Serine–Arginine Protein Kinase-like Protein, SrpKf, Stimulates Both Cellobiose-responsive and D-Xylose-Responsive Signaling Pathways in *Aspergillus Aculeatus*

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Serine–arginine protein kinase-like protein, SrpkF, stimulates both cellobiose-responsive and D-xylose-responsive signaling pathways in *Aspergillus aculeatus*

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Abstract

To elucidate the regulatory mechanisms of various cellulolytic enzyme genes in *Aspergillus aculeatus*, we identified one mutant that reduced the expression of FIII-avicelase (*chbI*) in response to cellulose from 12,000 *A. aculeatus* T-DNA-inserted mutants. The T-DNA inserted into a putative protein kinase gene similar to AN10082 in *A. nidulans*, the serine–arginine protein kinase F, SrpkF. The fold increase in *srpkF* gene expression in response to various carbon sources was 2.3 (D-xylose), 44 (Avicel®), 59 (Bacto™ Tryptone), and 98 (no carbon) compared with D-glucose. The deletion of *srpkF* in *A. aculeatus* resulted in a significant reduction in the cellulose-responsive expression of *chbI*, hydrocellulase (*cel7b*), and FIb-xylanase (*xynIb*) genes at an early induction phase. However, the *srpkF* deletion did not affect the expression of *xynIb* in response to D-xylose. Furthermore, the *srpkF*-overexpressing strain that expresses the *srpkF* gene at levels from four- to nine-fold higher than the control strain stimulated the expression of *chbI* and *cel7b* in response to cellobiose and the FI-carboxymethyl cellulase gene (*cmcI*) and *xynIb* in response to xylose. The expression of *chbI* and *cel7b* is regulated by a transcriptional activator, ManR, and the expression of *cmcI* and *xynIb* is regulated by XlnR. Our data demonstrate that SrpkF can stimulate both the ManR- and XlnR-dependent signaling pathways in response to cellobiose and D-xylose in *A. aculeatus*.

Keywords:

Gene regulation, Cellulase, ManR, XlnR, filamentous fungi, phosphorylation

Declarations

Funding

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Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material

All data generated or analyzed during this study are included in this published article and its Supplementary Information files. DNA sequences will be available at the DDBJ database when this article is released for publication.

Code availability

Not applicable.

Author contributions

S.T. and T.K. conceived and designed the project. R.K. and N.K. conducted the experiments. S.T wrote the paper. All authors read and approved the final manuscript.

Ethics approval

This article does not contain any studies that involve human participants or animals.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Introduction

Filamentous fungi are prominent producers of enzymes that degrade lignocellulose (Payne et al. 2015). Among filamentous fungi, *Trichoderma reesei* is well known to produce copious amounts of cellulolytic enzymes (Bischof et al. 2016). However, *T. reesei* glycoside hydrolases are being continuously improved to utilize lignocellulose for feedstock and generate biobased products. For example, β -glucosidase from *Aspergillus aculeatus* no. F-50 [NBRC 108789] was introduced into *T. reesei* to supplement its activities, which accelerated cellulose hydrolysis (Baba et al. 2015; Nakazawa et al. 2012). β -Glucosidase from *A. aculeatus* showed high compatibility in *T. reesei*, suggesting that *A. aculeatus* produces promising enzymes to liberate glucose from cellulose. However, cellulolytic enzymes of *A. aculeatus* are not utilized in industry because of low production levels. We aim to understand the regulatory mechanisms of the associated gene expressions and apply this knowledge to improving enzyme production in *A. aculeatus*.

Cellulolytic enzyme production in *Aspergillus* is regulated at the transcriptional level. The first identified transcriptional activator of cellulolytic and xylanolytic genes was XlnR, a Zn(II)₂Cys₆-type transcriptional activator that coordinates xylanolytic expression in *Aspergillus niger* (van Peij et al. 1998). Genetic analysis revealed that XlnR controls the expression of not only xylanolytic enzyme genes but also cellulolytic enzyme genes in *A. aculeatus* (Kunitake and Kobayashi 2017; Tani et al. 2014). XlnR regulates the expression of FIIb-xylanase (*xyn1b*) and FI-carboxymethyl cellulase (*cmc1*) genes, whereas FIII-avicelase (*cbh1*) and FII-carboxymethyl cellulase (*cmc2*) are under the control of the XlnR-independent signaling pathway in *A. aculeatus* (Tani et al. 2012). Clr-2 in *Neurospora crassa* and ClrB in *Aspergillus nidulans* were identified as Zn(II)₂Cys₆-type transcriptional activators controlling the expression of cellulolytic enzyme genes in response to cellulosic carbon sources (Coradetti et al. 2012). ManR, a ClrB ortholog, participates in the XlnR-independent signaling pathway in *Aspergillus oryzae* and *A. aculeatus* (Ogawa et al. 2013; Tsumura et al. 2021). Although regulators involved in the regulation mechanisms of cellulolytic and xylanolytic enzyme genes have been identified, the details of these regulation mechanisms have not been clarified.

