min; 26 cycles at 94 °C for 2 s, 66 °C for 20 s and 68 °C for 3 min, and a final cycle at 68 °C for 8 min. The PCR reactions resulted in PCR products of several fragment sizes that were purified and sequenced. Several rounds of PCR-walking extension in both directions were performed to isolate the entire *P. fusca TUB2* gene.

To determine the transcription start and termination sites and to verify the intron positions, total RNA was used to generate 5′ and 3′ rapid amplification of cDNA ends (RACE) products using the SMARTer™ RACE cDNA Amplification Kit (Clontech). The *P. fusca TUB2* gene was amplified from this adapter-ligated cDNA library using the primers BtubF and BtubR (Table 2), which were designed based on the 5′ and 3′ ends of the coding sequence. The Advantage 2 Polymerase Mix (Clontech) was used to amplify the cDNA fragments under the PCR conditions described by the manufacturer. After amplification, the PCR products were purified and used for direct sequencing.

Table 2 – Main primers used in this study.

Primer designation	Sequence (5′→3′)	Description
Target gene *P. fusca TUB2* (β-tubulin)		
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Primers used to amplify the first fragment of *PfTUB2*
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
BtubF	ATGCGTGAAATTGTTCATCT	Primers used to amplify *PfTUB2* cDNA and verify introns
BtubR	TTATTCTTCCGGTTGCATGGGTG	
IT1F	GGGCGCCAAGCCTTCACTCG	Primers used to amplify noncoding regions for intraspecific genetic diversity analysis
IR	AAAGGGACCAGCGCGAACAG	
Btub15F	TTCCCTGATCGAATGATGGCAACC	Primers used for quantitative gene expression analysis
Btub14R	CGTCGGAGTTTTCGACCAACTGATG	
E198F	CTAGTCGAAAACTCTGATGA	Set of primers developed for allele-specific PCR detection of the E198A mutation
A198F	CTAGTCGAAAACTCTGATGC	
A1981F	CTAGTCGAAAACTCTGATCC	
A1982F	CTAGTCGAAAACTCTGATTC	
A1983F	CTAGTCGAAAACTCTGATAC	
198R	CCGGTGTACCAATGCAAGAATG	
TubRT6F	CTGCACCTCGCGAAACTAAC	Primers used for molecular quantification of fungal growth
TubRT6R	CTACTAAACGCAGCGCAGTC	
Target gene *C. pepo ACT1* (actin)		
ActF	TGTCTGCAATACCAGGGAACAT	Plant primers used for molecular quantification of fungal growth
ActR	TGTGACGTAGATATCAGAAAGG	

Sequencing and sequence analysis

The PCR products were sequenced by Macrogen. The edited sequence data were subjected to NBCI BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) at both the nucleic acid and the amino acid levels for the identification of conserved domains. Potential open reading frames (ORFs), introns, and exons were predicted using FGENESH software from SoftBerry (http://linux1.softberry.com/berry.phtml). Amino acid alignments were performed with the CLC Main Workbench (Aarhus). Phylogenetic analyses were conducted using MEGA version 5 (Tamura *et al.* 2011).

Intraspecific genetic diversity analysis

To validate the usefulness of the *TUB2* gene as a marker for assessing intraspecific genetic diversity within populations of *Podosphaera fusca*, a 1379 bp fragment encompassing part of the promoter region and five of the six introns of the *TUB2* gene was amplified using the primer pair IT1F and IR (Table 2). PCR amplifications were conducted using genomic DNA as a template and proofreading *Pwo* SuperYield DNA Polymerase (Roche Diagnostics). The PCR amplification conditions consisted of an initial denaturation for 2 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min, and a final extension at 72 °C for 5 min. The PCR products were analysed by electrophoresis on 1 % agarose gels to verify the reactions and were subsequently sequenced.

Gene expression analysis

The expression of the *Podosphaera fusca TUB2* gene was analysed to validate the use of *TUB2* as an invariant endogenous control (reference gene) in quantitative gene expression studies. For each RNA extraction, 5 μg of total RNA was subjected to DNase treatment using DNase I FPLCpure (Amersham), according to the manufacturer's instructions. Reverse transcription reactions were performed in 20 μl volume using SuperScript III Reverse Transcriptase (Invitrogen) and Anchored Oligod(dT)$_{20}$ Primer (Invitrogen) following the manufacturer's recommendations. *Podosphaera fusca* cDNA was subjected to qPCR in a DNA Engine Opticon 2 Continuous Fluorescence Detector System (MJ Research) using the DNA stain SYBR Green JumpStart™ Taq ReadyMix™ (Invitrogen) and the primer pair Btub15F and Btub14R (Table 2). The PCR cycling conditions were as follows: 2 min of an initial denaturation step at 94 °C followed by 40 cycles that each consisted of 94 °C for 15 s, 67 °C for 45 s, and 72 °C for 1 min and a final extension step at 72 °C for 10 min. After amplifications, the data were analysed using the Opticon Monitor analysis software version 2.02.24 (MJ Research).

Fungicide sensitivity assay

A commercial formulation of the MBC fungicide thiophanate-methyl (Pelt, Nippon Soda) was used. Fungicide stock dispersions (10 mg ml^{-1}) were prepared as previously described (Fernández-Ortuño *et al.* 2006). Prior to each assay, stock solutions were diluted in sterile deionised water to yield solutions with the desired final concentrations ranging from 10 to 1000 μg ml^{-1}. For fungicide sensitivity testing, a leaf disc assay was conducted as previously reported (Fernández-Ortuño *et al.* 2006). Briefly, leaf discs obtained from cotyledons of 8-day-old zucchini plants were placed upside down in Petri dishes containing sterile filter paper imbibed with 3 ml of the corresponding fungicide dispersion. After 24 h of incubation, the discs were transferred onto sterile filter paper imbibed with sterile water deposited on agarised medium and inoculated on their upper side with conidia of the selected *Podosphaera fusca* isolate using a soft paintbrush or eyelash. After 8 d of incubation, powdery mildew growth on each leaf disc was recorded according to the previously described scale of values (Fernández-Ortuño *et al.* 2006). Minimal inhibitory concentrations (MIC), the fungicide concentrations inhibiting 50 % of growth (EC_{50}) and resistance factors (RF) were then calculated (Fernández-Ortuño *et al.* 2006). RF was calculated for resistant populations by dividing the mean of EC_{50} values of the resistant isolates by the mean of EC_{50} values of sensitive population.

