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Regulation of the cutinases expressed by *Aspergillus nidulans* and evaluation of their role in cutin degradation

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Abstract

Four cutinase genes are encoded in the genome of the saprophytic fungus *Aspergillus nidulans*, but only two of them have proven to codify for active cutinases. However, their overall roles in cutin degradation are unknown, and there is scarce information on the regulatory effectors of their expression. In this work, the expression of the cutinase genes was assayed by multiplex qRT-PCR in cultures grown in media containing both inducer and repressor carbon sources. The genes *ancut1* and *ancut2* were induced by cutin and its monomers, while *ancut3* was constitutively expressed. Besides, cutin induced *ancut4* only under oxidative stress conditions. An in silico analysis of the upstream regulatory sequences suggested binding regions for the lipid metabolism transcription factors (TF) FarA for *ancut1* and *ancut2* while FarB for *ancut3*. For *ancut4*, the analysis suggested binding to NapA (the stress response TF). These binding possibilities were experimentally tested by transcriptional analysis using the *A. nidulans* mutants ANΔ*farA*, ANΔ*farB*, and ANΔnapA. Regarding cutin degradation, spectroscopic and chromatographic methods showed similar products from ANCUT1 and ANCUT3. In addition, ANCUT1 produced 9,10-dihydroxy hexadecanoic acid, suggesting an endo-cleavage action of this enzyme. Regarding ANCUT2 and ANCUT4, they produced omega fatty acids. Our results confirmed the cutinolytic activity of the four cutinases, allowed identification of their specific roles in the cutinolytic system and highlighted their differences in the regulatory mechanisms and affinity towards natural substrates. This information is expected to impact the cutinase production processes and broaden their current biotechnological applications.

Keywords Cutinase · Expression · Carbon catabolite repression · Cutin degradation · Transcription factors · Oxidative stress · *Aspergillus nidulans*

Introduction

Cutin is a key component of the cuticle, covering the plant epidermis with a hydrophobic coating, present in nearly all above-ground parts of terrestrial plants. The primary function of this layer is to protect plants against desiccation and biotic stresses, acting as an interface between the plant and its environment (Martínez Rocha et al. 2008; Taiz and Zeiger 2002). The cutin is a polyester formed by long-chain fatty hydroxy acids, mainly derivatives of palmitic acid (16:0) and oleic acid (18:1), gathered by ester bonds forming a three-dimensional network stabilized by cross-linking (Ray and Stark 1998). These fatty acids can be hydroxylated or epoxylated at the middle of the carbon chain or in carbons closer to the double bond. The palmitic acid derivatives consist primarily of 16-hydroxy palmitic acid, and 9,16- or 10,16-dihydroxypalmitic acid, whereas oleic acid derivatives consist primarily of 18-hydroxyoleic acid, 9,10-epoxy-18-hydroxystearic acid, and

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9,10,18-trihydroxystearate (Kolattukudy 1980; Fernández et al. 2016).

Pathogenic microorganisms use enzymes such as lipases and cutinases to facilitate their penetration through the plant cuticle (Hynes et al. 2006; Kolattukudy 1985; Voigt et al. 2005). Both types of enzymes are carboxyl ester hydrolases (CEH), classified in the CAZy database (http://www.cazy.org) as part of the carbohydrate esterase families 3 and 5, but their underlying mechanisms remain poorly understood. It has been suggested that secreted cutinases and lipases cleave the plant polyester and the released cutin monomers can induce the expression of cutinase genes, stimulating fungal differentiation, e.g., conidial germination or appressorium formation (Serrano et al. 2014). Cutinase production is observed in many bacterial and fungal genera (pathogenic and non-pathogenic). A variable number of genes encoding these enzymes have been found, ranging from three to seventeen in a single organism (Skamnioti et al. 2008). Furthermore, there is a high heterogeneity in the regulatory mechanisms involved in their expression even in closely related taxonomic groups. For example, *Fusarium solani* sp. *pisi* has three cutinase genes and the expression of *cut1* is highly induced by cutin monomers and is positively regulated by the transcription regulator factor (TF) CTF1α. Likewise, *cut2* and *cut3* show basal expression levels and are regulated by CTF1β (Li et al. 2002). In *Fusarium oxysporum*, the transcription factor CTF is dispensable for virulence but regulates expression of a cutinase and other enzymes involved in fatty acid hydrolysis. In *Alternaria brassicicola*, one TF is involved in virulence and affects the expression of one out of its nine cutinase genes (Srivastava et al. 2012), while in *Aspergillus oryzae*, a Zn finger TF involved in lipid metabolism affects the expression levels of cutinase and other lipolytic enzymes (Garrido et al. 2012). In *Aspergillus nidulans*, the transcriptional factors FarA and FarB are homologous to those reported in *F. solani* and are part of the global regulatory system that controls the utilization of lipids as carbon source, depending on the fatty acid chain length (Hynes et al. 2006). Although these TFs are widely distributed and have been identified through sequence homology in various fungal species (Hynes et al. 2006), their precise role in the gene regulation of biotechnologically relevant enzymes has not been investigated. Another aspect largely overlooked is that the use of complex lipidic substrates, such as cutin, or even olive oil (Castro-Ochoa et al. 2012) may be regulated by the carbon catabolite repression (CCR) system. This is a complex regulatory process which depends not only on the presence of the CreA regulatory elements (Sánchez and Demain 2002; Ries et al. 2016), but also on the availability of the carbon source, which may act as a strong, intermediate, or de-repressor of the CCR (Mogensen et al. 2006). Carbon regulation is crucial to the microbial adaptation to the environment by affecting the physiology, virulence, and pathogenicity as well as the cell-cell communication (Adnan et al. 2018).

The *A. nidulans* genome encodes four cutinase genes (Galagan et al. 2005). The phylogenetic relationships among these genes and their relationship to other fungal or bacterial cutinases have been reported elsewhere (Castro-Ochoa et al. 2012; Skamnioti et al. 2008). However, as only one cutinase (ANCUT2) has been fully characterized (Castro-Ochoa et al. 2012; Bermúdez-García et al. 2017), the action of the other three enzymes on cutin degradation has been unveiled. Besides, whether they are expressed simultaneously or sequentially and if they constitute a system required for cutin degradation are all unanswered questions. Furthermore, there is no information currently available concerning the action mechanism of each cutinase or the precise regulatory factors involved in their expression. Induction by some nutritional factors like the plant polyester suberin has been reported (Martins et al. 2014). Also, a thermoalkaline cutinase (ANCUT2), producing methyl esters as biodiesel precursors (encoded by the AN7541 gene), was found in supernatants of cultures grown in media with olive oil (Castro-Ochoa et al. 2012; Bermúdez-García et al. 2017). To the best of our knowledge, the differential expression of the remaining three cutinase genes *ancut1*, *ancut3*, and *ancut4*, encoded by AN5309, AN7180, and AN10346, respectively, has not been reported to date.

