Fungal Genetics and Biology 45 (2008) 1479–1486

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

# A metabolomic approach to dissecting osmotic stress in the wheat pathogen *Stagonospora nodorum*

Rohan G.T. Lowe [a,1], Maryn Lord [a], Kasia Rybak [a], Robert D. Trengove [b,c], Richard P. Oliver [a,c], Peter S. Solomon [a,c,*]

[a] *Australian Centre for Necrotrophic Fungal Pathogens, SABC, Division of Health Sciences, Murdoch University, Perth, WA, 6150, Australia*
[b] *School of Pharmacy, Division of Health Sciences, Murdoch University, Perth, WA 6150, Australia*
[c] *Metabolomics Australia, Murdoch University, Perth, WA 6150, Australia*

ARTICLE INFO

*Article history:*
Received 20 June 2008
Accepted 21 August 2008
Available online 29 August 2008

*Keywords:*
Arabitol
Xylitol
Osmotic stress
Glycerol
*Stagonospora nodorum*
Metabolomics

ABSTRACT

A non-targeted metabolomics approach was used to identify significant changes in metabolism upon exposure of the wheat pathogen *Stagonospora nodorum* to 0.5 M NaCl. The polyol arabitol, and to a lesser extent glycerol, was found to accumulate in response to the osmotic stress treatment. Amino acid synthesis was strongly down-regulated whilst mannitol levels were unaffected. A reverse genetic approach was undertaken to dissect the role of arabitol metabolism during salt stress. Strains of *S. nodorum* lacking a gene encoding an L-arabitol dehydrogenase (*abd1*), a xylitol dehydrogenase (*xdh1*) and a double-mutant lacking both genes (*abd1xdh1*) were exposed to salt and the intracellular metabolites analysed. Arabitol levels were significantly up-regulated upon salt stress in the *xdh1* strains but were significantly lower than the wild-type. Arabitol was not significantly different in either the *abd1* or the *abd1xdh1* strains during osmotic stress but the concentration of glycerol was significantly higher indicating a compensatory mechanism in operation. Genome sequence analysis identified a second possible enzyme capable of synthesizing arabitol explaining the basal level of arabitol present in the *abd1xdh1* strains. This study identified that arabitol is the primary compatible solute in *S. nodorum* but in-built levels of redundancy are present allowing the fungus to tolerate osmotic stress.



## 1. Introduction

Fungi typically accumulate polyols in response to osmotic stress. Mannitol, glycerol, erythritol and arabitol have each been implicated in enabling filamentous fungi to withstand osmotic stress (De Vries et al., 2003; Dixon, 1999; Fujimura et al., 2000; Liepins et al., 2006; Ruijter et al., 2004; Stoop and Mooibroek, 1998). Arguably, the most comprehensive analysis on the response to osmotic stress in a filamentous fungus has been undertaken on *Aspergillus nidulans*. A combination of metabolite screening and reverse genetics have demonstrated that glycerol is the primary compatible solute and that a $NADP^+$-dependent glycerol dehydrogenase is required, not glycerol 3-phosphate dehydrogenase (De Vries et al., 2003; Fillinger et al., 2001). Glycerol has also been demonstrated as the primary solute accumulating in response to osmotic stress in *Aspergillus oyzae, Neurospora crassa* and *Hyprocea jecorina*.

Plant pathogenic fungi face a hostile growing environment during infection. Plant defence responses, nutrient limitation and oxidative stress are all obstacles that the invading fungus has to overcome. Osmotic stress is also touted as a potential barrier to an invading fungus and consequently the mechanisms enabling tolerance to low water activity are of particular interest. It is often considered that infecting fungal pathogens are likely to undergo osmotic stress during growth *in planta*. Surprisingly, there are only a handful of reports investigating the metabolic basis of osmotic stress tolerance in fungal plant pathogens. Dixon and co-workers identified that arabitol accumulates during external hyperosmotic stress in the rice pathogen *Magnaporthe grisea* (Fig. 1) (Dixon, 1999). Metabolite profiling of intracellular metabolites upon exposure of *Cladosporium fulvum* to salt *in vitro* revealed the accumulation of arabitol. Subsequent profiling during infection of tomato leaves highlighted an increased abundance of both arabitol and glycerol suggesting that osmoregulation and water acquisition is an important aspect of pathogenicity (Clark et al., 2003).

The ascomycete *Stagonospora* (syn. *Septoria) nodorum* (Berk.) Castell. and Germano [teleomorph *Phaeosphaeria* (syn. *Leptosphaeria) nodorum* (Müll.) Hedjar.] is a major pathogen on wheat, causing leaf and glume blotch diseases (Solomon et al., 2006a; Weber, 1922). Recent dissection of mannitol metabolism in *S. nodorum* has shown that the 6-carbon sugar alcohol has no direct role in osmotic stress (Solomon et al., 2007). This raises the ques-

* Corresponding author. Fax: +6189360303.
*E-mail address:* psolomon@murdoch.edu.au (P.S. Solomon).
[1] Present address: Rothamsted Research, Harpenden, Hertfordshire, AL5 2JQ, UK.

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doi:10.1016/j.fgb.2008.08.006

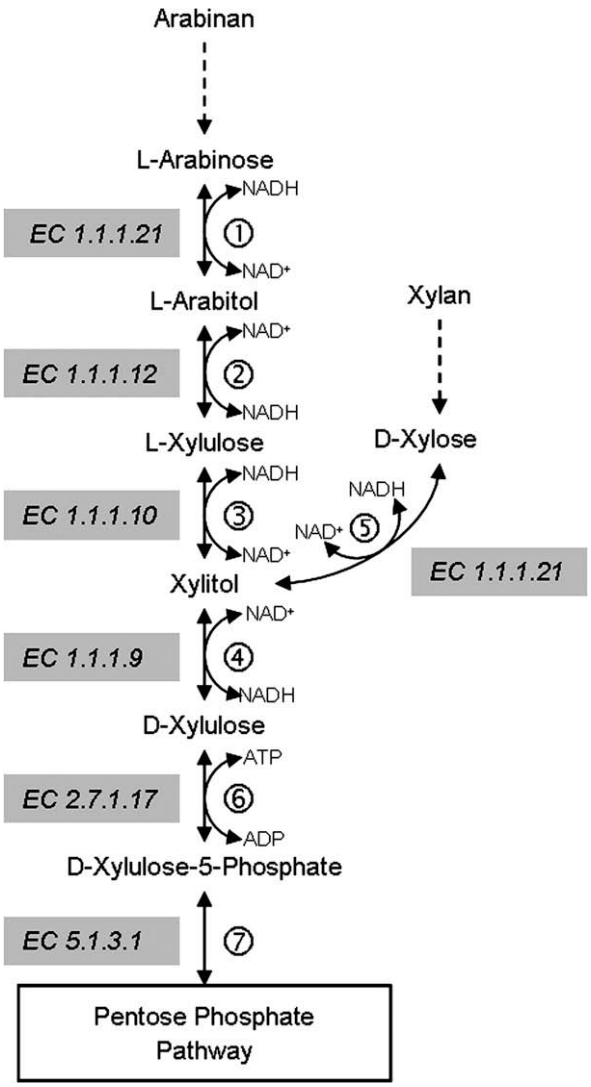


**Fig. 1.** The catabolism of D-xylose and L-arabinose in fungi. Xylose and arabinose can be catabolised in fungi by the above pathway. The enzyme activities listed are: 1, aldose reductase EC 1.1.1.21; 2, L-arabitol 4-dehydrogenase 1.1.1.12; 3, L-xylulose reductase EC 1.1.1.10; 4, xylitol dehydrogenase EC 1.1.1.9; 5, aldose reductase EC 1.1.1.21; 6, xylulokinase EC 2.7.1.17; 7, ribulose-phosphate 3-epimerase EC 5.1.3.1. Solid lines indicate one enzymatic step, dashed lines indicate multiple steps.

tion of how does *S. nodorum* respond to changes in the osmotic state of its growing environment. To address this, *S. nodorum* was exposed to strong saline conditions to induce osmotic stress and undertook a non-targeted metabolomics approach to understand the metabolic response. Arabitol was identified as the primary compatible solute in *S. nodorum*. The genetic tractability of *S. nodorum* was then exploited to dissect 5-carbon polyol metabolism and the role it plays during osmotic stress.

## 2. Materials and methods

### 2.1. Fungal strains and media

*Stagonospora nodorum* SN15 was provided by the Department of Agriculture, Western Australia. The fungus was routinely grown on CzV8CS (Czapek Dox agar (Oxoid) 45.4 g $l^{-1}$, agar 15.0 g $l^{-1}$, $CaCO_3$ 3.0 g $l^{-1}$, Campbell's V8 juice 200 ml $l^{-1}$, casamino acids 100 mg $l^{-1}$, peptone 100 mg $l^{-1}$, yeast extract 100 mg $l^{-1}$, adenine 15 mg $l^{-1}$, biotin 100 μg $l^{-1}$, nicotinic acid 100 μg $l^{-1}$, *p*-aminobenzoic acid 100 μg $l^{-1}$, pyridoxine 100 μg $l^{-1}$, thiamine 100 μg $l^{-1}$) (Solomon et al., 2006b). Plates were incubated at 22 °C in 12 h cycles of darkness and near-UV light (Phillips TL 40W/05). Liquid cultures were started with the addition of $10^7$ spores to 100 ml CzV8CS and also grown at 22 °C shaking at 130 rpm in the dark. For experiments that required defined growth conditions, *S. nodorum* SN15 was used to inoculate minimal medium (MM) which consisted of 6 g $l^{-1}$ glucose, 2 g $l^{-1}$ $NaNO_3^-$, 1.0 g $l^{-1}$ $K_2HPO_4$, 0.5 g $l^{-1}$ KCl, 0.5 g $l^{-1}$ $MgSO_4{\cdot}7H_2O$, 0.01 g $l^{-1}$ $ZnSO_4.7H_2O$, 0.01 g $l^{-1}$ $FeSO_4{\cdot}7H_2O$, 0.0025 g $l^{-1}$ $CuSO_4{\cdot}5H_2O$. 15 g $l^{-1}$ agarose was added if plates were required.

### 2.2. Plant material and infection conditions

Ten-cm diameter pots containing Perlite (P500) and Grade 2 vermiculite (The Perlite and Vermiculite Factory, WA, Australia) were seeded with 5 seeds of the wheat cultivar Amery and grown at 20 °C in a 12-h day/night cycle. Whole-plant spray infections were performed as described previously (Solomon et al., 2004). Seven days after infection the plants were scored for disease severity. The infections were given a score between 0 and 10 depending on the severity of the infection with 0 showing no symptoms of disease and 10 being a completely necrotic dead plant. Sporulation *in planta* was monitored by the latent period assay as previously described (Solomon et al., 2006b).

### 2.3. Construct development for Abd1 and Xdh1 disruption

The *Abd1* knockout construct was made by overlap PCR as previously described (Solomon et al., 2006b). The primers used for *Abd1* overlap construct were AbdKO5′f (5′-AATGACTCTTTTC-CATCGGC-3′), AbdKO5′r (5′-TGTGACTTTTGGTTACGCCGTCTTCTCC ACAACCTCCAGACTC-3′), AbdKO3′f (5′-TCTCCTATGAGTCGTTTACC CAGAACGGCGTTATTGAGCTGAGT-3′) and AbdKO3′r (5′-ACATGTT GAAGGGTCTGCC-3′). Amplification of SN15 gDNA with primers AbdKO5′r and AbdKO5′r produced the 5′ flank, while primers AbdKO3′f and AbdKO3′r produced the 3′ flank. The phleomycin resistance cassette was amplified from pAN8-1 with primers pAN8f (5′-TGTAGAAAAATGTGACGAACTCGTG-3′) and pAN8r (5′-TCTTTTACTTTCACCAGCGTTTCTG-3′). The final overlap PCR including all three fragments yielded a final construct of 4137 bp conferring phleomycin resistance that was used to transform *S. nodorum* SN15. Integration was screened for by PCR amplification of transformant gDNA using AbdScrnF (5′-TGTGGAACGCTTGCATAATGA-3′) and AbdScrnR (5′-AAAAACCGGCTTCACGAAGA-3′). Homologous recombination yielded a product of 4357 bp whilst and ectopic integration amplified the wild-type product of 3241 bp. Single integration events were screened for by determining the copy number of the phleomycin cassette as previously described (Solomon et al., 2008).

The *Xdh1* construct was developed using the splitmarker technique as previously described (Solomon et al., 2006b). The 5′ flank was amplified using the primers Xdh1KO5′f (5′- GCTTGAAAGCTC CATTATGC-3′) and Xdh1KO5′r (5′-GTACTGTGTAAGCGCCCACTTG TAGTCAGGGATGCGTGT-3′). The 3′ flank was amplified using Xdh1KO3′f (5′-TTGGGAGCTCGGTATAAGCCACCAAAGAGCTCAACGT C-3′) and Xdh1KO3′r (5′-TGAAGGATTGGGTTGTGTCT-3′). The flanks were fused to each half of the hygromycin resistance cassette amplified from pAN7-1. The resulting amplicons were used to transform SN15, and the transformants screened for by PCR using Xdh1KOscreenF (5′-CTTTAGTTCACCTCAGTTTGCC-3′) and Xdh1KO-screenR (5′- ATTCGAGAGACTCGGAGAGAG-3′). Homologous integration events were confirmed with detection of a 3814 bp amplicon whilst an ectopic integration yielded an amplicon of 1876 bp. Copy number was confirmed using primers specific for the hygromycin resistance cassette as previously described (Solomon et al., 2008).