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BIOLOGICS

Patulin transformation products and last intermediates in its biosynthetic pathway, E- and Z-ascladiol, are not toxic to human cells

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Abstract Patulin is the main mycotoxin contaminating apples. During the brewing of alcoholic beverages, this mycotoxin is degraded to ascladiol, which is also the last precursor of patulin. The present study aims (1) to characterize the last step of the patulin biosynthetic pathway and (2) to describe the toxicity of ascladiol. A *patE* deletion mutant was generated in *Penicillium expansum*. In contrast to the wild strain, this mutant does not produce patulin but accumulates high levels of E-ascladiol with few traces of Z-ascladiol. This confirms that *patE* encodes the patulin synthase involved in the conversion of E-ascladiol to patulin. After purification, cytotoxicities of patulin and E- and Z-ascladiol were investigated on human cell lines from liver, kidney, intestine, and immune system. Patulin was cytotoxic for these four cell lines in a dose-dependent manner. By contrast, both E- and Z-ascladiol were devoid of cytotoxicity. Microarray analyses on human intestinal cells treated with patulin and E-ascladiol showed that the latter, unlike patulin, did not alter the whole human transcription. These results demonstrate that E- and Z-ascladiol are not toxic and therefore patulin detoxification strategies leading to the accumulation of ascladiol are good approaches to limit the patulin risk.

Keywords Ascladiol · Patulin synthase · *Penicillium expansum* · *patE* gene · Cytotoxicity · Microarray analysis

Introduction

Patulin is a toxic secondary metabolite mainly produced by *Penicillium expansum*, a fungus known as the most serious apple post-harvest pathogen (Morales et al. 2008). Produced during fungal growth, patulin is not necessary for the installation of the blue mold decay disease but acts as a cultivar-dependent aggressiveness factor (Snini et al. 2016).

Due to the conjugated double bonds, patulin displays an electrophilic structure that allows it to interact irreversibly with cellular nucleophilic groups (Fliege and Metzler 2000a, b). This property can lead to chromosomal

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aberrations and micronucleus formation (Glaser and Stopper 2012) and hence confer to patulin cytotoxicity and genotoxicity potencies. However, the absence of significant carcinogenic effects in human studies led IARC to classify patulin in group 3 (not classifiable as carcinogenic to humans) (IARC 1986). Patulin was also shown to be a potent immunotoxin (Puel et al. 2010). Tolerable daily intakes have been fixed at 0.4 µg/kg body weight/day by the Joint WHO/FAO Committee on Food Additives (JECFA 1995).

All apple-based products such as juices, beverages, and compotes are susceptible to be contaminated by patulin. However, cider and other alcoholic beverages contain patulin at low level due to its transformation by yeasts during alcoholic fermentation (Moss and Long 2002). *Saccharomyces cerevisiae* converts patulin into two isomers of ascladiol, E-ascladiol and Z-ascladiol. Other microorganisms belonging to different taxa such as bacteria (Ricelli et al. 2007), ascomycetes (Dong et al. 2015), or basidiomycetes (Wright et al. 2007) are also able to degrade patulin into ascladiols. The use of these species as biocontrol agents to prevent patulin production in apple-based products is a promising alternative to chemical-based approaches that remain the main measure for the control of postharvest diseases in apples. Indeed, the utilization of synthetic fungicides appears to be not sustainable because of their health and environmental risks. Moreover, *P. expansum* has developed a resistance toward antifungal compounds currently used in the postharvest stage such as thiabendazol, fludioxonil, or pyrimethanil (Errampalli et al. 2006; Li and Xiao 2008; Caiazzo et al. 2014).

Biosynthetic pathways are also the major targets for reducing toxins' production. Patulin biosynthesis has been investigated for many years and a biosynthetic scheme has been proposed (Puel et al. 2010), although a lot of gray zones remain. This pathway requires at least ten enzymatic steps and involves fifteen genes, recently localized within a cluster on a 40-kb DNA fragment in *Aspergillus clavatus* and *P. expansum* (Artigot et al. 2009; Tannous et al. 2014). Similar to other polyketide mycotoxins, patulin derives from an acetate starter group with malonate being the chain extender (Steyn 1991). The four early steps of this pathway have been well characterized and lead to gentisyl alcohol synthesis (Beck et al. 1990; Artigot et al. 2009; Snini et al. 2014). The subsequent steps of this pathway are less understood and the mechanisms leading to the post-gentisyl alcohol precursors are more blurred. However, since the beginning of the eighties, it has been commonly accepted that E-ascladiol is the last intermediate in the patulin biosynthesis pathway; the last step being the oxidative ring closure of this diol to form patulin (Sekiguchi et al. 1983). So far, the gene involved in this step has not yet been identified.

In the aflatoxin biosynthetic pathway, a similar ring closure occurs during the step leading to the formation of versicolorin B from versiconal hemiacetal. This reaction was proved to be mediated by the versicolorin B synthase (VBS) in *Aspergillus parasiticus* (Silva et al. 1996). The amino acid sequence of this VBS protein was found to have the greatest homology with proteins in the glucose–methanol–choline (GMC) oxidoreductase family (Silva et al. 1996). Within the 40-kb patulin gene cluster, a putative GMC oxidoreductase gene was identified by BLASTP analysis (Artigot et al. 2009). In an analogical manner, this gene is suggested to have a potential role in the latest step of the metabolic pathway leading to patulin from ascladiol.

Since E- and Z-ascladiol are both the last patulin intermediates as well as its degradation products, they are key compounds in patulin control strategies. Blocking the transformation of ascladiol to patulin or promoting patulin degradation to ascladiol should be good strategies to reduce patulin toxicity, provided that ascladiol is devoid of toxicity. Unfortunately, the toxicity of the latter metabolite is poorly documented, the data being limited to a single two-page article where the toxicity is barely mentioned (Suzuki et al. 1971). The authors noted that after intraperitoneal administration in rodents, the acute toxicity of ascladiol was four times lower than that of patulin. Nonetheless, its toxicity has never been evaluated on human cells.

The objectives of the present paper were: (1) to identify the gene encoding the enzyme involved in the conversion of ascladiol into patulin in *P. expansum*, (2) to produce and purify the two ascladiol isomers from the mutant strain, and (3) to evaluate their toxicity on human cells.

Materials and methods

Chemicals

Patulin standard was purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The E-ascladiol was obtained by chemical synthesis using a method described by Shao et al. (2012). Briefly, patulin and sodium borohydrate at the ratio of 10:1.68 (w/w) were placed in 1 mL ethanol at 4 °C on ice for 3 min, and the reaction was quenched using glacial acetic acid. The reaction mixture was then dried under a stream of nitrogen. The obtained product was extracted with 4 mL ethyl acetate and further purified by silica gel column chromatography (ethyl acetate/hexane = 1:1 v/v) to afford the white solid. Its purity was confirmed by nuclear magnetic resonance (NMR). This chemical E-ascladiol was used as a standard in this study.

All solvents used in the extraction and high-performance liquid chromatography experiments were of

analytical grade and purchased from Fisher Scientific (Illkirch, France).