In the present work, the cutinase genes regulation was studied with two approaches: the effect of nutrients that could act as inducers or repressors, and the effect of TFs detected by in silico analysis of the regulatory regions of each gene. For this purpose, the expression levels of each cutinase gene were assayed in a wild-type strains and mutants deleted in the TF genes *far*A, *far*B, and *napA*. Finally, the role of each cutinase on cutin degradation was studied. In our results, the cutinolytic activity of the enzymes encoded by all four genes was detected, leading to understanding the physiological role of each cutinase on cutin degradation and showing how *A. nidulans* can express cutinases differentially, depending on the nutritional/growth conditions.

Materials and methods

Microorganism and growth conditions

All the strains used in this work were obtained by sexual crosses of strains derived from the original Glasgow strain FGSC A4. *Aspergillus nidulans* PW1 (*biA1*, *argB2*, *methG1*, *veA1*), a non-pathogenic arginine auxotroph (FGSC A1048) (Fungal Genetics Stock Center, Kansas City, MO), was used in this study as the wild-type strain. The *A. nidulans* mutants AN*Δfar*A (CFQ H 217) and AN*Δfar*B (CFQ H 218), affected in lipid transcription factors, and the stress sensitive strain *A. nidulans* AN*Δnap*A (CFQ H 216) were used to study the effect of TF with comparison purposes (Hynes et al. 2006;

Mendoza-Martinez et al. 2017) and are deposited with the Culture Collection of the Chemistry Faculty, UNAM, México (CFQ).

A basal medium was prepared as described by Käfer (1977) with pH adjusted to 6.5. For ANΔfarA, ANΔfarB, and ANΔnapA mutants cultivation, a vitamin stock was spiked as reported by Hynes et al. (2006). The basal medium contained 0.5% (*w*/*v*) glucose as carbon source, which in some conditions was replaced either by lipidic sources such as cutin, olive oil, 16-hidroxyhexadecanoate, propyl ricinoleate, triacetin, or tristearate, or non-lipidic ones including 1% glucose, starch and pectin, evaluated as inducers or repressors. All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). A medium lacking an inducer and repressor was formulated with proteose peptone as a carbon source (Difco, Beckton Dickinson, Heidelberg, Germany). Finally, to achieve oxidative stress conditions, 0.1 mM H_2O_2 was added to the cutin containing medium, according to Lee et al. (2010), who demonstrated that the expression of a cutinase from *Monilinia fructicola* was enhanced using 0.1–0.5 mM H_2O_2.

Fungal spores were produced on minimal nitrate agar plates; then the spores were washed with 0.1% (*v*/*v*) Tween 80 (Sigma Aldrich, St Louis MO, USA) solution, collected in sterile water and stored at 4 °C as previously described (Peña-Montes et al. 2008). A conidia stock containing 10^6 spores per milliliter was used to inoculate the growth media (50 mL); cultures were incubated for 24 h at 37 °C, under orbital agitation (300 rpm). Mycelia were harvested by filtration through Whatman paper no.1 (Sigma Aldrich, St Louis MO, USA), washed with sterile water and disrupted immediately by grinding with a pestle.

RNA isolation and cDNA synthesis

RNA was extracted using TRIzol (Life Technologies, Carlsbad, CA, USA) and precipitated with isopropyl alcohol and ethanol (Merck, México City, México). RNA integrity was verified electrophoretically: 2 μg was observed in 1.5% agarose gel containing 2.2 M formaldehyde (Merck, México City, México), and its purity was determined by the 260/280 nm ratio. RNA concentration was measured spectrophotometrically using a Take3 plate in an Epoch spectrophotometer (BioTek, Winooski, VT, USA).

DNA digestion of 1 mg total RNA was carried out with DNase I amplification grade (Thermo Scientific, Waltham, MA, USA) for 15 min at room temperature. DNase was inactivated by adding 1 mL of 25 mM EDTA to the reaction mixture followed by heating (65 °C, 10 min). Immediately afterwards, reverse transcription was performed with SuperScript II (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions and using dT Primers. Each experiment was run using biological triplicates.

Identification of cutinase genes in media with and without inducer by endpoint PCR

Specific primers were used for the amplification of the cutinase genes *ancut1* (*AN5309*), *ancut2* (*AN7541*), *ancut3* (*AN7180*), and *ancut4* (*AN10346*), and TF genes *farA* (*AN7050*) and *farB* (*AN1425*) by endpoint PCR. Primer sequences for cutinase genes are shown in Supplemental Table S1.

Analysis of transcription factors binding sites (TFBS)

The sequences found upstream the structural cutinase genes were analyzed in silico with the PROMO tool using version 8.3 of TRANSFACT (Messeguer et al. 2002; Farré et al. 2003) to detect TFBS.

Design and validation of expression assays (qRT-PCR)

Design of primers and probes

The PrimerQuest (IDT, Coralville, IA, USA) software was used for the design of qRT-PCR primers and probes with the following specifications: i) for primers—product size 75–150 pb, primer size 17–30 pb, GC 35–65%, Tm 59–65 °C; ii) for probes—probe size 20–30 pb, GC 40–60%, Tm 64–72 °C. Complete sequences of endpoint PCR products were used as templates for the design of primers and probes. Primer or probe sequences were selected to be complementary to an exon-exon junction, to ensure amplification from the cDNA template and not from genomic DNA. The efficiency of primers and probes from qPCR was tested for each gene using serial dilution curves (500–0.5 ng). Sequences of all genes used in this work were obtained from Aspergillus Genome Database (AspGD); the primers used are shown in Supplemental Table S2.