Allele-specific PCR assay

To develop a rapid diagnostic system to molecularly detect MBC resistance in *Podosphaera fusca*, a set of allele-specific primers was designed for the detection of the point mutation responsible for resistance (Table 2). Based on a single change from A to C at the codon position 198, the forward primers E198F and A198F were designed to match the A and the C nucleotides at the 3′-end in MBC-sensitive (MBC^S) and MBC-resistant (MBC^R) isolates, respectively. To improve the specificity of this method, an artificial mismatch base at the second nucleotide at the 3′-end of the primer A198F was introduced, resulting in the primers A1981F, A1982F, and A1983F. As a reverse primer, the 198R primer, designed based on the seventh exon of the *P. fusca TUB2* gene, was used. PCRs were developed with GoTaq Flexi DNA Polymerase (Promega), and the amplification conditions consisted in an initial denaturation for 2 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR products were analysed by electrophoresis on 1 % agarose gels in Tris-acetate-EDTA (TAE) buffer to verify the reactions.

Disease severity

Disease symptoms were estimated by percentage quantification of the leaf area covered with powdery mildew by an image analysis of pictures of powdery mildew-infected zucchini cotyledons. The image analysis software Visilog 4.1 (Noesis) was used according to Romero *et al.* (2003).

Molecular quantification of fungal growth

The *Podosphaera fusca TUB2* gene was also used as a molecular target in the quantification of fungal growth in host tissues by real-time PCR technology. Total DNA was isolated from infected leaf material as indicated above. Quantitative real-time PCR was conducted using a MiniOpticon™ Real-Time

PCR Detection System (Bio-Rad). Amplifications were set up in 20 µl volumes containing 1×iQ™SYBR® Green Supermix (Bio-Rad). Two different set of primers were used: TubRT6F/TubRT6R to amplify *PfTUB2* and ActF/ActR to amplify the plant actin gene *CpACT1* (Table 2). Real-time PCR reactions were performed using the following programme: 2 min 50 °C, 5 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 55 °C. A melting curve analysis was performed at the end of the PCR run over the range of 60–95 °C. The temperature was increased in a stepwise fashion by 0.5 °C for every 10 s dwell time to confirm the amplification of single amplicons.

A standard curve based on threshold cycles (C_t) for a 10-fold dilution series of fungal genomic DNA was constructed. C_t values were calculated using the Bio-Rad CFX Manager software V1.1 (Bio-Rad) to identify significant fluorescence signals above background during the early cycles of the exponentially growth phase of the PCR amplification process. A standard curve was obtained by plotting the C_t value against the logarithm of the concentration of each dilution series of fungal genomic DNA. The gene copy number was calculated using the equation proposed by Whelan *et al.* (2003). The resulting *PfTUB2* and *CpACT1* gene copy numbers were used to calculate the ratio *PfTUB2/CpACT1* to express the fungal content.

Data analysis

The expression stability of *PfTUB2* as a candidate reference gene was evaluated using the Microsoft Excel-based statistical tool BestKeeper (Pfaffl *et al.* 2004).

In order to compare the methods to assess powdery mildew infection, at the final time point, the standardised area of the disease curve progression over time (AUDPC) was calculated (Campbell & Madden 1990). Data obtained by each method were previously normalised to the time point that showed the highest value and that was considered as the 100 %, and the AUDPC values were then calculated. AUDPC data were statistically analysed by Student's *t*-test ($p = 0.05$) using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Isolation and sequence analysis of Podosphaera fusca TUB2 *gene*

To isolate the *P. fusca TUB2* gene, a PCR-based strategy was followed. Initially, a fragment of 402 bp, with high identity to β-tubulin proteins, was amplified. The sequence of this fragment was the starting point for the isolation of the complete gene by a genome-walking approach. In total, a 5.2 Kb fragment encompassing the *P. fusca TUB2* locus was isolated. DNA sequence analysis revealed that this fragment comprises 2.4 Kb upstream of the predicted ATG and 1.3 Kb of downstream noncoding sequence (Fig 1). The ORF of the *P. fusca TUB2* gene is 1657 bp long and is predicted to encode a 447 amino-acid protein with a theoretical molecular mass of 49.8 KDa. The coding region is interrupted by six introns that occur at approximately the same positions as the introns conserved among fungal β-tubulin genes and contains consensus splicing junctions adhering to the GT-AG rule. The introns and their positions were confirmed by sequencing the cDNA generated by RT-PCR.

A comparison of the *P. fusca TUB2* protein with the β-tubulin proteins of other fungi revealed a high identity at the amino acid level (Fig 2). The highly conserved glycine-rich peptide GGGTGAG, which is known to be part of the GTP-binding site, is present at amino acid positions 140–146. The KGHYTEG peptide, also implicated in the GTP-dependent assembly, is present at residues 103–109. This protein also contains the tetrapeptide MREI at the N-terminal region involved in the autoregulation of gene expression. At position 198 it is present the residue of glutamic acid changes to alanine and confers resistance to benzimidazole fungicides in many fungal pathogens.

The *P. fusca TUB2* gene promoter was also investigated for the occurrence of protein-binding motifs that may be related to the gene's regulation. Upstream of the putative start codon, sequence analysis revealed the presence of three TATA-like elements at positions -538, -1124, and -1819 and six CAAT-

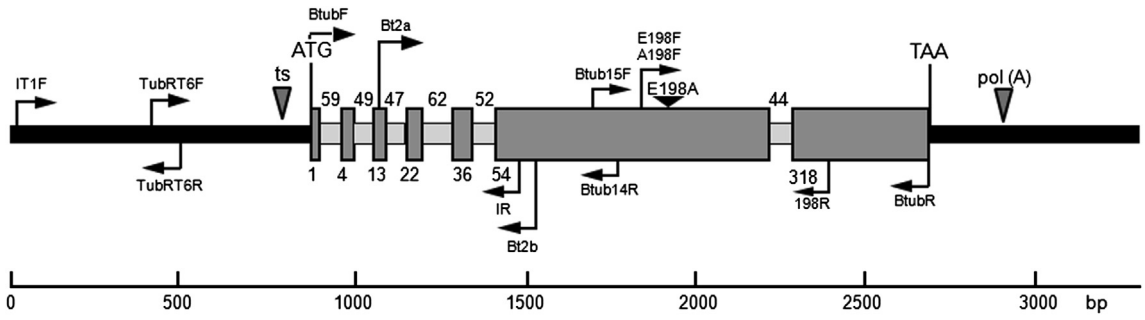

Fig 1 – Schematic representation of the cloned *P. fusca* β-tubulin gene (*PfTUB2*). The grey boxes represent coding regions, and the number below each exon refers to the amino acid position at the beginning of the exon. Introns are represented by soft grey boxes between exons. The number above each intron refers to the intron size in base pairs. The position of the longest 5′ transcript identified is labelled as 'ts'. The polyadenylation site is located downstream of the stop codon and is represented as 'pol(A)'. The line below the scheme acts as a reference for fragment size. The arrows indicate the sites of the most important PCR primers used in this study. The main mutation conferring resistance to MBC fungicides (E198A) is represented as an arrow head at the corresponding position over the gene scheme.