Microorganisms, media, and growth conditions

The *Penicillium expansum* strain NRRL 35695 (USDA-ARS National Center for Agricultural Utilization Research, Peoria, Ill.) was used in this study. It was cultured on Potato Dextrose Agar (PDA) medium (Sigma-Aldrich) at 25 °C for 7 days in dark to enhance sporulation. For the protoplast regeneration, 3×10^7 conidia were inoculated into 250 mL of yeast extract peptone dextrose (YPD) medium (20 g dextrose, 10 g yeast extract, 20 g bactopeptone per liter) followed by an incubation on an orbital shaker (150 rpm) at 25 °C for 12 h.

The *Saccharomyces cerevisiae* strain FY1679 (MATa/MATα ura3-52/ura3-52; trp1Δ 63/TRP1; leu2Δ 1/LEU2; his3Δ 200/HIS3; GAL2/GAL2) was used to generate the Pe*patE* gene disruption cassette by homologous recombination. This strain was grown on solid YPD medium at 30 °C for 5 days.

Generation and characterization of the PeΔ*patE* mutant strain

Construction of the ΔpatE disruption cassette and fungal transformation

In order to characterize the function of the *patE* gene (KF899892) in *P. expansum,* a gene deletion strategy was undertaken by replacing the coding region with the hygromycin selection marker, as previously described in a recent study (Snini et al. 2016). The schematic representation of the strategy employed is presented in Fig. S1, and all the primer sets used are listed in Table S1 (All details are presented in the Supplemental Material, Part 1). Genetic transformation of *P. expansum* was carried out following a procedure described in the study of Merhej et al. (2011).

Chemical characterization of the PeΔpatE mutant: HPLC–DAD analysis

To qualitatively determine the metabolites that were accumulated in transformant colonies, 10^6 spores of each purified transformant were centrally inoculated into plates containing 20 mL of PDA medium, known to support patulin production, and grown at 25 °C for 7 days. Three technical replicates were performed for each mutant strain. The wild-type NRRL 35695 strain was cultured following the same conditions and used as an experimental positive control. After the appropriate incubation period, metabolites produced by the mutant and the wild-type strains were extracted and analyzed by HPLC–DAD as described in our last study (Tannous et al. 2014). Patulin (Sigma-Aldrich) and purified E-ascladiol obtained by chemical synthesis were used as reference standards to highlight the accumulation of E-ascladiol in *patE* gene-disrupted transformants.

Molecular characterization of the PeΔpatE mutant

The *patE* disruption was validated by PCR screening and Southern blotting. Genomic DNA from the putative transformant and the parental strains was extracted following the protocol described by Puel et al. (2005). The extracted DNAs were subjected to PCR amplification with the primer pair dPatEF3/dPatER3. These primers yield a product of 6505 bp if the integration of the disruption cassette occurs at the *patE* locus and a 4409 bp product in the presence of the parental genomic DNA. These primers were designed outside the left and the right junctions of the disruption cassette. The Phusion High-Fidelity DNA Polymerase (Thermo scientific, Illkirch, France) was used for PCR reactions. For a further confirmation, PCR products were digested by *Eco*RI and the digestion products were resolved using agarose gel electrophoresis.

Southern blotting was performed using *Xho*I-digested genomic DNAs (20 µg) from wild-type *P. expansum* (NRRL 35695) and PeΔ*patE*19 strains. Southern blot conditions used were the same as previously described by Snini et al. (2016). Figure S2 (Supplemental Material, Part 1) is a schematic drawn to scale that illustrates the replacement of the *patE* ORF in *P. expansum.*

Purification and identification of the two ascladiol isomeric forms

The *patE* deficient strain was cultured on forty large PDA agar plates (140 mm) at 25 °C for 7 days in order to produce large amounts of the two isomeric forms of ascladiol, E-ascladiol and Z-ascladiol. At the end of the incubation period, the agar media were scraped off the Petri plates and macerated for 2 days with 100 mL ethyl acetate per plate. The organic filtrate was then concentrated and evaporated to dryness as described above. The final dry extract recovered from all the Petri plates was dissolved in 10 mL of a mix MeOH/H_2O (80/20).

The purification of both ascladiol isomers was performed on the same HPLC apparatus used above for analytical analysis. A semi-preparative Gemini C6- Phenyl column was used (250 mm length, 10 mm internal diameter, and 5 µm particular size; Phenomenex, Torrance, CA, USA). The separation of the two isomers was achieved by isocratic elution using water as mobile phase, at a flow rate of 4.3 mL/min. The preparative flow was pumped through a valve at the ultimate 3000 Fraction Collector (Dionex/Thermo Scientific, Courtaboeuf, France). Multiple

fractions were pooled, and the solvent was evaporated under reduced pressure. The dry residue was dissolved in 2 mL of Milli-Q water.

Prior to toxicity experiments, the identity of the purified metabolites was confirmed by high-resolution mass spectrometry and NMR. Details of these analyses are described in the Supplemental Material, Part 2.

Cytotoxicity assessment by CellTiter-Glo assay

Cytotoxicity was tested on four different cell lines. The human colon carcinoma cell line Caco-2 (TC7 subclone) was grown in Dulbecco's modified Eagles medium (DMEM), glutamax (Gibco, Saint-Aubin, France) supplemented with 0.5% gentamycin (10 mg/mL) (Eurobio, Les Ulis, France), 10% fetal bovine serum (FBS) (Eurobio), and 1% MEM non-essential amino acids (Sigma-Aldrich). The human embryonic kidney 293 cell line (HEK-293) and the human hepatocellular carcinoma cell line HepG2 were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 1% glutamine, and 1% penicillin/streptomycin. The human promyeloblast HL-60 cell line was grown in Iscove modified Dulbecco's medium (IMDM) supplemented with 30% fetal bovine serum and 1% penicillin/streptomycin.

The luminescent assay CellTiter-Glo (Promega, Madison, USA) was used to measure ATP as an indicator of cell viability. A decrease reflects a lower number of cells and/or a decrease in ATP production. EC50 value was defined as the concentration inducing a 50% decrease in ATP measurement when compared to the control. Measurements were done according to the manufacturer's instructions as previously described (Pierron et al. 2016). Details of these experiments are described in Supplemental Material Part 3.

Measurement of cell death

Apoptotic and necrotic cells were detected by flow cytometry using CellEvent™ Caspase3/7 Green Detection Reagent (Molecular Probes, Madison, USA) and Topro-3 (Molecular Probes), respectively.

In practice, 5×10^4 cells were seeded in 12-well plates (Greiner Bio-One, Les Ulis, France) and cultured for 2 days in DMEM supplemented with 10% FBS. The medium was then removed and cells were exposed for 5 and 24 h to free FCS medium supplemented with 3 or 30 µM of either patulin, E- or Z-ascladiol. Culture medium was collected and cells were harvested using Gibco® 0.25% trypsin—EDTA (Life Technologies, Paisley, UK). After centrifugation at $300\times g$ for 6 min, cells were suspended in 1 mL 10% FCS–DMEM medium containing 1.2 µM CellEvent™ Caspase3/7 Green and incubated 30 min at room temperature. Then, cells were suspended in 300 µL Running Buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). Topro-3 (0.2 µM) was added just before analysis. Cell counting and fluorescence were measured using a MACSQuant™ analyzer 10 cytometer (Miltenyi Biotec). Data were processed with MACSQuantify™ software (Miltenyi Biotec).

