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RESEARCH LETTER

D-Galactose uptake is nonfunctional in the conidiospores of *Aspergillus niger*

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Abstract

The majority of black Aspergilli (*Aspergillus* section Nigri), including *Aspergillus niger*, as well as many other *Ascomycetes* fail to germinate on D-galactose as a sole carbon source. Here, we provide evidence that the ability of *A. niger* to transport D-galactose is growth stage dependent, being absent in the conidiospores but present in the mycelia. Despite earlier claims, we could identify galactokinase activity in growing cells and all genes of the Leloir pathway (responsible for channelling D-galactose into the EMP pathway) are well induced on D-galactose (and also on lactose, D-xylose and L-arabinose) in the mycelial stage. Expression of all Leloir pathway genes was also detectable in conidiospores, although *galE* (encoding a galactokinase) and *galD* (encoding a galactose-1-phosphate uridylyl transferase) were expressed poorly. These results suggest that the D-galactose-negative phenotype of *A. niger* conidiospores may be due to the lack of inducer uptake.

Introduction

Plant cell wall polysaccharides – the most abundant organic compounds in nature – can be divided into three groups: cellulose, hemicellulose and pectin (de Vries & Visser, 2001). Three main hemicelluloses can be recognized: xylan, mannan and xyloglucan. Xylan contains a backbone of β-linked D-xylose residues that can be decorated with acetyl-, L-arabinose, D-galactose, (4-O-methyl-) D-glucuronic acid and ferulic acid. Mannan contains a β-linked D-mannose backbone that can be decorated with α- and β-linked D-galactose and, depending on the origin, can contain single D-glucose residues interrupting the mannose main chain (referred to as glucomannan). Xyloglucan contains a β-linked D-glucose backbone that is decorated with α-linked D-xylose residues. Attached to these residues are D-galactose, L-arabinose and/or L-fucose residues. D-Galactose is the only component common to all three hemicelluloses and is also found in pectin (Pauly & Keegstra, 2010). The enzymatic hydrolysis of these polysaccharides is subject to significant industrial interest, both in the food and feed as well as the wood-manufacturing sector (Bhat, 2000). Amongst microorganisms with an ability to produce plant cell wall degrading enzymes,

fungi are by far the most interesting group. Besides certain *Trichoderma* species, black Aspergilli such as *Aspergillus niger* are the most important organisms because of their high protein secretion capacity and wide range of cell wall degrading enzyme activity (de Vries & Visser, 2001).

In recent years, considerable knowledge has been accumulated on the enzyme systems and genes involved in degrading hemicelluloses to their monomers and also about the further metabolism of the hemicellulose monomers in fungi (Flipphi *et al.*, 2009). With respect to D-galactose, information has been obtained in *Trichoderma reesei* (Seiboth *et al.*, 2002, 2003, 2004; Karaffa *et al.*, 2006) and *Aspergillus nidulans* (Fekete *et al.*, 2004; Christensen *et al.*, 2011). In addition to the Leloir pathway, these fungi possess a second pathway for D-galactose catabolism, which, in analogy to the L-arabinose catabolic pathway, uses reductive and oxidative reactions to convert D-galactose into D-fructose-6-phosphate (Seiboth & Metz, 2011). Although genome information from *A. niger* has shown the presence of all genes/enzymes needed to degrade D-galactose (Flipphi *et al.*, 2009), only few experimental data are available on its metabolism (Mojzita *et al.*, 2011; Koivistoinen *et al.*, 2012). This may be due to the fact that with the exception of *Aspergillus brasiliensis*, D-galactose is considered a very poor carbon source for black Aspergilli including *A. niger* (Meijer *et al.*, 2011), which hampers efforts to cultivate it on D-galactose. Growth on D-galactose containing complex carbohydrates may also be affected, depending on which other carbon sources are present and the ratio of these and galactose in the carbohydrate. The aim of this study was to analyse and understand the physiological background of this phenomenon in *A. niger*.

Materials and methods

Fungal strain and cultivation conditions

Aspergillus niger N402 (FGSC A733; *cspA1*) was used in this study (Bos *et al.*, 1988). The conditions for strain maintenance have been described earlier as has the MM medium used for shake-flask cultivations (de Vries *et al.*, 2004). Carbon sources were used in 1% (w/v) final concentrations and are given at the respective results.