We previously established a positive screening method to identify regulators involved in the cellulose-responsive induction pathway in *A. aculeatus* by screening for 5-fluoroorotic acid (5-FOA)-resistant mutants on minimal medium (MM) with wheat brans as a sole carbon source (Kunitake et al. 2011; Kunitake et al. 2013). Using this method, we identified ClbR, dipeptidyl peptidase IV, and SepM as regulators stimulating the expression of cellulolytic enzyme genes in response to cellobiose (Kunitake et al. 2015; Kunitake et al. 2013; Tani et al. 2017; Tsumura et al. 2021). ClbR is a Zn(II)₂Cys₆-type transcriptional activator controlling the expression of cellulolytic enzyme genes in response to cellobiose (Kunitake, et al. 2013). SepM interacts with SepL, a putative kinase in the septation initiation network complex, which participates in septa formation and regulation mechanisms of the ManR-dependent signaling pathway (Tsumura et al. 2021). The molecular mechanisms of how the two different signaling

pathways (the ManR- and XlnR-dependent signaling pathways), control gene expression in response to cellulose remain unknown.

To comprehensively understand the regulation mechanisms of cellulolytic enzyme genes, we further screened for a new regulator. In this study, we identified a putative protein kinase, SrpKf, which participates in the ManR- and XlnR-dependent signaling pathways to induce gene expression in response to cellulosic carbon sources.

Materials and Methods

Strains, transformation, marker recycling, and T-DNA insertion

All *A. aculeatus* strains used in this study were derived from wild-type *A. aculeatus* no. F-50 [NBRC 108789]. Unless otherwise stated, all strains were propagated at 30°C in an appropriately supplemented MM (Adachi et al. 2009). *A. aculeatus* NCP2 (*niaD1::niaD::P_{CHBI}-pyrG; pyrG1*) was used to construct *A. aculeatus* strains for T-DNA insertion via *Agrobacterium tumefaciens*-mediated transformation. Counterselection on 5-FOA and marker recycling were performed as described previously (Kunitake et al. 2011; Kunitake, et al. 2013). *A. aculeatus* MR12 (*pyrG1; Δku80*) was used for the disruption and complementation of the *srpKf* gene (Tani et al. 2013). *Escherichia coli* DH5αF' was used for plasmid construction.

Disruption and complementation of srpKf

The *A. aculeatus* *srpKf*-deficient mutant (*pyrG1; Δku80; ΔsrpKf*) was created by replacing *srpKf* with the *A. nidulans* orotidine 5'-phosphate decarboxylase gene (*AnpyrG*) followed by marker recycling (Tani et al. 2013). To construct the *srpKf* deletion cassette, the 5' and 3' regions of *srpKf* (which play a key role in homologous recombination to replace *srpKf* with *AnpyrG*) were amplified from *A. aculeatus* genomic DNA using primer pairs 2.6k-5'srpKf/2.6k-5'srpKfR and L-kinaseF/L-kinaseR, respectively. The *AnpyrG* gene was amplified from *A. nidulans* genomic DNA using the primer pair AnpyrG-KF/AnpyrG-KR. The 3' flanking region of *sepM* was amplified using the primer pair 2.6k-MsrpKf/M-kinaseR to eliminate *AnpyrG* by intramolecular homologous recombination at the *srpKf* locus. The 5' region of *AnpyrG* (responsible for marker recycling) and the 3' region were fused via PCR using the primer pair 2.6k-5'srpKf/L-kinaseR and subcloned into the *EcoRV* site of pBluescriptIIKS(+) to yield pDsrpKf. The *srpKf* deletion cassette was amplified via PCR using the primer pair 2.6k-5'srpKf/L-kinaseR from pDsrpKf and introduced into MR12 (*pyrG1; Δku80*) by the protoplast-PEG method to yield the *A. aculeatus* *ΔsrpKf* plus *pyrG* strain (*pyrG1; Δku80; ΔsrpKf::AnpyrG*). Marker recycling was performed by spreading 1×10^4 transformant spores onto MM supplemented with 0.01% uridine and 1 mM 5-FOA. *A. aculeatus* *ΔsrpKf* (*pyrG1; Δku80; ΔsrpKf*) was obtained following monospore isolation (Supplementary Fig. S1). Supplementary Table S1 summarizes the primers used in this study.