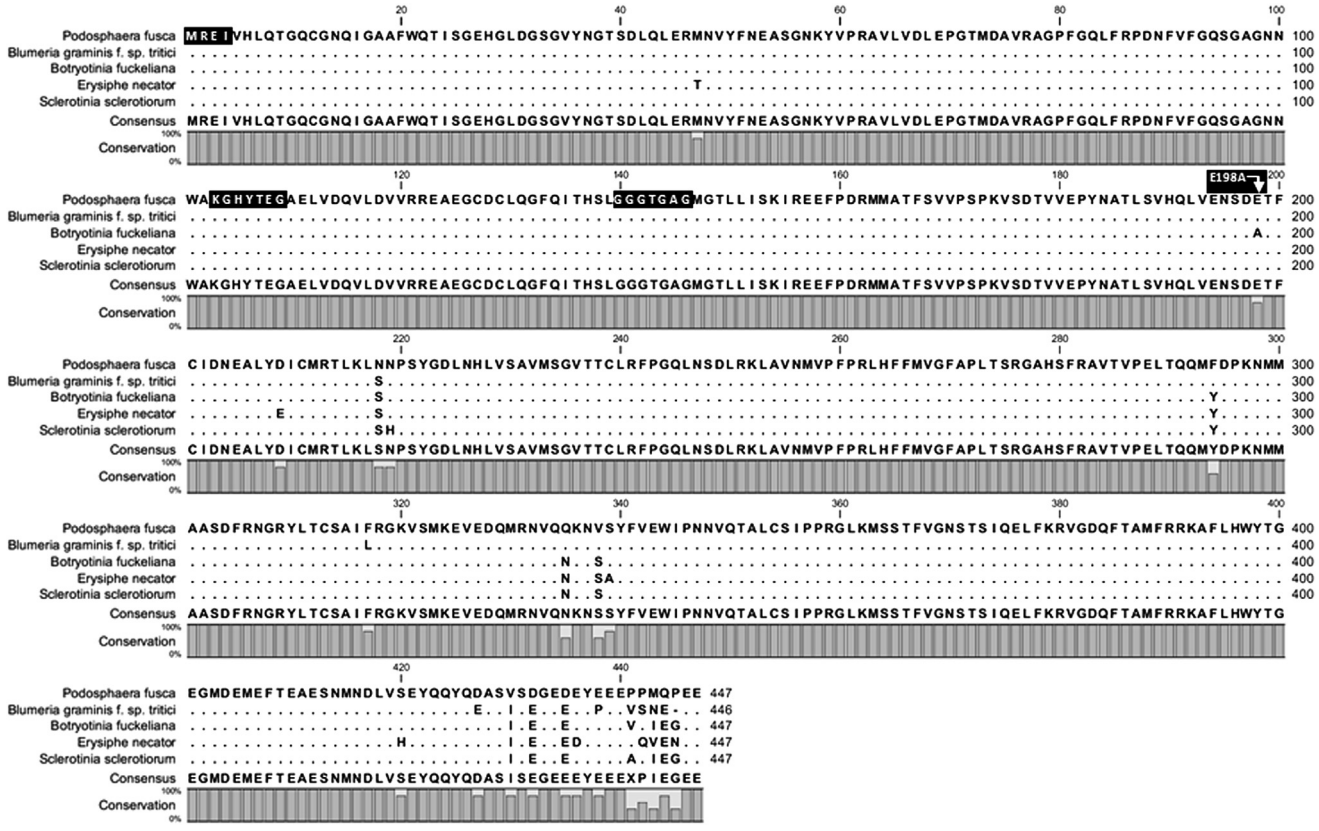

Fig 2 – Comparison of the complete amino acid sequence of β-tubulin from *P. fusca* with the sequences of β-tubulin proteins from other fungi. See Table 3 for details on accession numbers. For *P. fusca*, the amino acid sequence from accession no. AGH25506.1 corresponding to isolate 2086 was used. The numbers refer to amino acid positions in the β-tubulin protein sequence. The grey bars represent amino acid conservation. The amino acid alignment was obtained using CLC Main Workbench. Conserved motifs as well as the amino acid substitution responsible for resistance to MBC fungicides are highlighted in black boxes (see text for details).

box-related motifs at positions -178, -278, -308, -522, -654, and -694. Furthermore, the typical polyadenylation signal AATAAA was identified downstream of the putative stop codon at position +1912 (data not shown). This new gene was named *PfTUB2* and is deposited in GenBank under accession number KC333362.

Molecular phylogenetic analysis

A phylogenetic tree was constructed based on a multiple sequence alignment of various available β-tubulin sequences, thus allowing the relationships between different plant pathogenic fungi and oomycetes to be examined. Based on the β-tubulin sequence of different species of plant pathogens (Table 3), the sequence alignment analysis revealed the presence of three well-represented clusters in the neighbour-joining (NJ) phylogram (Fig 3). These clusters represented the three major taxa for which *TUB2* sequences were available: *Ascomycota*, *Basidiomycota*, and *Oomycota*. Within the ascomycetes, the three classes analysed, *Sordariomycetes*, *Dothideomycetes*, and *Leotiomycetes* were perfectly separated to form independent groups. However, within *Leotiomycetes* a discrepancy occurred, with the *Erysiphales* order branching away from the other two *Leotiomycetes* species used in the analysis. Within the *Erysiphales*, the *Blumeria*, and *Erysiphe/Golovinomyces* genera formed two clearly differentiated groups. *Podosphaera fusca* constituted an independent branch that was located closer to the group formed by the *Blumeria* species.

