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Detection of changes in mould cell wall stress-related gene expression by a novel reverse transcription real-time PCR method

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ABSTRACT

The cell wall integrity (CWI) pathway is activated in response to cell wall stresses due to different food-related environments. Rho1 is one of the main regulators within such pathway. The objective of this work was to design an easy-to-use RT-qPCR technique for the evaluation of the *Rho1* gene expression useful to measure responses to the presence of cell wall stressors such as the antifungal protein PgAFP. Two primer pairs were designed from published conserved regions. Their specificity initially was determined by *in silico* analysis for several fungal species. After optimising the qPCR, the primer pair *Rho1*-F1/R2 was selected due to the lowest Cq values obtained and its specificity. The qPCR method showed efficiencies between 97.5% and 100.5%. Applicability of the designed qPCR method was evaluated in the presence of the stressor PgAFP. The PgAFP-resistant *Penicillium polonicum* and the PgAFP-sensitive *Aspergillus flavus* showed *Rho1* gene over- and under- expression, respectively, indicating that the CWI pathway is activated in the former species but not activated in the latter one in response to the stress caused by PgAFP. This novel qPCR methodology able to detect changes in CWI-related gene expression in filamentous fungi will be useful in future studies to evaluate physiological mould responses to different food environmental challenges.

1. Introduction

Filamentous fungi are ubiquitous microorganisms that are able to colonise a wide range of ecological niches including soil, plants and foods. Ability to grow displayed by moulds in each environment is determined by a complex of interacting factors, which facilitate fungal adaptation to a specific ambient (Fuchs and Mylonakis, 2009; Magan, 2007). External conditions trigger different signal cascades within the fungal cell that culminate in changes at the transcriptional level, leading to the production of compatible solutes to enable the enzyme systems to function under the environmental conditions (Magan, 2007; Nesci et al., 2004; Ramos et al., 1999).

When filamentous fungi colonise foods, the production of secondary metabolites is a matter of concern due to the toxicity of some of them, including mycotoxins. Several studies have displayed the effect of abiotic environmental factors on the up or down regulation of specific genes involved in the biosynthesis of different mycotoxins such as carbon source, nitrogen source, oxidative stress, temperature, water activity (a_w), pH and preservative concentration (Magan, 2007; Medina et al., 2015; Schmidt-Heydt et al., 2007, 2013). This suggests that there may be important ecological reasons for the biosynthesis of mycotoxins as part of the evolution of physiological adaptation to certain environmental stress conditions. This ability to adapt to specific environments by adjusting the metabolite profile may be related to the sensitive regulation of the biosynthesis of these secondary metabolites depending on the surrounding conditions. For example, *Penicillium verrucosum* shifts its secondary metabolite profile towards citrinin under intense light conditions (oxidative stress), while ochratoxin A is the main secondary metabolite with increased concentrations of NaCl (osmotic stress) (Stoll et al., 2013).

In addition, moulds have other metabolic pathways that help to cope with unfavourable environmental conditions favouring colonisation of specific extreme environments. The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) pathways are particularly important in fungi for responding to osmotic, oxidative and cell wall stresses. Some authors have described that the activation of the stress

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response pathways can result in changes in mycotoxin production related pathways (Graf et al., 2012; Kohut et al., 2009; Ochiai et al., 2007; Stoll et al., 2013). Therefore, there is a need to understand the mechanism of adaptation of toxigenic fungal species to specific environments from a food safety point of view.

The CWI pathway is responsible for the maintenance of the cell wall by detecting cell wall stresses and responding mediating its biosynthesis, actin organization and other events. Overall, the CWI pathway is conserved among fungi, including yeasts and filamentous fungi (Fuchs and Mylonakis, 2009) and typically comprises cell wall sensors at the cell surface that detect environmental changes and initiates signals that are conveyed to the nucleus through guanine nucleotide exchange factors (GEFs), Rho GTPases, protein kinase C (PKC) and a mitogen-activated protein (MAP) kinase module. A basal activity of this pathway is required under normal growth conditions for maintaining ordinary wall biogenesis and cell integrity, but its activation occurs in response to stress which is sensed by transmembrane proteins (Dichtl et al., 2016).

Rho1 is considered the master regulator of CWI signaling (Levin, 2005). It is a small G protein coupled to a family of cell surface sensors, so its main function is to integrate signals from the cell membrane. Activation of Rho1 protein requires the switch between the inactive and cytosolic GDP-bound to the membrane-associated GTP-bound state (Xu et al., 2016). Rho GTPase then stimulates several downstream effectors, such as PKC, that regulates the mitogen-activated protein kinase (MAPK) cascade and a variety of outputs including β-1,3-glucan synthesis, cytoskeleton organization and polarized secretion (Dichtl et al., 2016; Levin, 2011). Attempts to delete *Rho1* in different fungal species (*Aspergillus niger*, *Candida albicans*, *Neurospora crassa*) failed, suggesting that this gene is essential for cell survival (Dichtl et al., 2012; Kwon et al., 2011; Richthammer et al., 2012; Vogt and Seiler, 2008). Besides, Rho GTPases have been also linked to other critical processes, such as morphogenesis and host infection (Xu et al., 2016).

The CWI pathway is involved in the tolerance of fungi to antifungal compounds used in food preservation (Hayes et al., 2014). It seems that the relative quantity of Rho1 protein in fungi changes in relation to their response against the antifungal treatment. Thus, Guest et al. (2004) stated that *Aspergillus nidulans* cells lacking RhoA (Rho1 homologue) display pronounced hypersensitivity to cell wall interfering drugs. In addition, it has been reported that *Aspergillus flavus* treated with PgAFP, an antifungal protein isolated from *Penicillium chrysogenum* strain RP42C (CECT 20922, Rodríguez-Martín et al., 2010), presented lower amounts of the Rho1 protein compared to the untreated control (Delgado et al., 2015b). However, when *Penicillium polonicum* grew in the presence of PgAFP exhibited higher relative amounts of Rho1 protein and chitin content than when it grew in the absence of PgAFP (Delgado et al., 2016). The same authors concluded that the higher Rho1 relative quantities, the better resistance of the fungus to the antifungal treatment. Although the above studies have examined the changing levels of the Rho1 protein in filamentous fungi, no reverse transcription real-time PCR (RT-qPCR) method based on the *Rho1* gene has been designed as a predictive tool to detect changes in CWI responses by filamentous fungi.

The objective of this study was to design a new RT-qPCR method based on the *Rho1* gene to detect changes triggered by cell wall instability. The suitability of the proposed RT-qPCR was evaluated by using the antifungal protein PgAFP, which is a proven cell wall stressor of filamentous fungi.

2. Materials and methods

2.1. Fungal strains

Three different strains from the Spanish Type Culture Collection (CECT, Spain) were used in this study: *A. flavus* CECT 2687, *P. polonicum* CECT 20933 and *P. chrysogenum* CECT 20922, producer of the antifungal protein PgAFP (Rodríguez-Martín et al., 2010).

The relative amounts of the Rho1 protein produced by *A. flavus* CECT 2687 and *P. polonicum* CECT 20933 in Potato Dextrose Broth (PDB; Scharlau Chemie, S.A., Spain) at 25 °C for 24 h, in either presence (10 μg/mL) or absence of PgAFP, were previously reported (Delgado et al., 2015b, 2016). These mould strains were selected due to their sensitivity and resistance to PgAFP, respectively (Delgado et al., 2015b, 2016). This protein has been previously used as biocontrol agent against toxigenic filamentous fungi in foods (Delgado et al., 2015a).

Table 1
Oligonucleotide sequences of primers designed in this study.

Primer pairs	Nucleotide sequences (5′-3′)	Position[a]
Rho1-F1	CTTTCCCCGAGGTCTACGTC	275
Rho1-R1	ACGGTCGTAATCCTCCTGAC	502
Rho1-R2	TCGTAATCCTCCTGACCAGC	498

PCR product size amplified with *Rho1*-F1/R1 is 119 bp.
PCR product size amplified with *Rho1*-F1/R2 is 115 bp.
[a] Positions are in accordance with the published sequence of the *Rho1* gene of *Fusarium oxysporum* (GenBank accession no. KC017392.1).

2.2. Primer design

Two primer pairs *Rho1*-F1/R1 and *Rho1*-F1/R2 (Table 1) were designed on the basis of 30 partial sequences of the *Rho1* gene of different mould species (*Fusarium graminearum*, *Fusarium oxysporum*, *Fusarium avenaceum*, *Fusarium pseudograminearum*, *Gibberela fujikuroi*, *Talaromyces marnefeii*, *Aspergillus fumigatus*, *Aspergillus clavatus*, *Aspergillus flavus*, *Aspergillus terreus*) retrieved from GenBank of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/; data not shown). Sequences were edited and aligned by the Clustal Omega resource from the EMBL European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalo/). Alignment showed conserved regions among all species, which were selected to design both primer pairs using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/; Rozen and Skaletsky, 2000). Primers were *in silico* tested by comparing their oligonucleotide sequences with the nucleotide sequence database in GenBank using the BLAST tool from the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The forward primer (*Rho1*-F1) crossed an exon-intron boundary and the reverse primers (*Rho1*-R1 and *Rho1*-R2) spanned an exon (Fig. 1).

2.3. Optimisation of RT-qPCR reactions

2.3.1. Inoculum preparation

To optimise the RT-qPCR method, *A. flavus* CECT 2687 and *P. polonicum* CECT 20933 were sub-cultured in PDB and incubated for five days at 25 °C in an orbital shaker at 200 rpm. Mycelia were filtered through Miracloth filter (Merck, Germany) and stored at −80 °C until extraction.

2.3.2. RNA extraction and cDNA synthesis

Mycelia were frozen with liquid nitrogen and ground with the aid of a mortar and pestle. RNA was extracted by using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Co. LLC., USA) following manufacturer's instructions (Protocol A) (Bernáldez et al., 2017). RNA concentration and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 μL aliquot on a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). The samples were diluted up to 0.1 μg/μL and treated with DNAse I (Thermo Fisher Scientific) to remove genomic DNA. RNA samples were stored at −80 °C until use. Next, the reverse transcription reaction of total RNA (500 ng) was carried out with the PrimeScript™ RT reagent Kit (Takara Bio Inc., Japan) as described by

Fig. 1. Design of the primers *Rho1*-F1, *Rho1*-R1 and *Rho1*-R2 based on the partial sequence of the *Rho1* gene of one of the targeted mould species (*Fusarium oxysporum*; GenBank accession no. KC017392.1). Exons regions are shaded. Primers are highlighted and underlined by an arrow.

the manufacturer (incubation at 37 °C for 15 min and 85 °C for 5 s). cDNA samples were stored at −20 °C until being used for qPCR.

2.3.3. qPCR reactions: optimisation, specificity and standard curves

The qPCR reactions were conducted in the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Life Technologies, USA) using the SYBR Green methodology. Reactions were prepared in 12.5 µL total volume mixtures in duplicate in MicroAmp fast reaction tubes. Two replicates of a non-template control were also included in all the runs. For testing the concentration of primers, several concentrations ranging from 50 to 300 nM were evaluated. The optimised reaction mixture consisted of 6.25 µL of SYBR® Premix Ex Taq™ (Tli RNAseH Plus; Takara Bio Inc.), 0.125 µL of ROX plus (Takara Bio Inc.), 200 nM of each primer, 2.5 µL of DNA template and 3.125 µL of Milli-Q water. To optimise qPCR conditions, different annealing temperatures were assayed (55, 58 and 60 °C). Quantification cycle (Cq) determination, which is the intersection between each fluorescence curve and a threshold line, was automatically performed by the instrument using default parameters of the Software ViiA7 RUO v.1.2.4. (Applied Biosystems).

After the PCR reaction, the specific binding of SYBR Green to the amplicon derived from the *Rho1* gene was tested. The specificity of the PCR product was checked by the construction of the melting curve by heating in a slow ramp between 60 and 95 °C in increments of 0.5 °C for 5 s. The melting temperature (Tm) was automatically calculated and compared with that deduced from the sequence of the expected fragment. In addition, the size of the PCR products was verified by electrophoresis in 2.5% agarose gels stained with 5 µL SafeView dye (NBS Biologicals, UK). The size of the PCR product was determined with GeneSnap v.7.09.02 image acquisition software (Syngene, UK), with the aid of a DNA molecular size marker of 2.1–0.15 kbp (Promega BioSciences, USA).

Standard curves were constructed using the qPCR amplicon obtained for *A. flavus* and *P. polonicum* using the selected primer pair *Rho1*-F1/R2. Firstly, the concentration of the obtained qPCR product was measured in the NanoDrop™ and the number of copies was then calculated. This stock solution was serially 10-fold diluted and an aliquot of each dilution was used as a transcript copy number standard for the qPCR by duplicate. The $\log_{10}$ of the number of gene copies was plotted against Cq values to obtain the standard curve. The criteria considered for reliability of the designed method were the regression coefficient from the linear fit (R^2) and the amplification efficiency calculated from the formula $E = (10^{-1/S}) - 1$, being S the slope of the linear fit in the standard curve (Rodríguez et al., 2015).

2.4. Applicability of the RT-qPCR method

To check the usefulness of the developed RT-qPCR method, the antifungal protein PgAFP produced by *P. chrysogenum* CECT 20922 (Rodríguez-Martín et al., 2010) was selected since its role as a cell wall stressor when used as biocontrol agent has been previously described.

2.4.1. Experimental settings

2.4.1.1. Inocula preparation. Spore suspensions of *A. flavus* and *P. polonicum* were obtained from Potato Dextrose Agar (PDA, Scharlau Chemie, S.A.)-7-day-old cultures. Five mL of Phosphate Buffered Saline (PBS pH 7.2, prepared as following: 0.32 g NaH_2PO_4, 1.0 g Na_2HPO_4, 9 g NaCl, 1 L H_2O) were added and spores were spread onto each colony surface with a sterile rod. Spore suspensions were disposed in sterile vials and counted using a Thoma chamber Blaubrand® (Brand, Germany). The concentration of the suspension for the experiment was adjusted to 10^6 spores/mL by diluting with PBS.

2.4.1.2. PgAFP purification. PgAFP was obtained from the *P. chrysogenum* strain liquid culture as described by Acosta et al. (2009). Briefly, this strain was grown in PDB, pH 4.5, at 25 °C for 21 days. The broth was filtered through a nitrocellulose 0.22-µm pore size (Sartorius, Germany) to obtain cell-free medium, which was applied to an ÄKTA FPLC with a cationic exchange column HiTrap SP HP (Amersham Biosciences, Sweden), equipped with a UV detector at 214 nm and a fraction collector FRAC-950 (Amersham Pharmacia Biotech, Sweden). By using the cationic exchange column, the fraction B5 from *P. chrysogenum* CECT 20922 produced just one peak. The resulting fraction containing PgAFP was then purified by gel filtration on a HiLoad 26/60 Superdex 75 column for FPLC (Amersham Biosciences). The fractions D5, D4, D3 were filtered and further tested against sensitive reference moulds. Next, the extracts containing the purified PgAFP from several batches were pooled in a stock solution, whose amount of protein was quantified by the Lowry method (Lowry et al., 1951). PgAFP stock solution was frozen at −20 °C and diluted as required to achieve the desired final concentration.

2.4.1.3. Inoculation, growth conditions and sampling. The experiments

were conducted in PDA plates supplemented with 10 μg/mL of PgAFP. The protein was aseptically added to the medium after autoclaving. Controls without PgAFP were also carried out in single PDA Petri dishes. Agar plates were centrally inoculated with 2 μL of the spore suspension and incubated at 25 °C for up to 9 days. Growth was daily measured and samples for RNA extraction were collected by days 3 and 9. All experiments were performed in triplicate.

2.4.2. Growth and sporulation assessment

Radial growth was measured in two directions at right angles to each other. Data was analysed using a primary model by plotting colony diameter (mm) against time (days). Data plots showed, after a lag phase, a linear trend with time. The slope of the regression line was used to obtain growth rate (μ_m, mm/day) (Garcia et al., 2009). Estimation of sporulation was performed macroscopically.

2.4.3. RNA extraction and cDNA synthesis

Mycelia were frozen in liquid nitrogen and ground in a frozen mortar to a fine powder. Total RNA extraction and subsequent cDNA synthesis were conducted as described in Section 2.3.2.

2.4.4. RT-qPCR assays and absolute gene expression

Data analysis was carried out using the Software ViiA7 RUO v.1.2.4. RT-qPCR experiments were performed with a 10-fold dilution of the cDNA synthesised for both strains. Concentrations of reagents and reaction conditions used were those optimised in the present work. Absolute expression levels of the *Rho1* gene from treated and untreated samples were extrapolated from the standard curves built as previously indicated (Section 2.3.3) by using the Cq values obtained for such samples.

2.5. Statistical analysis

Statistical analysis was performed using the IBM SPSS v.22.0 software (IBM Corporation, USA). Data sets of growth rates and *Rho1* gene expression were tested for normality using the Shapiro-Wilk test. Given that data sets failed the normality test, the analyses were performed using the non-parametric Kruskal-Wallis test to determine any significant differences. The Mann-Whitney *U* test was then applied to compare the obtained mean values. The statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Optimisation and specificity of qPCR

Annealing temperature was firstly optimised for both designed primer pairs (*Rho1*-F1/R1 and *Rho1*-F1/R2). No amplification was detected neither at 60 nor at 58 °C. Therefore, 55 °C was the annealing temperature chosen to detect changes in the *Rho1* gene expression by qPCR. Therefore, the optimised thermal cycling conditions consisted of one holding period at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 1 min.

From both primer pairs tested, *Rho1*-F1/R2 was selected for subsequent assays because non-template control (NTC) did not show unspecific amplification. In addition, Cq values observed with the latter were lower than those observed for the pair *Rho1*-F1/R1 at a particular cDNA concentration in both studied mould strains.

The optimal primer concentrations were chosen considering the lowest Cq value and the highest fluorescent signal for a fixed target concentration. Concretely, the concentration of each primer used for RT-qPCR was 200 nM.

When the melting curves were analysed, only one amplicon with Tm value of 84.12 °C for *P. polonicum* and of 85.90 °C for *A. flavus* was obtained with the primer pair *Rho1*-F1/R2 (Fig. 2). The specificity of the primer pair selected was also confirmed by agarose gel electrophoresis of the qPCR products, where only one ~115-bp amplicon was observed (data not shown).

3.2. Standard curves

An example of the standard curve constructed using the Cq values and the $\log_{10}$ of the transcript number of copies of the *Rho1* gene for the optimised method is shown in Fig. 3. Standard curves showed a high regression coefficient ($R^2 > 0.999$) confirming a reliable linear fit. The slopes of the standard curves were −3.38 and −3.31 that corresponds to amplification efficiencies of 97.5% and 100.5%, respectively.

3.3. Effect of PgAFP on growth and Rho1 expression levels of filamentous fungi

The mean growth rates and *Rho1* gene expression levels for *A. flavus* and *P. polonicum* on PDA with and without PgAFP are displayed in Fig. 4. Both fungal species showed strong differences between each other in their growth under the assayed experimental conditions, being *A. flavus* the one that grew faster. However, the presence of PgAFP in the culture medium caused a slight increment in the growth rate, but a decrease in the sporulation level in this fungus (Figs. 4 and 5). On the other hand, no differences were observed either in growth rate (Fig. 4) nor in sporulation level (data not shown) for *P. polonicum*.

When analysing the absolute expression of the *Rho1* gene for both strains evaluated after 3 days of incubation, non-significant differences were observed between treatments in *A. flavus* ($p > 0.05$; Fig. 4). On the contrary, a statistically significant increase in the absolute *Rho1* gene expression was detected for *P. polonicum* in the presence of PgAFP when comparing with the untreated sample ($p \leq 0.05$). The transcript number of copies of the *Rho1* gene in *P. polonicum* were much higher than that found in *A. flavus* (Fig. 4).

Non-significant differences were observed in the absolute *Rho1* gene expression values for both strains between 3 and 9 days of incubation (data not shown).

4. Discussion

Potential of filamentous fungi to adapt and colonise different food environmental conditions and composition has been extensively reported (Medina et al., 2015; Rodríguez et al., 2014; Schmidt-Heydt et al., 2007, 2013). This has been associated with the activation of specific intracellular mechanisms that allow fungi tolerating and overcoming diverse stresses (Fuchs and Mylonakis, 2009; Magan, 2007; Schmidt-Heydt et al., 2013). Even though specific proteins for several fungal species have been described, the understanding of the functioning of these specific pathways remains poorly elucidated (Hayes et al., 2014).

Among the stress-related pathways, the CWI is one of the main responsible for the maintenance of the cellular integrity through the reparation and/or fortification of the cell wall. In this study, a RT-qPCR methodology able to monitor the *Rho1* gene transcription levels in filamentous fungi has been developed for the first time. *Rho1* is a key gene within the pathway and is well conserved throughout the whole kingdom. Thus, the design of a qPCR based on such gene offers the possibility to study changes in CWI in fungi under diverse external conditions.

RT-qPCR has been reported as a powerful tool to examine in detail the impacts of environmental conditions on mycotoxin biosynthetic- and stress-related genes, which can be related to fungal growth and phenotypic mycotoxin production on different foods (Abdel-Hadi et al., 2010; Lozano-Ojalvo et al., 2013; Rodríguez et al., 2014, 2016). The development of this new RT-qPCR method would allow a better understanding of the role of the CWI pathway in the protection of fungi against adverse environmental food conditions as well as antifungal treatments.

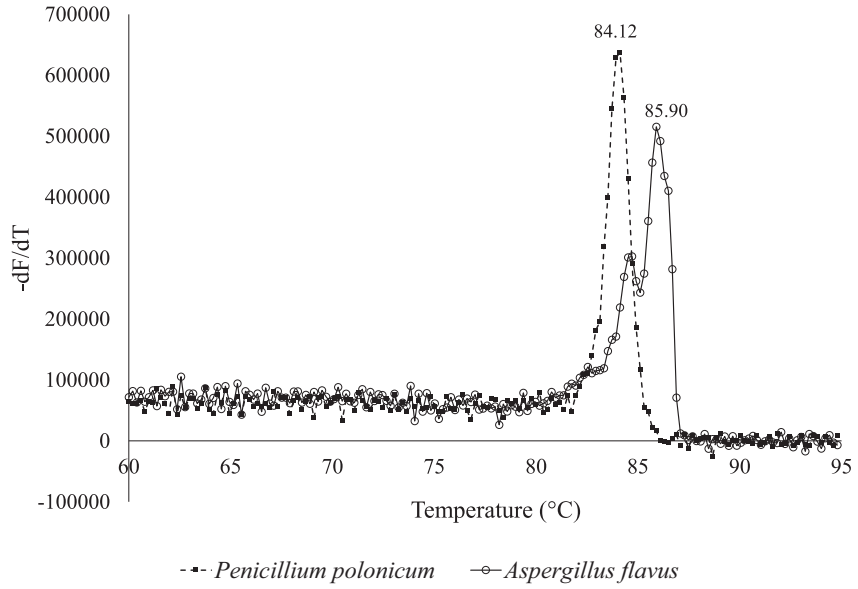

Fig. 2. Representative melting curves of the qPCR product of the *Rho1* gene with the specific primers *Rho1*-F1/R2. Numbers on the plot indicate the corresponding melting temperature (Tm).

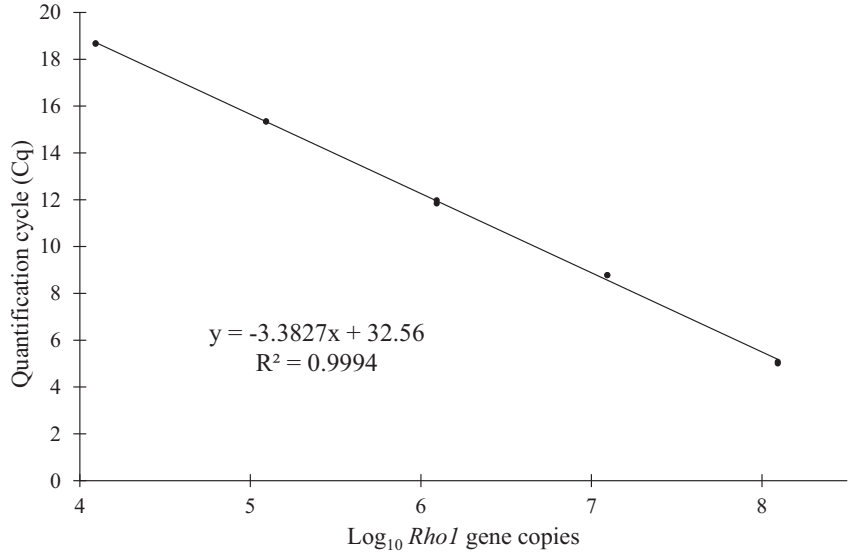

Fig. 3. Example of the standard curve for *Aspergillus flavus* showing the correlation between the number of copies of *Rho1* gene and quantification cycle (Cq) values.

Designing appropriate primer pairs is the first critical point in the successful development of a qPCR method. In our work, the sequences picked up by the primers designed were tested *in silico* in four fungal genera including *Aspergillus*, *Fusarium*, *Giberella* and *Talaromyces* (data not shown), confirming the selection of conserved regions of the gene.

The primer pair *Rho1*-F1/R2 was selected for qPCR optimisation since the Cq values obtained were lower than those with the primer pair *Rho1*-F1/R1 and the NTC did not show amplification. Furthermore, the primer pair *Rho1*-F1/R2 was adequate for qPCR, since primer dimers were not detected. This confirmed its specificity in spite of using SYBR Green.

The parameters obtained indicated that the optimised methodology is specific, sensitive and reproducible in different genera. In addition to the *Aspergillus* and *Penicillium* species analysed, the designed methodology was also tested with *Fusarium* and *Alternaria* strains (data not shown). Thus, the qPCR tool is applicable for the four most important toxigenic fungi in foods. The regression coefficients of the linear fit, the slopes and the efficiencies obtained were within the recommended range (Rodríguez et al., 2015). Thus, the developed qPCR technique met the premises to be a robust method able to appropriately detect and quantify fungal response to cell wall stressors.

