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The *Fusarium oxysporum sti35* gene functions in thiamine biosynthesis and oxidative stress response

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Abstract

The *sti35* gene of the vascular wilt fungus *Fusarium oxysporum* was originally identified based on induced expression under stress conditions. In this study, the transcriptional regulation and biological function of *sti35* were examined in the tomato pathogen *F. oxysporum* f.sp. *lycopersici*. Expression of *sti35* was repressed by thiamine and induced by high temperatures. *Sti35* transcripts were detected both during early and late stages of infection of tomato plants by *F. oxysporum*. Heterologous expression of the *sti35* cDNA restored thiamine prototrophy in a *Saccharomyces cerevisiae thi4* mutant and increased UV tolerance in a *uvr*$^-$ mutant of *Escherichia coli*. Targeted Δ*sti35* knockout mutants of *F. oxysporum* exhibited a thiamine auxotrophic phenotype and reduced tolerance to the superoxide-generating agent menadione, indicating that Sti35 has a dual role in thiamine biosynthesis and oxidative stress response. RT-PCR analysis revealed the presence of differential RNA splicing of the second 5′-UTR intron, suggesting that thiamine may regulate *sti35* expression via a post-transcriptional mechanism. *F. oxysporum* transformants carrying a transcriptional fusion of the *sti35* promoter to the *lacZ* reporter gene produced high levels of β-galactosidase activity when grown in the absence, but not in the presence of thiamine. Thus, the *sti35* promoter represents a useful tool for the controlled expression of genes of interest in *F. oxysporum*.

Keywords: *Fusarium oxysporum*; Thiamine biosynthesis; *sti35*; Gene regulation; Oxidative stress

1. Introduction

Thiamine (vitamin B1) is an essential vitamin that acts as a key cofactor of enzymes involved in basic processes of carbohydrate metabolism such as glycolysis, the citric acid cycle and the pentose-phosphate cycle. Thiamine biosynthesis occurs through a multistep pathway, involving two independent branches that synthesize the thiazole and pyrimidine moieties of thiamine, respectively (Hohmann and Meacock, 1998). The final products of the two branches, 2-methyl-4-amino-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP) and 4-methyl-5-(2-hydroxyethyl)thiazole monophosphate (TH-P), are condensed by thiamine-phosphate pyrophosphorylase (TMP-PPase) to produce the active thiamine pyrophosphate (TPP) (Hohmann and Meacock, 1998).

The *sti35* gene of the vascular wilt fungus *F. oxysporum*, which was originally identified as a stress-induced gene (Choi et al., 1990), encodes the founding member of a conserved eukaryotic protein family that functions in thiamine biosynthesis. The family includes Thi4p from

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Saccharomyces cerevisiae (Praekelt et al., 1994), Nmt2p from *Schizosaccharomyces pombe* (Manetti et al., 1994) and Thi1p from *Arabidopsis thaliana* (Machado et al., 1996), as well as others (Belanger et al., 1995; Ribeiro et al., 1996; Jacob-Wilk et al., 1997; Faou and Tropschug, 2003). Besides their role in thiamine biosynthesis, members of this family have been associated with additional, apparently unrelated cellular functions such as tolerance to DNA damage in *A. thaliana* (Machado et al., 1996) or maintenance of mitochondrial genome stability in *S. cerevisiae* (Machado et al., 1997). More recently, the *Neurospora crassa* orthologue of Sti35, CyPB37, was identified as a protein binding the CyP40-type cyclophilin NcCyP41 (Faou and Tropschug, 2003; Faou and Tropschug, 2004).

Like other genes of the thiamine biosynthesis pathway in yeasts and fungi, *sti35* orthologues are highly expressed and subject to transcriptional repression by thiamine (Maundrell, 1990; Praekelt et al., 1994; Faou and Tropschug, 2003; McColl et al., 2003). The molecular mechanism of thiamine repression in yeasts involves binding of a putative transcriptional repressor to a conserved regulatory region in the promoter (Zurlinden and Schweingruber, 1997). However, recent studies suggest the presence of a post-transcriptional mechanism of gene repression by thiamine. Analysis of *thiA*, the *Aspergillus oryzae* orthologue of *sti35*, identified conserved motifs in the promoter region which mediate differential splicing of an intron in the 5′-untranslated region (5′-UTR) (Kubodera et al., 2000; Kubodera et al., 2003). These regions bear structural similarity to TPP-triggered riboswitches that control thiamine biosynthetis genes in prokaryotes (Miranda-Rios et al., 2001; Winkler et al., 2002) and eukariotes (Thore et al., 2006; Cheah et al., 2007; Edwards et al., 2007). Riboswitches were initially discovered in the non-coding mRNA regions of bacteria and serve as metabolite-responsive genetic switches that modulate gene expression by interacting directly with the target metabolite (Lai, 2003). Consensus sequences similar to the TPP-binding riboswitch from *Escherichia coli* are also present in filamentous fungi and plants, suggesting that eukaryotes may also use riboswitches for genetic control (Sudarsan et al., 2003). Indeed, control of alternative RNA splicing and gene expression by thiamine was recently demonstrated for the *N. crassa nmt1* gene (Cheah et al., 2007).

The objective of the present work was to study expression and function of *sti35* in *F. oxysporum*. We find that *sti35* has a dual role in thiamine biosynthesis and general stress responses. Regulation of *sti35* by thiamine appears to be associated with alternative splicing of the second intron in the 5′-UTR, possibly mediated by a conserved region involved in forming an RNA secondary structure. Making use of the tight control by thiamine of the *sti35* promoter, we have developed a repressible promoter system as a useful tool for driving expression of endogenous and heterologous genes in *F. oxysporum*.

2. Materials and methods

2.1. Fungal isolates and culture conditions

Fusarium oxysporum f.sp. *lycopersici* wild type strain 4287 (race 2) was obtained from J. Tello, Universidad de Almeria, Spain. Fungal strains were stored as a microconidial suspension at −80 °C with 30% glycerol. For extraction of DNA and microconidial suspensions, cultures were grown in liquid potato dextrose broth (PDB) (Difco, Detroit, MI) at 28 °C with shaking at 170 rpm as described previously (Di Pietro and Roncero, 1998). For analysis of gene expression, freshly obtained microconidia were germinated for 14 h in PDB, washed twice in sterile water and transferred to minimal medium (SM) (Di Pietro and Roncero, 1998) supplemented with the appropriate compound(s).

2.2. Nucleic acid manipulations and library screening

Genomic DNA was extracted from fungal mycelium as described (Torres et al., 1993). Total RNA was isolated from powered mycelia using Tripure Isolation Reagent (Roche Diagnostics, SL), according to the manufacturer's recommendations. Southern and northern blot analyses and probe labelling were carried out as described (Di Pietro and Roncero, 1998) using the non-isotopic digoxigenin labelling Reagent kit (Roche Diagnostics, SL). Genomic DNA of *F. oxysporum* strain 4287 was used for PCR amplification with the primers sti18 and sti9 (Supplementary Table 1) derived from the *F. oxysporum* f.sp. *cucumerinum sti35* sequence available at the National Center for Biotechnology Information (Bethesda, MD) database (Accession No. AB033416). PCRs were routinely performed with the Long Template PCR system (Roche Diagnostics, SL). The amplified 2731 bp DNA fragment was cloned into the pGEM-T vector (Promega, Madison, WI). A cDNA library of *F. oxysporum* in λZAP (Roldan-Arjona et al., 1999) was screened with the genomic DNA fragment corresponding to the *sti35* gene of this fungus labelled with the non-isotopic digoxigenin labelling Reagent kit (Roche Diagnostics, SL), following standard protocols (Sambrook and Russell, 2001). Sequencing of both DNA strands of the obtained clones was performed at the Servicio de Secuenciación Automática de DNA, SCAI (Universidad de Córdoba, Spain) using the Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an ABI Prism 377 Genetic Analyzer apparatus (Applied Biosystems). DNA and protein sequence databases were searched at http://www.ncbi.nlm.nih.gov/BLAST/ using the BLAST algorithm (Altschul et al., 1990).