Intraspecific genetic diversity analysis

To determine the usefulness of the *TUB2* gene as a marker for assessing intraspecific genetic diversity in *Podosphaera fusca*, 17 isolates from different locations and years of isolation were selected from previous studies (Table 1). For sequence variation, fragments of 1379 bp from the 5′ end of the *TUB2* gene, including part of the promoter region and 269 bp corresponding to five of the six introns present in *PfTUB2*, were amplified with the primer pair ITF1/IR. Replicated sequencing of the forward and reverse strands (four sequences per isolate in total) was performed. Sequence analysis revealed the complete absence of genetic variation among the isolates. No polymorphism was detected within the sequenced fragments (data not shown).

Table 3 – List of sequences used in this study.

Species	GenBank accession no.	
	Nucleotide id.	Protein id.
Ascomycetes		
Blumeria graminis f. sp. *avenae*	AJ313153	
Blumeria graminis f. sp. *hordei*	AJ313149	
Blumeria graminis f. sp. *secalis*	AJ313154	
Blumeria graminis f. sp. *tritici*	AJ313150	CAC85614.1
Botryotinia fuckeliana	Z69263	CAA93254.1
Erysiphe necator	AY074934	AAM23017.1
Erysiphe pisi	X81961	
Fusarium oxysporum	EF450110	
Golovinomyces orontii	Go_V1_Contig9010	
Magnaporthe oryzae	XM_003718381	
Mycosphaerella graminicola	AY847870	
Podosphaera fusca	KC333362	AGH25506.1
Pyrenophora tritici	JQ314403	
Sclerotinia sclerotiorum	XM_001594794	XP_001594844.1
Verticillium dahliae	DQ166865	
Basidiomycetes		
Puccinia graminis f. sp. *tritici*	XM_003889743	
Uromyces appendiculatus	DQ983225	
Oomycetes		
Phytophthora infestans	XM_002908737	
Plasmopara viticola	DQ361159	

Expression analysis

In quantitative gene expression analysis, a reference gene for sample normalisation is required to determine target gene expression changes among experimental treatment groups. Typical 'housekeeping' genes, such as β-tubulin genes, are among the most commonly used normalisers. For a reference gene it is critical to be stably expressed also under various experimental settings. To validate the use of *Podosphaera fusca TUB2* as a normaliser, the expression of *PfTUB2* was studied at two different temperature conditions. The isolates of *P. fusca* 2086, SF60, 31426, 31856, and 311128 (Table 1) were grown on zucchini cotyledons maintained *in vitro* at two different temperatures, 20 °C and 25 °C. After 15 d of incubation, RNA was extracted and samples were adjusted to identical RNA concentrations. C_t values were then calculated for each isolate and temperature (data not shown), and stability in gene expression was analysed using BestKeeper. The BestKeeper tool expresses variation in gene expression as the standard deviation (SD) and coefficient of variance (CV) of the derived C_t values of the reference candidate gene. A low SD should be expected for most stably expressed genes. *PfTUB2* showed an SD of 0.64, which represents an acceptable 1.56-fold change in expression (Table 4). In conclusion, stability in gene expression was demonstrated, indicating that the expression of *PfTUB2* is consistent when equal amounts of starting template are used.

Detection of E198A mutation by allele-specific PCR

As indicated above, β-tubulins are the targets of MBC fungicides, and single-point mutations in their coding genes have been associated with MBC resistance in several phytopathogenic fungi. Because the MBC fungicide thiophanate-methyl is authorised in Spain for use against powdery mildew in cucurbits, in this work, we aimed to establish the role of mutations in the *TUB2* gene of *Podosphaera fusca* in resistance to MBC fungicides and to design an allele-specific PCR method to both detect these mutations and facilitate *P. fusca* MBC resistance monitoring. For this purpose, a set of 17 isolates from our laboratory collection was examined for thiophanate-methyl sensitivity using a leaf disc assay, and MIC and EC_{50} values were determined (Table 1). Two different groups of isolates were clearly identified. The first group was

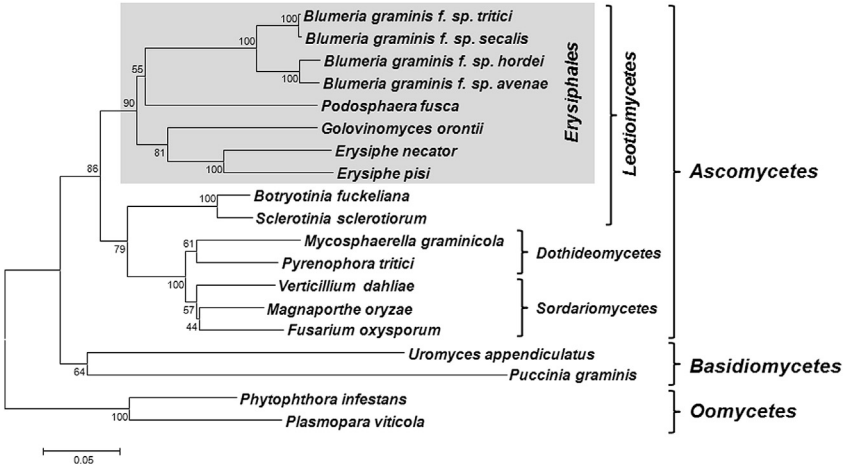

Fig 3 – Phylogenetic relatedness of plant pathogenic ascomycetes, basidiomycetes, and oomycetes based on the nucleotide sequences of the *TUB2* gene. The tree was constructed using NJ analysis. See Table 3 for details on accession numbers. For *P. fusca* the nucleotide sequence from accession no. KC333362 was used. Bootstrap values from 1000 replications are reported in the branches. Branch lengths are scaled in terms of evolutionary distances. The bar represents 0.05 substitutions per nucleotide.

Table 4 – BestKeeper analysis of *PfTUB2* gene expression.