To complement *A. aculeatus* Δ *srpkF*, the *srpkF* promoter, open reading frame, and 3'-untranslated region (UTR) were first amplified using the primer pair 2.6k-5'*srpkF*/M-kinaseR. *AnpyrG* and the 3' region required for homologous recombination at the *srpkF* locus were subsequently amplified with primer pairs AnpyrG-KF/AnpyrG-CR and C-kinaseF/C-kinaseR, respectively. The three DNA fragments were fused via PCR using the primer pair 2.6k-5'*srpkF*/C-kinaseR. Finally, *A. aculeatus* Δ *srpkF* was transformed with the amplified DNA fragments to yield the *srpkF*-complemented strain (*pyrG1*; Δ *ku80*; Δ *srpkF*::*srpkF*::*AnpyrG*) (Supplementary Fig. S1).

The *A. aculeatus* *SrpK* C-terminus deletion mutant was generated by introducing a stop codon at 1,129 nt. The ORF with 5' flanking region (-23-1,121 bp) and 3'-UTR of *srpkF* was amplified using primer pairs pK-F/*srpkF*stopMR-R and 3'UTRF/3'UTRR, respectively, and were fused via PCR using primer pair pK-F/3'UTRR. The region of *AnpyrG* for marker recycling and the 3' region required for homologous recombination at the *srpkF* locus were subsequently amplified with primer pair MRstop-*srpkF*-F/L-kinaseR using pD*srpkF* as a template. These fragments were fused via PCR using the primer pair pK-F/L-kinaseR. The fused fragments were introduced into MR12 (Supplementary Fig. S2). *A. aculeatus* Δ *CsrpkF* (*pyrG1*; Δ *ku80*; Δ *CsrpkF*₁₋₃₂₇) strain was obtained after marker recycling. The Δ *CsrpkF* strain was complemented by the same fragments used to complement Δ *srpkF* (Supplementary Fig. S2).

The *A. aculeatus* *srpkF*-overexpressing strain (*pyrG1*::*Ptef*-*srpkF*::*AapyrG*; Δ *ku80*; Δ *srpkF*) was generated by inserting an *srpkF*-overexpressing cassette into the *A. aculeatus* *pyrG* locus in Δ *srpkF*. To introduce the expression cassette into the *AapyrG* locus, the *AapyrG* ORF with its promoter and terminator region, and the *AapyrG* 3' region were amplified using primer pairs AapyrGORFF/AapyrGORFR and AapyrG3'F/AapyrG3'R, respectively. The promoter region of the translation elongation factor 1 α gene (*Ptef*) was amplified using primer set AaPtefF/AaPtefR and subsequently fused to the *AapyrG* 3' region via PCR using primer set AapyrG3'F/AaPtefR, followed by digestion with *NotI* and *SacI*. The amplified *AapyrG* ORF fragment was digested with *KpnI* and *XbaI* and subcloned into pBS *KpnI* and *SalI* sites. The plasmid was digested with *NotI* and *SacI* and ligated with the digested *AapyrG* 3' and *Ptef* fragments. The *srpkF*-overexpressing cassette was amplified using primer set 5'AapyrGF/3'AapyrGR. Finally, *A. aculeatus* Δ *srpkF* was transformed with the amplified DNA fragments to yield the *srpkF*-overexpressing strain (Supplementary Fig. S3).

Gene expression analysis by quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was used to quantify the expression of cellulase and hemicellulase genes on the basis of previous methods (Tani et al. 2017). In this study, 0.1% Bacto™ Tryptone (Thermo Fisher Scientific, Tokyo, Japan) was used as a neutral carbon source (noninducing condition). The indicated carbon sources were added to media supplemented with 0.1% Bacto™ Tryptone to investigate the expression of test genes. Total RNA (500 ng) was used to amplify cDNA with ReverTra Ace™ qPCR

RT-Master Mix (Toyobo, Tokyo, Japan). qRT-PCR was performed in a Thermal Cycler Dice™ Real-Time System (Takara, Kyoto, Japan). For amplification, a SYBR® Green I assay using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) was performed in a 20 µl reaction. The primers used for qRT-PCR are listed in Supplementary Table S1. Expression of the glyceraldehyde-3-phosphate dehydrogenase A gene (*gpdA*) was used as an internal control. The specificity of the PCR amplification was confirmed by melting curve analysis. The expression profile of each gene was analyzed using a delta–delta CT method. More than three biological replicates were performed for each experiment, and each was tested in triplicate.

