**Title:**

*Fusarium sambucinum astA* gene expressed during potato infection is a functional orthologue of *Aspergillus nidulans astA*.

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**Abbreviations:**

ORF – open reading frame

SMR – sulfur metabolite repression

PCR – polymerase chain reaction

EST – expressed sequence tag

**Abstract**

Sulfate assimilation plays a vital role in prototrophic organisms. Orthologues of the alternative sulfate transporter (AstA) gene from *Aspergillus nidulans* were identified in the fungal plant pathogens *Fusarium sambucinum* and *F. graminearum*. By physiological and biochemical analyses, the AstA orthologues were determined to be able to uptake sulfate from the environment. Similarly to *astA* in *A. nidulans*, the *FsastA* gene was found to be regulated by sulfur metabolite repression (SMR) in a sulfur-dependent manner. In contrast, the *FgastA* transcript was undetectable, however, when the *FgastA* gene was expressed heterologously in *A. nidulans*, the translated FgAstA protein acted as a sulfate transporter. Interestingly, *F. sambucinum astA* expression was remarkably augmented in infected potato tubers, despite the presence abundant sulfate and was found not to be correlated with plant resistance.

**Keywords:**
*Fusarium sambucinum*; plant pathogen; alternative sulfate transporter; sulfur starvation.

**Introduction**

Fungi assimilate sulfur in the form of sulfate which is one of the available sulfur form in nature. Sulfur-requiring molecules and amino acids can incorporate only reduced sulfur called sulfide, which demands the presence of appropriate biochemical mechanisms in the cell. After cell uptake, inorganic sulfate is phosphorylated *via* adenosine triphosphate (ATP) to generate 3'phosphoadenosine-5'phosphosulfate (PAPS). PAPS is then reduced in a two-step mechanism to sulfide. In the next stage, sulfide molecules condense with *O*-acetylserine to create cysteine, an intermediate used for further synthesis of methionine and *S*-adenosylmethionine (Paszewski et al. 1984, Marzluf, 1997, Piłsyk and Paszewski, 2009). Various sulfur-rich compounds, especially cysteine, methionine, and *S*-adenosylmethionine, are essential for the growth and activities of all organisms. Cysteine plays a crucial role in maintaining protein structure, stability and their catalytic function. Methionine, on the other hand, is responsible for protein synthesis initiation on the ribosomes, while *S*-adenosylmethionine plays a pivotal role in transferring methyl groups between biomolecules and plays an important role in polyamine biosynthesis (Marzluf, 1997). The ATP-dependent sulfate assimilation occurring in fungi is strictly connected with a large and ubiquitous family of transporter proteins called sulfate permeases (SulP) (Kertesz et al. 2001,

Loughlin et al. 2002, Piłsyk and Paszewski 2009, Alper and Sharma 2013). In *Neurospora crassa,* two sulfate transporters were widely described, *i.e.* CYS13 and CYS14. CYS14 is the leading high-affinity sulfate permease, which can be primarily found in mycelia, while CYS13 functions in conidia. Both *cys-13* and c*ys-14* genes are tightly regulated at the transcriptional level by the sulfur metabolite repression (SMR) system. SMR represses genes involved in ATP-dependent sulfate assimilation when favorable sulfur source like methionine or cysteine appears in excess (Ketter and Marzluf, 1988, Marzluf, 1997). In *N. crassa*, SMR consists of a positively-acting CYS3 transcription factor - a basic-region leucine zipper (bZIP), which is targeted by the negatively acting SCF ubiquitin ligase complex under repressing conditions. The bZIP domain of CYS3 binds to promoter elements with the consensus sequence 5'-ATGRYRYCAT-3', activating related genes. Chief components of SMR are common for *Aspergillus nidulans*, as positive acting MetR transcription regulator and orthologous SCF subunits were also identified (Natorff et al. 1998, 2003).

In *A. nidulans,* two sulfate transporters have also been described, although all strains do not possess both functional genes. *A. nidulans* strains originating in Glasgow England contain a single transporter of the sulfate permease (SulP) family, referred to as SB (Piłsyk et al. 2007). An *A. nidulans* strain isolated in Japan IAM2006, contains a second designated AstA (Piłsyk *et al*. 2007). *astA* was cloned as a suppressor complementing the sulfate permease-deficiency in the *A. nidulans sB* mutant and appears to be a member of a broad and poorly characterized family of allantoate permeases from the Major Facilitator Superfamily (MFS) (Piłsyk *et al*. 2007). In *A. nidulans* Glasgow strains, the *astA* is non-funcional (*e.g.* a pseudogene). As with *sB, astA* gene expression is tightly regulated by SMR (Piłsyk et al. 2007).

Screening available genomic sequences of fungi we found that putative AstA orthologues were present mostly in plant pathogens. Since this result suggested *astA* expression and sulfate demand by pathogen during host infection, therefore it was of interest to verify this hypothesis.

*Fusarium sambucinum* is one of the few species which contribute to the significant devastation of potato crops, increasing the cost of cultivation due to application of pesticides. In this study, we present in detail that *Fusarium* sp. genes similar to *A. nidulans astA* are its functional orthologues. Moreover, *F. sambucinum astA* is regulated by SMR just like *astA* from *Aspergillus* and is significantly derepressed during potato colonization.

## Materials and methods

### Fungal strains, growth conditions, plant material and tuber inoculation

*A. nidulans* strains from our collection carrying standard markers (Martinelli 1994) used in the study along with plasmids and the *Escherichia coli* strain used for plasmid propagation are listed in Table 1. The M111 strain derived from the Glasgow wild-type, carrying the *ΨastA* pseudogene and bearing the *sB43* mutation (sulfate permease-deficient mutant) was used for complementation test by *Fusarium astA* orthologues. M111 complemented with the *sB* gene was used as the *A. nidulans* reference strain. Listed strains and plasmids were obtained from the Fungal Genetics Stock Centre FGSC, Belgian Co-ordinated Collections of Microorganisms, Mycothèque de l'Université Catholique de Louvain (BCCM/MUCL).

For protoplast or DNA isolation from *A. nidulans*, mycelia were grown in complete (C) medium (Cove 1966), while *Fusarium* sp. were cultivated in Potato Dextrose Broth (PDB, BioShop).