Batch cultures were incubated on a rotary shaker (INFORS HT Multitron; 250 r.p.m.) at 30 °C in 500 mL Erlenmeyer flasks containing 100 mL of medium. Mycelia were pregrown in MM containing glycerol as a carbon source, harvested after 24 h by filtration on a sintered glass funnel, washed with cold sterile tap water and then transferred into fresh MM without glycerol, but supplemented with other carbon sources. For transcript analysis, samples were taken after 6 h of further incubation.

Aspergillus niger conidiospores are not formed on D-galactose containing solid medium. As a consequence, except where noted otherwise, we used glycerol as a sole carbon source to conidiate *A. niger* in the experiments aimed at investigating conidial stage events.

Nucleic acid isolation and hybridization

Fungal mycelia or conidia were harvested by filtration, washed with distilled water, frozen and ground under liquid nitrogen. For nucleic acid extraction, the Wizard Genomic DNA Purification Kit and SV Total RNA Isolation System (Promega) were used. Standard methods were used for electrophoresis, blotting and hybridization of nucleic acids (Sambrook *et al.*, 1989). Northern analysis was performed with the PCR DIG Probe Synthesis kit (Roche). 5.5 μg of RNA, respectively, was loaded into each lane. Primers for probe amplifications are given in Table 1.

Analytical methods

Mycelial dry mass was determined by withdrawing 2 × 5 mL aliquots from the culture, suction filtration through a preweighted glass wool filter and drying in an oven at 80 °C until constant weight. Data were averaged and deviated by not more than 14%.

The concentration of D-galactose in the growth medium was determined by HPLC analysis, using an H^+ exchange column (Bio-Rad Aminex HPX-H^+), employing 10 mM H_2SO_4 at 55 °C as mobile phase with isocratic elution and a refractive index detection.

To determinate the galactokinase activity, an HPLC method was used (Fekete *et al.*, 2002). Specific galactokinase activities are reported as mg protein, which was determined by means of a modification of the method of Lowry (Peterson, 1983) using BSA for calibration.

Measurement of D-galactose uptake

Mycelia were pregrown for 18 h on glycerol as a carbon source, harvested by gentle filtration and resuspended in 20 mL of carbon-free medium (MM) to give a final density of 1 mg mL^{-1}. MM was inoculated with 10^6, 10^7 and 10^9 spores mL^{-1}, respectively, when the D-galactose uptake of conidiospores were tested. After incubation at 30 °C for 60 min, 13.63 μL (0.2 mCi mL^{-1}) of D-galactose-1-^{14}C (G3143-14C; Sigma) was added to give 100 000–150 000 dpm per mL culture, and a further amount of cold D-galactose was added to give a final concentration of 1 mM. The cultures were incubated for

Table 1. Primers used for the amplification of the genes of the Leloir pathway in *Aspergillus niger*

Gene	Activity function	Locus	Oligonucleotide sequence	Amplicon size (bp)
galE	Galactokinase	An16g04160	galEforw: 5′TCTTGATTTGGCAGTAGTGTC-3′ galErev: 5′-CCTCAACCTGGGCAATAG-3′	1085
galD	Galactose-1-phosphate uridylyltransferase	An02g03590	galDforw: 5′-ACCCATTCAGAGAGGAGC-3′ galDrev: 5′-TTGCTCGGGTGTAATGTC-3′	1151
galF	UTP-hexose-1-phosphate uridylyltransferase	An12g00820	galFforw: 5′-ATAACTTCTTCGCCCTCTTCC-3′ galFrev: 5′-CCTCACCCTTCTTGTCAGC-3′	906
galG	UDP-galactose 4-epimerase	An14g03820	galGforw: 5′-ATTGACAGCGTTATCCAC-3′ galGrev: 5′-GGGTCATTATTTATTTCTGC-3′	1053
gpmB	Phosphoglucomutase	An02g07650	gpmBforw:: 5′-ACCACCATCGGCTCCAAGAC-3′ gpmBrev:: 5′-TAGGTGCGGACATCAGGCTC-3′	1175

further 6 h, and 1.0 mL of samples withdrawn in intervals of 30 or 60 min by immediately pipetting them into 1 mL of 1 M D-galactose and vigorous shaking. This treatment stopped further uptake of labelled D-galactose and also released ^{14}C-D-galactose adsorbed to the cells. The suspensions were then spun down in an Eppendorf centrifuge, and the radioactivity in the supernatant measured in a liquid scintillation counter (Wallac, Model 1409). OptiPhase HiSafe 3 (PerkinElmer) was used as a liquid scintillation cocktail.