Additional methods

Genomic DNA preparation and Southern blot analysis were performed as described previously (Kunitake et al. 2013). An in-house *A. aculeatus* draft genome database was used to obtain the genomic sequence of the *srpkF* gene. Two independently amplified cDNA fragments were analyzed to determine the *srpkF* cDNA sequence. Conidia from the *A. aculeatus* strains were collected with 0.1% Tween® 80/0.8% NaCl solution and counted with a hemocytometer. The number of conidia was normalized by colony area.

Nucleotide sequence data

The nucleotide sequence data from this study have been deposited in Japan's DNA Data Bank (DDBJ) Nucleotide Sequence Data Libraries. The accession number of *srpkF* in *A. aculeatus* is DDBJ Acc. no. LC638744.

Results

*Isolation of a cellulose-responsive induction-deficient mutant from an *A. aculeatus* T-DNA inserted mutant library*

We previously screened an *A. aculeatus* T-DNA inserted mutant library of approximately 12,000 transformants for strains that were 5-FOA-resistant and cellulose-responsive induction-deficient. We isolated five 5-FOA-resistant strains that showed reduced growth on media supplemented with 1% Avicel® but normal growth on media supplemented with 1% glucose, 1% beechwood xylan, or 1% D-xylose (Tani et al. 2017). In the present study, we further analyzed one 5-FOA-resistant strain (Q3) that showed a strong correlation between the function of the disrupted gene (via T-DNA insertion) and the cellulose-responsive induction-deficient phenotype.

We first investigated *cbhl* expression profiles in response to Avicel® in Q3 since the *cbhl* promoter was fused to the *pyrG* gene and was used as bait to screen for factors involved in the cellulose-induced signaling pathway with the 5-FOA-resistant phenotype (Kunitake et al. 2013). Expression of *cbhl* was induced in response to Avicel® at 9 h postinduction in the control strain (NCP2) but significantly reduced in Q3 to approximately 20% of NCP2 expression levels ($p < 0.05$, Student's *t*-test) (Fig. 1). Expression of

xynIb in response to Avicel® was also significantly reduced in Q3 to approximately 30% of NCP2 expression levels ($p < 0.05$, Student's *t*-test) (Fig. 1). These data suggest that the T-DNA insertion in Q3 disrupted a gene required for the induction of the test genes in response to Avicel®.

Serine–arginine protein kinase F participates in the early phase of cellulose induction

The T-DNA integration pattern in Q3 was analyzed by Southern blot because approaches for the recovery of T-DNA flanking sequences by inverse PCR differ depending on the integration pattern. Genomic DNA isolated from Q3 was digested with *EcoRI*, *XbaI* and *SpeI*, *HindIII*, and *PstI*. Single digestion by *EcoRI* (unique site in the T-DNA left flanking region), *HindIII* (absent from the vector), and *PstI* (unique site in the vector backbone) and double digestion with *XbaI* and *SpeI* (unique site in the T-DNA right flanking region and absent from the vector, respectively) all yielded a single band (Fig. 2a). These data demonstrate that one copy of the T-DNA integrated into the Q3 genome. We attempted to amplify the T-DNA flanking sequences via inverse PCR using Q3 genomic DNA digested with *EcoRI* and *XbaI/SpeI* to recover the right and left flanking sequences, respectively (Fig. 2b). We sequenced DNA fragments amplified via inverse PCR, which revealed that the T-DNA integrated into the ORF encoding the putative serine–threonine protein kinase (Fig. 2b). The right and left flanking sequences of the T-DNA were inserted at 1,122 and 1,137 nt from the translation start site of the gene with a 15 bp deletion in the recipient genome. The putative protein kinase gene was composed of 1,443 bp with five exons and encoded a 416-amino acid protein. Based on a homology search in FungiDB (<https://fungidb.org/fungidb/>), the putative protein kinase was most similar to ACLA_003920 in *Aspergillus clavatus* (E-value = 0.0; Identities = 81%). The gene was also similar to AN10082 in *A. nidulans*, *SrpKF* (E-value = 7e-100; Identities = 38%), which is a member of a family of serine–threonine protein kinases that includes an expanded group of seven serine–arginine protein kinases (SRPK) in *A. nidulans* (de Souza et al. 2013). Therefore, this gene is designated as *srpKF* in *A. aculeatus*. The T-DNA insertion at 1,122 nt from the translation start site of *srpKF* resulted in the production of a truncated protein of 327 amino acids of the original protein and seven additional amino acids (SNTDSL N) and a stop codon derived from the T-DNA fragment. These data suggest that *srpKF* is the candidate gene causing the cellulose-responsive induction-deficient phenotype of Q3.