Microarray analysis

In order to evaluate the impact of patulin and ascladiol on the global transcriptome, Caco-2 cell line was used for microarray analysis. Briefly, 1.5×10^5 cells were seeded into six-well flat-bottomed plates and incubated at 37 °C for 24 h. After incubation, the medium was replaced by a fresh one without serum containing 3 µM of patulin, 3 µM of E-ascladiol, or DMSO for the control condition. This concentration was chosen on the base of the results obtained in cytotoxicity experiments and represents the lowest patulin concentration, which has an effect on the different cell lines.

After 24 h of incubation, mRNA were extracted and the quality of these samples was assessed (Agilent RNA 6000 Nano Kit Quick, Agilent Bioanalyzer 2100); RNA Integrity Number (RIN) of these mRNA was superior to 9.

The Agilent SurePrint G3 Human GE 8 × 60 K microarray (Agilent technology, Santa Clara, USA) used in this experiment consisted of 62976 spots. For each sample, Cyanine-3 (Cy3)-labeled cRNA was prepared from 200 ng of total RNA using the One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer's instructions, followed by Agencourt RNAClean XP clean-up (Agencourt Bioscience Corporation, Beverly, USA). Six hundred nanograms of Cy3-labeled cRNA was hybridized on SurePrint G3 Human GE 8 × 60 K microarray following the manufacturer's instructions. Slides were scanned immediately after washing on an Agilent G2505C Microarray Scanner with Agilent Scan Control A.8.5.1 software. All experimental details are available in the Gene Expression Omnibus (GEO) database under accession number GSE75934. Microarray data from feature extraction software were analyzed with R (www.r-project.org, R v. 3.1.2), using Bioconductor packages (www.bioconductor.org, v 2.12).

Statistical analysis

For cytotoxicity assays, the statistical analysis was performed using GraphPad Prism 4 software. Following the Fischer test on equality of variance, one-way ANOVA was used to analyze the difference between the different molecules tested. Differences were considered to be statistically significant when the p value was lower than 0.05.

For microarray data, statistical analysis was performed with R 3.1.2 software as previously described (Pierron et al. 2016) and reported in the Supplemental Material,

Part 4. The log fold change (FC) of the expression value for each gene was then analyzed using Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Results

Sequence analysis of the putative GMC oxidoreductase from *P. expansum*

The *patE* gene sequence of *P. expansum,* located within a gene cluster involved in patulin biosynthesis, was identified in our previous study (Tannous et al. 2014). The translation of this gene's coding sequence results in a protein of 629 amino acids (GenBank AIG62134). A search in the Conserved Domain Database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) revealed that PATE, the protein encoded by the *patE* gene, belongs to a group of glucose–methanol–choline (GMC) flavoprotein oxidoreductases. Two Pfam conserved domains define the members of this protein family. The first one is the N-terminal GMC domain (Pfam 00732), involved in the binding of the Flavin Adenine Dinucleotide (FAD), a cofactor which provides the oxidative power required in the reactions of GMC-type oxidases. The second conserved domain is the C-terminal GMC domain (Pfam 05199) (Etxebeste et al. 2012). This domain of approximately 150 amino acids includes the active-site residues, comprising a strictly conserved histidine (Dijkman and Fraaije 2014). The conserved domains mentioned above are detected in GMC oxidoreductases including the versicolorin B synthase (VBS) which catalyzes the transformation of versiconal hemiacetal to versicolorin B, a key transformation in the aflatoxin biosynthetic pathway (Silva et al. 1996).

PATE was then subjected to a BLASTP analysis. The NCBI BLAST search revealed high similarities with a large number of GMCs, including a 60% of identity with the VBS. Similarities exist between the reactions leading to the closure of bisfuran ring in the aflatoxin biosynthetic pathway and the transformation of the patulin precursor, ascladiol into patulin (Supplemental Material, Part 5). Such structural similarities allow us to propose ascladiol as a plausible substrate of PATE.

Generation and characterization of the *patE*-deficient mutant

In order to study the function of the *patE* gene in the patulin biosynthetic pathway, the open reading frame (ORF) of *patE* gene in *P. expansum* was replaced by the hygromycin resistance marker.

A total of 29 independent transformants were picked randomly, streak purified, transferred to PDA plates and subsequently subjected to HPLC–DAD in order to measure patulin production. After the first round of screening, only one mutant strain out of 29 (PeΔ*patE*19) did not produce patulin when compared to the wild-type strain (Fig. 1). The absence of patulin in the PeΔ*patE*19 strain's extract was associated with the appearance of two new peaks that eluted before patulin, with retention times of 12 min and 14 min. The product eluted at 12 min was determined to be E-ascladiol according to the retention time and UV spectrum of the chemically prepared standard. The second product displayed a similar UV spectrum and was identified as Z-ascladiol (Fig. 1) after purification and HRMS and NMR analyzes. This mutant strain PeΔ*patE*19 was thus identified as promising candidate, having undergone the desired homologous integration. Molecular confirmations using PCR and Southern blot methods (described in the Supplemental Material, Part 6) endorsed that the PeΔ*patE*19 was the desired mutant. Taken together, these data provide consistent evidence that PATE catalyzes the last step of the patulin biosynthetic pathway, in which E-ascladiol is oxidized to patulin. Z-ascladiol is the non-enzymatic converted product of the E-ascladiol and is not directly involved in patulin biosynthesis (Sekiguchi et al. 1983).

Separation, identification, and confirmation of the two isomeric forms of ascladiol

Both E- and Z-ascladiol were purified from the PeΔ*patE*19 culture following the purification procedure described above.

The identity of the two ascladiols was confirmed by high-resolution mass spectrometry and NMR. All details of these analyses are presented in the Supplemental Material, Part 2.

Comparative toxicity of patulin and ascladiols on human cell lines

Overall, 24-h exposure to patulin caused a dose-dependent cytotoxic effect to the different human cell lines tested. On the contrary, E and Z-ascladiol did not show any effect whatever the type of cells and the concentration tested (Fig. 2). Intestinal and kidney cell lines were the most sensitive to patulin (Caco-2 $EC_{50} = 12.1\ \mu M \pm 1.6$; HEK 293 $EC_{50} = 13.1\ \mu M \pm 2.1$), and the human promyeloblasic cell line HL-60 was the most resistant. However, 100 µM patulin was fully cytotoxic for HL60 as already observed for other cell lines.