Chemicals

All chemicals were of analytical grade, and, except where noted otherwise, were purchased from Sigma-Aldrich Kft., Budapest, Hungary.

Reproducibility

All the results presented here are means of 3–5 independent experiments. The data were analysed and visualized by Sigmaplot (Jandel Scientific), and standard deviations (SDs) for each procedure were determined. The SD values were always < 14% of the means.

Results

Growth of *A. niger* on D-galactose

Conidiospores of *A. niger* were unable to germinate in submerged minimal medium with 1% D-galactose as a sole carbon source even after a prolonged incubation. Essentially similar results were obtained on solid medium. However, mycelia of *A. niger* pregrown on glycerol (or on any other carbon source tested such as D-glucose, peptone, L-arabinose, D-xylose) and transferred to fresh medium containing D-galactose as a sole carbon source were able to grow, although at a rate lower than other fungi such as *A. nidulans* (Fekete *et al.*, 2004) or *T. reesei* (Seiboth *et al.*, 2004) (Fig. 1).

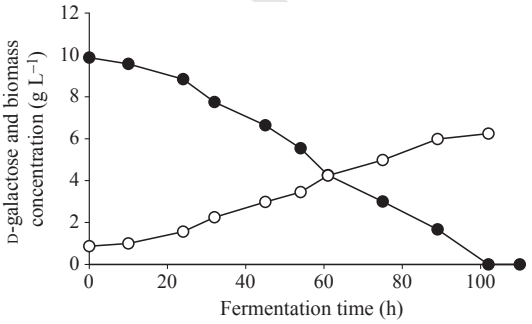

Fig. 1. Time-profile of biomass formation (○) and D-galactose consumption (●) of *Aspergillus niger* mycelia preformed on glycerol and transferred to minimal medium with D-galactose as a sole carbon source.

Uptake of D-galactose in *A. niger*

The above results suggested that *A. niger* can grow on D-galactose once the spores have germinated but its conidiospores fail to do so. This suggested to us that transport of D-galactose into the conidia may be nonfunctional. To investigate this hypothesis in more detail, we incubated mycelia and conidiospores, respectively, with ^{14}C-labelled D-galactose, and followed its uptake into the cells. Uptake by mycelia was related to dry weight. As it was practically impossible to determine biomass data for conidiospores in a reproducible way, we could not specify ^{14}C-labelled D-galactose uptake on the same basis in these two sets of experiments. Instead, we employed three different concentrations of conidia, namely 10^6, 10^7 and 10^9 spores mL^{-1}, respectively, under identical experimental conditions. Any D-galactose uptake was therefore expected to be proportional to the number of conidiospores present in the medium. Data obtained indeed showed that the mycelia preformed on glycerol were able to transport D-galactose (Fig. 2a). On the other hand, there was no ^{14}C-labelled D-galactose uptake by the conidiospores irrespective of their concentration (Fig. 2b), indicating the absence of D-galactose transport at this stage of growth in *A. niger*.

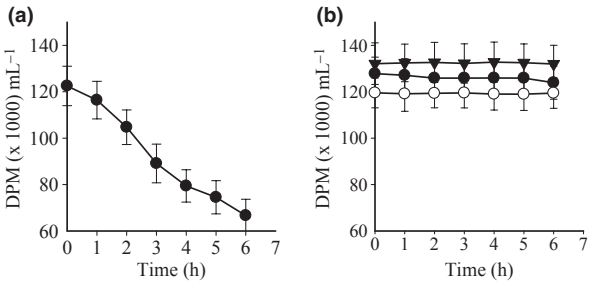

Fig. 2. (a) Time-course of extracellular ^{14}C-labelled D-galactose levels in *Aspergillus niger* culture supernatant inoculated with mycelium at a density of 1 mg mL^{-1}. Mycelium was pregrown on glycerol and biomass stayed constant during the 6 h of incubation. (b) Time-course of ^{14}C-labelled D-galactose uptake of *A. niger* conidiospores. (●), 10^6 spores mL^{-1}; (○), 10^7 spores mL^{-1}; (▲), 10^9 spores mL^{-1}.