BestKeeper parameters	*PfTUB2*
Number of samples	10[a]
Geometric mean (C_t)	19.17
Arithmetic mean (C_t)	19.18
Minimum (C_t)	18.38
Maximum (C_t)	20.35
SD [±C_t]	0.64[b]
CV [%C_t]	3.36
Minimum [×-fold]	−1.73
Maximum [×-fold]	2.26
SD [±×-fold]	1.56

a Expression was studied in five isolates of *P. fusca* that were grown at two temperature conditions, 20 °C and 25 °C.
b A candidate gene is considered suitable as a reference gene when the SD value is not higher than 1.0.

composed of six isolates that showed MIC and EC_{50} values of 10 μg ml^{-1} and <10 μg ml^{-1}, respectively. The second group consisted of 11 isolates showed MIC and EC_{50} values estimated as >1000 μg ml^{-1} in all cases. Based on these data and the maximum field application rate recommended by the manufacturer (675 g μml^{-1}), these 11 isolates were considered to be resistant to thiophanate-methyl, and their RFs were estimated to be >100.

Fragments encompassing the entire coding sequence of the *PfTUB2* gene were sequenced from the 17 isolates examined. As anticipated, the typical amino acid substitution E198A, which confers resistance to MBC fungicides in many fungal pathogens, was observed in all of the 11 thiophanate-methyl resistant isolates of *P. fusca* that were sequenced (Table 1). Based on these sequence data (Fig 4A), an allele-specific PCR method was used to detect this mutation. A set of specific primers were developed for this purpose. The primer pair E198F/198R was used to amplify a fragment of 670 bp, specifically from the MBC^S isolate 2086 (Fig 4B). No

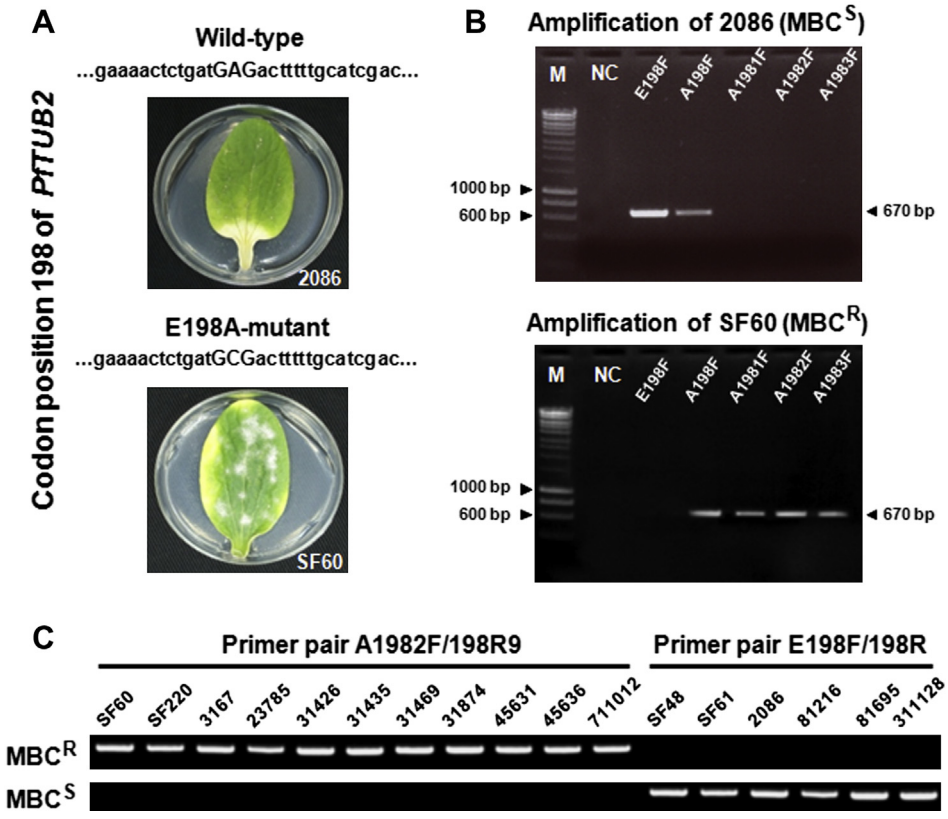

Fig 4 – Evaluation of mismatched allele-specific primers to discriminate between MBC-resistant (MBC^R) and MBC-sensitive (MBC^S) alleles in *P. fusca*. Details of the primers are given in Table 2. (A) A single point mutation at codon 198 of *PfTUB2* results in the E198A amino acid substitution, which confers resistance to MBC fungicides. Cotyledons were spread with thiophanate-methyl (500 μg ml^{-1}) to allow growth of the resistant isolate only. (B) Evaluation of mismatched allele-specific primers. Total DNA from MBC^S isolate 2086 (top panel) and from MBC^R isolate SF60 (bottom panel) was used as a template. 'M' indicates the marker lanes. 'NC' indicates the negative-control lanes. Rest of the lanes are labelled with the forward primer used. The reverse primer 198R was used in each amplification reaction. (C) Molecular detection of MBC resistance in *P. fusca*. The presence or absence of the E198A mutation was detected by allele-specific PCR in a collection of isolates that were resistant or sensitive to MBC. The isolate names are shown above the respective lanes. For MBC^R isolates, amplification was only achieved with the forward primer A1982F, whereas MBC^S isolates were detected only with the forward primer E198F. In all cases, the reverse primer 198R was used.

amplification was observed when DNA from the MBC^R isolate SF60 was used as a template, indicating that this primer pair was specific enough to detect sensitive isolates. When using the primer pair A198F/198R, in principle to detect only resistant isolates, fragments of 670 bp were obtained from MBC^R and MBC^S isolates (Fig 4B). To improve the specificity of the primer, an artificial mismatch base at the second nucleotide at the 3′ end of the primer A198F was introduced, resulting in the primers A1981F, A1982F, and A1983F. When using these primers, PCR products were obtained only from MBC^R isolates (Fig 4B). The primer A1982F was selected and tested, together with E198F, on the collection of isolates to verify specificity. As expected, MBC^R isolate amplification was obtained only with the primer combination A1982F/198R, whereas sensitive isolates were amplified only with the primer pair E198F/198R (Fig 4C). Therefore, the primer pairs E198F/198R and A1982F/198R were shown to be specific for detecting MBC^S and MBC^R phenotypes in *P. fusca*.

Molecular quantification of Podosphaera fusca *growth*

Powdery mildew symptoms are usually evaluated by the quantification of the leaf area covered by powdery mildew. Fungal biomass, however, is addressed by performing conidial counts under a microscope, which is very tedious. In this work, the *PfTUB2* gene was used as a molecular target to develop a rapid and accurate method for the molecular estimation of *P. fusca* growth. The method was validated in an experiment that assessed the growth of two *P. fusca* isolates in the presence of the MBC fungicide thiophanate-methyl in comparison with the typical method of estimating disease symptoms by image analysis. In the presence of the fungicide, the growth of the MBC^R isolate SF60 started to be visualised 5 d after inoculation, when small white dots of fungal mycelia spread onto the zucchini cotyledons were observed (Fig 5A). As the incubation proceeded, fungal growth progressively increased, resulting in great coverage of the leaf surface with

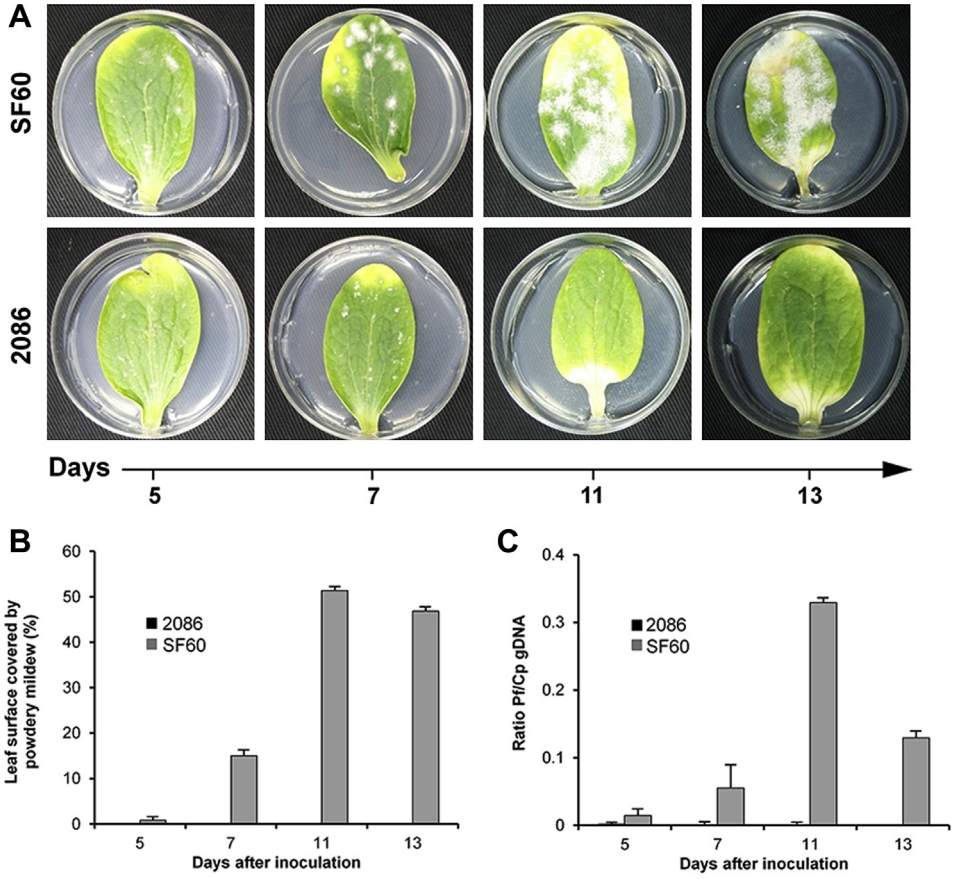

Fig 5 – Powdery mildew disease progression caused by *P. fusca* on zucchini cotyledons and evaluation of a molecular method for the quantification of fungal growth based on *PfTUB2*. (A) Development of two different isolates of *P. fusca*, SF60 (MBC^R) and 2086 (MBC^S), on zucchini cotyledons spread with thiophanate-methyl (500 μg ml^{-1}). Pictures were taken at the indicated time points. (B) Quantification of *P. fusca* growth on zucchini cotyledons treated with thiophanate-methyl by image analysis. Disease severity was expressed as the percentage of the leaf surface covered by powdery mildew as estimated by the image analysis software Visilog 4.1. The development of isolate 2086 could not be recorded due to its limited growth. The data represent the means of three independent experiments, and the bars show the SDs. A set of five cotyledons was analysed per time point. (C) qPCR analysis of *P. fusca* growth on zucchini cotyledons treated with thiophanate-methyl. Ratios of *P. fusca* to zucchini (*C. pepo*) gDNA were determined by qPCR with the primers TubRT6F/TubRT6R and ActF/ActR, respectively. Bars represent the mean ± SD of three DNA samples (each derived from five pooled cotyledons) with three technical replicates each. A statistical analysis of these data is shown in Table 5.

typical powdery mildew growth 13 d after inoculation. In contrast, no disease symptoms were visible when the fungicide-treated cotyledons were inoculated with the MBC^S isolate 2086.

Disease symptoms were estimated by image analysis (Fig 5B) and, in parallel, fungal growth was quantified by qPCR using *PfTBU2* as the target gene (Fig 5C). A standard curve was constructed using different concentrations of *P. fusca* genomic DNA, ranging from 1×10^2 to 1×10^{-2} ng μl^{-1}. Quantification showed a linear relationship ($r^2 = 0.996$) between log values of fungal genomic DNA and qPCR C_t over the range of examined DNA concentrations examined (data not shown). The results obtained by both methods were very similar, but the molecular method was more accurate. Indeed, the qPCR method revealed that fungal growth estimated by the *Pf/Cp* ratio gradually increased until the fungicide-resistant isolate covered most of the cotyledon surface, as shown for powdery mildew symptoms estimated by image analysis. However, in the case of the sensitive isolate, although no symptoms were observed on zucchini cotyledons, weak signals were detected by qPCR, confirming that no fungal growth occurred in the presence of fungicide. These differences in sensibility were corroborated by the statistical analysis of AUDPC data. As shown in Table 5, after analysis no statistically significant differences were found between the methods when infection by isolate SF60 resistant to thiophanate-methyl was evaluated. However, when infection by the sensitive isolate 2086 in the presence of fungicide was evaluated, comparison between the methods could not be established due to the lower resolution of the image analysis technique.

Discussion

In this study, we have isolated the β-tubulin gene from *Podosphaera fusca*, the main causal agent of cucurbit powdery mildew (Pérez-García *et al.* 2009). The cloning strategy, which was the genome-walking technique (Zhang & Gurr 2000), resulted in the isolation of 5.2 Kb of coding and noncoding flanking sequences of the *PfTUB2* locus. From this species, only two complete genes have been isolated to date with both isolations using this approach: *CYP51* (López-Ruiz 2009) and *TUB2*. Although the genomes of powdery mildew fungi are littered with retrotransposable elements (Spanu *et al.* 2010), this PCR-based technique offers a good alternative for the isolation of genes from species with complex genomes such as powdery mildew fungi, when gene libraries or genome sequences are unavailable.

The β-tubulin proteins are essential components of cells, and similar to other 'housekeeping' genes, the β-tubulin genes are highly conserved. As expected, the predicted protein of the *PfTUB2* gene was nearly identical to the deduced proteins of *Blumeria graminis* f. sp. *hordei*, *Botryotinia* fuckeliana, *Erysiphe necator*, and *Magnaporthe grisea* (Dean *et al.* 2005; Amrani & Corio-Costet 2006; Rowe & Kliebenstein 2007) and exhibited the typical domains of β-tubulins. These domains include the highly conserved glycine-rich domain GGGTGAG, which is known to be part of the GTP-binding site (Nogales *et al.* 1998); the peptide KGHYTEG, also implicated in the GTP-dependent assembly (Burns & Farrell 1996); and an N-terminal tetrapeptide involved in the autoregulation of gene expression (Bachurski *et al.* 1994). In addition, most of the amino acid changes were concentrated at the C-terminus of the predicted protein as observed in other fungal β-tubulin genes. The highly variable carboxyl terminal, which was rich in glutamic acid residues, is important for binding of the microtubule-associated proteins MAP2 and MAPT (Cross *et al.* 1994).

Regarding noncoding sequences, several TATA-like elements and CAAT-box-related motifs were identified in the promoter region of the *PfTUB2* gene. TATA boxes were found to be strictly conserved and are essential for transcription initiation from all protein-coding genes, from yeast to man (Smale & Kadonaga 2003). The CAAT motifs are found in the promoters of many eukaryotic genes and play a pivotal role in determining the efficiency of a promoter (Msiska & Morton 2009). Furthermore, the consensus sequence AATAAA, which is thought to be involved in the 3′-end formation and polyadenylation of the precursor mRNA (Msiska & Morton 2009), was also found downstream of the putative stop codon.

Apart from the basic structural functions of β-tubulins, the β-tubulin genes have been particularly useful as molecular tools for different research purposes, such as phylogenetic, population genetics and gene expression analyses. The aim of this study was to clone the *PfTUB2* gene to validate the use of the β-tubulin gene as a molecular tool for addressing fundamental questions about *P. fusca* biology. Thus, we first used *TUB2* as a tool to explore the phylogeny of pathogenic fungi. The nucleotide sequence of *PfTUB2* was used to study the relatedness between *P. fusca* and other species of powdery mildew fungi, plant pathogenic fungi, and oomycetes. The phylogenetic position of *P. fusca* inferred based on *PfTUB2* was concordant with the phylogenetic history of *Erysiphales* which was reconstructed using ITS-rDNA (Takamatsu 2004). All of the powdery mildew species were clustered together, with *P. fusca* more closely related to *Blumeria* than to *Golovinomyces/Erysiphe* species. However, in relation to the other species of *Leotiomycetes* used in the analysis, a discrepancy occurred: all of the *Erysiphales* species branched apart as an independent group. *Leotiomycetes* phylogeny has recently been

Table 5 – Statistical analysis of the methods used in this work to assess powdery mildew infection. Powdery mildew disease progression caused by *P. fusca* on zucchini cotyledons was evaluated by qPCR and image analysis. Development of the *P. fusca* isolates SF60 (MBC^R) and 2086 (MBC^S) on zucchini cotyledons spread with thiophanate-methyl (500 $\mu g\ ml^{-1}$) was recorded.

Method	Experiment 1		Experiment 2		Experiment 3	
	2086	SF60	2086	SF60	2086	SF60
qPCR	0.77	228.03a	1.44	252.13a	0.61	210.59a
Image analysis	ND	269.2a	ND	202.66a	ND	291.19a

Values shown are the area under the disease progress curve (AUDPC) and represent the mean of five cotyledons.
Numbers followed by the same letter indicate that they were not significantly different according to the Student's t-test ($p = 0.05$).
ND: Not detected.

inferred using rDNA data (Wang et al. 2006). The results showed that *Leotiomycetes* is a well-defined class that includes the *Erysiphales* among other groups. Whereas certain authors discourage the use of ITS regions to perform phylogenetic and evolutionary analyses (Wyand & Brown 2003), the use of β-tubulin sequences has increased (De Jong et al. 2001). The discrepancy observed between rDNA and *TUB2* phylograms reinforces the use of multigene phylogenies to address questions of fungal systematics. A good example is the genome-wide phylogenetic analysis of the major classes of the *Ascomycota* that was performed by Robbertse et al. (2006). Our results suggest that *TUB2* could be useful for this type of analysis.

The β-tubulin gene is a typical molecular marker for addressing intraspecific genetic diversity in population genetics studies of powdery mildew fungi (Inuma et al. 2007; Brewer & Milgroom 2010). To validate the use of *PfTUB2* as a marker for genetic diversity, 17 isolates of *P. fusca*, which were obtained from different hosts, locations, and years of isolation, were examined for genetic variation at the *TUB2* locus. Sequence analysis showed that the 17 isolates analysed were identical. Two opposite possibilities may explain this result. First, in contrast to other powdery mildew species, such as *Blumeria graminis* or *E. necator* (Inuma et al. 2007; Brewer & Milgroom 2010), the β-tubulin gene is not informative for genetic variation in *P. fusca*, as already shown for ITS regions in a previous study (Fernández-Ortuño 2007). Second, the lack of genetic diversity within the β-tubulin gene and ITS regions reflects that the European populations of *P. fusca* constitute a single clonal population, which is not surprising considering the high sporulation rate and efficient aerial dispersal of this type of pathogen (Brown & Hovmøller 2002). In any case, to test both hypotheses, a multilocus sequence typing (MLST) approach (Brewer & Milgroom 2010) with additional marker genes and a higher number of isolates should be undertaken.

Another typical use of β-tubulin genes is related to gene expression analysis by modern real-time, quantitative RT-PCR technologies. Similar to other 'housekeeping' genes, it is generally assumed that the β-tubulin genes are constitutively expressed (Matsuda et al. 2005). Therefore, these genes are commonly used as reference genes in expression studies (Zhang et al. 2000). To confirm the usefulness of *TUB2* as a normaliser gene in quantitative gene expression experiments, the expression of *PfTUB2* was studied in 17 isolates of *P. fusca*, and expression stability was statistically analysed by the BestKeeper test. No differences in gene expression were observed among the isolates, confirming that this 'housekeeping' gene can be used with total confidence as a reference in gene expression analyses.

Although great efforts have been invested in plant breeding programmes, growers still have important concerns about disease control, and the application of fungicides continues to be the principal practice for the management of powdery mildew in most cucurbit crops (McGrath 2001; Pérez-García et al. 2009). The impact of chemical control, however, has been very much tempered by the ease with which *P. fusca* develops resistance, quickly rendering many systemic fungicides ineffective. The cucurbit powdery mildew fungus has exhibited a high potential for developing resistance in many areas of the world to several fungicide classes, including MBCs, sterol demethylation inhibitors (DMIs), morpholines, organophosphates, hydroxypyrimidines, Qo inhibitors (QoIs), and quinoxalines (McGrath 2001; Fernández-Ortuño et al. 2006; López-Ruiz et al. 2010). Microtubule assembly is the target of benzimidazole fungicides, and single-point mutations in the β-tubulin gene are known to be responsible for resistance to these compounds in several fungi (Ma & Michailides 2005). Whereas benzimidazole resistance has been widely documented in *P. fusca* (McGrath 2001), the molecular basis of such resistance has not been reported to date. After a comparison of *PfTUB2* sequences from isolates that are sensitive and resistant to thiophanate-methyl, our results showed that in *P. fusca*, the main mechanism that confers resistance to MBC fungicides is the typical E198A amino acid substitution at β-tubulin, as previously described in other filamentous fungi (Albertini et al. 1999; Ma & Michailides 2005). Moreover, the isolates resistant to thiophanate-methyl were also resistant to carbendazim (data not shown), another MBC fungicide, thus confirming the crossresistance phenotype conferred by the E198A mutation.

The MBC fungicide thiophanate-methyl is still authorised in Spain for their use against powdery mildew in cucurbits. To properly combat this disease, fungicide resistance monitoring is vital, not only to determine whether resistance is the cause of a lack of disease control but also to check whether resistance management strategies are working. Conventional bioassays for detecting fungicide resistance in obligate parasites, such as powdery mildews, are very resource intensive (Fernández-Ortuño et al. 2006; López-Ruiz et al. 2010). However, advances in molecular biology have provided new opportunities for rapidly detecting fungicide-resistant genotypes once mechanisms of resistance are elucidated at the molecular level (Ma & Michailides 2005). To date, several PCR-based methods have been developed to identify SNP markers and mutations associated with fungicide resistance in many plant pathogenic fungi (Fraaije et al. 2002; Avenot et al. 2008; Chen et al. 2009; Yin et al. 2010; Liu et al. 2012). Once the role of the E198A amino acid substitution in resistance to MBC fungicides was established for *P. fusca*, an allele-specific PCR assay was developed to detect isolates resistant to these compounds by detecting such mutation. In these assays the specificity of the primers is essential. In our case, the addition of an extra mismatch base was necessary to produce a significant increase in specificity for the allele-specific primers, which was subsequently validated by the molecular analysis of the collection of 17 MBC^S and MBC^R isolates of *P. fusca*. The next step will be the development of an allele-specific real time PCR assay for the large scale, high-throughput monitoring of MBC resistance in the field, such as the assay recently developed for *Sclerotinia sclerotiorum* (Yin et al. 2010). The timely detection of MBC^R alleles in *P. fusca* populations could help cucurbit growers to make proper decisions about resistance management programmes and to more efficiently control powdery mildew.

Since the first reports of DNA-mediated transformation in model fungi, many commercially and agriculturally important fungal species have been transformed. Unfortunately, powdery mildew fungi have proven to be recalcitrant to transformation. Although the stable transformation of *B. graminis* was reported more than 10 y ago (Chaure et al. 2000), the genetic transformation of powdery fungi is still a pending issue.

Hygromycin B resistance is one of the most popular selectable markers for transformation of filamentous fungi (Ruiz-Diez 2002); however, zucchini cotyledons, which are widely used for *P. fusca* cultivation, are very sensitive to this antibiotic (Vela-Corcía unpublished). By contrast, MBC fungicides, such as benomyl, seem to be more appropriate for *in planta* selection (Chaure *et al.* 2000). In this sense, another potential application of *PfTUB2* with the MBC^R allele could be its use in transformation vectors that, together with thiophanate-methyl selection, could be used to transform *P. fusca* by several of the existing transformation methods.

The quantification of powdery mildew infections in plants is currently based on three major methods: macroscopic categorisation, microscopy-based penetration and conidiophore/conidial counts, all of which have certain limitations. Whereas microscopic determinations are very tedious and time-consuming, semiquantitative macroscopic categorisations are prone to subjectivity (Weβling & Panstruga 2012). Although image analysis software (Romero *et al.* 2003) is helping to solve this problem, accurate methods for quantifying fungal growth that are suitable for high-throughput analysis are needed. In this study, we have developed a qPCR assay to quantify *P. fusca* growth using *PfTUB2* as a molecular target. We have validated this method by testing the growth of two isolates of *P. fusca*, one resistant and the other sensitive to thiophanate-methyl, in zucchini cotyledons spread with the fungicide and by comparing the method with disease quantification by image analysis. The results obtained from both methods were very similar, but the molecular assay seems to be more accurate when fungal growth is scarce. A similar method has recently been developed for the *Arabidopsis* powdery mildew *Golovinomyces orontii* using other target fungal genes (Weβling & Panstruga 2012). We anticipate that these methods will be valuable tools to quantify plant-powdery mildew interactions in the near future.

The isolation of the *P. fusca* β-tubulin gene has allowed us to develop a number of molecular tools with different research applications. Although *P. fusca* represents one of the most serious threats to cucurbit production worldwide, very little is known about *P. fusca* biology especially as regards the factors governing the intimate molecular dialogue between host and pathogen and the pathogen's lifestyle as an obligate biotroph (Pérez-García *et al.* 2009). To mitigate this situation, sequencing of the *P. fusca* transcriptome is currently underway. A detailed analysis of the sequence data should reveal new insights into *P. fusca* biology. In relation to this analysis, the molecular tools developed in this work should be very useful for further research on key aspects of plant-powdery mildew interactions.

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