The ability of patulin and ascladiols to induce apoptosis was further evaluated on Caco-2 cells. A short exposure (5 h) to 30 µM patulin induced a strong decrease in

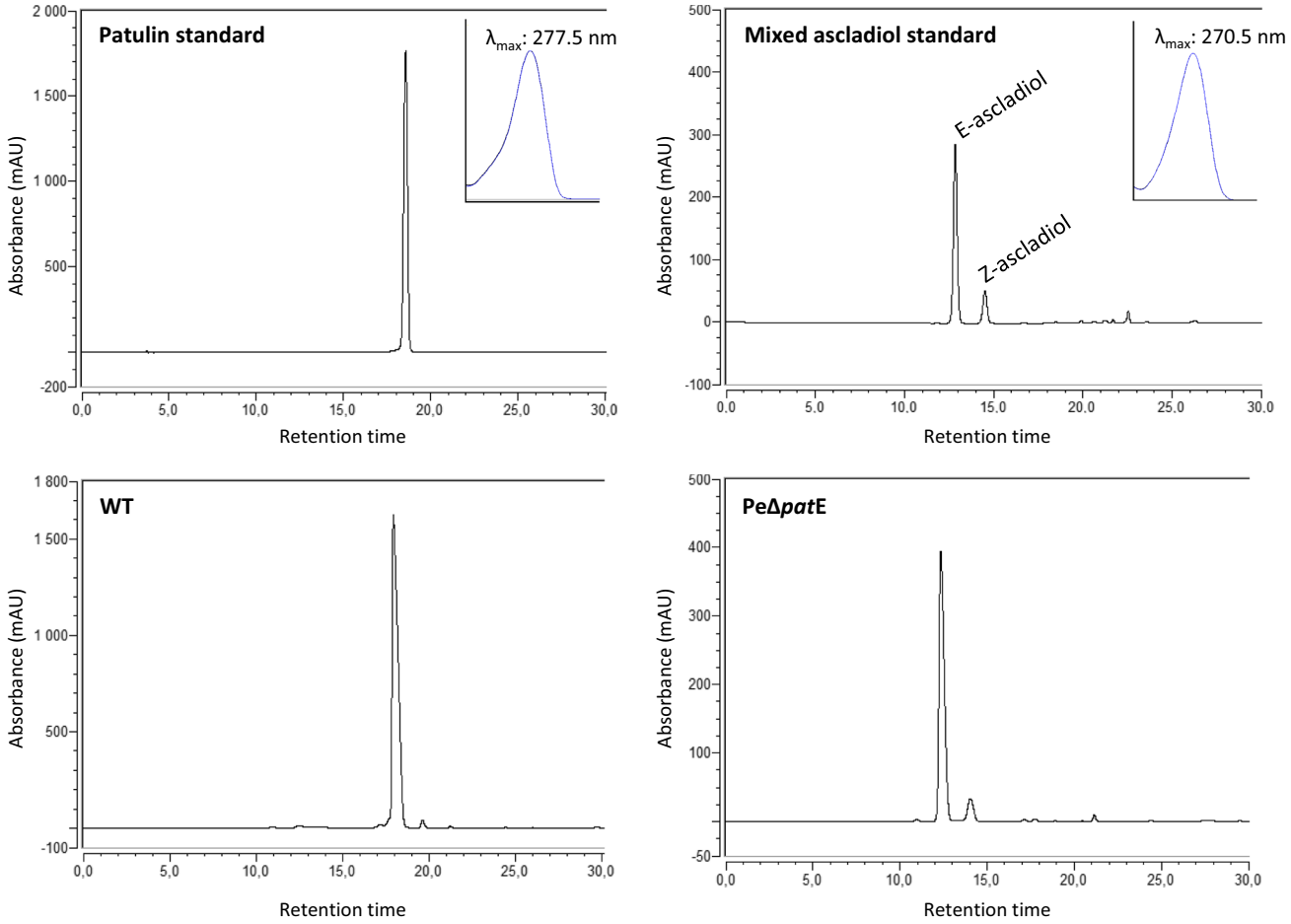

Fig. 1 High-performance liquid chromatography (HPLC)-UV chromatograms at 277 nm of the patulin and ascladiol standard solutions, the wild-type NRRL 35695 extract and the PeΔ*patE*19 extract

the number of cells (Fig. 3a) and the viability rate (Fig. 3b), indicating an inhibition of the proliferation and an induction of cell death. These effects were even more pronounced after 24 h of exposure (Fig. 3d, e). Cell death was due to necrosis as shown by the high Topro-3 staining and the low level of CellEvent Caspase3/7 Green staining. The main part of the necrotic cells displayed low caspase activation (Fig. 4), which did not prevent the cells from undergoing necrotic cell death. Conversely, cytometry analysis showed that E- and Z-ascladiol did not induce any cell death in Caco-2 cells (Supplemental Material Part 7). The inability of patulin to trigger apoptosis was confirmed by the shape of the nuclei observed by microscopy (Supplemental Material Part 8): cells treated for 24 h with 30 µM patulin did not show a significant increase in condensed and fragmented nuclei compared to control cells. Globally, the combined effect of 30 µM patulin on both proliferation and viability led to a high cytotoxic effect with 65 and 85% decrease in live cells after 5- and 24-h exposures, respectively (Fig. 3c, f). On the contrary, E-ascladiol did not trigger any toxic effect even at high concentration (30 µM). It is worthy to note that 30 µM Z-ascladiol induced a slight decrease in cellular proliferation (20%) after 24-h exposure (Fig. 3d) but did not affect the cell viability (Fig. 3e).

Transcriptomic analysis

To further confirm the low toxicity of ascladiol, a genome-wide transcriptomic analysis was performed on Caco-2 cells exposed to diluent, patulin or E-ascladiol, the most prevalent isomer issued from the degradation of patulin by yeast. A set of 6124 probes (corresponding 5360 genes) was identified as significantly differentially expressed (FDR < 5%) mainly between control and patulin treated cells. The Venn diagram (Fig. 5a) illustrates that 2811 and 2612 genes were over and under expressed in response to patulin treatment, respectively. In contrast, only nine genes were differentially expressed (DE) in response to