Aspergillus niger is able to phosphorylate D-galactose

The D-galactose-negative phenotype of *A. niger* was earlier speculated to be the consequence of a lack of galactokinase activity (Elshafei & Abdel-Fatah, 2001). In contrast, cell-free extracts of *A. niger* mycelia prepared by us were able to phosphorylate D-galactose, resulting in a specific galactokinase activity similar in value to that of *A. nidulans* (Ilyés *et al.*, 2004). Galactokinase activity showed a basically constitutive nature, although it was higher during growth on pentoses (notably on D-xylose) and D-galactose than on D-glucose (Table 2). This finding is corroborated by the fact that the genome of *A. niger* contains a locus (An16g04160; *galE*) with obvious similarity to other fungal galactokinases (Flipphi *et al.*, 2009). Northern analysis performed with the respective gene as a probe showed that the gene was transcribed on all carbon sources investigated. Expression on D-galactose was higher than on D-glucose or glycerol, however, lower than on L-arabinose or D-xylose (Fig. 3a).

Aspergillus niger has an intact Leloir pathway

The finding that galactokinase was active prompted us to study whether a full Leloir pathway is operating in *A. niger*. *In silico* data revealed that the *A. niger* genome contains orthologs for each gene of this pathway (Flipphi *et al.*, 2009). Expression studies showed that they are all expressed in a fashion similar to galactokinase, for example, transcripts were formed on all carbon sources studied, but their transcript levels were higher on pentoses (L-arabinose, D-xylose) and on D-galactose (Fig. 3a). The reason for the higher expression of Leloir pathway genes on L-arabinose and D-xylose than on D-galactose remains unclear at this point and will require further study. Most notably, however, results obtained from conidiospores formed on glycerol or D-glucose showed that while all transcripts of the Leloir pathway genes were also present in conidiospores, *galE* (encoding a galactokinase) and *galD* (encoding an UTP-galactose-1-phosphate uridylyltransferase) were very poorly expressed (Fig. 3b), indicating that the potential to convert D-galactose into an intermediate of the EMP pathway may be dependent on the growth stage of the fungus.

Discussion

Aspergillus niger has a prominent position amongst microorganisms employed in industrial biotechnology, thus it is not surprising that numerous studies have been devoted to its biology (Andersen *et al.*, 2011). However, its metabolic relationship with D-galactose remained obscure; although this hexose is a major component of hemicelluloses and pectin, whose enzymatic hydrolysis is subject to considerable industrial interest. 3

In this article, we have provided evidence that the D-galactose-negative phenotype of *A. niger* is growth stage dependent, being complete in the conidiospores but only partial in mycelia germinated on any other carbon source. This result required that a D-galactose transporter system needs to be present in *A. niger*. In the yeast *Kluyveromyces lactis*, D-galactose and lactose transport are mediated by the same protein (Baruffini *et al.*, 2006), while in the related species *A. nidulans*, transport of these two sugars are independent (Fekete *et al.*, unpublished data). Galac- 4
tose permeases from *A. niger* have not yet been identified but BLAST analysis with the *Saccharomyces cerevisiae GAL2* gene as a query identified at least four unknown hexose permeases that shared high similarity to Gal2p (< e−120), that were contained in neighbouring phylogenetic clades in a phylogenetic tree, with the *S. cerevisiae* Gal2p being the basal protein (Fekete *et al.*, unpublished). Their function is currently investigated by us. In any case, it is clear from our experiments, however, that the transport of

Table 2. Intracellular galactokinase activity as a function of carbon source in *Aspergillus niger*

	Carbon source					
	D-galactose	Lactose	L-arabinose	Glycerol	D-glucose	D-xylose
Galactokinase activity (U per mg protein)	0.28 ± 0.014	0.19 ± 0.012	0.20 ± 0.01	0.11 ± 0.006	0.08 ± 0.05	0.31 ± 0.015