*The *srpKF* gene expressed under the carbon starvation condition*

The expression profiles of *srpKF* were assessed under inducing (1% Avicel® or 1% D-xylose), repressing (1% D-glucose), carbon neutral (0.1% Bacto™ Tryptone), and carbon starvation conditions of the cellulolytic and xylanolytic enzyme genes. Transcripts of *srpKF* were quantified using RNA prepared from the *A. aculeatus* wild-type strain grown under the following conditions: 1% D-glucose medium for 3 h, 1% D-xylose medium for 3 h, 1% Avicel® medium for 9 h, 0.1% Bacto™ Tryptone medium for 3 h, and no carbon source for 3 h. The fold increases in *srpKF* gene expression compared with D-glucose

medium were 2.3 (D-xylose), 44 (Avicel®), 59 (Bacto™ Tryptone), and 98 (no carbon) (Fig. 3). *A. aculeatus* grew poorly on the 1% Avicel® medium, 0.1% Bacto™ Tryptone medium, and under the no carbon condition, whereas it grew well on 1% glucose and 1% D-xylose media, indicating that *A. aculeatus* could not acquire sufficient carbon from 1% Avicel® and 0.1% Bacto™ Tryptone media. These data demonstrate that *srpkF* is expressed under conditions in which the carbon source is limited.

Functional analysis of SrpkF in A. aculeatus

To genetically analyze the function of *srpkF*, the whole *srpkF* gene was deleted by homologous recombination ($\Delta srpkF$, *pyrG*⁺) followed by marker recycling to yield an *A. aculeatus srpkF* deletion mutant ($\Delta srpkF$, *pyrG*⁻). Since the *srpkF* deletion mutant had never been isolated using the DNA fragment corresponding to the 1522 nt region upstream from the translation start site that was used for homologous recombination, the DNA fragment from 1,523 to 2,613 nt upstream was used for targeting. Furthermore, the 3' flanking region of *srpkF* was deleted after marker recycling in $\Delta srpkF$. Thus, the generated *srpkF* mutant deleted *srpkF* with 1.5 kb upstream and 1.3 kb downstream flanking sequences. $\Delta srpkF$ was transformed with *srpkF* DNA fragment yielding a complemented strain (*srpkF*⁺) (Supplementary Fig. S1). To generate a C-terminal deletion mutant of SrpkF composed of 327 amino acids ($\Delta CsrpkF$) in Q3, a stop codon was introduced at 1,129 nt from the translation start site of the ORF followed by marker recycling of *pyrG* to yield $\Delta CsrpkF$ (*SrpkF*₁₋₃₂₇, *pyrG*⁻). $\Delta CsrpkF$ was also complemented by the *srpkF* DNA fragment. We confirmed that gene replacement and complementation occurred as expected by Southern blot analysis (Supplementary Fig. S2). MR12, $\Delta srpkF$, $\Delta CsrpkF$, and *srpkF*⁺ grew equally well on MM supplemented with 1% D-glucose, 1% D-xylose, and 1% Avicel® (data not shown). Since the expression profiles of test genes in the complemented $\Delta CsrpkF$ strain were no different from those in MR12 and *srpkF*⁺, we used *srpkF*⁺ as a control strain in this study (data not shown).

We investigated the effect of *srpkF* deletion on gene expression in response to cellulosic carbon sources. Transcripts of *cbhI* and *xynIb* were quantified at 6 and 9 h postinduction in the MR12, $\Delta CsrpkF$, $\Delta srpkF$, and *srpkF*⁺ strains. The expression of *cbhI* and *xynIb* was reduced in $\Delta CsrpkF$ and $\Delta srpkF$ only under inducing conditions. The fold induction of *cbhI* in response to Avicel® decreased significantly compared with MR12 at 9 h postinduction to 50% in $\Delta srpkF$ and 31% in $\Delta CsrpkF$ ($p < 0.05$, Student's *t*-test). Similarly, the fold induction of *xynIb* significantly decreased to 26% in $\Delta srpkF$ and 32% in $\Delta CsrpkF$ ($p < 0.05$, Student's *t*-test) (Supplementary Fig. S4 and Fig. 4). These data demonstrated that SrpkF is involved in the expression of both genes in response to Avicel®. Since their expression levels decreased similarly in both $\Delta CsrpkF$ and $\Delta srpkF$ and were restored by *srpkF* complementation, we further analyzed the SrpkF function using only $\Delta srpkF$. Expression of *cel7b* significantly decreased to 55% at 9 h postinduction in $\Delta srpkF$ compared with MR12 ($p < 0.05$, Student's *t*-test), which was restored

in *srpkF*⁺ (Fig. 4). The expression of *cmc2*, *cmc1*, and *xyn1a*, decreased to approximately 60%, but this reduction was not statistically significant (Fig. 4).

Overexpression of srpkF

To assess the effect of overexpression of *srpkF* on the expression of genes encoding cellulosic biomass-degrading enzymes in *A. aculeatus*, we constructed an *srpkF*-overexpressing strain that constitutively expresses *srpkF* under the control of the translation elongation factor 1 α gene promoter (*Ptef*), a high-level constitutive promoter in *Aspergillus* (Kunitake et al. 2015). The *srpkF*-overexpressing cassette was introduced into the *pyrG* locus in Δ *srpkF* as a single copy by homologous recombination, as confirmed by Southern blot analysis (Supplementary Fig. S3), yielding the *srpkF*-overexpressing strain (OE*srpkF*). The expression of *srpkF* increased four to seven times in the presence of cellobiose with 1-deoxynojirimycin (DNJ) and approximately nine times in the presence of D-xylose (Supplementary Fig. S5). Since *A. aculeatus* produces β -glucosidase, which effectively hydrolyzes cellobiose to glucose, DNJ was added as a β -glucosidase inhibitor (Tani et al. 2012). The physiological phenotype of the OE*srpkF* strain was no different from MR12, Δ C*srpkF*, and Δ *srpkF* (data not shown).

We assessed the effect of *srpkF* overexpression on the expression of cellulosic biomass-degrading enzyme genes in response to physiological inducers. The expression of *cbhI*, *cmc2*, and *cel7b* is induced in response to cellobiose by the ManR-dependent signaling pathway. Under the inducing condition, the expression of *cbhI*, *cmc2*, and *cel7b* generally increased in OE*srpkF* compared with MR12 (Fig. 5 a and b); however, their expression profiles varied. The expression of *cbhI* was induced at 2, 3, and 4 h postinduction in OE*srpkF* only under the inducing condition, resulted in a significant increase of the fold induction (Fig. 5 a and b). By contrast, the overexpression of *srpkF* did not significantly increase either the expression level or fold induction of *cmc2* (Fig. 5 a and b). The expression of *cel7b* significantly stimulated under both inducing and noninducing conditions such that there was no significant difference in the fold induction, indicating that SprkF stimulated the expression of *cel7b* at a basal level (Fig. 5 a and b).

The expression of *xyn1b* and *cmc1* is induced in response to D-xylose by the XlnR-dependent signaling pathway. The expression of *xyn1b* and *cmc1* was enhanced markedly at 1.5 and 3.0 h postinduction in OE*srpkF* under the D-xylose-inducing condition (Fig. 5c and d). These data confirm that SprkF participates in not only the cellulose-responsive signaling pathway but also in the xylose-responsive signaling pathway.

Discussion

In this study, we demonstrated that deletion of *srpkF* resulted in the reduction of cellulase and xylanase gene expression in response to cellulosic carbon sources. Since the overexpression of *srpkF* promoted the

gene expression in the early induction stages in response to cellobiose and D-xylose, we confirmed that *SrpkF* in *A. aculeatus* is a positive regulator in response to cellulosic and xylanolytic carbon sources.

A comparison of amino acid sequences using the FASTA algorithm revealed that *srpkF* orthologs are highly conserved in the *Aspergillus* section *Nigri*. Orthologs are also present in a few strains from other sections and other genera, such as *A. nidulans* (AN10082, E-value = 2e-91; Identities = 42%), *Penicillium rubens* (Pc12g16110, E-value = 1e-104; Identities = 47%), and *Coccidioides immitis* (CIMG_04484, E-value = 6e-103; Identities = 44%) (Fig. 6). AN10082 in *A. nidulans* encodes a serine-arginine protein kinase F (*SrpkF*) for which a deletion mutant did not show a distinguishing phenotype (de Souza et al. 2013). SRPK was first identified as a cell cycle-regulated kinase specific for SR proteins, which are a family of pre-mRNA splicing factors containing SR domains that consist largely of serine/arginine repeats (Gui et al. 1994b; Gui et al. 1994c). Members of this kinase family are known to phosphorylate serines within the SR domain and are widely conserved in eukaryotes. Although *S. cerevisiae* encodes a single SRPK family member, *Sky1*, *A. nidulans* and *A. aculeatus* encode seven SRPKs from *SrpkA* to *G* (de Souza et al. 2013) and four SRPKs, respectively. *SrpkA* proteins in *Aspergillus* are highly conserved with *S. cerevisiae* *Sky1*, suggesting that they modulate the subcellular localization and function of Ser-Arg rich splicing-factor proteins (Dagher and Fu 2001; Gui et al. 1994a). However, other SRPKs possess various domains, suggesting functional diversification. Few nonsplicing functions of SRPKs have been reported (Gou et al. 2020; Hong et al. 2012; Wang et al. 2017), and it remains unclear whether SRPKs have evolved further regulatory roles (Bustos et al. 2020). The functions of SRPKs might be illuminated by investigating their gene expression profiles since some genes exhibit highly tissue-specific profiles (Nakagawa et al. 2005; Wang et al. 1998).