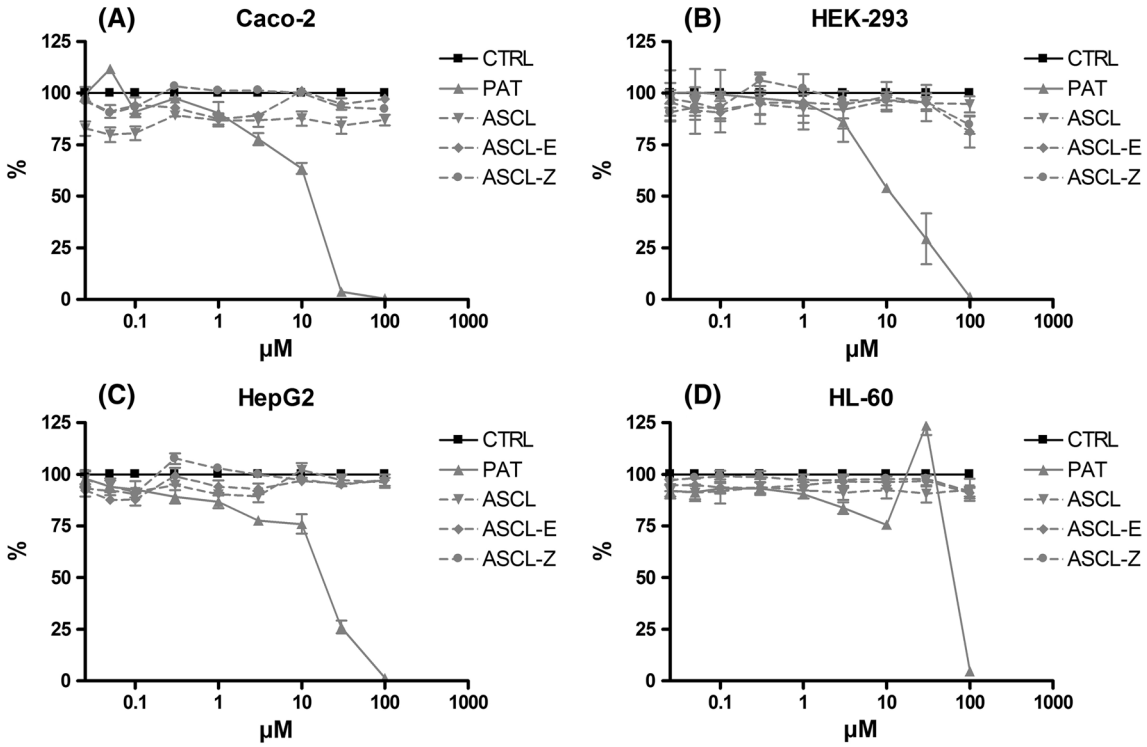

Fig. 2 Effect of patulin and ascladiols on Caco-2, HEK-293, HepG2, and HL-60 cell lines. Proliferative cells were incubated with increasing concentrations of patulin (PAT), standard ascladiol (ASCL), purified E-ascladiol (ASCL-E), and purified Z-ascladiol (ASCL-Z) for 24 h. Cell viability evaluated by measurement of ATP is expressed as % of control cells. Graphs show the mean ± standard error of the mean (SEM) from six replicates

E-ascladiol treatment. Among them, two were down-regulated and common to the response to patulin treatment. The first one is the CLK1 gene (FC patulin treatment = 0.86; FC E-ascladiol treatment = 0.71) and the second is the LOC100507579 gene (FC patulin treatment = 0.68; FC E-ascladiol treatment = 0.75). The seven remaining genes were RND3, NR0B2, DUSP1, ED1, CYR61, FOS, and EGR2. These genes were specific to E-ascladiol treatment and were very weakly up-regulated with the respective fold change: 1.29; 1.38; 1.46; 1.49; 1.53; 1.96; 2.23.

A hierarchical clustering of individual gene expression values was performed on the significantly regulated transcripts (Fig. 5b). It reveals a similar global expression pattern between control and E-ascladiol-treated cells. In contrast, patulin treated cells display a distinct expression pattern. Looking at the clustering dendrogram shown in Fig. 5b, when compared to E-ascladiol, four gene clusters presenting specific expression profiles were identified for patulin. Cluster 1 contains 163 genes that are up-regulated by patulin and slightly down-regulated by E-ascladiol, cluster 2 contains 948 genes that are up-regulated by patulin and slightly up-regulated by E-ascladiol, cluster 3 contains 490 genes that are down-regulated by patulin and slightly down-regulated by E-ascladiol, and cluster 4 contains 800 genes that are down-regulated by patulin and up-regulated by E-ascladiol.

The Ingenuity Pathway Analysis software (IPA), using the core analysis function was used to analyze the biological functions of DE genes. First, IPA Upstream Regulator Analysis was used to identify upstream regulators (molecule that can affect the expression of another molecule) that may be responsible for gene expression changes observed in our experimental data set. Briefly, 35 upstream regulators were identified to be inhibited (23/35) or activated (12/35) by patulin. Among them, the tumor protein p53 and the CTNNB1 transcription factor were inhibited (Table S2). The first one encoded a protein that responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, or DNA repair. The second encoded a protein, which is part of a protein complex that constitutes adherent junctions. Theses junctions are necessary for the epithelium maintenance by regulating cell growth and adhesion between cells. On the contrary, no upstream regulator was identified to be inhibited or activated by E-ascladiol.

In order to visualize the relationship between molecules, networks of DE genes based on defined relationships between genes in the Ingenuity Knowledge Base