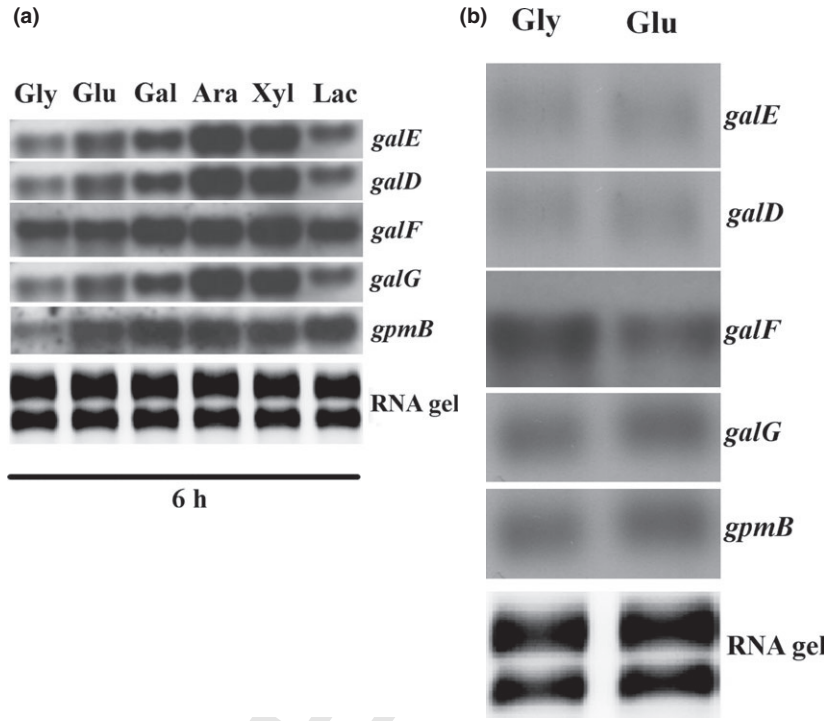

Fig. 3. Expression analysis of the genes comprising the Leloir pathway in *Aspergillus niger* on different carbon sources. (a) Expression in mycelia and (b) expression in conidiospores. Gly, glycerol; Glu, D-glucose; Gal, D-galactose; Ara, L-arabinose; Xyl, D-xylose; Lac, lactose. 18S rRNA was used as control.

D-galactose is not functional in the conidiospores of *A. niger*. While the reason for this unknown, our data suggest that D-galactose uptake in *A. niger* is growth stage dependent; for example, it is expressed in mycelia but not in resting conidia, resembling the behaviour of certain permeases from *T. reesei* (Metz *et al.*, 2011) and *A. nidulans* (Tazebay *et al.*, 1997; Amillis *et al.*, 2004; Pantazopoulou *et al.*, 2007).

D-Galactose metabolism via the Leloir pathway is a ubiquitous trait in pro- and eukaryotic cells (Frey, 1996). It involves an ATP-dependent galactokinase (EC 2.7.1.6) to form D-galactose 1-phosphate, which is subsequently transferred to UDP-glucose in exchange with D-glucose 1-phosphate by D-galactose 1-phosphate uridylyltransferase (EC 2.7.7.12). The resulting UDP-galactose is a substrate for the reaction catalysed by UDP-galactose 4-epimerase (EC 5.1.3.2), resulting in UDP-glucose. While we did not determine specific enzyme activities apart from that of galactokinase, gene expression data strongly suggest that the Leloir pathway is readily available to convert D-galactose once this sugar is inside of the cell, which occurs only in the mycelial stage of *A. niger*. In the conidiosporal stage, however, expression of the genes encoding the first two enzymes of the Leloir pathway was hardly detected, and weak expression was observed for the other three genes of the pathway as well. As we demonstrated that the conidia are unable to transport D-galactose, we conclude that the D-galactose-negative phenotype of the *A. niger* is unlikely to be caused by a lack of D-galactose catabolism. Rather, the phenomenon seems to be mainly uptake related in conidiospores. Therefore, the reduced expression observed for the Leloir genes in conidiospores may be due to the lack of inducer (D-galactose) uptake and appears to be a secondary effect rather than the cause of the nongrowth phenotype. Future studies will address this in more detail.