2.3. Reverse transcription-polymerase chain reaction

To determine *sti35* expression during the fungus–plant interaction, roots of 3-week-old tomato plants were

submerged in SM with 1% sucrose, containing 2.5×10^6 spores ml^{-1} of *F. oxysporum* and incubated in a shaker at 28 °C for 12, 24, 36 and 48 h. Root samples were frozen and total RNA was isolated and used as a template for reverse transcription-polymerase chain reaction (RT-PCR) as previously reported (Di Pietro and Roncero, 1998). Briefly, total RNA was treated with RNase-free DNase (Roche Biochemicals, SL) and reverse transcribed into cDNA with M-MLV reverse transcriptase (Invitrogen S.A., Spain) using a poli-dT antisense primer. The cDNA was then used for PCR amplification with the sense primer sti10 and the antisense primer sti2 (Supplementary Table 1).

To analyse the splicing of the introns in the 5′-UTR region of the *sti35* gene, total RNA was reverse transcribed into cDNA and used for PCR amplification with the sense primer sti26 and the antisense primer sti5 (Supplementary Table 1).

2.4. Construction of plasmid vectors and fungal transformation

Gene replacement vector pD*sti35* was constructed as follows: a BamHI site was introduced at position 530 of the *sti35* coding region using the overlapping method (Sambrook and Russell, 2001) with the primers sti13BamHI and sti14BamHI (Supplementary Table 1) and the final product was cloned into pGEM-T. A 0.53 kb BamHI-fragment containing the 3′ coding region of *sti35* gene was replaced with a 1.8 kb BamHI fragment containing the hygromycin B resistance gene under the control of a *Cochliobolus heterostrophus* promoter (Turgeon et al., 1987). A linear fragment containing the interrupted *sti35* allele was amplified with primers sti8 and sti9 (Supplementary Table 1) and used to transform protoplasts of *F. oxysporum* as described (Di Pietro and Roncero, 1998). Hygromycin-resistant transformants were subjected to two consecutive rounds of single sporing and stored as microconidial suspension at −80° C. For complementation experiments, a 2.7 kb DNA fragment encompassing the entire *sti35* gene was obtained by PCR with primers sti18 and sti9 (Supplementary Table 1) using *F. oxysporum* genomic DNA as a template. The obtained fragment was introduced into protoplasts of a Δ*sti35* mutant by cotransformation with the plasmid pAN8-1 carrying the phleomycin resistance cassette (Mattern et al., 1988) as previously described (Di Pietro et al., 2001).

2.5. Expression of the sti35 cDNA in bacteria and in yeast

The *sti35* cDNA was used for PCR amplification using specific primers sti1BamH1 and sti2BamH1 (Supplementary Table 1) and cloned into the BamH1 site of the bacterial expression vector pSE420 (Invitrogen S.A., Spain) or the BamH1 site of the yeast expression vector pRS426 (Mumberg et al., 1994). The cDNA was cloned in both orientations. The same vectors without the *sti35* cDNA were used as controls. The bacterial strains used for the complementation experiments were the wild type for DNA repair functions, *E. coli* K12 strain AB1157 (*F- thi1 his4d* (*gpt-proA*) *argE3 thr1 leuB6 ara14 lacY1 galK2 xyl5 mtl1 tsc33 supE44 rpsL31*) and its derivative *uvrA*$^-$ DNA repair mutant AB1886 (Bachmann, 1972). The yeast recombinant vector (pRS426-sti35) was transfected into the *S. cerevisiae thi4::kanMX4* disruption strain (EUROSCARF). The genotype of this strain is *BY4741*; *Mat a*; *his3Δ1*; *leu2Δ0*, *met15Δ0*; *ura3Δ0*; *YGR144w::kanMX4*. Minimal medium was prepared as described (Wickerman, 1951) with the omission of thiamine and uracil, unless indicated otherwise. Transformants were recovered at 30 °C on medium lacking uracil, but containing thiamine, for vector selection.

2.6. Construction of the Psti35::lacZ fusion

The *sti35* promoter region (−731 to +18) of *F. oxysporum* (P*sti35*) was amplified from genomic DNA using primers sti20 and stirevBamHI (Supplementary Table 1). The amplified fragment was cloned into pGEM-T to obtain plasmid pGEM-T*sti*. A *lacZ* allele lacking the first nine amino acids of the coding region was obtained by PCR amplification from plasmid pTRAN3 (Punt et al., 1991), using primers lacZ2 and lacZ3BamH1 (Supplementary Table 1), and cloned into pGEM-T. The BamHI/NotI fragment from pGEM-T*sti* and the ApaI/BamHI fragment from pGEM-T*lacZ* were inserted by 3-way ligation into the pAN7blue3 transformation vector linearized with ApaI–NotI (Bowyer et al., 1995). The resulting plasmid was used to transform protoplasts of *F. oxysporum* wild type strain as described (Di Pietro and Roncero, 1998).

2.7. β-Galactosidase assays

β-Galactosidase activity in mycelial extracts was measured at 37 °C as described previously (Miller, 1992), with modifications (Marui et al., 2002), using *o*-nitrophenol-β-D-galctopyranoside as a substrate ($\varepsilon = 0.0045$ ml/nmol/cm at 37 °C. Briefly, mycelia harvested by filtration were washed with water, frozen rapidly, ground to a fine powder under liquid nitrogen, suspended in extraction buffer [50 mM NaH_2PO_4 (pH 7.0), 5 mM EDTA, 10 mM β-mercaptoethanol, 25 mg of phenylmethylsulfonyl fluoride per mililiter] and kept for 10 min on ice. The suspension was centrifuged for 15 min and the supernatant was directly used for the β-galactosidase assay. Activity was expressed in Miller units per mg of protein (Miller, 1992). Protein concentration was determined as described using bovine serum albumin as a standard (Bradford, 1976).

3. Results and discussion

3.1. Cloning and sequence analysis of the sti35 gene of F. oxysporum f.sp. lycopersici

The *sti35* gene of *F. oxysporum* f.sp. *lycopersici* strain 4287 was amplified by PCR from genomic DNA, using

specific primers derived from the *sti35* sequence of *F. oxysporum* f.sp. *cucumerinum* (EMBL Accession No. AB033416). A DNA fragment of 2731 nucleotides was obtained, cloned into pGEM-T and sequenced. The fragment contained the complete *sti35* coding region of 1012 bp together with 1123 bp of 5′- and 596 bp of 3′-flanking region. The deduced Sti35 protein of 320 amino acids showed high similarity to other proteins required for the biosynthesis of thiamine in fungi and plants, including the GxGxxG signature that is characteristic of the general dinucleotide binding protein family (Godoi et al., 2006) (Fig. 1). Inspection of the *sti35* promoter region (Fig. 2a) revealed a putative TATA box at position −425 respective to the ATG codon, several putative heat shock elements (HSEs) containing the NAGAAN motif (Bienz and Pelham, 1987), as well as two stress