In order to acquire RNA, *A. nidulans* was grown in a minimal sulfur-free (MM-S) medium (Lukaszkiewicz and Paszewski 1976), where sulfate salts were replaced by chloride salts, while *Fusarium* sp. was incubated in a modified sulfur-free Defined Fusarium Medium (DFM-S) (based on Sørensen et al. 2014), where sulfate salts were also replaced by chlorides.

Both MM-S and DFM-S minimal media were supplemented with 0.1 mM sulfate (low inorganic sulfur), 5 mM sulfate (high inorganic sulfur), 250 μM or 50 μM L-methionine (low organic sulfur, derepressing conditions) as a sole sulfur source. The MM-S medium was also supplemented according to the auxotrophic requirements of the strain employed.

For liquid cultures, 100 ml of referred minimal medium were used. *A. nidulans* was cultured at 37°C for 18 h, while *Fusarium* sp. at 28°C for 72 h. *Escherichia coli* was grown in standard LB medium supplemented with antibiotics, as required (Sambrook et al. 1989). All cultures mentioned above were placed in a rotary shaker (200 rpm) for an appropriate amount of time.

Twelve potato cultivars were obtained from: Agnes (Europlant GmbH, Germany), Anabelle (HZPC, Holland), Bard, Denar, Finezja, Justa and Ruta (HZ, Zamarte), Bzura and Hinga (PMHZ, Szczekęcino), Cekin, Irga and Irys (HR, Szyldak). Tubers were cultured *in vitro* under standard conditions for 4-6 weeks until they were appropriate for fungal inoculation. In aseptic conditions tubers were wounded by ∅ 2 mm rod bar and inoculated with 50 μl of *F.*

*sambucinum* conidia suspension in concentration of 100-140/ml. Infected tubers were incubated at 16°C with 95-100% humidity for a period of 21 days.

**Nucleic acids isolations and manipulations**

Standard procedures for plasmid propagation and isolation were performed according to Sambrook et al. (1989). DNA from *A. nidulans* and *Fusarium* sp. was isolated by the salting-out method. In the first step, mycelia samples were frozen in liquid nitrogen and ground in a mortar and pestle. This step was followed by immediate suspension of obtained mycelia powder in warm STEN buffer (1% SDS, 100 mM Tris pH 7.5, 50 mM EDTA pH 8, 100 mM NaCl) (Sambrook et al. 1989).

Total RNA was isolated from powdered mycelia using the TRI Reagent (Molecular Research Center), according to the manufacturer's protocol (Chomczyński 1993) and subsequently precipitated with lithium chloride added to a final concentration of 3.42 M, as described by Barlow et al. (1963). RNA from *F. sambucinum*-infected potato tubers was isolated according to Kumar et al. (2007), followed by TRI Reagent and lithium chloride additional clean-up, as described above.

DNA was sequenced and primers were synthesized by the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, PAS. Sequences of the primers used are provided in supplementary Table S1.

Reverse transcription was performed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit, according to the manufacturer's protocol (Thermo Scientific, Fermentas).

Both *FgastA* and *FsastA* genes were PCR-cloned (see: primers in Table S1) and ligated into the pGEM-T easy vector (Promega).

**Transformation of *A. nidulans***

Mycelia for transformation were harvested by filtration on Schott funnel, washed with 0.6 M KCl and finally suspended in 0.6 M KCl solution buffered with potassium phosphate (10 mM, pH 6.5) and containing lytic enzymes: 15-20 mg/ml of Glucanex® 200G (Novozymes), 2 mg/ml of Driselase® (Sigma-Aldrich) and 1 mg/ml of snail acetone powder (Sigma-Aldrich). Protoplasts were prepared and transformed by the PEG method (Kuwano et al. 2008). Transformants were selected for sulfur prototrophy on MM-S medium supplemented with 0.1 mM sulfate and 1.2 M sorbitol.

**Sulfate uptake assay**

For sulfate uptake analysis cultures were grown in 100 ml of MM-S liquid medium supplemented with 50 μM methionine (derepressing conditions) for 18 h at 37°C on a rotary shaker (215 rpm). 15 μCi of [$^{35}$S]-labeled $H_2SO_4$ (Hartmann Analytic) was added to each culture along with cold sodium sulfate to a final concentration of 1 mM. Cultures were kept in a rotary shaker (215 rpm) at 37°C and 10-ml aliquots were taken after 30 and 60 min of incubation. Sulfate uptake was stopped by adding 5 ml of cold 2 mM sodium sulfate containing 10% trichloroacetic acid and 0.5 mM potassium chromate. Mycelia samples were collected by filtration through paper discs, washed with 100 ml of 1 mM sodium sulfate followed by 300 ml of tap water rinse, and dried at 80°C to a constant weight. Dried samples were placed in Packard scintillation vials containing 5 ml of liquid scintillator (Akwascynt, BioCare) and radioactivity was measured in a 1209 Rackbeta Vallac scintillation counter (LKB). Transport rates [nmol/mg dry weight] were estimated on the basis of at least three measurements taken at different times.

To estimate the Michaelis constant of transport ($K_\tau$), cold sulfate was added to various final concentrations (0.166, 0.2, 0.25, 0.33, 0.5 and 1 mM). Mycelia were treated as described above and the initial rate of transport [μmol/min/mg dry weight] was calculated for each concentration of sulfate. These data served to create the Hanes-Woolf plot and linear regression was used for calculation of $V_{max}$ from the slope and $K_\tau$ from the ordinate axis intercept.

**Quantitative real-time RT-PCR**

Real-time RT-PCR was performed using the LightCycler®480 System (Roche Laboratories) with SYBR Green detection, according to the manufacturer's instructions. Primers used to quantify expression of target genes (Supplementary Table S1) were designed using Primer3 software (Untergasser et al. 2012). Primer specificity and qRT-PCR analysis was performed as described previously (Piłsyk et al. 2014). The *act1* γ-actin gene (FGSG_07335) was used as the normalization reference (internal control) for target gene expression ratios. Average cycle thresholds were calculated and the Pfaffl method (Pfaffl 2001) was applied to calculate relative expression with respect to that of actin.