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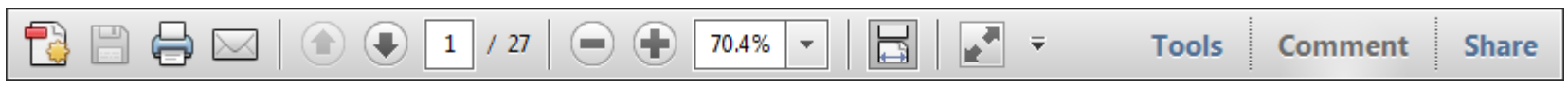
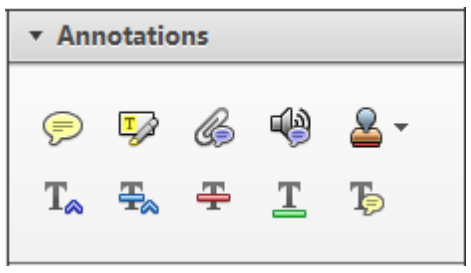


This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

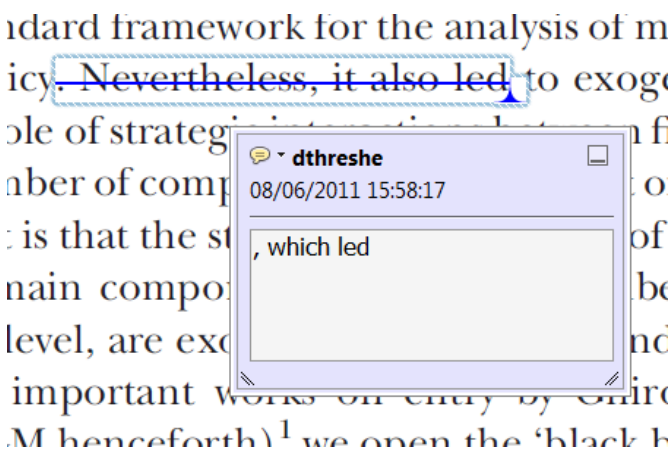


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

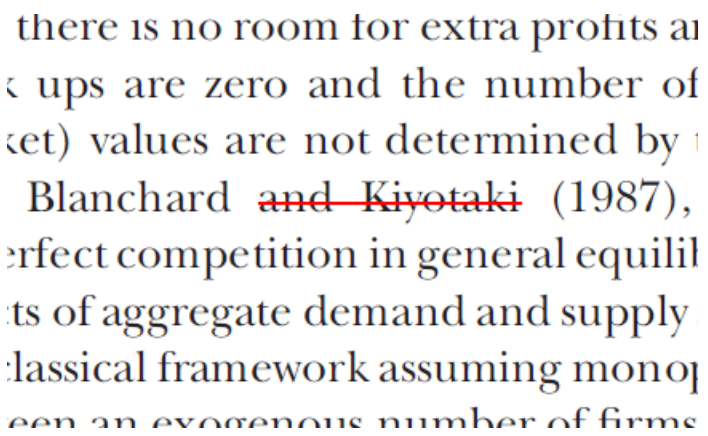


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

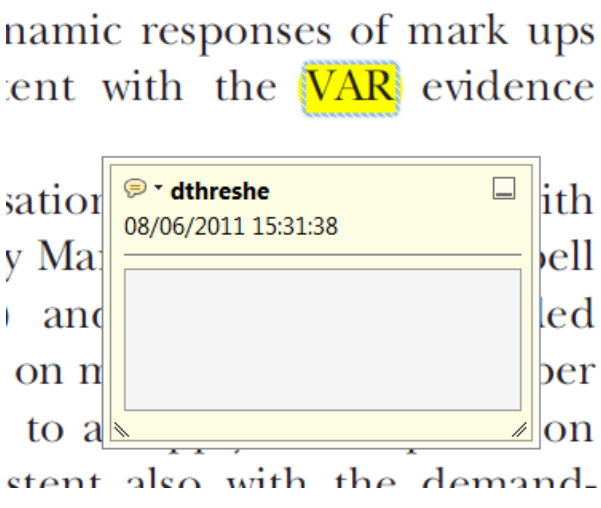


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