qPCR conditions and analysis

Real-time PCR was performed in the 7500 Real-Time PCR System (Thermo Scientific, Waltham, MA, USA), using the TaqMan Gene Expression Master Mix (Thermo Scientific, Waltham, MA, USA). The qPCR assays were run following the default amplification protocol (50 °C for 2 min, 90 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min). In all experiments, appropriate negative controls containing no cDNA template were amplified to detect potential cross-contamination. Each experiment used three biological replicates, each with three technical replicates. Data were normalized according to the Pfaffl modification of the Double Delta formula (Pfaffl 2001) and using the selected endogenous control. Minimal media (glucose 0.5%) were used as the control condition for all assays. Real-time PCR was performed as 1 uniplex and 2 duplex assays, using each set of primers and

probe for the genes under investigation (refer to Supplemental Table S3 for the final concentrations of primers and probes). For the duplex assays, the fluorophores chosen were FAM, Cy3, and Cy5. Efficiency curves were drawn for each gene in the multiplex and uniplex assays. The results obtained were analyzed using the Applied Biosystems 7300–7500 SDS Software (Thermo Scientific, Waltham, MA, USA) and the expression level was calculated using equation 1:

$$R = \frac{E_{target}^{\Delta Ct\ target(control-sample)}}{E_{ref}^{\Delta Ct\ ref\ (control-sample)}} \quad (1)$$

where E_{target} and E_{ref} were the efficiencies obtained for each gene in the study and the endogenous gene, respectively, and ΔCt was the difference between the Ct achieved in the control condition and the Ct achieved in the different media. The data reported here are the average of three separate experiments run in triplicate. In the "Results" section, data are plotted as log(R), where zero values mean no change in expression vs. control; positive values indicate an increased expression and negative values point to downregulation.

Reference gene selection

The genes encoding β-tubulin (*tubC*) and ubiquitin (*ubq1*) (AN6838 and AN4872, respectively) were selected as suitable reference genes for the qPCR analyses according to the literature (Semighini et al. 2002; Noventa-Jordao et al. 2000). Efficiency curves were drawn for each gene to optimize amplification and both genes yielded similar amplification efficiencies (ca. 90–95%). qPCR assays were carried out using the cDNA obtained from mycelia grown in different media as a template. The Cts for both reference genes were analyzed statistically to test for significant differences between conditions using one-way ANOVA with a 95% significance. Statistical analyses were carried out using the GraphPad Prism 5 software (GraphPad, La Jolla, CA, USA). The gene *ubq1* was used in subsequent analyses as its Ct showed less variability under different growth conditions.

The next step was the optimization of the conditions to perform two multiplex and one uniplex assays for analyzing the expression levels of genes *ancut1*, *ancut2*, *ancut3*, and *ancut4*. The genes *ancut1* and *ubq1* were detected in the first assay and *ancut3*, and *ancut4* in the second one. For its part, *ancut2* was analyzed in a uniplex assay. To define the appropriate concentrations of each set of primers, amplification curves were constructed using successive dilutions of cDNA ranging from 500 to 0.5 ng as a template. The optimal concentrations of primers and probes (i.e., 90–110% efficiency) are listed in Supplemental Table S3.

Enzymatic assays

Quantification of carboxylesterase activity

Carboxylesterase activity was quantified by measuring, in a microtiter test at 410 nm, the conversion of the substrate *p*-nitrophenyl acetate (*p*-NPA, Sigma-Aldrich, St Louis, MO, USA) to *p*-nitrophenol (*p*-NP): 170 μL of 50 mM phosphate buffer pH 7.2 + 20 μL of 1 mM *p*-NPA in ethanol + 10 μL of enzyme extract. In the negative (abiotic) control, the enzyme extract was replaced by the buffer. Each enzymatic assay was performed in triplicate. The yield of the reaction was measured at 1-min intervals over 10 min. A programmed protocol in the software Gen5 1.10 provided with the Epoch spectrophotometer (BioTeK, Winooski, VT, USA) was used. One activity unit was defined as the amount of enzyme required to convert 1 μmol *p*-NPA to *p*-NP per minute under the specified conditions. A calibration curve correlating optical density with *p*-NP concentration was used to estimate the formation of *p*-NP. The standard curve was prepared in ethanol with *p*-NP concentrations ranging from 25–200 μmol, and a molar extinction coefficient of 4.900 cm^{-1} M^{-1} was obtained.

SDS-PAGE and zymograms

Proteins were recovered from the supernatant fraction of fungal cultures using Amicon Ultra-15 centrifugal filter units (Merck Millipore, Burlington, MA, USA). Protein concentration was determined using the Bradford protein assay kit (Bio-Rad Laboratories, Irvine, CA, USA) according to the manufacturer's instructions for the microplate format. Bovine serum albumin was used as the protein standard (Bradford 1976), at 595 nm, using the protocol provided with the Gen5 1.10 spectrophotometer software (BioTek, Winooski, VT, USA).

SDS-PAGE was carried out using 14% T acrylamide gels, as previously described (Laemmli 1970). The molecular mass of proteins was determined by comparing their mobility relative to that of a low-range protein marker containing a mixture of six proteins ranging in size from 14 to 97 kDa (Bio-Rad Laboratories, Irvine, CA, USA). Samples (50 μg per lane), diluted in Laemmli 4× sample buffer devoid of β-mercaptoethanol, were not heated. As controls, the electrophoretic protein profiles were also visualized with either silver or Coomassie blue, documented using the Gel Doc imaging system and analyzed with the ImageLab 4.0 software (Bio-Rad Laboratories, CA, USA) (data not shown). After the electrophoretic separation, esterase activity against α-naphtyl acetate was detected using zymography (Castro-Ochoa et al. 2012); activity is evidenced by the formation of dark red bands in the gel.

Cutin isolation and characterization

Golden Delicious apple cutin was prepared as described by Walton and Kolattukudy (1972). The chemical composition of cutin was determined spectrometrically using ATR-FTIR. Spectral measurements were recorded on a Brüker IFS66/S FTIR spectrometer (Brüker Daltonics, Billerica, MA, USA) using a single reflection ATR cell (DuraDisk, fitted with a diamond crystal). Data were recorded at room temperature within the range of 4000–600 cm^{-1} by accumulating 258 scans with a 4 cm^{-1} resolution. Five spectrum replicates of each sample were recorded to evaluate reproducibility (OPUS v6.5, Bruker, Billerica, MA, USA) (Supplemental material Fig. S1).