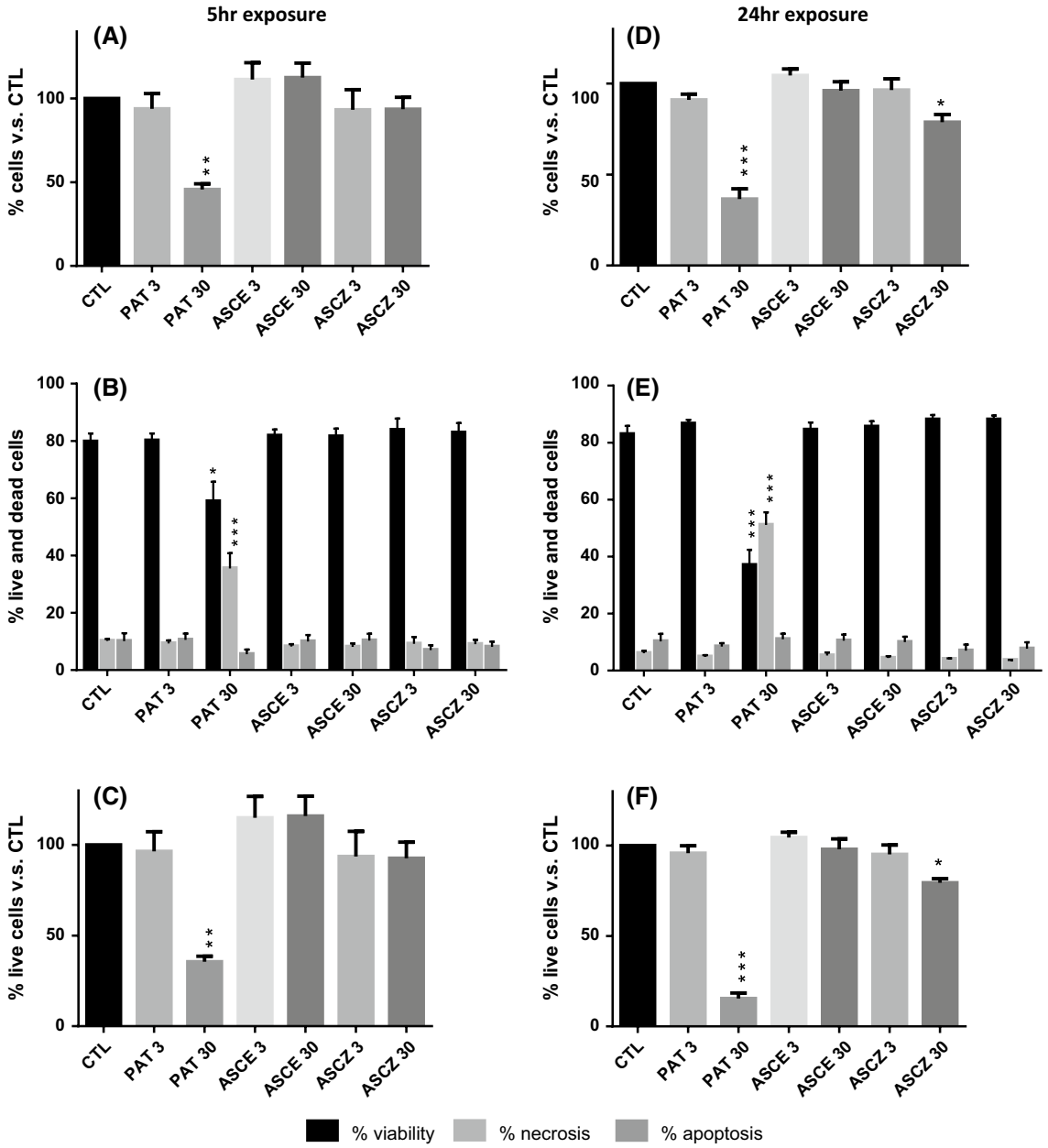

Fig. 3 Effect of patulin and ascladiols on Caco-2 cell death. Caco-2 cells were exposed to 3 and 30 µM of each mycotoxin for 5 h (**a**–**c**) and 24 h (**d**–**f**). After treatment, cells were stained with Topro-3 and CellEvent™ Caspase3/7 Green to identify necrotic (Topro-3 positive) and apoptotic cells (CellEvent Green positive, Topro-3 negative). Viable cells correspond to CellEvent Green negative-Topro-3 negative cells. Cell counting and fluorescence were recorded by flow cytometry. **a**, **d** Cell counting; data are expressed as percentage of cells compared to the not treated cells (CTL). **b**, **e** Distribution of viable, necrotic and apoptotic cells among the cell population. **c**, **f** % of live cells versus not treated cells taking into account cell counting and % of viable cells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Graphs show the mean ± standard error of the mean (SEM) from five replicates. Patulin (PAT), E-ascladiol (ASCE) and Z-ascladiol (ASCZ)

(IKB), were generated. With the same experimental data set, 25 networks were identified (Table S3). As an example, the network No. 1 (Fig. 6) is composed of 134 molecules located in different cellular compartments which are involved in the development of cancer and hematological and immunological diseases.

Discussion

E-ascladiol was identified as a direct precursor of patulin in cell-free extracts from *Penicillium griseofulvum* patulin-minus mutants (Sekiguchi et al. 1983). Nevertheless, the gene responsible for this final step of the patulin

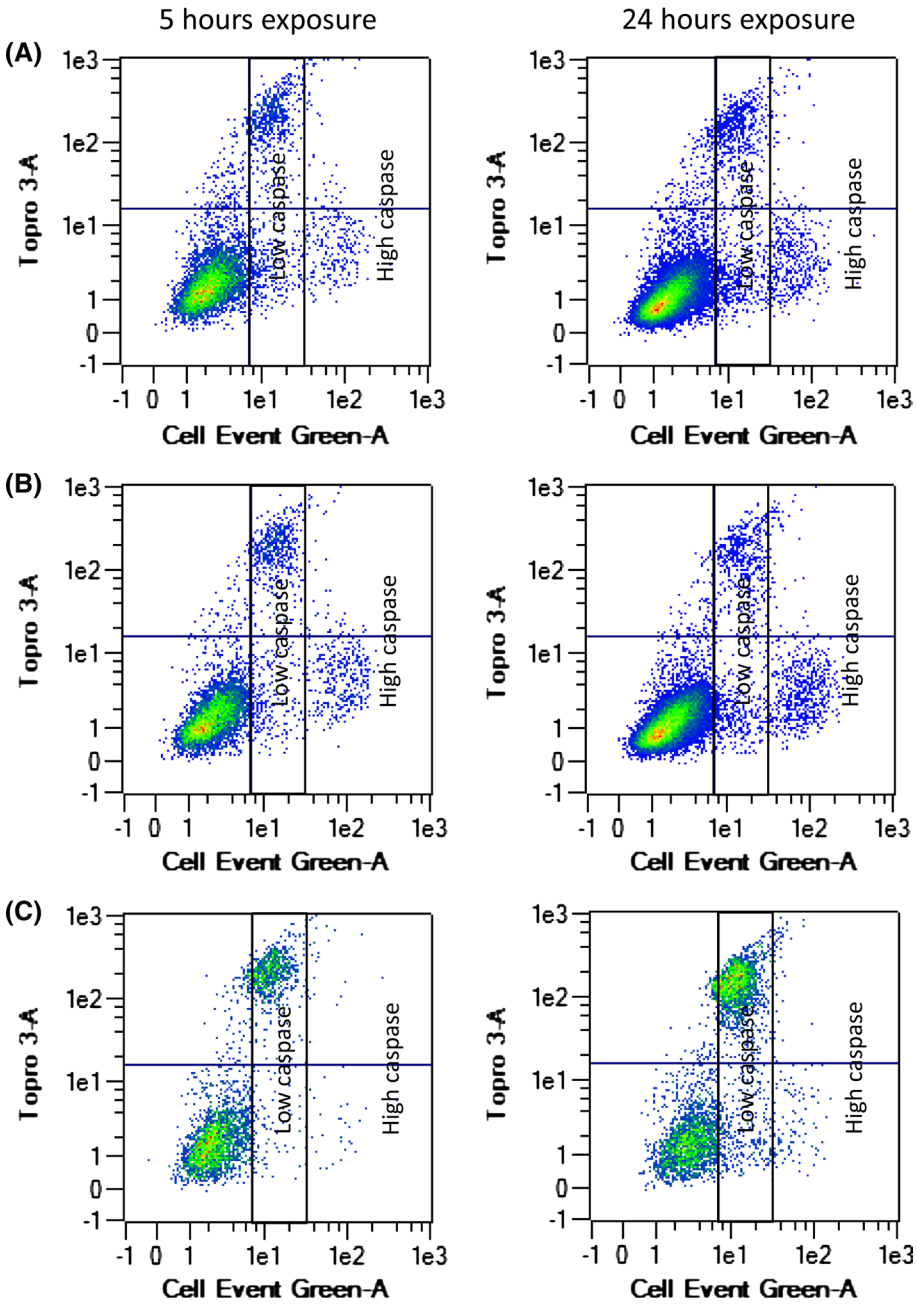

Fig. 4 Induction of "low-caspase" cells after 30 µM patulin exposure. Caco-2 cells were exposed for 5 and 24 h to 3 and 30 µM patulin concentrations. After staining with Topro-3 and CellEvent™ Caspase3/7 *Green*, cytometry analysis allows to distinguish low and high caspase positive cells from caspase-negative viable cells. The dot-blot is representative of 5 independent experiments. **a** not treated; **b** 3 µM; **c** 30 µM (color figure online)

pathway has remained unidentified. In the current study, we reported the target deletion of the patulin biosynthesis gene *patE*, which encodes a putative GMC oxidoreductase. The resulting knockout mutant PeΔ*patE*19 shows a lack of patulin-producing ability along with a high accumulation of E-ascladiol. This finding provides clear evidence that the last step in the patulin biosynthetic pathway is catalyzed by the PATE enzyme and confirms the earliest findings of Sekiguchi et al. (1983) that E-ascladiol is the last intermediate in the patulin biosynthetic pathway.