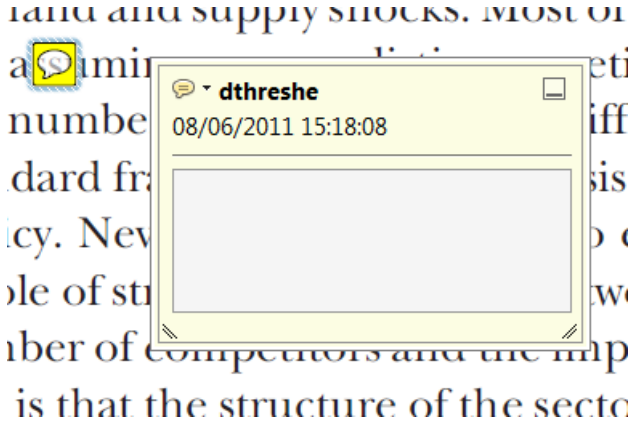


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



WILEY-BLACKWELL

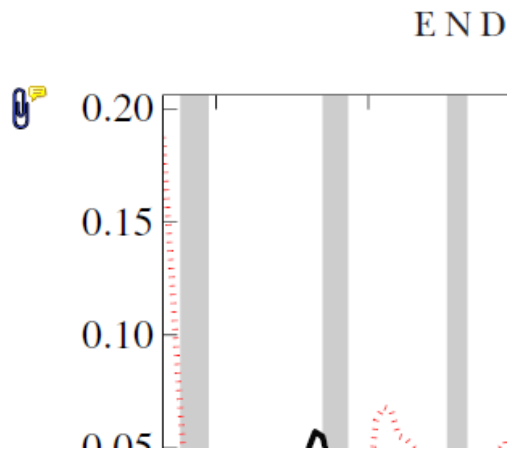
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

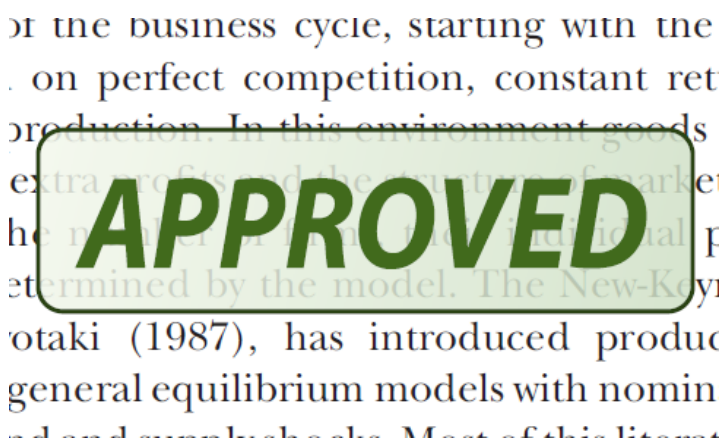


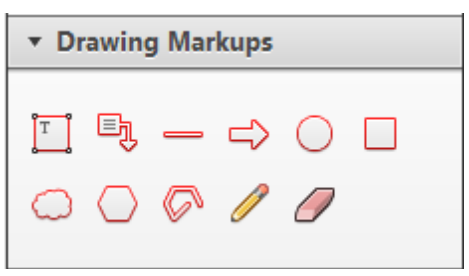
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

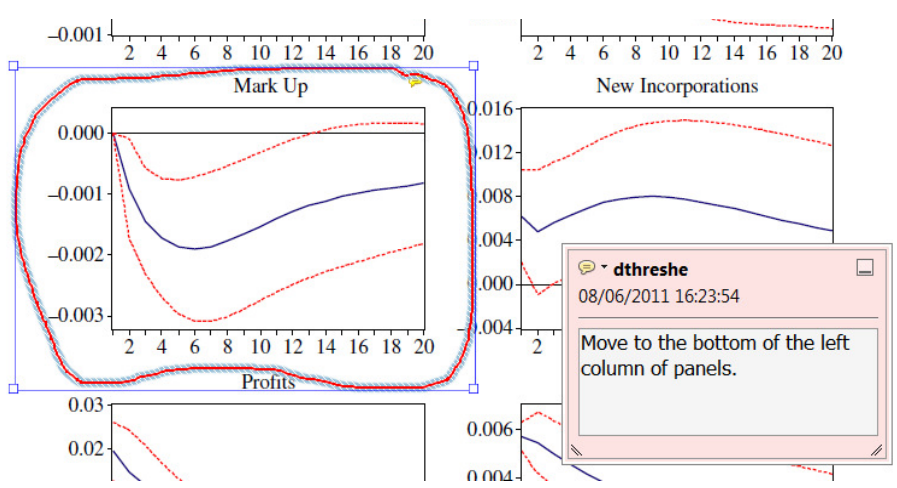
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: