δ -Aminolaevulinic acid synthesis is required for virulence of the wheat pathogen *Stagonospora nodorum*

Peter S. Solomon, Cordula I. Jörgens and Richard P. Oliver

Correspondence
Peter S. Solomon
psolomon@murdoch.edu.au

Australian Centre for Necrotrophic Fungal Pathogens, SABC, Division of Health Sciences, Murdoch University, Perth 6150, Australia

δ -Aminolaevulinic acid (ALA) is synthesized in fungi by ALA synthase, a key enzyme in the synthesis of haem. The requirement for ALA synthase in *Stagonospora nodorum* to cause disease in wheat was investigated. The single gene encoding ALA synthase (*Als1*) was cloned and characterized. Expression analysis determined that *Als1* transcription was up-regulated during germination and also towards the latter stages of the infection. The *Als1* gene was further characterized by homologous gene replacement. The inactivation of *Als1* resulted in strains producing severely stunted germ tubes leading quickly to death. The strains could be recovered by supplementation with 33 μ M ALA. Pathogenicity assays revealed the *als1* strains were essentially non-pathogenic, inferring a key role for the synthesis of ALA during *in planta* growth. Supplementing the strains with ALA restored growth *in vitro* and also pathogenicity for up to 5 days after inoculation. Further examination by inoculating the *als1* strains onto wounded leaves found that pathogenicity was only partially restored, suggesting that host-derived *in planta* levels of ALA are not sufficient to support growth. This study has identified a key role for fungal ALA synthesis during infection and revealed its potential as an antifungal target.

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INTRODUCTION

The infection cycle of most phytopathogenic fungi involves three distinct stages, both in terms of development and also metabolic activity (Solomon *et al.*, 2003a). The first stage of the infection cycle begins on the leaf surface where the fungus is reliant on the catabolism of intracellular nutrient sources for germination and growth prior to penetration (Idnurm & Howlett, 2002; Solomon *et al.*, 2004; Thines *et al.*, 2000). The second phase of the infection cycle begins after the fungus has penetrated the host and initiates a period of vegetative growth whilst accessing the rich carbon and nitrogen sources (Solomon & Oliver, 2001). The current dogma is that once these nutrient sources have been utilized, the fungus then completes the infection cycle by sporulating (Solomon *et al.*, 2005). The role of primary metabolism in these three stages of the infection cycle has been investigated to varying degrees. In most systems, it is not known which host-derived metabolites are utilized by the fungus, although several key biosynthetic pathways have been determined to be required for *in planta* growth of various pathogenic fungi (Solomon *et al.*, 2003a).

An area of primary metabolism in fungal–plant interactions that has yet to be investigated is that of porphyrin and

haem biosynthesis. A key intermediate in the synthesis of porphyrins is δ -aminolaevulinic acid (5-aminolaevulinic acid; ALA) which is known to occur via two distinct pathways in nature (Beale, 1978). The synthesis of porphyrins in animals and fungi begins with the condensation of glycine and succinyl-CoA by ALA acid synthase succinyl-CoA:glycine C-succinyl transferase (decarboxylating, EC 2.3.1.37) to form ALA (Kikuchi *et al.*, 1958). All other organisms, including plants, synthesize ALA from glutamate via glutamyl-tRNA and glutamate 1-semialdehyde and do not use ALA synthase (Beale & Castelfranco, 1974). This clear difference in the synthesis of ALA between fungi and plants makes the pathway a logical choice for further study.

ALA synthases in fungi have received little attention. Whilst many orthologues of genes predicted to encode ALA synthases have been described from various fungal genome sequencing projects, only enzymes from the yeast *Saccharomyces cerevisiae* and the basidiomycete *Ustilago maydis* have been scrutinised in detail (Schneegurt, 2005; Urban-Grimal & Labbe-Bois, 1981; Urban-Grimal *et al.*, 1986). The gene encoding ALA synthase in *Aspergillus oryzae* (*HemA*) has been disrupted for the purpose of its development as a selectable marker in subsequent transformation (Elrod *et al.*, 2000). The resulting *hemA* strain was found to be auxotrophic for ALA and could be complemented with the addition of exogenous ALA.

Abbreviation: ALA, δ -aminolaevulinic acid.