Analysis of the degradation products generated by each cutinase

Supernatants from media containing only one of the studied cutinases as the main enzyme were incubated with 500 mg cutin as described previously (Castro-Ochoa et al. 2012). The soluble products of the enzymatic hydrolysis of cutin were obtained from filtrates using a hexane:ethyl acetate extraction. Negative controls, i.e., non-inoculated cutin media incubated under the same conditions as the enzymatic reactions, were also tested. All the organic extracts were dried under soft vacuum, resuspended in methanol, and analyzed by ultra-high-performance liquid chromatography-electrospray-high resolution mass spectrometry-mass spectrometry (UHPLC-ESI-HRMS/MS) using an Agilent 6560 Ion Mobility Q-TOF LC/MS system (Agilent Technologies, La Jolla, CA, USA) fitted with an electrospray ionization source (ESI-II). Chromatographic separation was carried out in an UHPLC Agilent 1260 Infinity II LC system (Agilent Technologies, La Jolla, CA, USA) system using an Agilent Technologies column (150 × 2.1 mm, 2.7 μm particle size) from Supelco Inc. (Bellefonte, PA, USA). The mobile phase at 500 μL/min flow rate, consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B), sets as follows: 10% B for 1 minute, followed by a linear gradient of 10–95% B over 4.7 min, 1.3 min to reach 100% B, 3 min at 100% B, 0.5 min to return to the initial conditions, and 5.5 min to re-equilibrate the column. ESI-II was operated in the positive ionization mode. Nitrogen was used as sheath gas, sweep gas, and auxiliary gas at flow rates of 10 a.u. (arbitrary units) in all cases. Capillary temperature and electrospray voltage were set at 300 °C and − 2.5 kV, respectively. An S-Lens RF level of 50 V was used. The HRMS instrument was operated in full MS scan with *m/z* ranging from 100 to 1700. The mass resolution was tuned to 70,000 full-width half maximum (FWHM) at *m/z* 200, with an automatic gain control (AGC) target (the number of ions to fill C-Trap) of 5.0E5 and a maximum injection time (IT) of 200 ms. The full MS scan was followed by a data-dependent scan operated in the All Ion Fragmentation (AIF) mode with a 30-eV fragmentation energy applied to the high-energy collision dissociation (HCD) cell. At this stage, the mass resolution was set at 17,500 FWHM at *m/z* 200, AGC target at 5.0E5, maximum IT at 200 ms, with a scan range from *m/z* 100 to 1700. MS data were processed with the MassHunter™ 2.0 software (Agilent Technologies, La Jolla, CA, USA) by applying the METLIN Lipids database list. Several parameters, such as retention time, accurate mass errors, and isotopic pattern matches, were used to confirm the identity of compounds. When available, standards were also analyzed by UHPLC-ESI-MS for further confirmation of identity.

Results

Differential expression of the cutinolytic system induced by lipidic substrates

The sequences of the four cutinase genes and their encoded proteins in the *A. nidulans* genome, obtained from the AspGD, were analyzed. A high homology (60%) was found between the protein sequences encoded by genes AN5309 (*ancut1*) and AN4571 (*ancut2*), while AN10346 (*ancut4*) had the lowest.

The effect of lipidic and non-lipidic carbon sources on the expression levels of *ancut1*, *ancut2*, and *ancut3* was measured by qRT-PCR in *A. nidulans* grown in media containing different carbon sources. The expression of *ancut4* was not detected under any of these conditions. As a negative control, cultures were incubated in 0.5% glucose, in the absence of cutin, a condition where no cutinase activity was previously detected (Castro-Ochoa et al. 2012).

The highest expression of the cutinase genes, especially *ancut1* (ca. A 10,000-fold increase), was observed when the fungus was grown in cutin-containing media (Fig. 1a). The presence of lipidic substrates, either carrying long carbon chains (≥C16) or triglycerides containing short and long aliphatic carbon chains, resulted in 10- to 100-fold increase in the expression of *ancut1*. In contrast, the *ancut2* expression levels underwent upregulation in the presence of either cutin or olive oil (2 to 8-fold increase) but were virtually unaltered by either 16-hydroxyhexadecanoate or propyl ricinolate, considered synthetic cutin monomers nor by any of the triglycerides tested herein. Finally, *ancut3* expression levels were not significantly altered by any of the lipidic carbon sources used in this assay.

Non-lipidic carbon sources, such as 1% glucose, strongly repressed the expression of *ancut1* and *ancut2* (Fig. 1b).

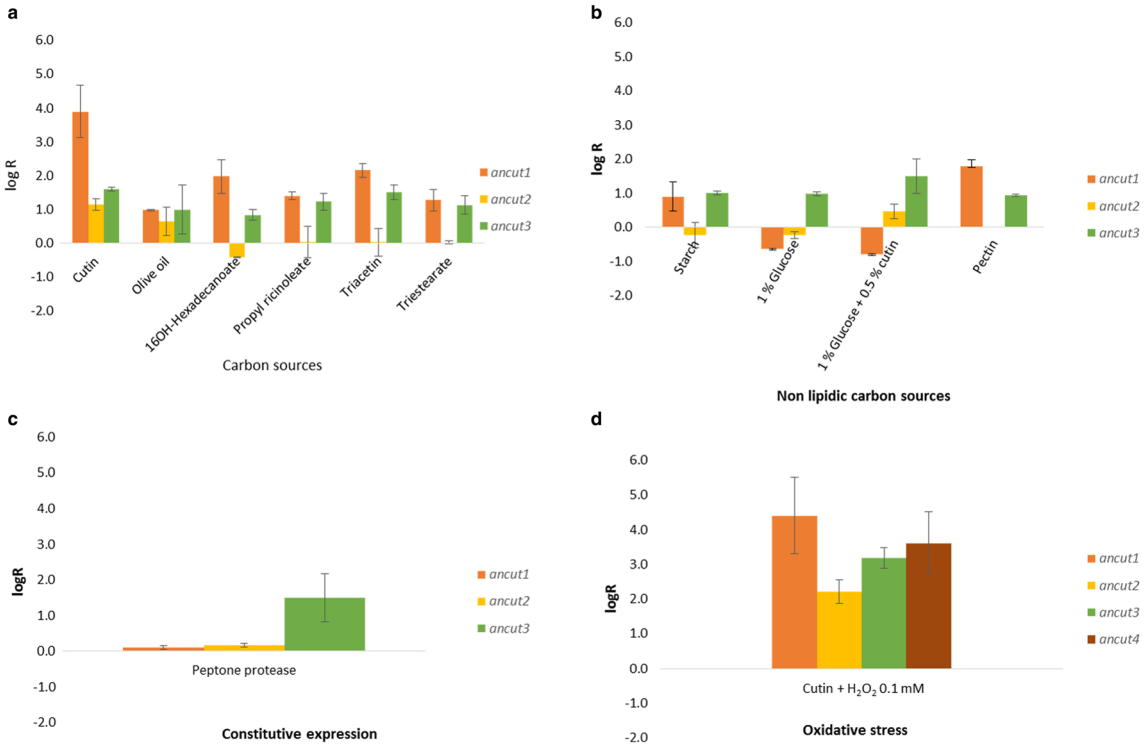

Fig. 1 Expression levels of the cutinase genes *ancut1*, *ancut2*, and *ancut3*, relative to the expression of the reference endogenous gene selected, when *A. nidulans* was grown for 24 h in different conditions: lipidic carbon sources as possible inducers (**a**), non-lipidic carbon sources as possible repressors (**b**), under constitutive expression conditions (**c**), and under oxidative stress conditions (**d**). logR is the average expression by qRT-PCR of three biological replicates, each analyzed in three technical replicates