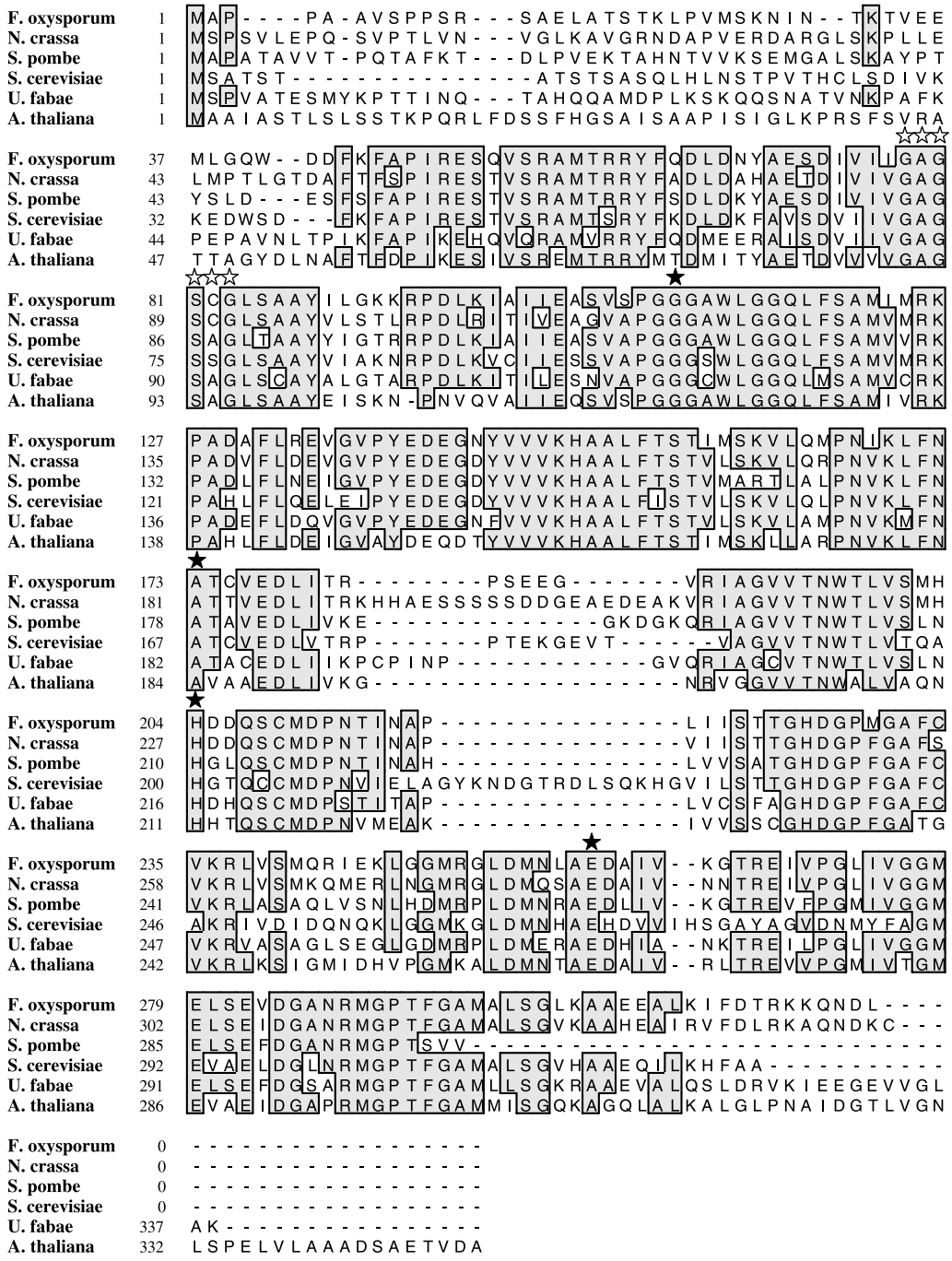

Fig. 1. Amino acid alignment of the deduced *Fusarium oxysporum* Sti35 (BAA85305) with *Neurospora crassa* CyPBP37 (CAC03570), *Schizosaccharomyces pombe* Nmt2 (CAA5779), *Saccharomyces cerevisiae* THI4 (CAA97157), *Uromyces fabae* Thi4 (Q9UVF8) and *Arabidopsis thaliana* Thi1 (NP_200288). The general dinucleotide binding protein signature GxGxxG is indicated by empty asterisks. The four conserved amino acid residues essential for thiazole biosynthetic activity and/or mitochondrial DNA damage tolerance in *A. thaliana* Thi1 (Godoi et al., 2006) are marked by filled asterisks.

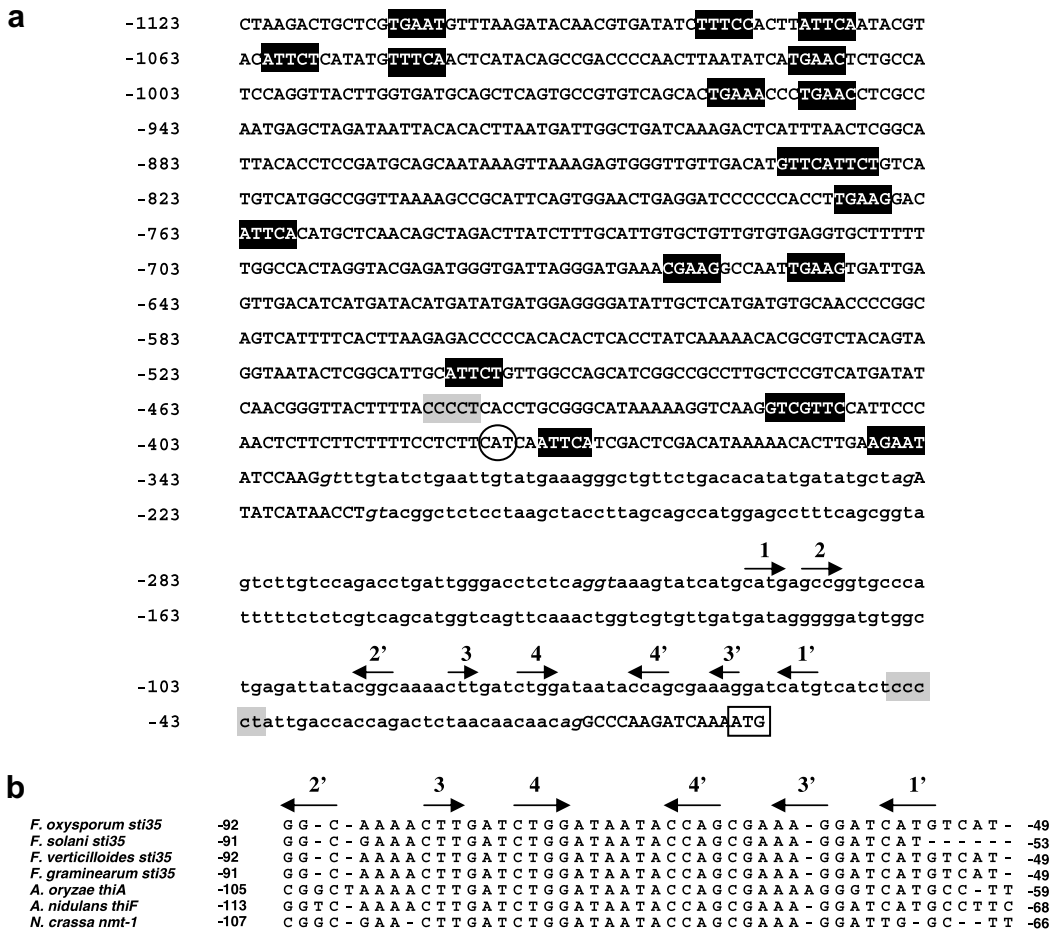

Fig. 2. The 5′ untranslated region (UTR) of the *Fusarium oxysporum sti35* gene. (a) Nucleotide sequence of the 5′-UTR of the *sti35* gene. The putative transcription start site is indicated by a circle and the start codon is marked with an empty box. Lower case letters represent introns. Consensus splicing sites are in italics. Black boxes indicate heat shock elements (HSE) and grey boxes indicate stress-response elements (STRE). Inverted repeats with putative function in hairpin structure formation are indicated by numbered arrows, where the same numbers correspond to complementary sequences. (b) Nucleotide sequence alignments of a conserved region in the 5′-UTR of the *sti35* orthologues from different fungi. Inverted repeats with putative function in hairpin structure formation are indicated by numbered arrows (see above).

response elements (STREs) (Martinez-Pastor et al., 1996).

A *F. oxysporum* cDNA library was screened with a *sti35* probe and 3 hybridizing clones were isolated. Sequence analysis of these clones suggested that the putative transcription start site is localised at position −381 (Fig. 2a). Comparison of the genomic and cDNA clones revealed the existence of three introns, one located in the coding region and two in the 5′-UTR (Fig. 2a). The position of the 5′-UTR introns was highly conserved relative to the *sti35* orthologues of *N. crassa* and *A. nidulans* (Kubodera et al., 2003; McColl et al., 2003). The second of the 5′-UTR introns of *F. oxysporum sti35* contained sequence motifs that are conserved among microbial genes involved in thiamine biosynthesis (Kubodera et al., 2003) (Fig. 2b) and have been suggested to function as a thiamine pyrophosphate-binding riboswitch-like domain (Sudarsan et al., 2003; Thore et al., 2006; Cheah et al., 2007). Comparison of the *sti35* promoter with the orthologous region of *Fusarium graminearum* revealed a 100% sequence identity within these two 5′-UTR introns (Fig. 2b).

3.2. Regulation of sti35 expression in culture and in planta

Northern analysis of mycelia grown in the absence of thiamine at 28 °C detected an abundant transcript of approximately 1 kb, consistent with the expected size of the *sti35* gene product (Fig. 3a). By contrast, *sti35* transcript levels were basically undetectable in mycelia grown in the presence of 10 mM thiamine. Thus, *F. oxysporum sti35* is highly expressed during vegetative growth and subject to repression by thiamine. This regulatory pattern resembles that of the *S. pombe* and *S. cerevisiae* orthologues, although the concentrations of thiamine required for complete repression of *sti35* in *Fusarium* are somewhat higher than those reported for the yeasts (Maundrell, 1990; Praekelt et al., 1994). Since *F. oxysporum sti35* was originally identified as a stress-induced gene (Choi et al.,

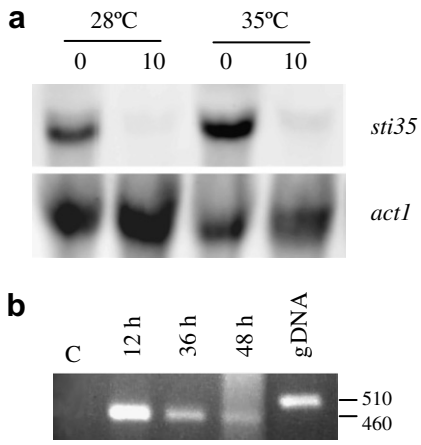

Fig. 3. Expression of *sti35* is downregulated by thiamine and upregulated by temperature stress. (a) Effect of thiamine and temperature on *sti35* expression. Northern hybridization analysis was performed using total RNA obtained from mycelia of *F. oxysporum* germinated 14 h in PDB at 28 °C, transferred to minimal medium without thiamine (0) or with 10 mM thiamine (10) and incubated for 8 h either at 28 °C or at 35 °C. The upper panel shows hybridization with the *sti35* probe; the lower panel shows hybridization with the *act1* (actin gene) probe. (b) RT-PCR analysis of *sti35* expression *in planta*. Total RNA obtained from roots of 2-week-old tomato plants submerged for the indicated time periods in SM containing 2.5×10^6 spores ml^{-1} of *F. oxysporum*, was used as a template for RT-PCR with *sti35* specific primers flanking the third intron (see Section 2). Molecular sizes of the amplified fragments in bp are indicated. C, uninfected control plants. gDNA, genomic DNA used as PCR template.

1990), we determined transcripts in *F. oxysporum* mycelia grown at high temperature. *Sti35* transcript levels in the absence of thiamine were significantly increased during growth at 35 °C, as compared to 28 °C, although expression was still highly repressed by thiamine (Fig. 3a). This result points to a possible role of *sti35* in heat stress response. In agreement with this finding, the *thi-4* mutant of *S. cerevisiae* exhibited increased induction of petite mutants compared to the wild type upon chronic exposure to 37 °C, as well as a strong oxidative enzymatic response and protein oxidations, suggesting a potential role for the Thi4 protein in protecting yeast from heat stress (Medina-Silva et al., 2006). Transcription of the *N. crassa sti35* orthologue, CyPBP37, which was identified as a protein binding to the cyclophilin NcCy41, was also induced by a variety of stresses including high temperature (Faou and Tropschug, 2004).

To determine expression of *sti35* during early stages of plant infection, RT-PCR analysis was performed using total RNA obtained from tomato roots inoculated with a microconidial suspension of *F. oxysporum* (see Section 2). The presence of *sti35* transcripts was detected during the three time points sampled (12, 36 and 48 h after inoculation), with the highest intensity at 12 h (Fig. 3b). Similar experiments were also conducted at later stages of infection, revealing the presence of the *sti35* transcript at 3 and 7 days after inoculation in infected roots and stems but not in the uninoculated control plants (data not shown). Thus, *sti35* is expressed by *F. oxysporum* during plant infection. In agreement with this finding, a recent microarray analysis in *F. oxysporum* f.sp. *vasinfectum* identified *sti35* as one of the genes expressed during infection of cotton roots (Dowd et al., 2004). Interestingly, high expression levels of two thiamine biosynthetic genes were also detected in haustoria of the biotrophic pathogen *Uromyces fabae* (Sohn et al., 2000).

3.3. Dual role of Sti35 in thiamine biosynthesis and general stress response

Previous studies indicated that the yeast Thi4p and its plant orthologue, Thi1p, have two distinct cellular functions, one in thiamine biosynthesis and the other in DNA damage tolerance (Machado et al., 1996) and maintenance of mitochondrial genomic stability (Machado et al., 1997). To test whether *F. oxysporum* Sti35 is also able to carry out this dual role, we performed functional complementation experiments with a Δ*thi4* mutant of *S. cerevisiae* and with a DNA repair-deficient strain of *E. coli*. The *sti35* cDNA was cloned both in sense and antisense orientation into the yeast expression vector pRS460 under control of the *gal1* promoter. The constructs were transformed into the *S. cerevisiae thi4*$^-$ mutant (YGR144W), which is unable to synthesize the thiazol precursor of thiamine and therefore has a thiamine auxotrophic phenotype. Transformants were selected on media lacking uracil and subsequently tested for growth on medium lacking thiamine. Only those yeast transformants carrying the *sti35* cDNA in the sense orientation could grow in the absence of thiamine, whereas those carrying the antisense cDNA or the empty vector did not (Fig. 4a). By contrast, all types of transformants were able to grow on thiamine-supplemented medium. These results indicate that *F. oxysporum sti35* encodes a functional homologue of the yeast *thi4* gene.

To determine the role in DNA damage tolerance, the *sti35* cDNA was cloned into the bacterial expression vector pSE420 and transformed into both *E. coli*, wild type strain, AB1157 and the *uvrA*$^-$ mutant strain AB1886 which is deficient in the *uvrABC* DNA repair mechanism (Bachmann, 1972). Cells of the different bacterial strains were UV-irradiated with the increasing doses and the surviving colonies were determined (Fig 4b). Expression of the *sti35* cDNA in *E. coli* significantly increased survival of the DNA repair-deficient *uvrA* strain, similar to the *A. thaliana thi1* gene (Machado et al., 1996).

Taken together, these complementation studies suggest that *F. oxysporum sti35* has a dual function in thiamine biosynthesis and tolerance of DNA damage. This finding confirms earlier studies on the THI1 protein from *A. thaliana* which suggested a dual role of THI1 in thiazole biosynthesis and mitochondrial DNA damage tolerance in plants and yeast (Machado et al., 1996). Interestingly, four conserved amino acid residues whose site-directed mutagenesis in THI1 suppressed either both activities or only the thiazole biosynthetic activity (Godoi et al., 2006) are also present in the deduced *F. oxysporum* Sti35 protein (Fig. 1).

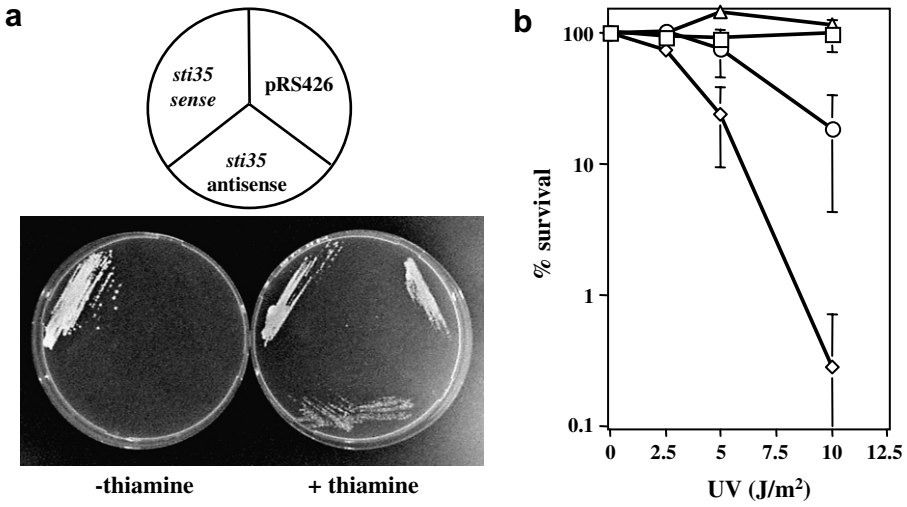

Fig. 4. *Fusarium oxysporum sti35* complements the defects in thiamine biosynthesis and DNA damage tolerance in mutants of *Saccharomyces cerevisiae* and *Escherichia coli*. (a) Functional complementation of a *S. cerevisiae thi4*$^-$ mutant with the *F. oxysporum sti35* cDNA. The *thi4*$^-$ deficient strain was transformed with expression vector pRS426 containing the *sti35* gene either in the sense or antisense orientation under control of the *gal1* promoter, or with the empty vector. Transformants were streaked on minimal medium with galactose, in the presence or absence thiamine. Plates were incubated at 30 °C for 3 days. (b) Survival of different *E. coli* strains after UV treatment. Wild type strain AB1157 (triangles), AB1157 transformed with the empty plasmid pSE420 (squares), *uvrA*$^-$ mutant strain AB1886 transformed with empty plasmid pSE420 (diamonds) and AB1886 transformed with pSE420 containing the *sti35* cDNA (circles) were inoculated in 10 ml of LB medium with ampicillin and IPTG, incubated with shaking at 37 °C until reaching the exponential growth phase (OD_{600} 0.5), centrifuged, resuspended in 10 mM $MgSO_4$ and UV-irradiated with 2.5, 5, 7.5 or 10 J/m^2. Cells were plated on LA, incubated overnight at 37 °C and individual bacterial colonies were counted. Mean values and standard errors are from two independent biological experiments, each with four sample replicates.

To further study the role of *sti35*, we generated a *F. oxysporum* strain lacking a functional copy of the gene. The gene replacement vector p*Dsti*35 (Fig. 5a) was introduced in the wild type strain (see Section 2 for details). Among 108 hygromicin-resistant transformants whose genomic DNA was analysed by PCR with gene-specific primers, a single transformant, named Δ*sti35*, produced an amplification product indicative of a homologous integration-mediated gene replacement, whereas all the other transformants contained ectopic insertion copies of the construct (Fig. 5b and data not shown). Functional complementation of the Δ*sti35* strain was performed by co-transformation of the phleomycine cassette with a 2.7 kb genomic DNA fragment containing the complete coding region of *sti35* together with 1123 bp of 5′- and 596 bp of 3′-flanking region. Seventeen phleomycin resistant transformants were analyzed by PCR with primers sti8 and sti9, and one of them gave rise to an amplification product whose size was consistent with that of the wild type *sti35* allele (Fig. 5b). This complemented strain was named Δ*sti35* + *sti35*.

To determine the role of Sti35 in thiamine biosynthesis, the strains were grown on minimal medium with or without thiamine (Fig. 5c). In contrast to the wild type, the Δ*sti35* strain grew poorly on minimal medium without thiamine, while showing wild type growth in the presence of the vitamin. Thus, similar to *S. cerevisiae* (Machado et al., 1997), deletion of the *sti35* gene in *F. oxysporum* confers thiamine auxotrophy. As expected, growth in the absence of thiamine was restored to wild type levels in the Δ*sti35* + *sti35* strain (Fig. 5c).

As previously mentioned, *sti35* transcript levels are increased during growth at 35 °C (Fig. 3a). Because chronic exposure to high temperatures in yeast is known to generate increased levels of reactive oxygen species (ROS) (Davidson and Schiestl, 2001), we tested whether Sti35 plays a role in protection of *F. oxysporum* against the superoxide-generating agent menadione. As shown in Fig. 5d, the Δ*sti35* mutant was more sensitive to menadione than the wild type and the Δ*sti35* + *sti35* strain. The menadione-sensitive phenotype of the Δ*sti35* strain is unlikely to be directly associated with thiamine deficiency, since the experiments were conducted on thiamine-supplemented medium. Rather, these results suggest that *F. oxysporum* Sti35 plays an additional, thiamine-independent role in tolerance of oxidative stress whose underlying mechanism is currently unknown.

To test the role of Sti35 in virulence of *F. oxysporum*, infection assays were performed by immersing the roots of tomato plants in microconidial suspensions. Severity of wilt symptoms in plants inoculated with the wild type strain increased steadily throughout the experiment (data not shown). Initially, leaf tips turned yellow and curved, then the stalk weakened and decayed, and the plants were dead 20 days after inoculation. Progression of disease symptoms in plants inoculated with the Δ*sti35* mutant was not significantly different from that of the wild type strain, suggesting that Sti35 is not essential for virulence of *F. oxysporum* (data not shown). This result is in agreement with a previous study, in which targeted disruption of *sti35* in *F. oxysporum* f.sp. *cucumerinum* had no detectable effect on pathogenicity (Thanonkeo et al., 2000).

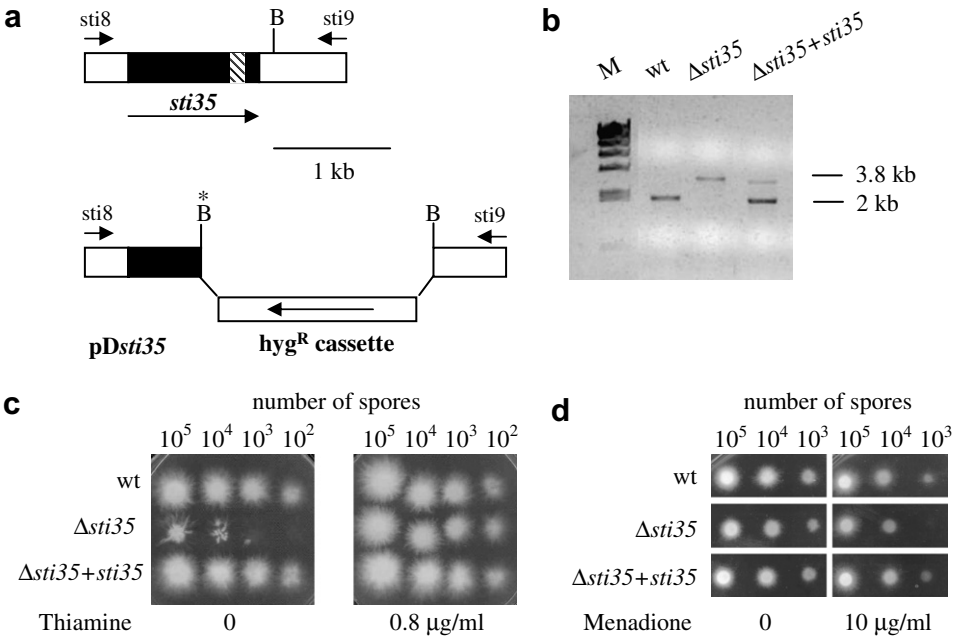

Fig. 5. Sti35 has a dual function in thiamine biosynthesis and tolerance to oxidative stress. (a) Physical maps of the *sti35* locus and the gene replacement vector pD*sti35*. B, BamHI. Direction of transcription is indicated by an arrow. The new BamHI site introduced by site-directed mutagenesis is marked with an asterisk. Primers used for PCR amplification in 5B are indicated. (b) PCR analysis of different *F. oxysporum* strains. Genomic DNA from wt, Δ*sti35* and Δ*sti35* + *sti35* strains was amplified using primers sti8 and sti9. M: HindIII digested lambda DNA markers. The size of the amplified fragments is indicated on the right. (c) Deletion of *sti35* causes thiamine auxotrophy in *F. oxysporum*. Serial dilutions of microconidia of the wt, Δ*sti35* and Δ*sti35* + *sti35* strains were spotted onto minimal medium with or without 0.8 µg/ml thiamine. Plates were incubated at 28 °C for 3 days. (d) Deletion of *sti35* increases sensitivity of *F. oxysporum* to menadione. Serial dilutions of microconidia of the wt, Δ*sti35* and Δ*sti35* + *sti35* strains were spotted onto minimal medium containing 0.8 µg/ml thiamine, with or without 10 µg/ml menadione. Plates were incubated at 28 °C for 2 days.

3.4. Evidence for post-transcriptional regulation of sti35 expression

The comparison of the *sti35* cDNA and genomic clones revealed the existence of two introns in the 5′-UTR (Fig. 2a). The position of the second intron is highly conserved with respect to other fungal *sti35* orthologues and contains inverted repeat regions that show homology to those reported in bacterial, fungal and plant thiamine biosynthesis genes (Fig. 2b) (Sudarsan et al., 2003; Thore et al., 2006; Cheah et al., 2007). Correct splicing of this second intron is required for gene expression in *A. oryzae* (Kubodera et al., 2003) and *N. crassa* (Cheah et al., 2007).

Splicing of the 5′-UTR introns of *F. oxysporum sti35* was determined by RT-PCR, using total RNA obtained from mycelium grown under inducing (no thiamine) or repressing conditions (1 or 10 mM thiamine) at 28 or 35 °C. At both growth temperatures, a single amplification band (named a), was obtained from mycelium grown in the absence of thiamine (Fig. 6a). Cloning and sequencing of the fragment "a" confirmed that it corresponded to the fully processed transcript with the two 5′-UTR introns correctly spliced. Conversely, fragment "a" was not detected in mycelium grown in the presence of either 1 or 10 mM thiamine. Instead, at least three higher molecular weight RT-PCR products (named b, c and d) were observed. Cloning and sequencing of fragments b and d confirmed that in fragment "b" the second intron was only partially spliced starting at a cryptic 5′ donor splicing site located at −192 (Fig. 6b) and in fragment "d" the entire second intron was present. The faint band "c" which could not be cloned and sequenced, may correspond to the transcript in which only the first part of the second intron was spliced. This hypothesis is supported by the fact that GT at the cryptic 5′ splicing site is preceeded by AG, thus creating two putative head to tail introns of 80 and 179 nucleotides which might be processed sequentially (Fig. 6b).

These results prompt us to speculate that thiamine may interfere with the splicing of the second 5′-UTR intron, suggesting the presence of a post-transcriptional regulation mechanism of *sti35* by thiamine which is active both at 28 and at 35 °C. This hypothesis is supported by a recent report demonstrating that thiamine controls alternative RNA splicing and expression of the orthologous *N. crassa nmt1* gene (Cheah et al., 2007).

In *A. thaliana*, an inverted repeat structure in the 3′-UTR of the putative thiamine biosynthesis gene *thiC* was shown to form a stem-loop structure that acts as a riboswitch by directly binding thiamine pyrophosphate (Thore et al., 2006). Likewise, differential splicing of *sti35* may be controlled by thiamine binding to the conserved inverted repeat region in the *sti35* pre-mRNA (Fig. 2b). Indeed, analysis of this region using the Mfold program available at http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-

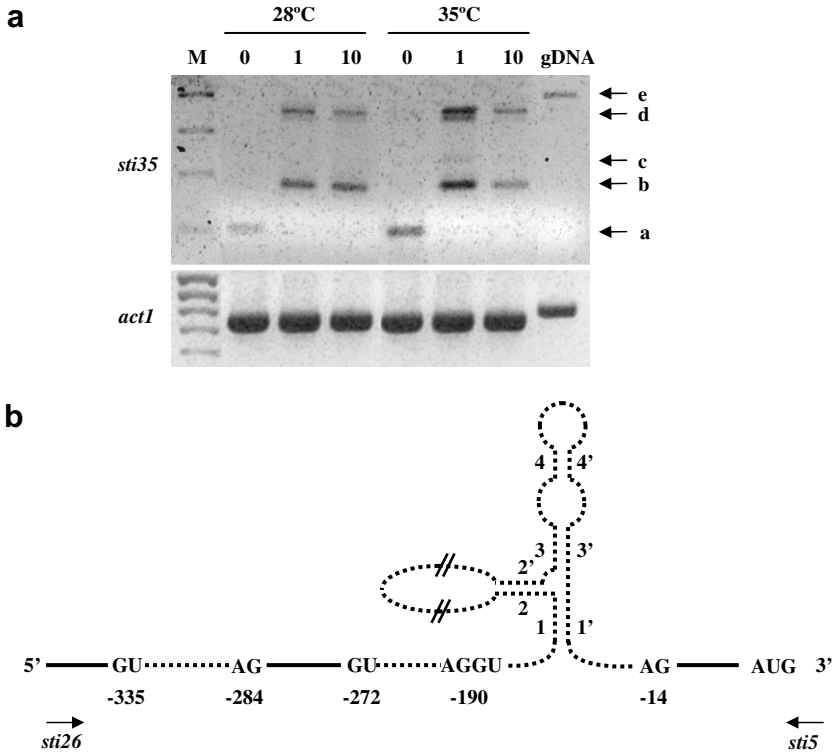

Fig. 6. Effect of thiamine on splicing of the second *sti35* 5′-UTR intron. (a) RT-PCR analysis of *sti35* expression and splicing. Total RNA obtained from the wild type strain grown in SM with or without 10 mM thiamine at the indicated temperature was subjected to RT-PCR analysis using the specific primers sti26 and sti5 shown in (b). Amplified fragments a (100 bp), b (179 bp), c (estimated 240 bp) and d (357 bp) are indicated. gDNA (408 bp), genomic DNA used as PCR template. The lower panel shows RT-PCR products with the same cDNAs, using specific primers for the *act1* (actin) gene. (b) Schematic illustration of the 5′-untranslated region (UTR) of *F. oxysporum sti35*. Numbers indicate nucleotide positions relative to the start codon. Introns are indicated by a dashed line. Relative positions of the primers used for RT-PCR are indicated by arrows. The hairpin structure of the putative thiamine riboswitch in the second intron is shown. Complementary inverted repeats are marked with the same number (see also Fig. 2a).

bin/rna-form1.cgi (Lyngso et al., 1999) predicted a putative RNA secondary structure similar to reported thiamine riboswitches (Rodionov et al., 2002) (schematically depicted in Fig. 6b). Besides reducing the level of mature mRNA, splicing from the cryptic site at −192 generates a small upstream ORF (uORF), MEPFSGSLVQT which is out of frame with the consensus *sti35* coding sequence and thus may lead to a decrease in the efficiency of the translation machinery, as shown for the *nmt-1* gene of *N. crassa* (McColl et al., 2003; Cheah et al., 2007). Thus, multiple post-transcriptional mechanisms may be contributing to the regulation of *sti35* expression by thiamine in *F. oxysporum*.

3.5. Use of Psti35 as a thiamine-repressible promoter system

Fusarium oxysporum is an emerging model for studying the molecular bases of fungal pathogenicity on plant and mammalian hosts (Di Pietro et al., 2003; Ortoneda et al., 2004). However, this model species currently lacks a reliable promoter system that allows the controlled expression of genes of interest. Given its tight regulation by thiamine, the *sti35* promoter represents a potential tool for the construction of gene expression vectors, similar to those based on the orthologous *nmt1* promoter in the *S. pombe* (Maundrell, 1993). To test whether the *sti35* promoter is able to confer thiamine-regulated expression of a downstream gene, a DNA fragment containing 749 bp of the *sti35* promoter (P*sti*35) was transcriptionally fused to the *lacZ* reporter gene (Fig. 7a). Protoplasts of the *F. oxysporum* wild type strain were transformed with a vector containing P*sti35::lacZ* and the hygromycin resistance marker, and hygromicin resistant transformants were selected and analysed for presence and number of copies of the P*sti35::lacZ* construct, using PCR and Southern analysis (data not shown). Three independent transformants harbouring a single copy of the construct were grown in minimal medium with or without 10 mM thiamine, and total mycelial extracts were analyzed for β-galactosidase activity. High levels of β-galactosidase activity were detected in mycelia of the three transformants grown in the absence of thiamine (Fig. 7b). Absolute activity levels varied between the different transformants, possibly due to positional effects at the site of insertion. By contrast, β-galactosidase activities in the transformants grown in the presence of 10 mM thiamine were very low (similar to the background levels in the wild type strain), suggesting that thiamine-mediated repression of the *lacZ* reporter gene was functional *in vivo*. Thus, besides its interest as a gene with dual function in thiamine biosynthesis and stress

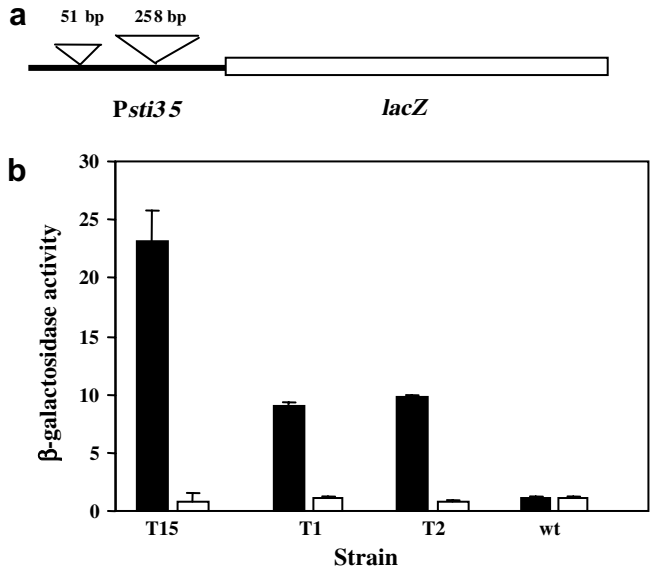

Fig. 7. Thiamine-dependent expression of the *lacZ* reporter gene fused to the *sti35* promoter. (a) Schematic illustration of the P*sti35::lacZ* fusion construct. Introns in the 5′-UTR of P*sti35* are indicated as triangles. (b) β-Galactosidase activities (expressed in Miller units) of three independent transformants carrying a single copy of the P*sti35::lacZ* construct. Transformants were grown for 8 h in minimal medium with (white bars) or without 10 mM thiamine (black bars) and β-galactosidase activity in mycelial extracts was determined. The wild type strain was included as a negative control. Mean values and standard errors are from two independent biological experiments, each with three sample repeats.

response, P*sti35* represents a useful tool for controlled expression of genes of interest in the fungal model pathogen *F. oxysporum*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2007.09.003.

References

Altschul, S.F., Gish, W., Miller, W., Myers, C.W., Lipman, D.L., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Bachmann, B.J., 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. 36, 525–557.

Belanger, F.C., Leustek, T., Chu, B., Kriz, A.L., 1995. Evidence for the thiamine biosynthetic pathway in higher-plant plastids and its developmental regulation. Plant Mol. Biol. 29, 809–821.

Bienz, M., Pelham, H.R., 1987. Mechanisms of heat-shock gene activation in higher eukaryotes. Adv. Genet. 24, 31–72.

Bowyer, P., Clarke, B.R., Lunness, P., Daniels, M.J., Osbourn, A.E., 1995. Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267, 371–374.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cheah, M.T., Wachter, A., Sudarsan, N., Breaker, R.R., 2007. Control of alternative RNA splicing and gene expression by eukaryotic riboswitches. Nature 447, 497–500.

Choi, G.H., Marek, E.T., Schardl, C.L., Richey, M.G., Chang, S.Y., Smith, D.A., 1990. sti35, a stress-responsive gene in *Fusarium* spp. J. Bacteriol. 172, 4522–4528.

Davidson, J.F., Schiestl, R.H., 2001. Mitochondrial respiratory electron carriers are involved in oxidative stress during heat stress in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 21, 8483–8489.

Di Pietro, A., García-Maceira, F.I., Meglecz, E., Roncero, M.I.G., 2001. *Fusarium oxysporum* is essential for root penetration and pathogenesis. Mol. Microbiol. 39, 1140–1152.

Di Pietro, A., Madrid, M.P., Caracuel, Z., Delgado-Jarana, J., Roncero, M.I.G., 2003. *Fusarium oxysporum*: exploring the molecular arsenal of a vascular wilt fungus. Mol. Plant Pathol. 4, 315–326.

DiPietro, A., Roncero, M.I., 1998. Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. Mol. Plant Microbe Interact. 11, 91–98.

Dowd, C., Wilson, I.W., McFadden, H., 2004. Gene expression profile changes in cotton root and hypocotyl tissues in response to infection with *Fusarium oxysporum* f sp. *vasinfectum*. Mol. Plant Microbe Interact. 17, 654–667.

Edwards, T.E., Klein, D.J., Ferre-D'Amare, A.R., 2007. Riboswitches: small-molecule recognition by gene regulatory RNAs. Curr. Opin. Struct. Biol.

Faou, P., Tropschug, M., 2003. A novel binding protein for a member of CyP40-type Cyclophilins: *N. crassa* CyPBP37, a growth and thiamine regulated protein homolog to yeast Thi4p. J. Mol. Biol. 333, 831–844.

Faou, P., Tropschug, M., 2004. *Neurospora crassa* CyPBP37: a cytosolic stress protein that is able to replace yeast Thi4p function in the synthesis of vitamin B1. J. Mol. Biol. 344, 1147–1157.

Godoi, P.H., Galhardo, R.S., Luche, D.D., Van Sluys, M.A., Menck, C.F., Oliva, G., 2006. Structure of the thiazole biosynthetic enzyme THI1 from *Arabidopsis thaliana*. J. Biol. Chem. 281, 30957–30966.

Hohmann, S., Meacock, P.A., 1998. Thiamin metabolism and thiamin diphosphate-dependent enzymes in the yeast *Saccharomyces cerevisiae*: genetic regulation. Biochim. Biophys. Acta 1385, 201–219.

Jacob-Wilk, D., Goldschmidt, E.E., Riov, J., Sadka, A., Holland, D., 1997. Induction of a Citrus gene highly homologous to plant and yeast thi genes involved in thiamine biosynthesis during natural and ethylene-induced fruit maturation. Plant Mol. Biol. 35, 661–666.

Kubodera, T., Watanabe, M., Yoshiuchi, K., Yamashita, N., Nishimura, A., Nakai, S., Gomi, K., Hanamoto, H., 2003. Thiamine-regulated gene expression of *Aspergillus oryzae* thiA requires splicing of the intron containing a riboswitch-like domain in the 5'-UTR. FEBS Lett. 555, 516–520.

Kubodera, T., Yamashita, N., Nishimura, A., 2000. Pyrithiamine resistance gene (ptrA) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci. Biotechnol. Biochem. 64, 1416–1421.

Lai, E.C., 2003. RNA sensors and riboswitches: self-regulating messages. Curr. Biol. 13, R285–R291.

Lyngso, R.B., Zuker, M., Pedersen, C.N., 1999. Fast evaluation of internal loops in RNA secondary structure prediction. Bioinformatics 15, 440–445.

Machado, C.R., de Oliveira, R.L., Boiteux, S., Praekelt, U.M., Meacock, P.A., Menck, C.F., 1996. Thi1, a thiamine biosynthetic gene in *Arabidopsis thaliana*, complements bacterial defects in DNA repair. Plant Mol. Biol. 31, 585–593.

Machado, C.R., Praekelt, U.M., de Oliveira, R.C., Barbosa, A.C., Byrne, K.L., Meacock, P.A., Menck, C.F., 1997. Dual role for the yeast THI4

gene in thiamine biosynthesis and DNA damage tolerance. J. Mol. Biol. 273, 114–121.

Manetti, A.G., Rosetto, M., Maundrell, K.G., 1994. nmt2 of fission yeast: a second thiamine-repressible gene co-ordinately regulated with nmt1. Yeast 10, 1075–1082.

Martinez-Pastor, M.T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H., Estruch, F., 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). Embo J. 15, 2227–2235.

Marui, J., Tanaka, A., Mimura, S., de Graaff, L.H., Visser, J., Kitamoto, N., Kato, M., Kobayashi, T., Tsukagoshi, N., 2002. A transcriptional activator, AoXlnR, controls the expression of genes encoding xylanolytic enzymes in *Aspergillus oryzae*. Fungal Genet. Biol. 35, 157–169.

Mattern, I.E., Punt, P.J., van den Hondel, C.A., 1988. Aspergillus transformation conferring phleomycin resistance. Fungal Genet. Newsl. 35, 25.

Maundrell, K., 1990. nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J. Biol. Chem. 265, 10857–10864.

Maundrell, K., 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 123, 127–130.

McColl, D., Valencia, C.A., Vierula, P.J., 2003. Characterization and expression of the *Neurospora crassa* nmt-1 gene. Curr. Genet. 44, 216–223.

Medina-Silva, R., Barros, M.P., Galhardo, R.S., Netto, L.E., Colepicolo, P., Menck, C.F., 2006. Heat stress promotes mitochondrial instability and oxidative responses in yeast deficient in thiazole biosynthesis. Res. Microbiol. 157, 275–281.

Miller, J.H., 1992. A Short Course in Bacterial genetics: A laboratory Manual and Handbook for Escherichia coli and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Miranda-Rios, J., Navarro, M., Soberon, M., 2001. A conserved RNA structure (thi box) is involved in regulation of thiamin biosynthetic gene expression in bacteria. Proc. Natl. Acad. Sci. USA 98, 9736–9741.

Mumberg, D., Muller, R., Funk, M., 1994. Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res. 22, 5767–5768.

Ortoneda, M., Guarro, J., Madrid, M.P., Caracuel, Z., Roncero, M.I., Mayayo, E., Di Pietro, A., 2004. *Fusarium oxysporum* as a multihost model for the genetic dissection of fungal virulence in plants and mammals. Infect. Immun. 72, 1760–1766.

Praekelt, U.M., Byrne, K.L., Meacock, P.A., 1994. Regulation of THI4 (MOL1), a thiamine-biosynthetic gene of *Saccharomyces cerevisiae*. Yeast 10, 481–490.

Punt, P.J., Greaves, P.A., Kuyvenhoven, A., van Deutekom, J.C., Kinghorn, J.R., Pouwels, P.H., van den Hondel, C.A., 1991. A twin-reporter vector for simultaneous analysis of expression signals of divergently transcribed, contiguous genes in filamentous fungi. Gene 104, 119–122.

Ribeiro, A., Praekelt, U., Akkermans, A.D., Meacock, P.A., van Kammen, A., Bisseling, T., Pawlowski, K., 1996. Identification of agthi1, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of Alnus glutinosa. Plant J. 10, 361–368.

Rodionov, D.A., Vitreschak, A.G., Mironov, A.A., Gelfand, M.S., 2002. Comparative genomics of thiamin biosynthesis in procaryotes New genes and regulatory mechanisms. J. Biol. Chem. 277, 48949–48959.

Roldan-Arjona, T., Perez-Espinosa, A., Ruiz-Rubio, M., 1999. Tomatinase from *Fusarium oxysporum* f sp. *lycopersici* defines a new class of saponinases. Mol. Plant Microbe Interact. 12, 852–861.

Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sohn, J., Voegele, R.T., Mendgen, K., Hahn, M., 2000. High level activation of vitamin B1 biosynthesis genes in haustoria of the rust fungus Uromyces fabae. Mol. Plant Microbe Interact. 13, 629–636.

Sudarsan, N., Barrick, J.E., Breaker, R.R., 2003. Metabolite-binding RNA domains are present in the genes of eukaryotes. Rna 9, 644–647.

Thanonkeo, P., Akiyama, K., Jain, S., Takata, R., 2000. Targeted disruption of sti35, a stress-responsive gene in phytopathogenic fungus *Fusarium oxysporum*. Curr. Microbiol. 41, 284–289.

Thore, S., Leibundgut, M., Ban, N., 2006. Structure of the eukaryotic thiamine pyrophosphate riboswitch with its regulatory ligand. Science 312, 1208–1211.

Torres, A.M., Weeden, N.F., Martí, A., 1993. Linkage among isozyme, RFLP and RAPD markers in Vicia faba. Theor. Appl. Genet. 85, 937–945.

Turgeon, B.G., Garber, R.C., Yoder, O.C., 1987. Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell Biol. 7, 3297–3305.

Wickerman, L.J., 1951. Taxonomy of yeasts. US Dept. Agric. Tech. Bull. 1029, 11–56.

Winkler, W., Nahvi, A., Breaker, R.R., 2002. Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. Nature 419, 952–956.

Zurlinden, A., Schweingruber, A.M., 1997. Identification of a DNA element in the fission yeast *Schizosaccharomyces pombe* nmt1 (thi3) promoter involved in thiamine-regulated gene expression. J. Bacteriol. 179, 5956–5958.