Whilst the genes and resulting proteins have been characterized in some fungi, there have been no reports on the role of ALA synthesis during *in planta* growth for phytopathogens. The ascomycete *Stagonospora* (syn. *Septoria*) *nodorum* (Berk.) Castell. & Germano [teleomorph *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (Müll.) Hedjar.] is a major pathogen on wheat, causing leaf and glume blotch diseases (Weber, 1922). In Western Australia, *S. nodorum* causes 5–20 % crop losses per annum (Murray & Brown, 1987). To investigate the requirement of *in vivo* ALA synthesis during pathogenicity, the encoding gene in *S. nodorum* has been cloned and characterized by homologous gene replacement.

METHODS

Fungal strains and media. *Stagonospora nodorum* SN15 was provided by the Department of Agriculture, Western Australia. All media and growth conditions were as described previously except where supplemented with ALA (Solomon *et al.*, 2004).

RNA extraction and transcript analysis. Total RNA was isolated from fungal mycelium and plant material using Trizol Reagent (Invitrogen). RNA (1 µg) was reverse-transcribed with iScript (Bio-Rad) as per the manufacturer's protocol. The resulting cDNA was then quantified using a RotorGene (Corbett Research, Sydney, Australia). The PCR contained 10 µl iSybr quantitative premix (Bio-Rad), 5 µl diluted cDNA and 5 µl 1.2 µM primers. The conditions used were 95 °C for 3 min, then 94 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s for 35 cycles with data collected at each extension stage. Primers used for quantitative PCR were *Als1*-qPCRf (5'-GACTATCTTGGAATGGGTCGCA-3') and *Als1*-qPCRR (5'-CGCACATGCTATAGACACTCTCAA-3'), and *Actin*-qPCRf (5'-CTGCTTTGAGATCCACAT-3') and *Actin*-qPCRR (5'-GTCACCACTTCAACTCC-3').

All sequencing was performed using a Perkin Elmer PCR sequencing premix and analysed on an ABI 373 or 377 automatic DNA sequencer.

Development of *S. nodorum als1*. The *Als1* cDNA was disrupted by insertional mutagenesis as described previously (Solomon *et al.*, 2003b). The resulting insert was amplified using standard M13 and M13R primers and purified using a QIAquick PCR purification kit (Qiagen). The amplified insert was eluted from the QIAquick column with STC buffer (1.2 M sorbitol, 10 mM calcium chloride, 10 mM Tris, pH 7.5) and used directly for transformation. Transformation of *S. nodorum* SN15 to create *S. nodorum als1* was performed as described by Solomon *et al.* (2003b).

***S. nodorum* growth assays.** Fungal growth assays were performed in 96-well microtitre plates. To each well, 160 µl minimal medium was added together with 20 µl of the appropriate 10 × concentration of ALA, if required. The wells were inoculated with the addition of 20 µl 1×10^6 spores ml⁻¹ and the absorbance of the plates was read at 600 nm using a microplate reader (model 3550-UV; Bio-Rad). The microtitre plates were then wrapped in Parafilm and incubated without agitation at 22 °C. After 7 days, a second absorbance reading was taken at 600 nm. To calculate growth over the 7-day period, the initial absorbance reading was subtracted from the final reading.

***S. nodorum* germination assays.** The germination of *S. nodorum* was assayed as follows. Agarose slides were prepared by pipetting a small amount of 0.7 % molten agarose in the centre of a standard glass microscope slide. Two thin pieces of transparency film were placed on the outer edges of the slide on either side of the agarose. Whilst the agarose was molten, a second microscope slide was placed over the top of the first with the width of the transparency

film determining the thickness of the agarose. After the agarose had set, the top slide was removed leaving a thin layer of agarose on the bottom slide.

The slides were inoculated with a 20 µl spore suspension containing 2000 spores. The inoculated slides were placed in square plastic Petri dishes containing wet tissue to ensure the slides did not over dry. For all assays undertaken, three fields containing 100 spores were counted and subjected to ANOVA analysis to determine statistical significance.

Plant material and infection conditions. Ten centimetre diameter pots containing Perlite (P500) and Grade 2 vermiculite (The Perlite and Vermiculite Factory, WA, Australia) were seeded with five 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 being uninfected and no symptoms of disease and 10 being a completely necrotic dead plant.

Pathogenicity of the mutants was also investigated using a detached-leaf assay. This involved placing 2-week-old wheat leaves on 0.15 % benzimidazol plates with the ends of the leaves embedded into the agar. The leaves were inoculated with 5 µl drops of inoculum containing 10⁴ spores in 0.02 % Tween 20. The plates were wrapped in Parafilm and incubated at 20 °C in a 12-h day/night cycle. Disease severity was assessed by measuring the amount of necrotic tissue 6 days post-inoculation (p.i.).

Leaf clearing and staining. Lesions on wheat leaves caused by *S. nodorum* were examined by light microscopy. Diseased leaf tissue was stained and cleared with trypan blue using a method modified from those described elsewhere (Bruzzese & Hasan, 1983; Shipton & Brown, 1962). The tissue was boiled with trypan blue stain for 5 min, then de-stained and stored in a 2.5 g chloral hydrate ml⁻¹ solution.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *Als1*

The sequencing of an EST library cloned from *S. nodorum* growing on wheat cell walls has been described previously (Solomon *et al.*, 2003b). One gene, with a partial ORF showing notable similarity to 5-aminolevulinate synthase, was identified. The partial cDNA sequence was used to identify a full-length ORF, named *Als1*, within the *S. nodorum* genome sequence (SNU09357.1; GenBank DQ167577; www.broad.mit.edu/annotation/fungi/stagonospora_nodorum/). Annotation of the ORF from the genome sequence reported the *Als1* gene to contain only one intron located at 332–428 bp. Comparative analysis with the *Als1* cDNA identified a second intron at 1635–1685 bp. The translated product was predicted to be 620 aa. BLASTP analysis against the nr database (www.ncbi.nlm.nih.gov/blast/) identified the predicted protein to be 70 % identical to a hypothetical protein from *Magnaporthe grisea* and 65 % identical to a characterized 5-aminolevulinate synthase from *Aspergillus oryzae* (Elrod *et al.*, 2000). Motif searching of the predicted protein sequence identified a 5-aminolevulinate synthase PFAM domain (pfam02490) from aa 141 to 211 and an amino-transferase class I and II PFAM domain (pfam0015) from aa 215 to 540.

Als1 is expressed during infection

To determine if *Als1* may play a role during *in planta* growth, gene expression during infection was examined by quantitative PCR (Fig. 1). Expression of *Als1* was apparent throughout all stages of infection. The pattern of expression is interesting in that it implies the heaviest requirement for *in vivo* synthesis occurs during germination, during the latter period of vegetative growth and also during sporulation. The expression levels during germination suggest that the fungus has only very limited, if any, stored ALA available for use and thus is reliant on *de novo* synthesis. The lower level of expression at 2 days p.i. corresponds to when the fungus has penetrated the leaf surface and has begun vegetative growth. It could be that upon penetrating the leaf, the fungus gains access to host-derived ALA and thus *in vivo* synthesis decreases. Another possibility is that when the fungus gains access to rich plant-derived carbon, it may go into a fermentative mode of growth and consequently the requirement for haem biosynthesis would decrease. After 2 days p.i. though, the *Als1* expression levels again increase, implying that host-derived levels of ALA are not adequate to supply the rapidly growing fungus or that the fungus is more reliant on respiration and consequently *in vivo* synthesis is required. Note that whilst the data reported above rely on normalization with actin, data on *Als1* expression normalized against both EF1 α and β -tubulin expression were collected and found to be consistent with the actin normalization results (data not shown). On the basis of this *Als1* expression pattern, it was decided to further characterize the function of the *Als1* gene by homologous gene replacement.

Development of *S. nodorum als1* strains

The development of the insertional inactivation construct was undertaken using *Als1* cDNA as the target for a novel transposon containing the phleomycin selectable marker.

Fig. 1. Analysis of *Als1* expression during infection. *Als1* transcript levels are normalized to those of actin. Note that the expression of *Als1* in ungerminated spores is represented at the zero time point. The experiment used two biological replicates, each consisting of two technical replicates. Different letters (a, ab, b, c) indicate significant ($P=0.05$) differences in enzyme activity by ANOVA analysis.

After the transposition reaction and subsequent transformation into *E. coli* XL1Blue, 18 colonies were obtained. Six of these colonies were selected at random for plasmid extraction. Three of the six plasmids appeared to have the transposon inserted into the *Als1* cDNA as determined by restriction enzyme analysis. Sequencing of these three plasmids using primers homologous to the end of the transposon confirmed that the transposon for the three constructs had inserted at the same location, between bases 939 and 940 of the genomic sequence. One of the constructs was selected and named pGPSP-*als1*.

The pGPSP-*als1* construct was used to transform *S. nodorum* SN15 to create *als1* strains. Thirty-five transformants were obtained of which 28 were found to require ALA for growth. Further screening by PCR identified these 28 transformants to have undergone homologous recombination with the pGPSP-*als1* construct. This was confirmed in several strains by Southern analysis (data not shown) and *S. nodorum als1-3* and *als1-4* were chosen for further analysis. *S. nodorum Als1-18* was also chosen as an ectopic control.

in vitro characterization of the *als1* strains

The strains lacking *Als1* were characterized *in vitro*. The transformation and subsequent subculturing suggested that the disruption of *Als1* rendered the fungus auxotrophic for ALA. For further characterization, growth tests were performed on a defined growth medium with varied amounts of ALA (Fig. 2a). There was no discernable difference in growth observed compared to wild-type when the mutants were grown in the presence of ALA. Concentrations as low as 33 μ M were able to fully complement growth. However, growth of the mutants was severely restricted in the absence of ALA, confirming that the *als1* strains were auxotrophic for ALA.

To determine the viability of the *als1* strains in the absence of exogenous ALA, a growth experiment was undertaken where ALA was added to the *als1* strains at various times after inoculation into minimal medium (Fig. 2b). The growth tests revealed that the *als1* germlings remained viable and able to be complemented with exogenous ALA for up to 5 days after inoculation. After 5 days, no growth was observed after the addition of ALA, confirming that the strains were no longer viable.

Germination was further investigated using assays performed on agarose slides in the presence and absence of ALA (Fig. 3). *S. nodorum* SN15 appeared to germinate typically with germ tubes extending approximately 85 μ m on average. For spores lacking *als1*, the rate of germination was not significantly different than that of the wild-type; however, the length of the germ tubes averaged only 17 μ m. The addition of 100 μ M ALA had no effect on the germination rate of wild-type spores; however, the mean germ tube length of the *als1* spores in the presence of 100 μ M ALA increased to 80 μ m, suggesting the ALA was able to complement the *als1* genotype.

Fig. 2. (a) Growth test to determine the level of ALA required to complement growth. Light grey bars indicate growth of *S. nodorum* SN15, the white bars represent the growth of the ectopic strain *Als1-18* and the growth of *als1-3* and *als1-4* are represented by the dark grey and black bars, respectively. (b) A microtitre plate growth assay determining the viability of *als1-3* in the absence of exogenous ALA. ALA was added at a final concentration of 100 µM to the cultures during inoculation (□) and at 1 (○), 2 (×), 4 (●) and 6 days p.i. (△). Two biological replicates were used with eight technical replicates assayed. Bars show SD.

Pathogenicity assays

The virulence of the *als1* strains was investigated to determine the requirement of the *Als1* gene for pathogenicity. For the seedling assay, the disease scores for both *S. nodorum als1-3* and *als1-4* were not significantly different from those of the Tween control, recording scores between 2 and 3 (Fig. 4). The wild-type and ectopic strains were both scored at 9, indicating a high degree of pathogenicity. The *als1* strains were also assayed using the detached-leaf system (Fig. 5). Strong lesions were evident for both *S. nodorum* SN15 and *Als1-18*, whilst symptoms of disease were essentially absent for leaves inoculated with *als1-3* and *als1-4*. Microscopic analysis of the infections showed typically strong growth at 2 days p.i. for wild-type with multiple penetration attempts observed (Fig. 5i, j). In contrast, much less growth was observed for the *als1* strains (only *als1-3* shown) with many of the spores producing stunted germ tubes.

Complementation was attempted via the addition of ALA to the inoculum (Fig. 5c, d). Curiously, the addition of ALA to the wild-type and ectopic strains appeared to inhibit pathogenicity. This was attributed to the previously described toxicity of ALA in the presence of light (Rebeiz *et al.*, 1984). The assay was reattempted by placing the leaves in complete darkness for 2 days following inoculation (Fig. 5e, f).

Fig. 3. Microscopic examination at 1 day p.i. of thin agarose slides inoculated with (a) *S. nodorum* SN15, (b) *S. nodorum* SN15 with 100 µM ALA, (c) *S. nodorum als1-3* and (d) *S. nodorum als1-3* with 100 µM ALA. Approximately 100 spores of each strain were counted with typical examples being shown. Bar, 30 µm.

Increased lesion sizes were apparent for the wild-type and ectopic strains, confirming the phototoxic effect of ALA. The restoration of pathogenicity of the *als1* strains was also apparent when inoculating with 1 mM ALA, either in the light or the dark, implying that the synthesis of ALA by *S. nodorum* during infection is a requirement for *in planta* growth. To determine whether the requirement for ALA is limited to only the leaf surface or if *in planta* ALA synthesis is also required for vegetative growth, the strains were inoculated onto wounded leaves in the absence of ALA (Fig. 5g, h). Pathogenicity was at least partially restored, suggesting that the fungus was able to enter the leaf through the artificial openings and was able to at least initiate a vegetative growth state. However, disease progression was halted at an early stage, suggesting there may be sufficient amounts of ALA available to support a small amount of fungal growth, but the fungus soon outgrows the supply and the disease is halted. These results compare well with measured *Als1* expression levels and suggest that ALA is immediately

Fig. 4. Seedling pathogenicity assay. A score of 0 indicates no obvious symptoms whilst 10 represents a completely dead leaf. The assay was performed twice with eight biological replicates used for each experiment. Bars show SD.

Fig. 5. Detached-leaf pathogenicity assays. The leaves in (a) were inoculated in the absence of ALA, whilst 1 mM ALA was included in the inoculum used in (c). (e) shows leaves inoculated in the presence of 1 mM ALA and incubated in darkness for 48 h, whilst the leaves in (g) were wounded by gently puncturing eight times with a 25-gauge sterile needle. The panels within (a), (c), (e) and (g) refer to leaves inoculated with *S. nodorum* *als1-3* (1), *als1-4* (2), *Als1-18* (3) and SN15 (4), and the Tween control (5). The graphs in (b), (d), (f) and (h) depict lesion sizes for the infections in (a), (c), (e) and (g), respectively. Note that the graphs shown represent two biological replicates with each containing five technical replicates. (i) and (j) are trypan-blue-stained samples at 2 days p.i. of detached wheat leaves infected with *S. nodorum* SN15 and *als1-3*, respectively. Bars in (i) and (j), 30 μ m.

Fig. 6. Examination of the viability of *als1-3* on the leaf surface. Two microlitres of 100 μ M ALA was applied to the site of inoculation at the following times: 0 (□), 1 (○), 3 (×), 5 (●) and 6 days p.i. (△). Two biological replicates were used with eight technical replicates assayed. Bars show SD.

available once the fungus first penetrates the leaf, but there are soon insufficient levels of ALA to support growth and consequently the fungus is reliant on *in vivo* synthesis.

The viability of the *als1* germplings on the leaf surface was also examined. Exogenous ALA was added at various times after inoculation and lesion size was measured (Fig. 6). As mentioned above, the inclusion of ALA in the initial inoculum restored pathogenicity. The additional of ALA to the surface of the leaf at the site of the inoculation was able to restore pathogenicity for up to 5 days p.i.. After 5 days, the addition of ALA could not initiate *in planta* growth strongly, suggesting that the *als1* germplings were no longer viable. To confirm this, the site of the inoculation was removed from the leaf at various times after inoculation and placed on a minimal medium plate containing 1 mM ALA. Growth was consistently apparent after inoculation for up to 5 days, but not for longer than this, confirming that the *als1* strains were no longer viable after this time (data not shown). The period of 5 days is consistent with the *in vitro* observations in minimal medium. Based on the assumption that the leaf surface is essentially devoid of nutrients, the consistency of the results, comparing what was observed on the leaf surface and *in vitro* observations, indicates that the nutritional status of the environment has no impact on the viability of the *als1* strains.

The nutritional requirements of fungal phytopathogens growing *in planta* are poorly understood. The elucidation of pathways that fungi require for *in planta* growth will enable a better understanding of disease. This study has identified for the first time that the fungus *S. nodorum* is not supplied with the key intermediate ALA during *in planta* growth and therefore is reliant on *in vivo* synthesis. Given that the synthesis of ALA in plants occurs via distinct mechanisms to those in fungi, the pathway represents an attractive antifungal target. Experiments are now under way to exploit this.

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REFERENCES

- Beale, S. I. (1978). δ -Aminolevulinic acid in plants: its biosynthesis, regulation, and role in plastid development. *Annu Rev Plant Physiol* **29**, 95–120.
- Beale, S. I. & Castelfranco, P. A. (1974). The biosynthesis of δ -aminolevulinic acid in higher plants. II. Formation of ^{14}C - δ -aminolevulinic acid from labelled precursors in greening plant tissues. *Plant Phys* **53**, 297–303.
- Bruzzese, E. & Hasan, S. (1983). A whole leaf clearing and staining technique for host specificity studies of rust fungi. *Plant Pathol* **32**, 335–338.
- Elrod, S. L., Jones, A., Berka, R. M. & Cherry, J. R. (2000). Cloning of the *Aspergillus oryzae* 5-aminolevulinate synthase gene and its use as a selectable marker. *Curr Genet* **38**, 291–298.
- Idnurm, A. & Howlett, B. J. (2002). Isocitrate lyase is essential for the pathogenicity of the fungus *Leptosphaeria maculans* to canola (*Brassica napus*). *Eukaryot Cell* **1**, 719–724.
- Kikuchi, G., Kumar, A., Talmage, P. & Shemin, D. (1958). The enzymatic synthesis of δ -aminolevulinic acid. *J Biol Chem* **233**, 1214–1219.
- Murray, G. M. & Brown, J. F. (1987). The incidence and relative importance of wheat diseases in Australia. *Aust Plant Pathol* **16**, 34–37.
- Rebeiz, C. A., Montazer-Zouhoor, A., Hoppen, H. J. & Wu, S. M. (1984). Photodynamic herbicides: 1. Concept and phenomenology. *Enzyme Microb Technol* **6**, 390–401.
- Schneegurt, M. A. (2005). δ -Aminolevulinic acid biosynthesis in *Ustilago maydis*. *J Basic Microbiol* **45**, 155–159.
- Shipton, W. A. & Brown, J. F. (1962). A whole-leaf clearing and staining technique to demonstrate host–pathogen relationships of wheat stem rust. *Phytopathology* **52**, 1313–1318.
- Solomon, P. S. & Oliver, R. P. (2001). The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. *Planta* **213**, 241–249.
- Solomon, P. S., Tan, K. C. & Oliver, R. P. (2003a). The nutrient supply of pathogenic fungi; a fertile field for study. *Mol Plant Pathol* **4**, 203–210.
- Solomon, P. S., Thomas, S. W., Spanu, P. & Oliver, R. P. (2003b). The utilisation of di/tripeptides by *Stagonospora nodorum* is dispensable for wheat infection. *Physiol Mol Plant Pathol* **63**, 191–199.
- Solomon, P. S., Lee, R. C., Wilson, T. J. G. & Oliver, R. P. (2004). Pathogenicity of *Stagonospora nodorum* requires malate synthase. *Mol Microbiol* **53**, 1065–1073.
- Solomon, P. S., Tan, K.-C. & Oliver, R. P. (2005). Mannitol 1-phosphate metabolism is required for sporulation *in planta* of the wheat pathogen *Stagonospora nodorum*. *Mol Plant Microbe Interact* **18**, 110–115.
- Thines, E., Weber, R. W. S. & Talbot, N. J. (2000). MAP kinase and protein kinase A-dependent mobilization of triacylglycerol and glycogen during appressorium turgor generation by *Magnaporthe grisea*. *Plant Cell* **12**, 1703–1718.
- Urban-Grimal, D. & Labbe-Bois, R. (1981). Genetic and biochemical characterisation of mutants of *Saccharomyces cerevisiae* blocked at six different steps of heme biosynthesis. *Mol Gen Genet* **183**, 85–92.
- Urban-Grimal, D., Volland, C., Garnier, T., Dehoux, P. & Labbe-Bois, R. (1986). The nucleotide sequence of the *HEM1* gene and evidence for a precursor of the mitochondrial 5-aminolevulinate synthase in *Saccharomyces cerevisiae*. *Eur J Biochem* **156**, 511–519.
- Weber, G. F. (1922). Septoria diseases of wheat. *Phytopathology* **12**, 537–585.