The expression of *srpkF* increased under carbon-limited conditions, such as the no carbon and Avicel® conditions (Fig. 3), which showed a strong correlation between *SrpkF* function in cellulose-responsive induction and expression profiles. To narrow the possible pathways involving *SrpkF*, we focused on two signaling pathways involved in cellulose-responsive induction in *A. aculeatus*. One is the ManR-dependent signaling pathway that induces expression of *cbhI*, *cmc2*, and *cel7b*. Contrary to our assumptions, the effects of deletion and overexpression of *srpkF* were not consistent among the three genes. Complex regulations were observed in our previous study as well. The overexpression of *clbR*, a putative transcription factor involved in cellobiose-responsive induction in *A. aculeatus*, did not affect the expression of *cbhI* and *cmc2* but reduced the expression of *cel7b*; nevertheless, the deletion of *clbR* reduced their expression levels in response to Avicel® (Kunitake et al. 2015). These data demonstrate that various factors are involved in the ManR-dependent signaling pathway in response to cellulosic carbon sources in *A. aculeatus*.

Interestingly, the overexpression of *srpkF* promoted xylanase gene expression in response to D-xylose, whereas no effect of the *srpkF* deletion was observed on the xylose-responsive expression of the xylanase gene, indicating that the function of *SrpkF* is critically regulated at the transcription level. Note that *xynIb*

and *cmc1* are under the control of XlnR (Tani et al. 2012). XlnR is constitutively expressed in *Aspergillus oryzae* and is phosphorylated in the presence of D-xylose while rapidly dephosphorylated by removing D-xylose from the media (Noguchi et al. 2011). Although it is unknown if ManR is phosphorylated, SrpK is a candidate kinase to phosphorylate factors involved in the ManR- and XlnR-dependent signaling pathways.

Various protein kinases are involved in establishing a complex regulatory network to maintain the precise balance of carbon sources required for growth and hydrolytic enzyme production. *Trichoderma* mitogen-activated protein kinase, Tmk2, is involved in cell wall integrity, sporulation, and cellulase production (Wang et al. 2014). The cyclic AMP-dependent protein kinase A (PKA) affects cellulase gene expression in response to light in *T. reesei* (Schuster et al. 2012). PKA in *A. nidulans* is also involved in the repression of cellulolytic enzyme genes in response to carbon sources (Kunitake et al. 2019). However, the molecular mechanisms of how their kinases control the expression of cellulolytic enzyme genes remain unknown.

A recent study revealed the levels of conservation and diversity in the regulatory mechanisms of cellulolytic enzyme genes in Ascomycete fungi (Kunitake and Kobayashi 2017). XlnR mainly regulates the expression of xylanolytic enzyme genes and is only slightly involved in the expression of cellulolytic enzyme genes in response to cellulose in *Aspergillus oryzae* and *A. aculeatus* (Marui et al. 2002; Tani et al. 2012). By contrast, Xyr1, an XlnR ortholog in *T. reesei*, is a master regulator governing the expression of xylanolytic and cellulolytic enzyme genes in response to various carbon sources such as D-xylose, sophorose, galactose, and lactose (Stricker et al. 2006; Stricker et al. 2007). XLR-1 in *N. crassa* is involved in the induction of xylanolytic enzyme genes but not significantly involved in the expression of cellulolytic enzyme genes (Sun et al. 2012). The expression of cellulolytic enzyme genes is mainly regulated by ManR in *A. oryzae* and *A. aculeatus* (Ogawa et al. 2013; Tsumura et al. 2021) and its orthologs ClrB in *A. nidulans* and CLR-2 in *N. crassa* (Coradetti et al. 2012; Coradetti et al. 2013). Orthologous genes of *srpK* were absent from the genomes of genera of cellulase-producing fungi, such as *Trichoderma* and *Neurospora* species. Since the acquisition of paralogous genes establishes new signal transduction pathways (Baker et al. 2013), the acquisition of *srpK* in *Aspergillus* might lead to a different regulation mechanism of cellulolytic enzyme genes. The logical next step is to identify the target protein of SrpK, which will help to understand the complex regulatory mechanisms of cellulolytic enzyme genes in *Aspergillus*.