The applicability of the designed qPCR method was tested by using the antifungal protein PgAFP as cell wall stressor since both tested fungal species showed different behaviour when grew in the presence of such compound. It has been reported that PgAFP can alter chitin synthesis leading to cell death *in A. flavus* or restoring cell wall structure in PgAFP-resistant *P. polonicum* (Delgado et al., 2015b, 2016). These changes in cell wall chitin deposition were related to differential levels of the Rho1 protein in those moulds (Delgado et al., 2015b, 2016). Our results have also shown different expression levels of the *Rho1* gene in response to PgAFP for both studied strains. For *P. polonicum*, an over-expression of *Rho1* gene was observed, which is in accordance with previously reported results for the corresponding protein (Delgado et al., 2016).

For this species, differences were observed in growth rates and sporulation between the PgAFP-treated batch and the control one. This

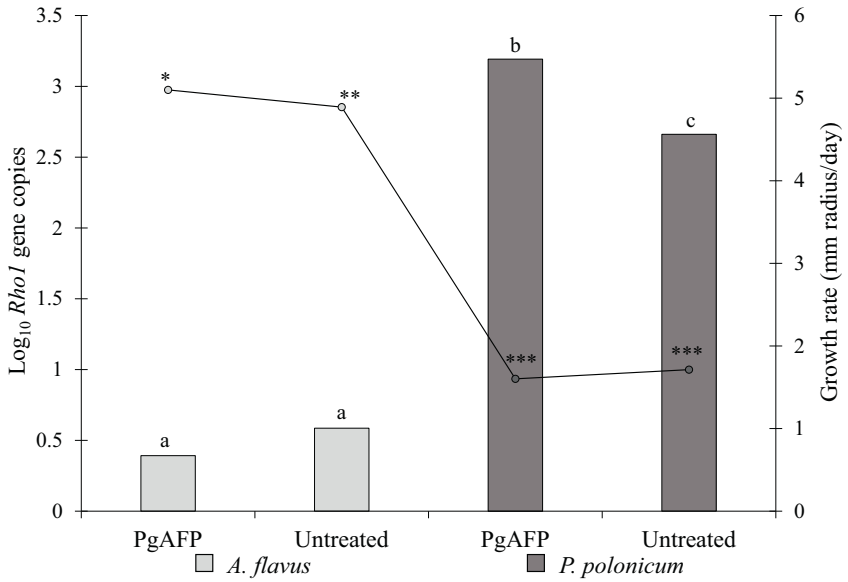

Fig. 4. Growth rate (in line) and absolute *Rho1* gene expression levels (in bars) for *Aspergillus flavus* and *Penicillium polonicum* strains on PDA untreated and treated with 10 μg/mL PgAFP after 3 days of incubation. Significant differences between treatments and strains are pointed with different letters for gene expression and different number of asterisks for growth rate ($p \leq 0.05$).

result supports the hypothesis that in *P. polonicum* the CWI pathway is active in the presence of PgAFP, allowing the fungus to overcome such treatment. Regarding *A. flavus* growth, samples treated with PgAFP presented a slightly but significantly higher growth rate than control samples. This was not expected; however, it must be highlighted that the colony diameter as the sole indicator of fungal growth might not be appropriate to reflect the whole influence of a given factor in a study (Schmidt-Heydt et al., 2012). The biomass and sporulation of the colony are also important parameters to take into account to measure fungal growth. In fact, PgAFP-treated *A. flavus* colony presented less aerial mycelium and a dramatic decrease of sporulation (Fig. 5) in spite of its higher growth rate. These findings are in accordance with a previous study that demonstrated that a conditional *Rho1* mutant of *A. fumigatus* showed strongly reduced sporulation when cultured under suppressive conditions (Dichtl et al., 2012). Regarding the *Rho1* gene expression, it was expected the fact that non-significant differences between the control and PgAFP-treated samples were detected. This seems to indicate that the CWI pathway is not activated in this species under this external condition, which is in accordance with previous studies reporting the susceptibility of the tested mould species to the PgAFP-antifungal treatment (Delgado et al., 2015b, 2016).

To our knowledge, this is the first study that develops a qPCR methodology to evaluate changes in CWI-related gene expression in filamentous fungi. The structure of fungal cell wall is unique, different from mammals and other eukaryotes, being a common target for antifungal treatments (Jabes et al., 2016). The understanding of the regulatory mechanisms involved in its biosynthesis and maintenance is crucial to understand the adaptation of fungi to different food environments and for optimisation of existing antifungal treatments and development of new ones. The technique designed in the present work might be useful for these purposes. Moreover, toxigenic fungi produce toxic metabolites under different food ambient conditions, according to the species and compounds involved (Graf et al., 2012). Some of these secondary metabolites are used by the fungus as an adaptation strategy in unfavourable environments (Schmidt-Heydt et al., 2012). Therefore, stressful ecological factors may trigger the accumulation of secondary metabolites and compatible solutes in foods. The CWI pathway could be activated against external stimuli that may occur in a food-related ambient, such as heat shock, pH changes, limited nitrogen sources and oxidative stress (Fuchs and Mylonakis, 2009) or the presence of fungicides (Hayes et al., 2014). Monitoring the expression of the *Rho1* gene would provide valuable information about fungal physiology during fungal growth and secondary metabolite production. This implies that, for example, the qPCR could be used to evaluate the effect of different environmental conditions (*e.g.* pH, nutrients, water activity), storage conditions (*e.g.* temperature, relative humidity), the nature and dose of the antifungal substance used, the predominant fungal contaminants and even mycotoxins themselves. Thus, from a food safety point of

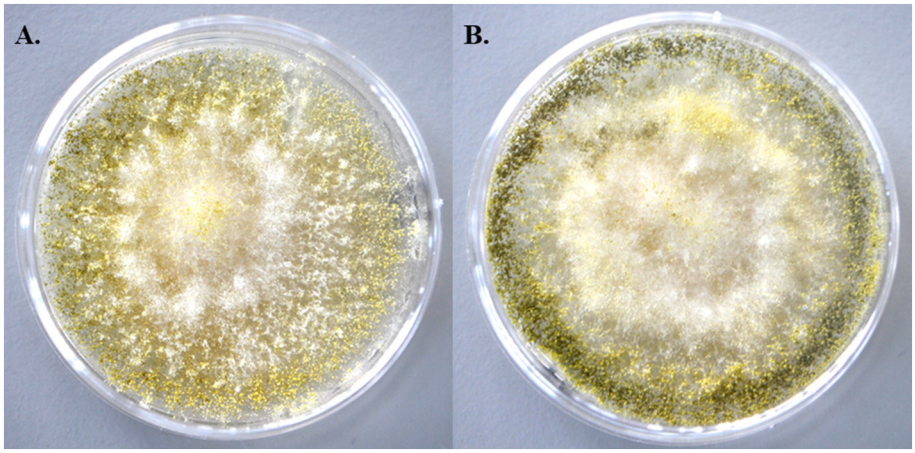

Fig. 5. *Aspergillus flavus* growth and sporulation level after 9 days in PDA plates. A: treated with 10 μg/mL PgAFP; B: control (untreated).

view, successful strategies to prevent mycotoxin-contaminated food and feed in the market could be developed.

5. Conclusions

An easy-to-use tool to evaluate the CWI pathway activation in filamentous fungi at diverse food environments has been developed. Absolute expression levels of the *Rho1* gene in fungi have been analysed in an attempt to test intracellular response to a cell wall stressor linked to the activation of the CWI pathway. An in-depth understanding of these mechanisms will be useful in future studies to evaluate physiological fungal responses to specific food conditions that may lead to adaptation of spoiling and toxigenic fungi to such niches.

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References

Abdel-Hadi, A., Carter, D., Magan, N., 2010. Temporal monitoring of the *nor-1* (*aflD*) gene of *Aspergillus flavus* in relation to aflatoxin B1 production during storage of peanuts under different water activity levels. J. Appl. Microbiol. 109, 1914–1922.

Acosta, R., Rodríguez-Martín, A., Martín, A., Núñez, F., Asensio, M.A., 2009. Selection of antifungal protein-producing molds from dry-cured meat products. Int. J. Food Microbiol. 135, 39–46.

Bernáldez, V., Rodríguez, A., Rodríguez, M., Sánchez-Montero, L., Córdoba, J.J., 2017. Evaluation of different RNA extraction methods of filamentous fungi in various food matrices. LWT Food Sci. Technol. 78, 47–53.

Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F., Asensio, M.A., 2015a. Growth inhibition and stability of PgAFP from *Penicillium chrysogenum* against fungi common on dry-ripened meat products. Int. J. Food Microbiol. 205, 23–29.

Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2015b. Impact of the antifungal protein PgAFP from *Penicillium chrysogenum* on the protein profile in *Aspergillus flavus*. Appl. Microbiol. Biotechnol. 99, 8701–8715.

Delgado, J., Owens, R.A., Doyle, S., Asensio, M.A., Núñez, F., 2016. Increased chitin biosynthesis contributes to the resistance of *Penicillium polonicum* against the antifungal protein PgAFP. Appl. Microbiol. Biotechnol. 100, 371–383.

Dichtl, K., Helmschrott, C., Dirr, F., Wagener, J., 2012. Deciphering cell wall integrity signalling in *Aspergillus fumigatus*: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol. Microbiol. 83, 506–519.

Dichtl, K., Samantaray, S., Wagener, J., 2016. Cell wall integrity signalling in human pathogenic fungi. Cell. Microbiol. 18, 1228–1238.

Fuchs, B.B., Mylonakis, E., 2009. Our paths might cross: the role of the fungal cell wall integrity pathway in stress response and cross talk with other stress response pathways. Eukaryot. Cell 8, 1616–1625.

Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2009. Predicting mycotoxins in foods: a review. Food Microbiol. 26, 757–769.

Graf, E., Schmidt-Heydt, M., Geisen, R., 2012. HOG MAP kinase regulation of alternariol biosynthesis in *Alternaria alternata* is important for substrate colonization. Int. J. Food Microbiol. 157, 353–359.

Guest, G.M., Lin, X., Momany, M., 2004. *Aspergillus nidulans* RhoA is involved in polar growth, branching, and cell wall synthesis. Fungal Genet. Biol. 41, 13–22.

Hayes, B.M.E., Anderson, M.A., Traven, A., van der Weerden, N.L., Bleackley, M.R., 2014. Activation of stress signalling pathways enhances tolerance of fungi to chemical fungicides and antifungal proteins. Cell. Mol. Life Sci. 71, 2651–2666.

Jabes, D.L., Oliveira, A.C.F., Alencar, V.C., Menegidio, F.B., Reno, D.L.S., Santos, D.S., Barbosa, D.A., Vilas Boas, R.O., de Oliveira Rodrigues Cunha, R.L., de Oliveira, R.C., Nunes, L.R., 2016. Thioridazine inhibits gene expression control of the cell wall signaling pathway (CWI) in the human pathogenic fungus *Paracoccidioides brasiliensis*. Mol. Gen. Genomics. 291, 1347–1362.