**Fluorescent microscopy**

Infected tuber slides for microscopic observations were treated o/n with 10% potassium hydroxide followed by washing with 70% ethanol. Next, samples were stained with propidium iodide (Sigma-Aldrich) in order to observe potato cell walls. For fungi visualization an Atto488 labeling dye conjugated with wheat germ lectin (Sigma-Aldrich) which binds to chitin in the fungal cell wall were applied. Samples were examined using a Zeiss LSM 880 microscope (Jena, Germany) at 40× and 100× magnification.

**Bioinformatic tools**

Nucleic acid sequence alignments were constructed using MUSCLE software (URL: http://www.drive5.com/muscle/) (Edgar 2004). Homology searches were carried out against the GenBank (release 95.0) database with BLAST algorithm (Altschul and Lipman 1990).

**Results**

**The *F. sambucinum* and *F. graminearum astA* gene complements the *A. nidulans sB* mutation and restore prototrophy**

We cloned the *astA* genes from two closely related plant pathogen *Fusarium* species: *F. graminearum* and *F. sambucinum*, which differ in host specificity. *F. graminearum* colonizes graminoids, while *F. sambucinum* is a common pathogen of potato crops, widely cultivated in central Europe.

The entire *FgastA* locus sequence was PCR-cloned with the use of specific primers designed based on the known genomic sequence of the FGSC#9075 strain.

The *FsastA* gene was amplified by PCR using degenerate primers $U_{CYS3}$ and $L_{TAG}$ designed based on the consensus sequence of respectively, the putative CYS3-binding site present in the promoter region of the gene (Fig. 1 A) and 3' ORF sequence encompassing the stop codon, obtained based on the sequences of the following *Fusarium* species: *F. graminearum* (*Giberella zeae*), *F. verticillioides* (*G. moniliformis*), *F. oxysporum fsp. lycopersici*, *F. solani* (*Nectria haematococca*).

Both PCR products, *FgastA* and *FsastA* were ligated independently into the pGEM T-easy vector, yielding respectively, pGEM:*FgastA*14 and pGEM:*FsastA*4, and then sequenced.
Subsequently, functionality of both *astA* genes cloned was verified. For this, we transformed the *A. nidulans sB* strain deprived of sulfate permease with recombinant pGEMT-easy plasmids carrying the *FgastA* and *FsastA* genes. Obtained transformants were selected on culture medium enriched with 0.1 mM sulfate.
Both, *FgastA* and *FsastA* genes complement the *A. nidulans sB* mutant, which confirmed the sulfate transporter function of the respective proteins (Fig. 1 B).
Sequence analysis of the 1557-bp open reading frame (ORF) of the *F. sambucinum astA* gene showed 87% identity with *F. graminearum astA* of the same length. Detailed analysis of the respective *FgastA* and *FsastA* sequences confirmed that both genes are interrupted by six introns as determined by comparison of the RT-PCR-generated cDNA obtained on the template of mRNA with the genomic sequence. Since *FgastA* mRNA was undetectable in wild type *F. graminearum*, total RNA was isolated from *A. nidulans* [*FgastA*$^{+}$] transformant and the cDNA copy for *FgastA* was then amplified. Six introns are located in conserved positions in both of the analyzed *Fusarium* genomes. Moreover, five of them reside in the exact positions as in *A. nidulans astA*. The resulting *F. sambucinum* and *F. graminearum* AstA proteins consist of 518 amino acids. The FsAstA protein shares 94% identity and 96% similarity with the FgAstA protein and 72% identity and 81% similarity with the *A. nidulans* AstA protein. Sequences of the *FgastA* and *FsastA* genes are available at the EMBL/GenBank database under accession numbers KM588204 and KM588205, respectively.

**Differences in the kinetics of sulfate uptake by FgAstA and FsAstA versus *A. nidulans* AstA when expressed in the *A. nidulans sB* mutant**

The *A. nidulans sB* mutant transformed separately with *F. graminearum* and *F. sambucinum astA* genes was cultivated in MM-S supplemented with 50 μM methionine to derepress expression of genes encoding sulfate transporters. As control, the *A. nidulans sB* mutant bearing the *astA* gene from the Japanese *A. nidulans* IAM2006 strain was used. In order to evaluate substrate uptake for each type of transformant, radioactive sulfate was added to the cultures and its level was measured in the mycelia after 30 and 60 min of incubation (Fig. 2 A).

The $K_\tau$ values determined for FgAstA and FsAstA were 35.6 and 37.1 μM, respectively, which was more than two-fold lower than for *A. nidulans* AstA (85 μM) (Fig. 2 B). The $V_{max}$ was 0.889 for FgAstA and 0.856 for FsAstA (nmol/min) and were almost two times lower than the $V_{max}$ calculated for *A. nidulans* AstA, *i.e.* 1.53 nmol/min. However, such differences did not affect the sulfate uptake rate.

### *FsastA* in *F. sambucinum* is regulated by SMR in the same manner as *astA* in *A. nidulans*

The putative CYS3 (MetR)-binding area in the promoter region of *Fusarium astA* genes (Fig. 1 A) resembles that of *N. crassa* 5'-ATGRYRYCAT-3'. Therefore, we examined expression of *FsastA* in *F. sambucinum* expecting that its regulation by sulfur compounds will be similar as with the *astA* orthologue from *A. nidulans*.

To accomplish this aim, *F. sambucinum* was cultivated in DFM-S medium supplemented with 0.1 and 5 mM sulfate and 50 μM methionine. Quantitative real-time PCR analysis of *FsastA* expression showed that in the presence of 0.1 mM sulfate as the sole sulfur source, the transcript level was over 260-fold higher compared to the reference level acquired in 5 mM sulfate culture conditions (Fig. 3 A). Even stronger derepression of 3500-fold was observed when the cultivation medium was supplemented with 50 μM methionine as the sole sulfur source. These results confirmed that expression of the *F. sambucinum astA* gene is regulated by available sulfur sources and is under the control of SMR.

It is interesting that in *F. graminearum* cultivated under defined conditions of sulfur starvation, the *FgastA* transcript is not detected by sqRT-PCR (not shown). However, mRNA for *FgastA* is detectable in heterologous system (Fig. 3 B).

### SMR controls two sulfate permeases from the SulP family in *F. sambucinum*

As it seems unlikely for AstA to be the only sulfate transporter in *F. sambucinum*, we investigated whether this fungus possesses, besides FsAstA, a sulfate permease from the SulP family that would be regulated by SMR. Genomic analysis of the closest genetic relative, *i.e. F. graminearum*, revealed that at least three genes: FGSG_02163, FGSG_01066 and FGSG_11293, encode proteins which are orthologous to the *A. nidulans* sulfate permease SB (e-value equal 0.0). Based on the significant nucleotide sequence homology between both *Fusarium* species, a set of primer pairs were designed and used in sqRT-PCR analysis of the

three genes encoding these proteins in *F. sambucinum*. At least two of them were found to be orthologous to FGSG_02163 and FGSG_01066 proteins and regulated by available sulfur sources (Fig. 3 C).

### *FsastA* expression is significantly derepressed in infected potato tuber tissue

To examine the demand for sulfate of *F. sambucinum* during potato colonization, expression study of genes encoding chosen sulfate transporters was performed.

Twelve potato cultivars: Agnes, Anabelle, Bard, Bzura, Cekin, Denar, Finezja, Hinga, Irga, Irys, Justa and Ruta, were selected for susceptibility tests. In standard culture conditions (see Materials and Methods), *F. sambucinum* very quickly necrotized and decomposed tuber tissue. Therefore, due to this fact, susceptibility to the pathogen was approximated on a three-point scale. Potato cultivar was claimed to be resistant when necrosis caused by *F. sambucinum* was limited to the inoculation area (Fig. 4 A, cultivars: Agnes, Bard, Finezja), moderately resistant when rotten area did not cover 100% of the tuber (Fig. 4 B, cultivars: Anabelle, Cekin, Irga, Ruta) or susceptible when the entire tuber was rotten (Fig. 4 C, Bzura, Denar, Hinga, Irys, Justa). It appeared that the most commonly cultivated potatoes in Poland: Irga, Cekin, Irys, Denar and Justa, were moderately or fully susceptible to *F. sambucinum* infection.

In our experiments, *F. sambucinum* started to colonize potatoes by entering wounds and scars present in the tubers, spreading subsequently in the periplasmic space of the parenchymal cells (Fig. 4 D). At the same time, fungal haustoria were formed and quickly after the entire tuber was penetrated and necrotized.

For sqRT-PCR analysis, four infected tubers were selected depending on their resistance to *F. sambucinum* infection: one resistant cultivar (Finezja), one moderately resistant (Anabelle) and two susceptible (Hinga, Irys). Interestingly, significant *FsastA* expression was detected in all infected tubers examined, independently of plant susceptibility (Fig. 4 E). Obtained results have shown expression of the *FsastA* gene from *F. sambucinum* as well as significant expression of genes encoding two putative sulfate permeases.

## Discussion

By screening available genomic sequences of fungi, we identified numerous species possessing genes homologous to *A. nidulans astA*, encoding an alternative sulfate transporter (AstA).

*Fusarium* sp. fungi, like *F. solani*, *F. oxysporum* and *F. sambucinum*, contribute to significant devastation of potato crops and increase the cost of cultivation due to the need to apply pesticides. *F. sambucinum* and *F. graminearum* belong to the *F. sambucinum* complex species, but despite a high level of similarity, they occupy different ecological niches and infect different crops. *F. sambucinum* is a common potato pathogen, while *F. graminearum* often devastates maize and various cereals. Homologous *astA* genes from both *Fusarium* sp. were PCR-cloned and confirmed to act as sulfate transporters by complementing the *A. nidulans* sulfate permease mutant. Kinetic studies showed that AstA proteins derived from *Fusarium* sp. had transportation parameters comparable to that of *A. nidulans* AstA. Hence, despite the far evolutionary distance between these genera, high sequence homology among the AstA proteins seems to reflect a similar function in sulfate transport.

We have shown that expression of *FsastA* in *F. sambucinum* was strictly regulated by sulfur sources. Such expression pattern is similar to the *A. nidulans astA* gene, which is controlled by SMR (Piłsyk et al. 2007). However, in closely related *F. graminearum* the *FgastA* transcript was undetectable, which suggests that the *FgastA* gene is expressed under other, currently unknown conditions. The fifth intron of *FgastA* was found to contain a non-canonical GC-AG splice site rarely found in *F. graminearum* and other fungi (Rep et al. 2006). However, it is unlikely that this would be the cause of lack of transcript as heterologous expression of the *FgastA* gene leads to complementation of a sulfate transporter-deficient *A. nidulans* strain, where *FgastA* mRNA is spliced properly.

A notable high expression of the *F. sambucinum FsastA* gene was detected in infected potato tubers. Transcripts were detected in all examined potato species and their levels were not correlated with tuber susceptibility to the pathogen. Furthermore, expression of two of the three genes encoding putative sulfate permeases in *F. sambucinum* were detected during potato infection. The inability to detect transcripts of third putative sulfate permease might result from the fact that this gene is expressed under other conditions or is a pseudogene, or, alternatively, the encoded protein in engaged in transport of another substrate.

Despite the low content of readily available methionine (c.a. 10 nM, Dancs et al. 2008), potato tubers are abundantly rich in sulfate, which concentrations in the homogenates of some potato cultivars ranging from 18 to 19 mM (Abah et al. 2008). Hence, sulfate assimilation should be repressed, particularly in respect to genes coding for sulfate transporters. In this aspect, the presented results seems to be contradictory. However, during infection and overgrowing of tubers by the fungus, the local sulfate concentration might decrease enough to derepress transcription of genes encoding sulfate transporters. In our

experiments, a significant bulk of mycelia in rotten potato was spread as a net in a cavity formed in tubers during infection. Such mycelia had limited access to sulfate present in potato tissues; thus, existence of two distinctive mechanisms which are responsible for sulfate uptake seems to be crucial for fungus survival. Moreover, the kinetic mechanism of MFS transporters is based simply on the substrate or electrochemical gradient, while SulP members require ATP-derived energy for transport (Law et al. 2008).

It is interesting to note that expression of the *astA* gene orthologue, *i.e.* FVEG_12081, was also found in the cDNA library (EST1020224) constructed from maize root infected by *F. verticillioides* (Brown et al. 2005). This finding together with our results suggests that during fungal infection local concentration of sulfate might decrease so significantly that expression of various sulfate transporters is activated.

These results lean us towards an hypothesis that AstA might be a sulfate transporter of obligate parasites, like *Fusarium*. Due to the fact that AstA is present only in some fungi, its physiological function in the cell is still uncertain. Nonetheless, existence of an extremely active sulfate transporter AstA in *F. sambucinum* may be the result of adaptation to the sulfate starvation conditions created during infection in the necrotized host tissue.

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**Figures**

**Fig. 1. A)** Alignment of promoter sequences of *astA* orthologues from known *Fusarium* sp.. Conserved putative CYS3 (MetR)-binding area is underlined and bolded. Conserved amino acids are marked in dark and grey shaded, the consensus sequence is outlined below. **B)** Growth of the *A. nidulans sB43* mutant transformed with *astA* from *F. graminearum* (*FgastA*$^{+}$) and *F. sambucinum* (*FsastA*$^{+}$). Solid minimal sulfur-free medium (MM-S) was supplemented with 0.1 mM sulfate or 0.25 mM methionine. The sulfate permease-deficient recipient strain (*sB43*) and transformants with the wild type *sB* or the *astA* gene from the Japanese *A. nidulans* IAM2006 strain (*AnastA*$^{+}$), are included as controls.

**Fig. 2. A)** Kinetics of sulfate uptake by mycelia of *A. nidulans sB43* mutant expressing FgAstA and FsAstA proteins compared to the *A. nidulans* AstA transporter. Sulfate uptake was measured as described in Materials and Methods. Incubations were carried out for 1 h. Uptake rates were determined at sulfate concentrations ranging from 0.166 to 1 mM. **B)**

Sulfate uptake by *A. nidulans* transformants measured as influx of $^{35}SO_4^{2-}$. Mycelia with the *astA* gene (wild type *A. nidulans* AstA transporter) and *Fusarium astA* alleles were grown overnight at 37°C in MM-S medium containing 50 μM methionine as a derepressing sulfur source. Radioactive 1 mM sulfate was added and aliquots of the mycelia were collected for measurement at indicated times as described in Materials and Methods.

**Fig. 3. A)** Expression of the *FsastA* gene in *F. sambucinum* growing under various sulfur conditions in minimal Defined Fusarium Medium (DFM). Transcript levels obtained from cells cultured under described conditions were compared by qPCR to those obtained from cultures where 5 mM methionine was used as a sulfur source. **B)** RT-PCR on cDNA isolated from *A. nidulans* heterologously expressing the *FgastA* gene from *F. graminearum*. Lanes: 1 – size range standard, 2 – *FgastA* RT-PCR on the cDNA template from *A. nidulans* transformant, 3 – *FgastA* PCR on the genomic DNA template from *F. graminearum* (control; PCR product is longer due to presence of intron). **C)** Semiquantitative RT-PCR performed on cDNA templates of *F. sambucinum* genes putatively encoding sulfate permeases. At least two of the examined genes (FGSG_02163 and FGSG_01066 orthologue) are regulated by sulfur sources.

**Fig. 4.** *F. sambucinum* during potato tuber infection. **A)** Potato cultivars resistant to *F. sambucinum*: Agnes, Bard, Finezja, respectively **B)** Moderately resistant cultivars: Anabelle, Cekin, Irga, Ruta, respectively **C)** Susceptible cultivars: Bzura, Denar, Hinga, Irys, Justa, respectively **D)** Penetration and colonization of potato tuber cells by *F. sambucinum* observed under fluorescent microscope (potato cell wall stained red with propidium iodide and fungal cell wall stained green with Atto488 labeling dye conjugated with wheat germ lectin). **E)** Semiquantitative RT-PCR on cDNAs from the wild type *F. sambucinum* strain (control) and from *F. sambucinum* isolated from infected potato tissue. Four potato cultivars have been selected for this experiment: Hinga, Irys, Finezja, Anabelle, due to their diverse resistance to *Fusarium* infection. In all of them, the *FsastA* transcript has been detected as well as transcripts of the genes encoding putative sulfate permeases orthologous to FGSG_02163 and FGSG_01066 from *F. graminearum*. (PCR *FsastA* product acquired from the genomic DNA template derived from *F. sambucinum* is longer due to presence of an unspliced intron).

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**Table 1.** List of strains and plasmids

| Designation | Genotype or relevant features | Reference or source |
|---|---|---|
| ***E. coli*** strain XL1 Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*$^{q}$ *ZΔM15* Tn10 (Tetr)]$^{c}$ | Stratagene |
| ***Emericella*** strains | | |
| *Aspergillus nidulans* M111 [*pyr-4*$^{+}$] | *sB43 pyrG89* [ *Ncpyr-4*$^{+}$] *pyroA4 yA2* | laboratory collection |
| *Aspergillus nidulans* M111 [*astA*$^{+}$, *pyr-4*$^{+}$] | *sB43 pyrG89* [*astA*$^{+}$ *Ncpyr-4*$^{+}$] *pyroA4 yA2* | laboratory collection |
| ***Fusarium*** strains | | |
| MUCL 31741 | *Fusarium sambucinum* wild type, isolated from Charlotte potato cultivar | BCCM/MUCL |
| FGSC#9075 | *Fusarium graminearum* wild type | FGSC |
| **Plasmids** | description | |
| PGEM:FsastA4 | $U_{CYS3}$-$L_{TAG}$ PCR fragment of *FsastA* ligated into pGEM T-easy vector | this study |
| PGEM:FgastA14 | $U_{UTR}$-$L_{UTR}$ PCR fragment of *FgastA* ligated into pGEM T-easy vector | this study |
| kPMS11-52 | BamHI-SalI insert bearing *astA* gene in pBluescript KS(–) vector | Piłsyk et al. 2007 |

**Table S1.** Oligonucleotides used as primers for PCR amplification and sequencing.

| name | sequence (5'→3') | description |
|---|---|---|
| $U_{CYS3}$ | CAGCCCCGGCACTTGGTSTATKGCGTGCTYACTTGGC | Primer pair for cloning of the *FsastA* gene |
| $L_{TAG}$ | CTATGTTGGCTTTGCAAKGGRATTCAATKCGGCTGG | KM588205 from *F. sambucinum* |
| $U_{UTR}$ | CCAAGCTCTCACAAGAGACAGGCCAAGG | Primer pair for cloning of the *FsastA* gene from |
| $L_{UTR}$ | ATGTATAGCCTAAGCGATAAGAAT | *F. graminearum* |
| Fs.*astAq*-U1 | TCAGCGTGGTTTTGACACAT | KM588205 *FsastA* realtime qPCR primer pair |
| Fs.*astAq*-L1 | AACCCAGCCATGACAGTCTC | |
| UFg*astA* | CGCCTCTGTTGGTCTCTTTC | KM588204 *FgastA* realtime qPCR primer pair |
| LFg*astA* | CATCACAGGCCAATGACATC | |
| UFg*sB1* | TTCACTCATGCTGCTGTTCC | FGSG_02163 (*sB1*) sqPCR primer pair |
| LFg*sB1* | TGACTTGATGGCAGTTCGAG | |
| UFg*sB2* | TCGGGGTACCCAAAATATCA | FGSG_01066 (sB2) sqPCR primer pair |
| LFg*sB2* | TGCGACTGAAAGATCCTGTG | |
| UFg*sB3* | AACACAGACGACCCCAAGAC | FGSG_11293 (sB3) sqPCR primer pair |
| LFg*sB3* | ATCGGGGCGTCTAGTATGTG | |
| Faktq-U3 | CACCGGTATCGTTCTGGACT | FGSG_07335 *Fusarium* γ-actin realtime qPCR |
| Faktq-L3 | GCAGTCTGGATCTCCTGCTC | primer pair |

Fig. 1

A

```
F. solani          : GTGCTCCCGGCACTTGCTGGTATTGCGTGCTCGAATTCTGC
F. graminearum     : CAGC-CCCGGCACTTGGT-CTATGGCGTGCTT--ACTTGGC
F. verticillioides : CAGC-CCCGGCACTTGGT-GTATTGCGTGCTC--ACTTGGC
F. oxysporum       : CAGC-CCCGGCACTTGGG-GTATTGCGTGCTC--ACTTGGC
                     caGC CCCGGCACTTGgt gTATtGCGTGCTc  AcTtgGC
```

B

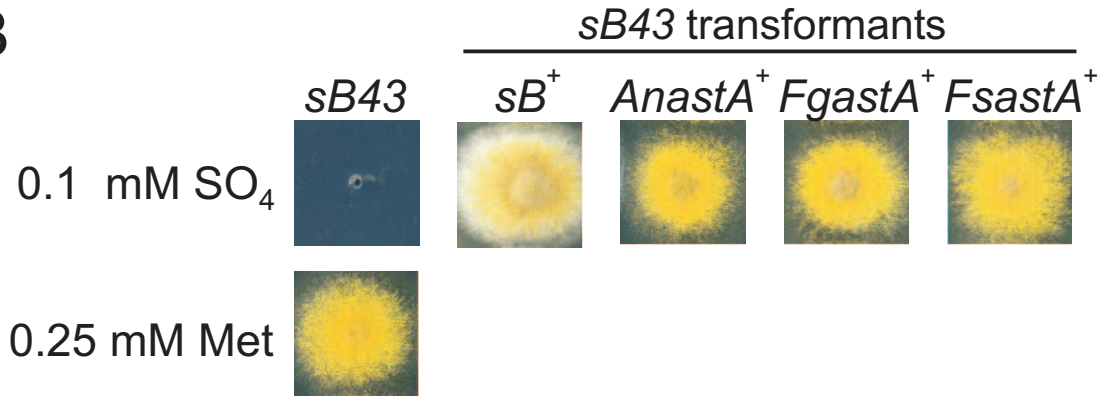
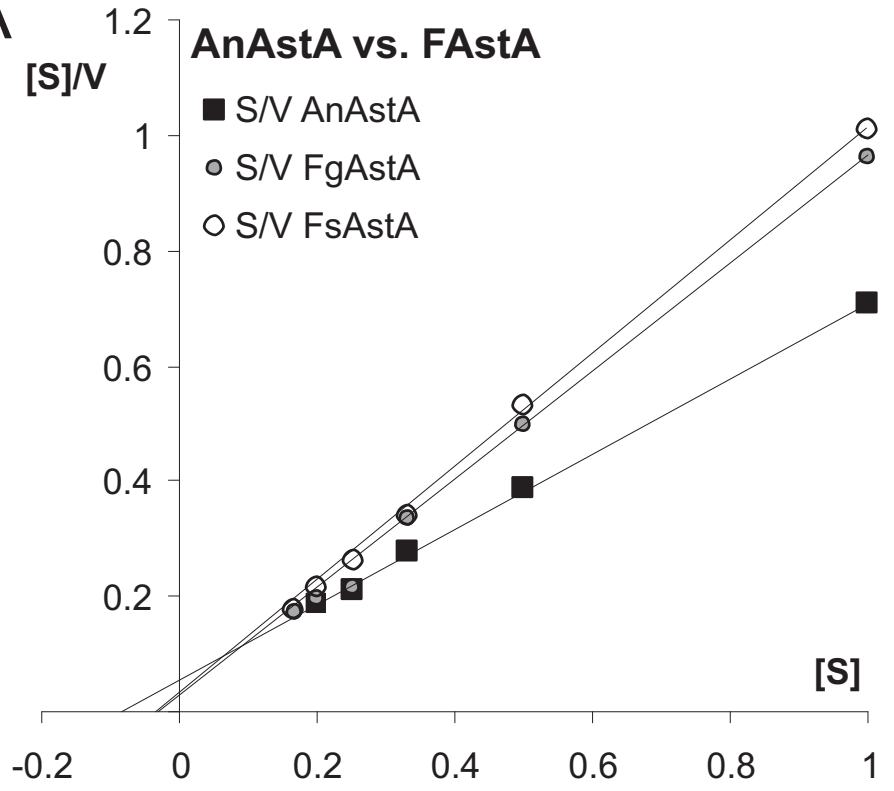


Fig. 2

A


AnAstA vs. FAstA
[S]/V
S/V AnAstA
S/V FgAstA
S/V FsAstA
[S]
1.2
1
0.8
0.6
0.4
0.2
-0.2
0
0.2
0.4
0.6
0.8
1

B

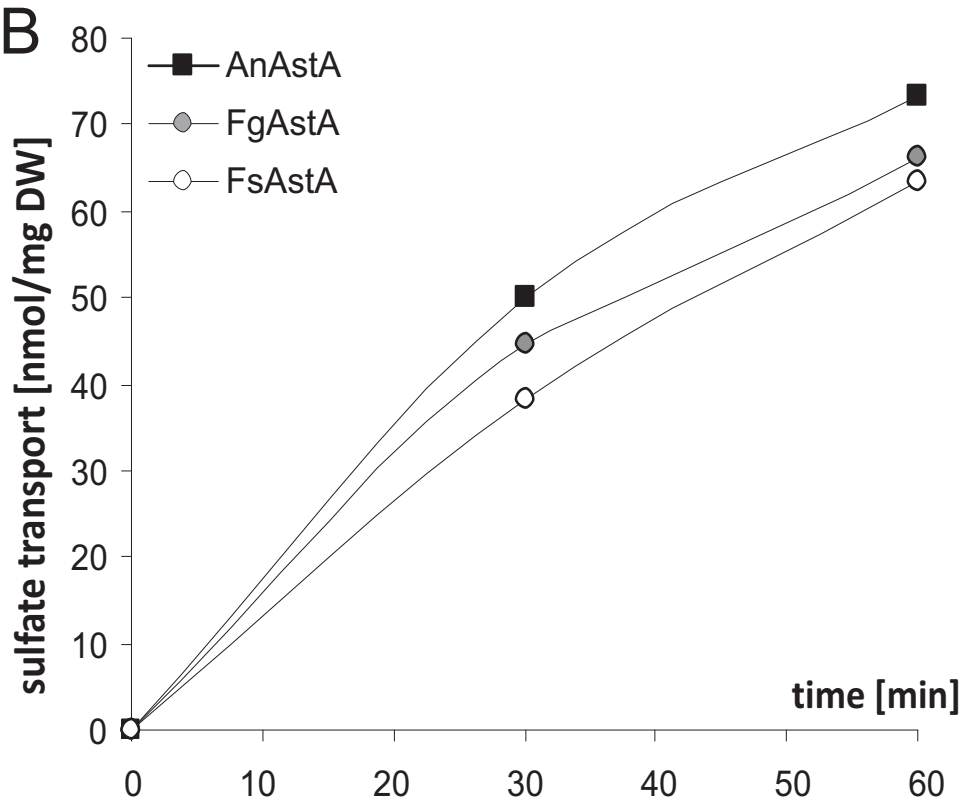
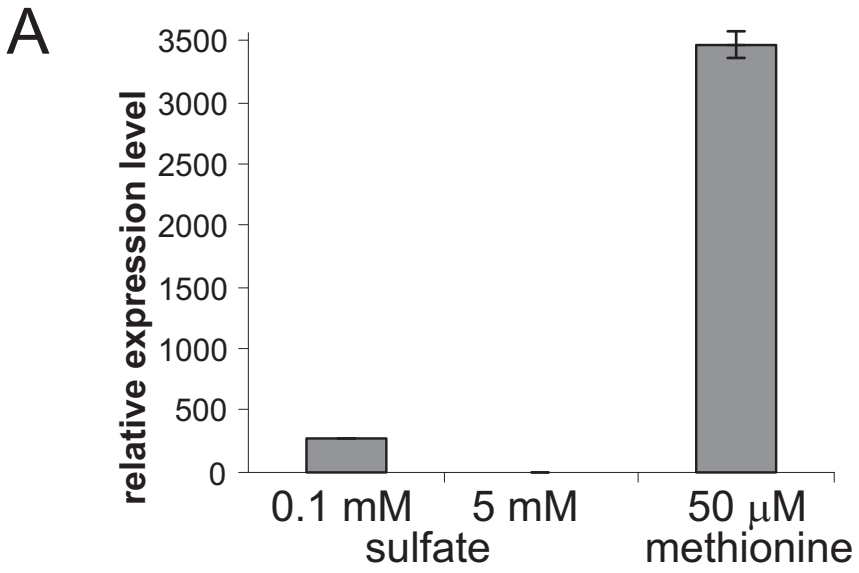
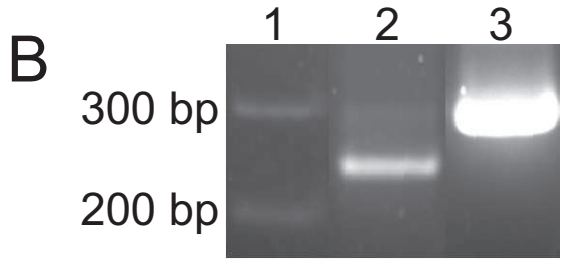

AnAstA
FgAstA
FsAstA
sulfate transport [nmol/mg DW]
time [min]
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60

Fig. 3


A
relative expression level
3500
3000
2500
2000
1500
1000
500
0
0.1 mM sulfate
5 mM
50 μM methionine


B
1
2
3
300 bp
200 bp

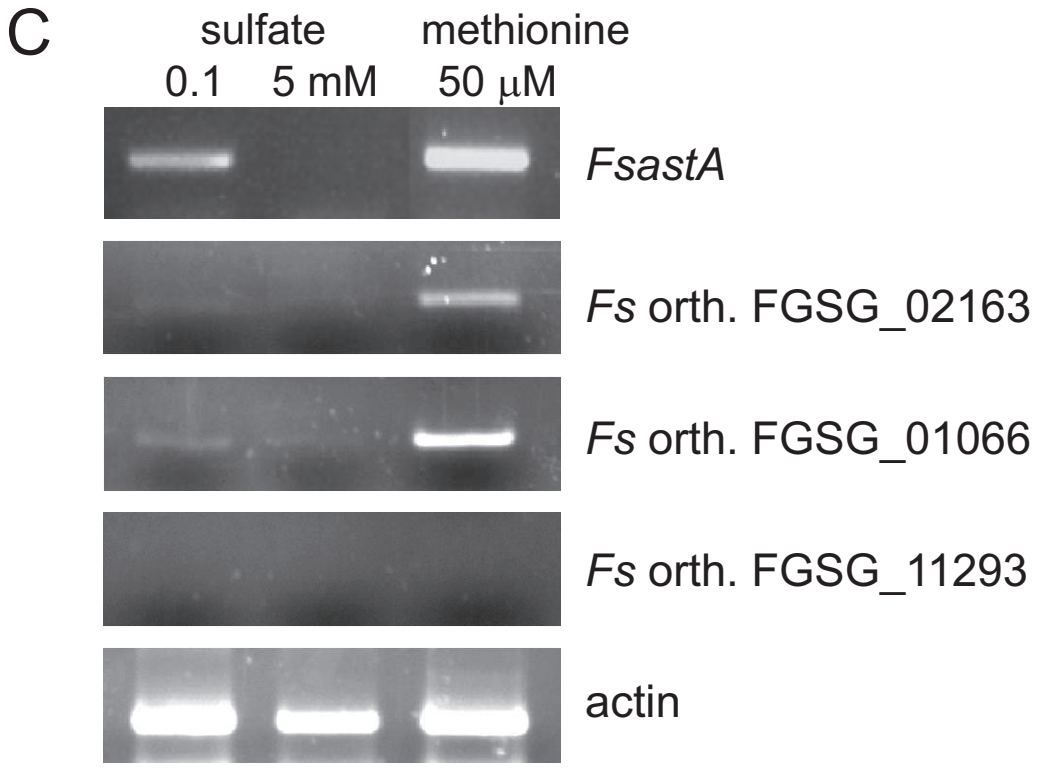
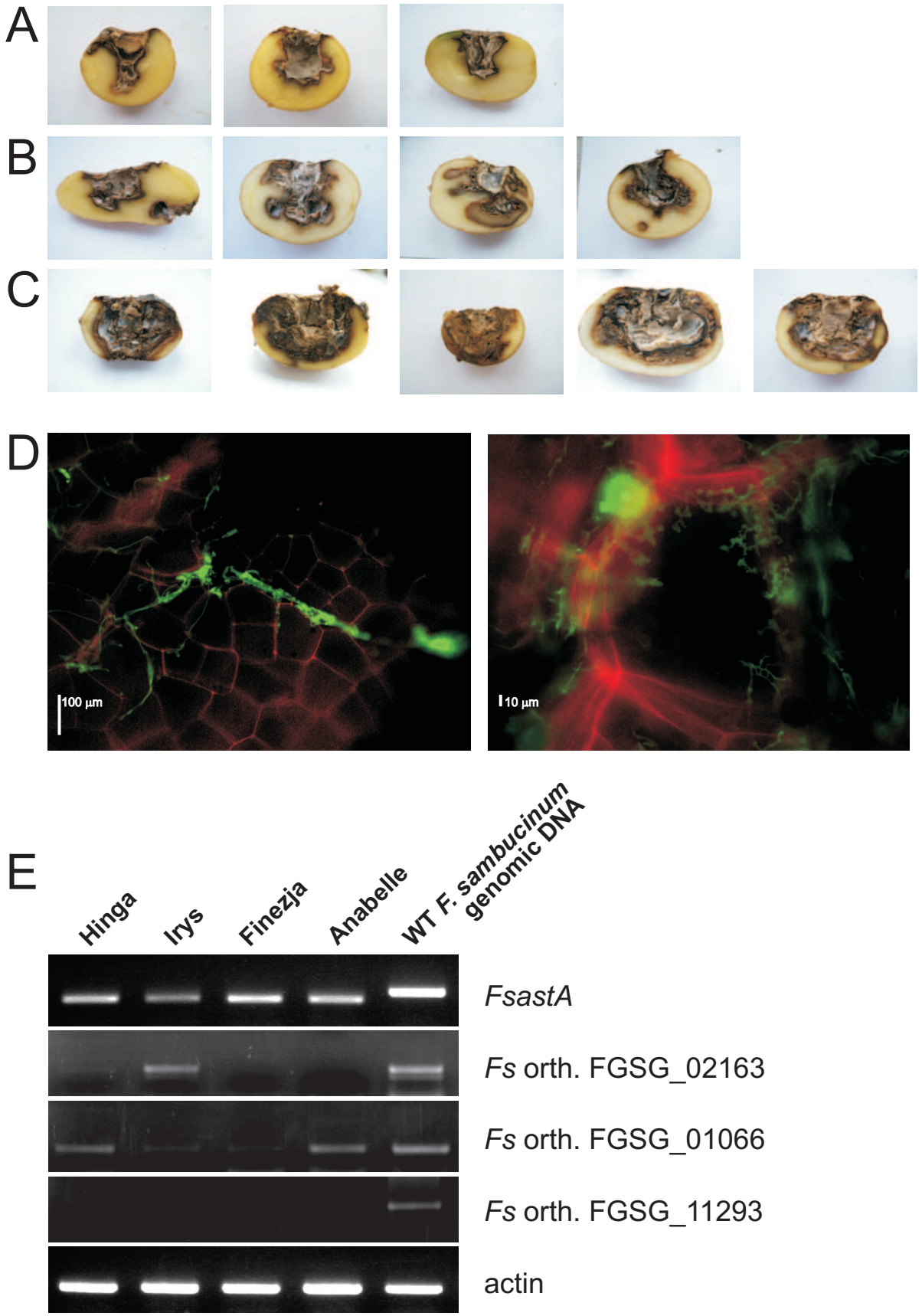

C
sulfate
methionine
0.1
5 mM
50 μM
FsastA
Fs orth. FGSG_02163
Fs orth. FGSG_01066
Fs orth. FGSG_11293
actin

Fig. 4


A
B
C
D
100 µm
10 µm
E
Hinga
Irys
Finezja
Anabelle
WT F. sambucinum genomic DNA
FsastA
Fs orth. FGSG_02163
Fs orth. FGSG_01066
Fs orth. FGSG_11293
actin