In addition to its biosynthetic role as a direct precursor, several studies revealed that E-ascladiol is a degradation product of patulin. The degradation of patulin into E-ascladiol was described during the fermentation of apple juice to cider using the commercial yeast *S. cerevisiae*, where traces of Z-ascladiol were detected (Moss and Long 2002). This transformation was also encountered by other fungal species belonging to *Ascomyceta* and *Basidiomyceta* phyla. In a recent study, a *Sporobolomyces* sp. strain from the subphylum *Pucciniomycotina* was found to be able to degrade patulin and form two major breakdown products, desoxypatulinic acid and Z-ascladiol, whereas small amounts of E-ascladiol were detected (Ianiri et al. 2013). Some bacterial species such as *Gluconobacter*

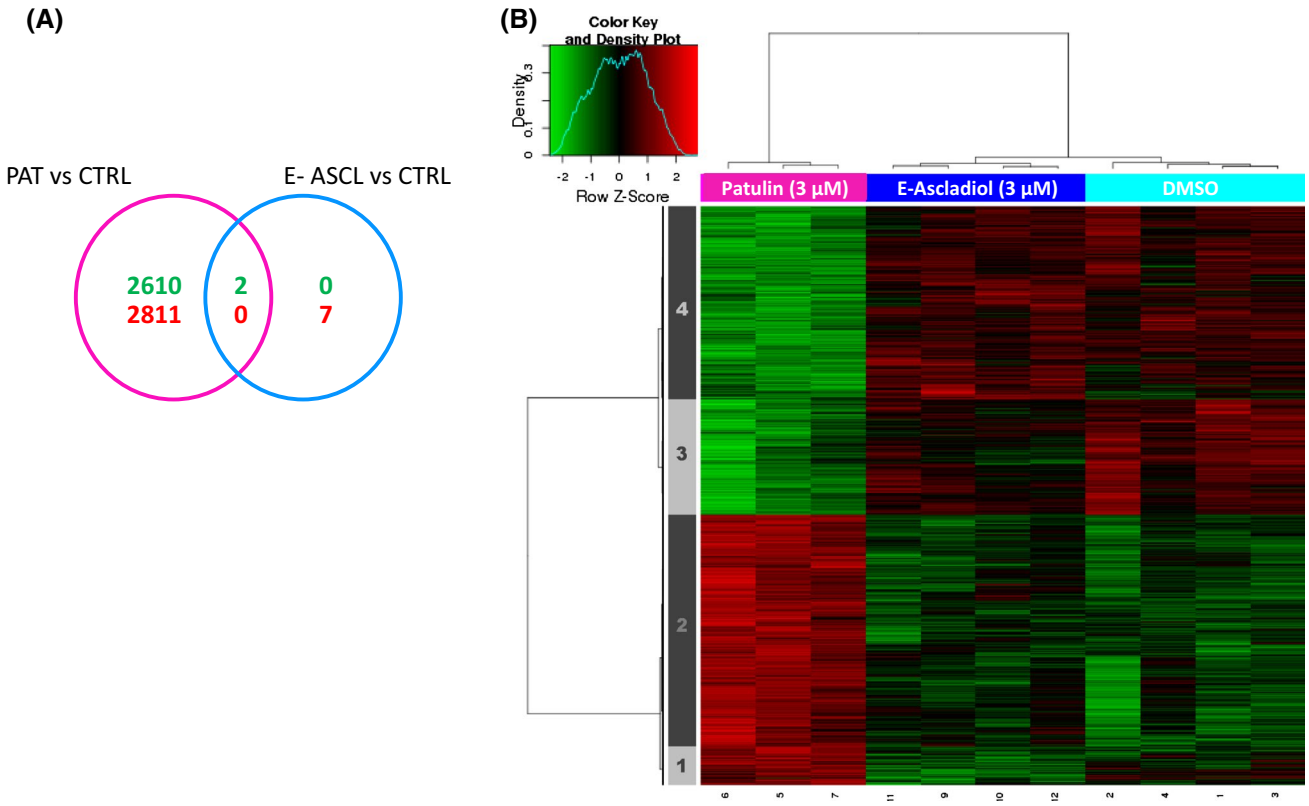

Fig. 5 Gene expression profile of Caco-2 cell line exposed to patulin or ascladiol. Transcriptome analyses of the Caco-2 cell line treated with patulin, E-ascladiol, and DMSO (control) were performed using Agilent SurePrint G3 Human GE 8 × 60 K microarray. Each group comprised four replicated cell cultures (except for patulin, $n = 3$). About 6124 probes with FDR < 5% (BH procedure) were considered significantly regulated between any of treated and DMSO cells. **a** Venn diagram illustrating the overlaps between the genes significantly up- or down-regulated in response to patulin or E-ascladiol treatment. **b** A hierarchical clustering was obtained from individual's expression values of 2000 most significantly regulated probes over each comparison. *Red* and *green colors* presented in the heat map indicate values above and below the row (probe) mean centered and scaled expression values (Z-score), respectively. *Black* indicates values close to the mean. The probes clustering and individuals clustering are illustrated on *left* and the *top* panel dendrogram, respectively. The *dark* and *light gray boxes* on the probe dendrogram illustrate the 4 probes clusters showing a specific expression pattern along conditions (color figure online)

oxydans (Ricelli et al. 2007) were also reported to be able to produce both isomeric forms of ascladiol from patulin. Another patulin degradation product by yeast *Rhodosporidium kratochvilavae* LS11 strain was detected in vitro and identified as desoxypatulinic acid (DPA) (Castoria et al. 2011). This compound lacks both hemiacetal and lactone functions and thus is unable to react with thiol groups. This biodegradation compound was shown to be significantly less toxic than patulin against human lymphocytes (Castoria et al. 2011). Unlike DPA, the E-ascladiol has no hemiacetal group but retains the integrity of the lactone ring in its chemical structure. Could this lack of hemiacetal functional group lead to a lower toxicity of ascladiol as compared to patulin?

In order to answer this question, we explored and compared the toxicity of patulin and both ascladiol isomers on a panel of human cell lines. Three carcinoma cells derived from different tissues (intestine, liver and lymphocytes) were resistant to E- and Z-ascladiol, while they were sensitive to patulin from 3 µM. Considering that carcinoma cell lines may be more resistant to cytotoxic agents compared to normal cells (Shoemaker et al. 1983), the non-toxicity of E- and Z-ascladiol was also demonstrated on the immortalized HEK-293 kidney cell line. Additionally, we confirmed these results on a normal intestinal line treated with 30 or 100 µM of either patulin, E- or Z-ascladiol (Supplemental material, part 10). It is noteworthy that the non-toxicity property of E-ascladiol has also been shown in an ex vivo porcine intestinal model (Maidana et al. 2016).

The patulin cytotoxicity reported in the current work matches with previous studies. The exposure of Caco-2 cell line to increasing concentrations of patulin (1–100 µM) during 24 h resulted in a dose-dependent decrease in the transepithelial resistance (TER), while the lactate

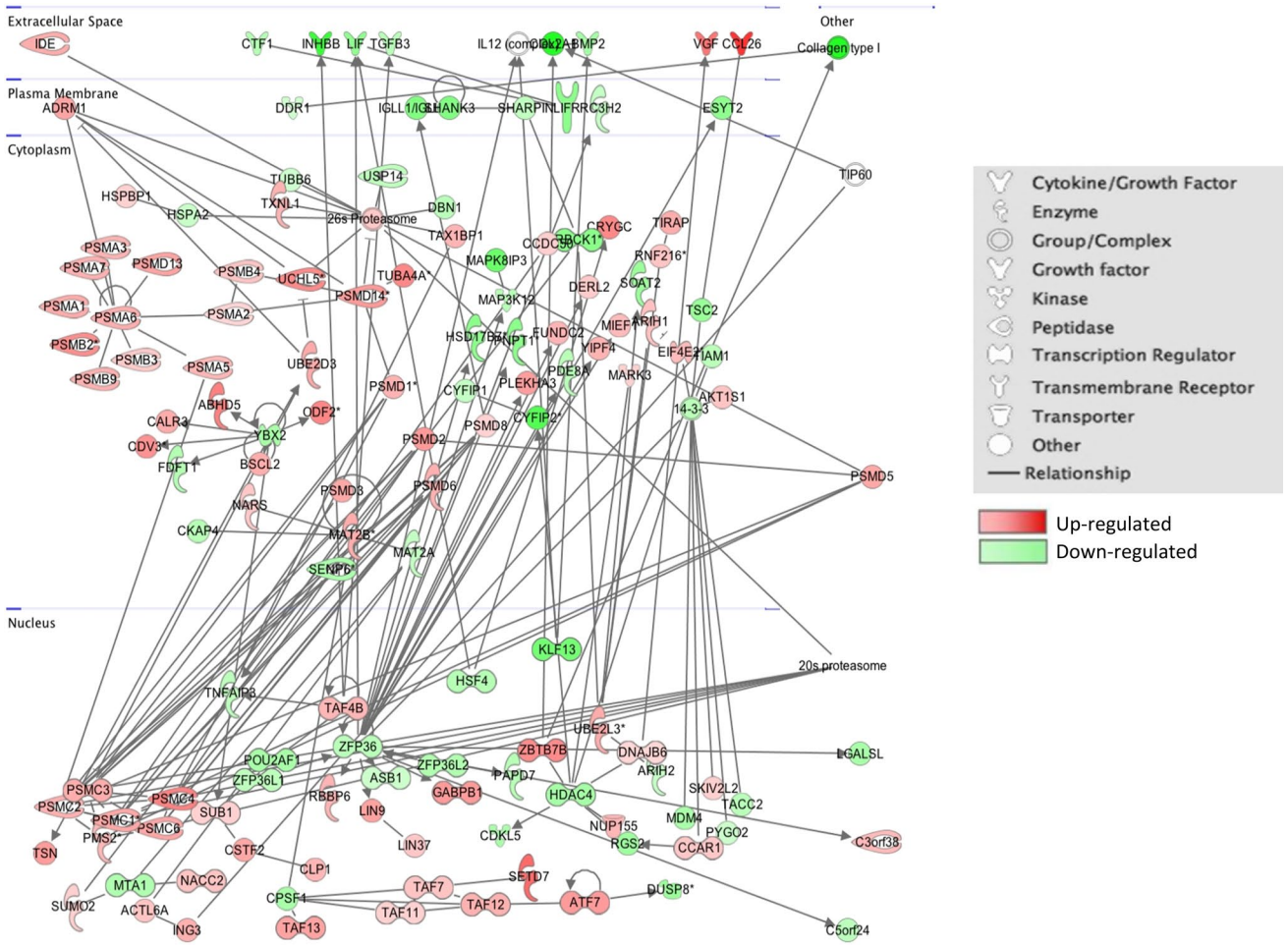

Fig. 6 Patulin-related gene network identified by the IPA system. Network presents up-regulated and down-regulated genes identified upon exposure to patulin and their known relationship as defined in the Ingenuity Knowledge Base (IKB). Node (gene) and edge (gene relationship) *symbols* are described in the bottom part of the figure. The intensity of the node color (*red*) indicates the degree of up-regulation. The intensity of the node color (*green*) indicates the degree of down-regulation. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. Node (gene) and edge (gene relationship) *symbols* are described in the bottom part of the figure. The node *shapes* denote enzymes, kinases, peptidases, transmembrane receptor, cytokines, growth factor, transporter, translation factor, nuclear receptor, transcription factor and others. Molecules with an *asterisk* are involved in others pathways (color figure online)

dehydrogenase assay did not report cell lysis (Mahfoud et al. 2002). This finding has been later reproduced in subsequent studies (McLaughlin et al. 2009; Kawauchiya et al. 2011). It was previously shown that 5 and 7.5 µM patulin decreased the viability of human embryonic kidney cells (HEK 293) of 30 and 45% respectively (Wu et al. 2008). Another study showed that 100 µM patulin was also toxic to human lymphocytes (Castoria et al. 2011) as demonstrated by a trypan blue exclusion assay. Interestingly, these analyses provide the first evidence that ascladiol has no toxic effect on human cell lines even at a high concentration (100 µM). The toxicity of ascladiol was only investigated in a study from 1971 on rodents (Suzuki et al. 1971) and has not been confirmed in humans.

Conclusion

In this study, the status of E-ascladiol as an intermediate of patulin biosynthesis was confirmed by the disruption of *patE* gene encoding the patulin synthase, the enzyme involved in the ultimate step of patulin biosynthesis pathway in *P. expansum*. Results showed that E- and Z-ascladiols are non-cytotoxic whereas patulin presents high cytotoxic potential for four human cell lines in a dose-dependent manner. Microarray analysis strengthened this absence of non-toxicity of ascladiol. Evidence of lower toxicity of a biodegradation product over the parent mycotoxin has been reported in many cases (aflatoxins, zearalenone, citrinin) (El-Sharkaway et al. 1991; Alberts et al. 2006;

Devi et al. 2006). Similarly, we observed in this study, that microbial degradation of patulin to E- and Z-ascladiol, greatly reduces its toxicity.

On the basis of these results, we can reasonably conclude that the risk generated by patulin contamination of apples in cider industry is only limited to ciders with high patulin levels. Furthermore, this study reinforces the idea that blocking the transformation of ascladiol to patulin or promoting patulin degradation to ascladiol are good strategies in mitigating patulin contamination risks.

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Compliance with ethical standards

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

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