Kohut, G., Ádám, A.L., Fazekas, B., Hornok, L., 2009. N-starvation stress induced FUM gene expression and fumonisin production is mediated via the HOG-type MAPK pathway in *Fusarium proliferatum*. Int. J. Food Microbiol. 130, 65–69.

Kwon, M.J., Arentshorst, M., Roos, E.D., van den Hondel, C.A., Meyer, V., Ram, A.F., 2011. Functional characterization of Rho GTPases in *Aspergillus niger* uncovers conserved and diverged roles of Rho proteins within filamentous fungi. Mol. Microbiol. 79, 1151–1167.

Levin, D.E., 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 69, 262–291.

Levin, D.E., 2011. Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genet 189, 1145–1175.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lozano-Ojalvo, D., Rodríguez, A., Bernáldez, V., Córdoba, J.J., Rodríguez, M., 2013. Influence of temperature and substrate conditions on the omt-1 gene expression of *Aspergillus parasiticus* in relation to its aflatoxin production. Int. J. Food Microbiol. 166, 263–269.

Magan, N., 2007. Fungi in extreme environments. In: Kubicek, C.P., Druzhinina, I.S. (Eds.), Environmental and Microbial Relationships. Springer, Berlin, Heidelberg, pp. 85–103.

Medina, A., Schmidt-Heydt, M., Rodríguez, A., Parra, R., Geisen, R., Magan, N., 2015. Impacts of environmental stress on growth, secondary metabolite biosynthetic gene clusters and metabolite production of xerotolerant/xerophilic fungi. Curr. Genet. 61, 325–334.

Nesci, A., Etcheverry, M., Magan, N., 2004. Osmotic and matric potential effects on growth, sugar alcohol and sugar accumulation by *Aspergillus* section *Flavi* strains from Argentina. J. Appl. Microbiol. 96, 965–972.

Ochiai, N., Tokai, T., Takahashi-Ando, N., Fujimura, M., Kimura, M., 2007. Genetically engineered *Fusarium* as a tool to evaluate the effects of environmental factors on initiation of trichothecene biosynthesis. FEMS Microbiol. Lett. 275, 53–61.

Ramos, A.J., Magan, N., Sanchis, V., 1999. Osmotic and matric potential effects on growth, sclerotia and partitioning of polyols and sugars in colonies and spores of *Aspergillus ochraceus*. Mycol. Res. 103, 141–147.

Richthammer, C., Enseleit, M., Sanchez-Leon, E., März, S., Heilig, Y., Riquelme, M., Seiler, S., 2012. RHO1 and RHO2 share partially overlapping functions in the regulation of cell wall integrity and hyphal polarity in *Neurospora crassa*. Mol. Microbiol. 85, 716–733.

Rodríguez, A., Medina, Á., Córdoba, J.J., Magan, N., 2014. The influence of salt (NaCl) on ochratoxin A biosynthetic genes, growth and ochratoxin A production by three strains of *Penicillium nordicum* on a dry-cured ham-based medium. Int. J. Food Microbiol. 178, 113–119.

Rodríguez, A., Rodríguez, M., Córdoba, J.J., Andrade, M.J., 2015. Design of primers and probes for quantitative Real-Time PCR methods. In: Basu, C. (Ed.), PCR Primer Design. Springer, New York, pp. 31–56.

Rodríguez, A., Medina, Á., Córdoba, J.J., Magan, N., 2016. Development of a HOG-based real-time PCR method to detect stress response changes in mycotoxigenic moulds. Food Microbiol. 57, 109–115.

Rodríguez-Martín, A., Acosta, R., Liddell, S., Núñez, F., Benito, M.J., Asensio, M.A., 2010. Characterization of the novel antifungal protein PgAFP and the encoding gene of *Penicillium chrysogenum*. Peptides 31, 541–547.

Rozen, S., Skaletsky, H., 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. 132, 365–386.

Schmidt-Heydt, M., Baxter, E., Geisen, R., Magan, N., 2007. Physiological relationship between food preservatives, environmental factors, ochratoxin and *otapks*PV gene expression by *Penicillium verrucosum*. Int. J. Food Microbiol. 119, 277–283.

Schmidt-Heydt, M., Graf, E., Stoll, D., Geisen, R., 2012. The biosynthesis of ochratoxin A by *Penicillium* as one mechanism for adaptation to NaCl rich foods. Food Microbiol. 29, 233–241.

Schmidt-Heydt, M., Stoll, D.A., Mrohs, J., Geisen, R., 2013. Intraspecific variability of HOG1 phosphorylation in *Penicillium verrucosum* reflects different adaptation levels to salt rich habitats. Int. J. Food Microbiol. 165, 246–250.

Stoll, D., Schmidt-Heydt, M., Geisen, R., 2013. Differences in the regulation of ochratoxin A by the HOG pathway in *Penicillium* and *Aspergillus* in response to high osmolar environments. Toxins 5, 1282.

Vogt, N., Seiler, S., 2008. The RHO1-specific GTPase-activating Protein LRG1 regulates polar tip growth in parallel to ndr kinase signaling in *Neurospora*. Mol. Biol. Cell 19, 4554–4569.

Xu, X., Wang, Y., Tian, C., Liang, Y., 2016. The *Colletotrichum gloeosporioides* RhoB regulates cAMP and stress response pathways and is required for pathogenesis. Fungal Genet. Biol. 96, 12–24.