However, this sugar had virtually no effect on the expression levels of *ancut3*. In addition, when cutin was added to the 1% glucose medium, *ancut1* expression levels were extremely repressed (ca. three orders of magnitude), and the repression of this gene was more intense relative to that shown by *ancut2*. Pectin and starch caused a slight repression of *ancut2* (ca. 0.5 times), but no significant response was observed for *ancut1*. A constitutive expression of *ancut3* was suggested as the enzyme levels were affected neither by inducer nor repressors. This assumption was corroborated by measuring *ancut3* in cells grown in a medium lacking both inducers and repressors (Fig. 1c).

Detection of *ancut4* was possible when cutin was used as a carbon source and H_2O_2 was added to generate oxidative stress, as described in the "Materials and methods" section. Under these conditions, the expression of *ancut4* was increased almost 4000-fold (Fig. 1d).

An in silico analysis was performed to identify the putative regulatory sequences present upstream of each gene. The recognized sites by FarA and FarB were identified in all cases, except for *ancut4*. In this last case, sequences responding to transcriptional factors involved in the oxidative stress such as NapA were detected (Mendoza-Martinez et al. 2017). Additionally, recognition sites for CreA were identified to corroborate the CCR effect (Table 1).

Differential expression of cutinases in mutants affected in the transcription factors

To explore whether the constitutive or inducible expression of the cutinase genes was regulated by the transcriptional factors suggested by in silico analysis, FarA or FarB (involved in lipid metabolism), or NapA (which regulates the oxidative stress response), the expression of the four *ancut* genes was analyzed in *A. nidulans* mutants deleted in each one of the TF genes: ANΔ*far*A, ANΔ*far*B, and ANΔ*napA*. As shown in Fig. 2, the expression of a*ncut1* and *ancut2* was repressed in the ANΔ*far*A mutant, even when grown in the presence of cutinase inducers. The *ancut1* expression levels in the ANΔ*far*A mutant showed a three orders of magnitude decrease compared to levels obtained in the wild-type strain PW1 in the presence of cutin. The expression of *ancut3* remained unaltered in the presence of cutin but was repressed when olive oil and glucose were used as carbon sources (Fig. 2a).

In media containing cutin, deletion of *far*B led to the partial repression of *ancut1* and *ancut2*, and total repression of *ancut3*, while in olive oil, only *ancut1* underwent upregulation (Fig. 2b). Under oxidative stress conditions, the mutants lacking the NapA TF expressed *ancut1*, *ancut2*, and *ancut3* while *ancut4* was totally repressed. It

Table 1 Analysis of the transcription factors potential binding sites in the 5′ region of the cutinase genes

Sequence motif	TF	Gene	Name	5′ region position
CCTGCC/GGCAGG	FarA, FarB	AN5309	*ancut1*	-222, -370, -496
		AN7541	*ancut2*	-245, -389, -640
		AN7180	*ancut3*	-212, -629
		AN10451	*ancut4*	NI
CCGGGG	CreA	AN5309	*ancut1*	-963, -557, -526
		AN7541	*ancut2*	-390, -329
		AN7180	*ancut3*	-970, -750
		AN10451	*ancut4*	-606
GGAATTGGGGCATTGG	NapA/NF-Y1	AN10451	*ancut4*	-28, -124,-238, -370

The analysis was conducted with the PROMO tool using version 8.3 of TRANSFACT (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 / Messeguer et al. 2002)

NI not identified

must be noted that the expression level of *ancut1* was 100-fold lower in the AN*ΔnapA* mutant than in the PW1 strain grown under the same conditions. However, no change in the expression pattern of *ancut2* and *ancut3* was observed (Fig. 2c).

CEH activity in crude extracts and zymograms

The effect of lipidic and non-lipidic carbon sources on esterase activity was assayed. As shown in Fig. 3, the highest esterase activity was achieved when cutin was used as carbon source, followed by those obtained in olive oil and pectin and finally, the other complex carbon sources present in plants, like cutin monomers or starch. The culture media which showed less activity were those where glucose 1% was present.

The cutinase enzymes were visualized by zymogram assays. As seen in Fig. 4, when the PW1 strain was grown in cutin or in its monomer, 16-hydroxyhexadecanoate, the molecular mass of the detected bands matched that of ANCUT1, while no activity was detected in extracts from a glucose-containing medium. In addition, when olive oil was used as inducer, a different activity band was detected, which matched the molecular mass of ANCUT2. These results were consistent with those previously reported, where ANCUT1 and ANCUT2 were identified by mass spectrometry (Castro-Ochoa et al. 2012). The constitutive expression of ANCUT3 was verified in peptone cultures lacking cutin or any other lipidic source. Finally, when H_2O_2 was added to cutin media, ANCUT4 activity was detected (Fig. 4). Both ANCUT3 and ANCUT4 were identified by mass spectrometry as shown in the Supplemental Table S4. In pectin-containing medium, a high molecular weight band with esterase activity was detected, but it does not correspond to any of the four cutinases encoded by *A. nidulans*.

Cutinase activities and identification of the cutin degradation products

The ability of fungal cultures to degrade cutin was assayed by incubating the *A. nidulans* mycelium with cutin. The AT-FTIR spectra shown in Supplemental Fig. S1 indicate that cutin was degraded into several products.

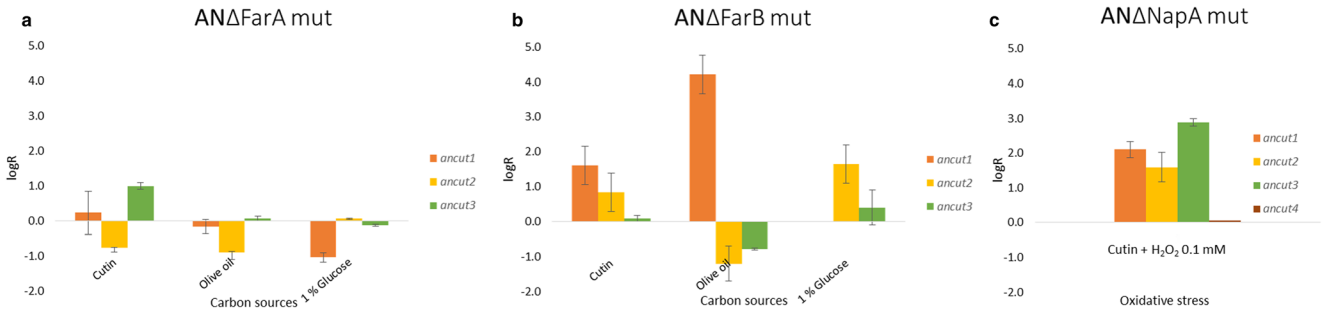

Fig. 2 Expression levels of the *A. nidulans* cutinase genes *ancut1*, *ancut2*, and a*ncut3* in the AN*ΔfarA* (**a**), AN*ΔfarB* (**b**), and ANΔnapA (**c**) mutants relative to the expression of the endogenous gene selected. The Far mutants cDNA was obtained from *A. nidulans* strains grown in media supplemented with cutin or olive oil (inducers), or 1% glucose (repressor). The NapA mutants cDNA was obtained from *A. nidulans* strains grown in media supplemented with cutin and induced with 0.1 mM H_2O_2. logR is the average expression by qRT-PCR of three biological replicates, each analyzed in three technical replicates

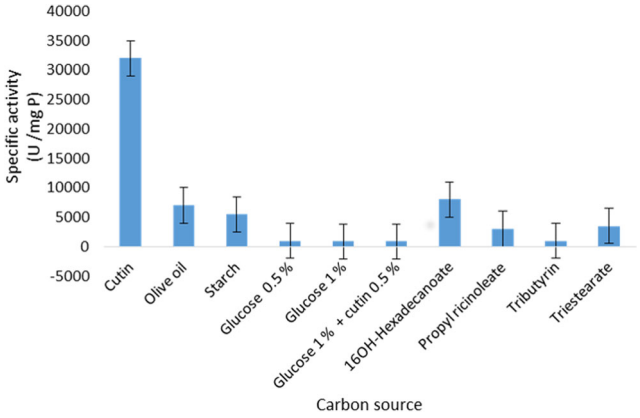

Fig. 3 Specific carboxyl esterase activity of *Aspergillus nidulans* PW1 extracts grown in media supplemented with different carbon sources, either lipidic or non-lipidic. Activity was measured spectrophotometrically using *p*-NPA as substrate. One activity unit is defined as the amount of enzyme (in mg) necessary to obtain 1 mmol *p*-NP per time unit

To gain a deeper insight into the role of each cutinase on cutin degradation, extracts from media allowing production of only one cutinase, or at least a preponderant one, were incubated with cutin. The products released were analyzed by U-HPLC-MS-MS. As shown in Table 2, the products yielded by ANCUT1 and ANCUT3 were similar and corresponded to the main reported cutin constituents (Hernández-Velasco et al. 2017). However, the presence of 9,10-dihydroxyhexadecanoic acid in samples incubated with ANCUT1 suggests that the enzyme cleaves the cross-linking bonds, which are formed by the secondary alcohol esters; therefore, ANCUT1 could be considered as an endo cutinase. The products yielded by ANCUT2 and ANCUT4 were abundant in 18-C chains and corresponded to ω fatty acids. However, the achieved degradation levels of ANCUT 4 were lower compared to the other three cutinases.

Discussion

The variability in the number of cutinases codified by different fungal species has raised questions about the biological significance of these enzymes. The divergent evolution of the encoding genes could lead to more efficient enzymes and to a better adaptation to different niches. If differences appear in the regulatory regions, the possibility of colonizing different environments is enhanced (Skamnioti et al. 2008; Adnan et al. 2018). Furthermore, the biotechnological applications of enzymes with different physicochemical properties would also be different.

These questions were explored in the four cutinases encoded by *A. nidulans*. The analysis of the coding regions indicated that all of them shared both the cut-1 motif (GYSQG), containing the cutinase active serine, and the cut-2 motif that carries the aspartate and histidine residues of the active site (Ettinger et al. 1987). The homology found among the different cutinase genes was consistent with that reported by other authors (Skamnioti et al. 2008; Castro-Ochoa et al. 2012), suggesting evolutionary differentiation.

On the contrary, important differences were found among the regulatory regions. These included the presence of binding sites for different transcription factors that resulted in their expression under different conditions. As expected, most of the genes required cutin to induce their expression but the upregulation levels were different for each gene: *ancut1* underwent a 10,000-fold increase, while *ancut2* underwent just a 10-fold increase and the expression levels of *ancut2* were similar in both cutin and olive oil–supplemented media. This difference in behavior cannot be explained only by differences in the regulatory sequences, as both responded to the presence of FarA, the main regulator of the metabolism of long-chain fatty acids (Hynes et al. 2006). FarA is

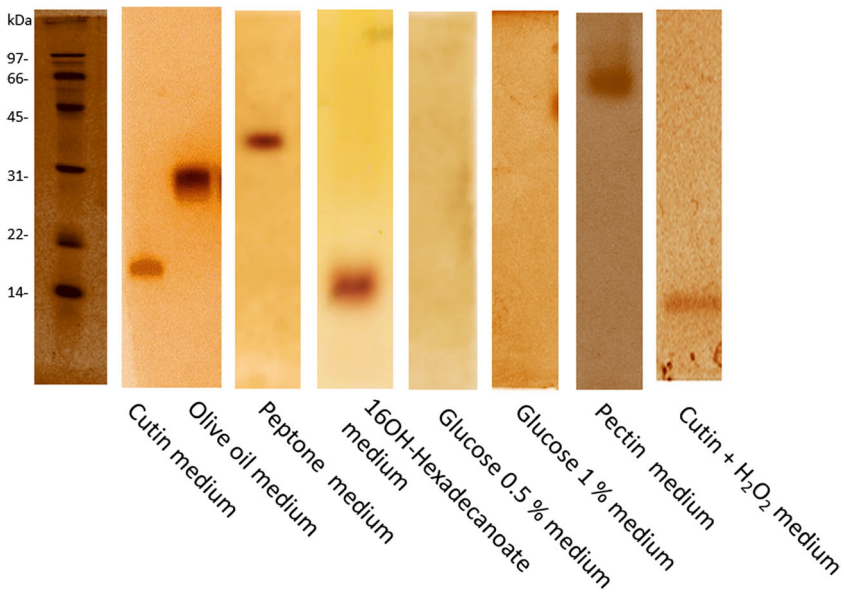

Fig. 4 Zymograms from cultures of the wild strain (PW1) grown in different carbon sources. Broad Range Molecular Weight Marker was used as a protein size standard. The protein electrophoretic profiles were also visualized with either silver staining or Coomassie blue (data not shown)

Table 2 Cutin degradation products obtained after enzymatic hydrolysis

Enzyme	Compounds	Formula	$MW_{detected}$	Error*
ANCUT1	Phloionolic acid	$C_{18}H_{36}O_5$	332.2569	1.9700
	7-Keto palmitic acid	$C_{16}H_{30}O_3$	270.2198	1.1000
	9,10-Dihydroxy-hexadecanoic acid	$C_{16}H_{32}O_4$	288.231	3.380
	10-Hydroxy-16-oxo-hexadecanoic acid	$C_{16}H_{30}O_4$	286.2138	2.1600
	9-Hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	294.2199	1.2300
ANCUT2	Phloionolic acid	$C_{18}H_{36}O_5$	332.2565	0.5400
	10-Hydroxy-16-oxo-hexadecanoic acid	$C_{16}H_{30}O_4$	286.2149	1.8900
	9-Hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	294.2194	0.2700
	9-Hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	294.22	1.72
ANCUT3	Phloionolic acid	$C_{18}H_{36}O_5$	332.256	0.900
	7-Keto palmitic acid	$C_{16}H_{30}O_3$	270.2181	4.9900
	10-Hydroxy-16-oxo-hexadecanoic acid	$C_{16}H_{30}O_4$	286.2142	0.5600
	9-Hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	294.2194	0.2500
ANCUT4	Phloionolic acid	$C_{18}H_{36}O_5$	332.2554	2.5100
	9-Hydroxy-10E-octadecen-12-ynoic acid	$C_{18}H_{30}O_3$	294.2195	0.0700

*Error, absolute value of the deviation between measured mass and theoretical mass of the selected peak in ppm

homologous to a TF that proved to be key in the regulation of *cut1* from *F. solani* (Li et al. 2002). A different behavior was found for *ancut3* expression, as this was constitutively produced, while *ancut4* was induced by cutin but only under oxidative stress conditions.

The overall behavior in the presence of non-lipidic carbon sources was consistent with the CCR mechanism proposed for cellulases in filamentous fungi (Gutiérrez-Rojas et al. 2015). Our results evidenced that either glucose or starch strongly represses expression of *ancut1* and *ancut2*, while pectin had a stronger repressive effect only on *ancut2*. On the contrary, non-lipidic carbon sources did not affect *ancut3* expression.

An explanation for the different responses to inducers may be found in the experiments performed with the mutants deleted in the TFs. In the mutants ANΔ*far*A and ANΔ*far*B, cutinase gene expression decreased in media supplemented with cutin, while no cutinase activity was observed in the respective zymograms. Similar to previous studies, the ANΔ*far*A mutant failed to grow in media containing the long-chain carbon source 16-hydroxyhexadecanoate (Hynes et al. 2006). The expression of *ancut1* and *ancut2* decreased up to 100-fold in ANΔ*far*A cultures grown in cutin media while the expression levels of *ancut3* were maintained, meaning that FarA did not influence the expression of *ancut3* when cutin was present. However, when olive oil, rich in long-chain fatty acids, was used as a carbon source, the expression of all three cutinase genes was diminished. Even *ancut3* showed a 10-fold decreased expression, which could signify that when the TF FarA is missing, all the lipidic metabolism is affected. When the ANΔ*farA* mutant was grown in 1% glucose medium, the expression levels of *ancut1* and *ancut2* were less repressed than in the wild-type strain but the expression of *ancut3* was negatively affected perhaps due to other TF involved in CCR regulation. These results reinforce the hypothesis that there is a relationship between the regulation of the cutinolytic system and CCR where FarA plays a key role but the overall mechanism is not yet fully understood (Li et al. 2002) (Fig. 2).

In the case of the ANΔ*farB* mutant, the expression levels of *ancut3* decreased significantly under all tested conditions, suggesting that FarB may regulate the constitutive expression of *ancut3* in *A. nidulans*. Interestingly, in the ANΔ*farB* mutant, the expression levels of *ancut1* and *ancut2* were partially decreased in cutin medium, although *ancut1* levels were increased in olive oil medium. The likely explanation is that in the absence of ANCUT3 in the cutin medium, there were no monomers to induce the expression of *ancut1* or *ancut2*. In the presence of the repressor glucose (1%), the relative expression levels of both cutinase genes were apparently higher in the ANΔ*farB* mutant compared to the wild-type strain (Figs. 1b and 2). This result suggested an attenuation of the CCR mechanisms when the FarB regulator was not expressed.

The in silico analysis of the upstream sequence of *ancut4* (Table 1) revealed binding sites for TF involved in oxidative stress responses, such as AP-1-like elements, transcriptional factors that are activated after the generation of reactive oxygen species, involved in defense and repair in yeast, fungi, and mammals (Toone et al. 2001), as previously reported for other cutinases or cell wall–degrading enzymes (CWDE) (Lee et al. 2010). In previous reports, when the transcriptional factors Yap1/Pap1/NapA were deleted, the fungus lost its ability to resist oxidative stress caused by H_2O_2 leading to downregulation of *ancut4* (Mendoza-Martinez et al. 2017). In the

present study, the expression of *ancut4* was only detected in assays performed under oxidative stress while in the deletion mutant ANΔ*napA*, no expression of this cutinase gene was detected. This result confirmed that the expression of this fourth cutinase was related to the response to stress conditions. As mentioned above, *A. nidulans* has a saprophytic way of life. Therefore, the first three cutinase genes would allow the fungus to grow on decayed plant material, while ANCUT4 would be more useful when colonizing living plants, where stress is more likely to occur.

Although the expression of several genes could be detected in different culture media, zymograms from each medium showed only a single activity band; these results were confirmed when proteins were identified by LC-MS (Supplemental Table S4). These results suggested a post-transcriptional or post-translational mechanism that would explain, for example, why ANCUT3 is not observed in the zymograms even if it is expressed constitutively and is not affected by CCR. Probably, this cutinase acts as the trigger of the infection and its activity was only detected before penetration, as previously shown in the oomycete *Phytophthora capsica*, which expressed constitutive cutinases only upon host contact (Munoz and Bailey 1998). Some of the post-transcriptional/translational modifications that could lead to the protein inactivation could be phosphorylation or ubiquitination, as observed before for *Saccharomyces cerevisiae* (Oliveira and Sauer 2011). However, more experiments should be performed over ANCUT3 to answer this question.

The fact that a culture condition specific for each cutinase was established allowed an analysis of the cutin degradation pattern. The results indicate that the type of monomers released likely depends on the mechanism of action of each cutinase. ANCUT2 and ANCUT3 release fatty acids typically found in the ends of the molecule, which are involved in the formation of primary esters. As already mentioned, 9,10-dihydroxyhexadecanoic acid is a precursor in the formation of cross-linking bonds, which are inaccessible to most enzymes, making cutin more resistant. The fact that ANCUT1 is the only enzyme of the four cutinases produced by *A. nidulans* that has the ability to release this monomer could explain why it exhibits the highest expression levels in the

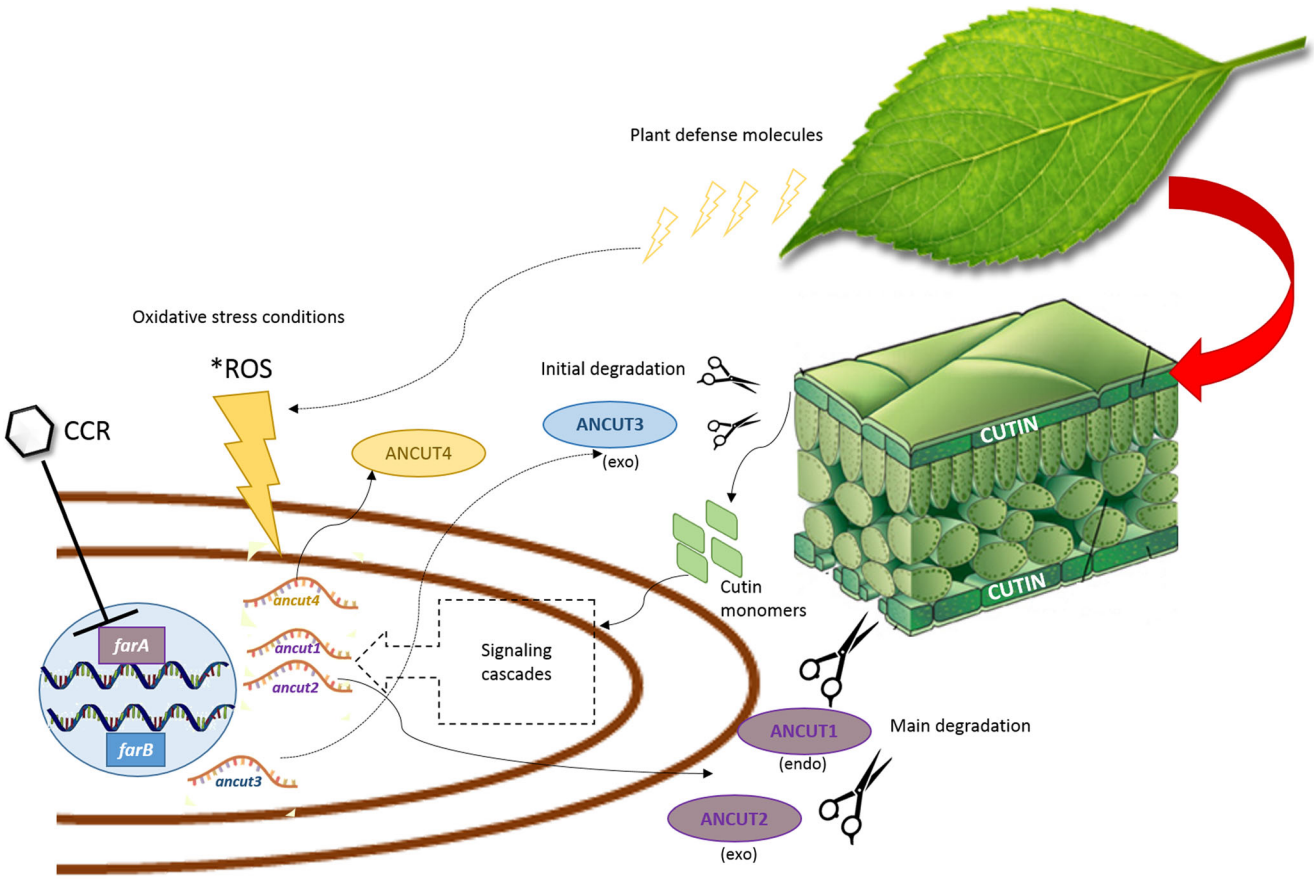

Fig. 5 Proposed regulatory mechanism for the cutinolytic system of *A. nidulans*. Far regulators affect the expression of three cutinase genes. These allow the fungus to detect cutin even in the presence of repressors, hence initiating its degradation, consistent with the increased expression of *ancut3*. This initial degradation releases cutin monomers that induce *farA* and, consequently, the expression of genes *ancut1* and *ancut2*. The expression of *ancut4* occurs under oxidative stress conditions as a mechanism to avoid plant defenses

presence of cutin. The activity of ANCUT4 was limited to the release of ω fatty acids. It must be stated that even if no pure enzymes were used to obtain these results, the degradation patterns of cutin after treatment with each native cutinase correspond to those obtained with supernatants from recombinant strains expressing each one of the four cutinases independently (data not shown, manuscript in preparation). All the above information led to the proposal of the integrated model of regulation shown in Fig. 5. However, there are no reports explaining the mechanisms of enzymatic cutin degradation, as most of the works have been performed with chemical methods, so it is difficult to compare these results with those obtained from other systems.

The results presented in this work showed that the four cutinase genes encoded by the *A. nidulans* genome are no longer hypothetical cutinases as they were independently expressed and hydrolyzed cutin to yield different products. One of them, ANCUT1, seems to cleave the polymer in an endolytic way, a feature not previously reported. The four genes displayed a different response to global regulators: three of the genes seem to constitute a cutinolytic system which is globally regulated by both CCR and by TF associated with lipid metabolism. The fungus may detect cutin even in the presence of repressors and start its degradation to produce cutin monomers because ANCUT3 has a basal expression, regulated by FarB, and is unaffected by CCR. These monomers induced FarA and later cutin could be degraded either by ANCUT1 or ANCUT2 as illustrated by the mechanistic proposed model shown in Fig. 5. Both genes were induced by different lipidic sources, and their enzymes generated different degradation products. The functional role of *ancut4*, phylogenetically more distant to the other three and whose regulation is performed under stress conditions, requires further analysis. As a final remark, this work offered an initial insight about how structural and regulatory gene divergence led to a comprehensive strategy to colonize cutin-containing niches. This divergence also resulted in cutinases with different biochemical properties that may find various biotechnological uses, such as ANCUT1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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