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Supplementary Table S1

Name	Sequence (5' to 3')
<i>For srpkF deletion</i>	
2.6k-5'srpK	TACTGCCTTGGTCGTGACAATCG
2.6k-5'srpK	CCGATGACAAAGTACGTAGTCCGCGAAACC
2.6k-MsrpK	CTACGTACTTTGTTCATCGGATTCAGTCAAGC
M-kinaseR	TCTGCGGCAAGCCAGAGCCACTTAACTG
AnpyrG-KF	CTCTGGCTTGCCGCAGACAATGCTCTC
AnpyrG-KR	GTCCTTGCCATATCGTTCAGAGCTGGTCAC
L-kinaseF	AACGATATGGCAAGGACCTAATCTGATCG
L-kinaseR	GAGTATGGTGGAGGCTAGATTG
<i>For srpkF and ΔCsrpK complementation</i>	
2.6k-5'srpK	TACTGCCTTGGTCGTGACAATCG
C2.6k-5'srpK	TTGCGACCAAAGTACGTAGTCCGCGAAACC
C4k-srpK	CTACGTACTTTGGTCGCAAGACTGAAACTG
M-kinase-R	TCTGCGGCAAGCCAGAGCCACTTAACTG
AnpyrG-KF	CTCTGGCTTGCCGCAGACAATGCTCTC
AnpyrG-CR	AGACTGTAAACATATCGTTCAGAGCTGGTCAC
C-kinaseF	GAACGATATGTTTACAGTCTTGGCCTTCCC
C-kinaseR	CAGGACAACGAAAGCATGAG
<i>For ΔCsrpK</i>	
pK-F	GATTTCCCCACACATCAACC
srpKstopMR-R	CCAAGGATCTCAATTAGAGAACGCGGAGGTATC
3'UTRF	CGTTCTCTAATTGAGATCCTTGGTTCCAGGAATAG
3'UTRR	CCGATGACAATTGTGGTGGATTTGCAGA
MRstop-srpK-F	CCACCACAATTGTTCATCGGATTCAGTCAAGC
L-kinaseR	GAGTATGGTGGAGGCTAGATTG
<i>For Ptef plasmid</i>	
AapyrGORFF	AAGAGCTCTTGTATGCGACTGGGCTTTC
AapyrGORFR	GGTACTGTACCGCTTGCAGGATGTTGTTTAG
AaPtefF	CCTGCAAGCGGTACAGTACCTACGTGCTGCAATC
AaPtefR	AATGCGGCCGCGGTGACGGTGTGTGAAGTG
AapyrG3'F	ATACTCGAGCGGATTCAGATCTGGAGG
AapyrG3'R	AAGGTACCCCTGTTCTGGTTGG
<i>For srpkF overexpression</i>	
OE-srpK	TAAGCGGCCGCATGACGGCACTATTGAGCTCG

OE-srpkR	ATTGTGGTGGATTTGCAGACC
5'AapyrGF	AAGGTACCGCGTCGCGAAGGAGTTGT
3'AapyrGR	AAGAGCTCTTGTATGCGACTGGGCTTTC

For qRT-PCR

QgpdA-F	CTCCTCCACTGGTGCTGCT
QgpdA-R	GACTTCTCGAGACGGCAGG
QcbhI-F	ACCATGTGCACAGGCGATAC
QcbhI-R	TGCTGTTGTGACGGTCTTT
Qcel7b-F	ACCAGGGACTCACCAAGATG
Qcel7b-R	GGCGTTGGTGGGGTAG
Qcmc1-F	TGGCTCGCAGAAGACCTACA
Qcmc1-R	ACCAGTTCGAGACGGAGAGC
Qcmc2-F	CCCTGCTGCAGAGCTATGTG
Qcmc2-R	GGCAGTGGAGGTGGAGGTAG
QxynIb-F	GAACTTCGTCGGTGGAAAGG
QxynIb-R	CCAGGGTTGTACTCCCCGTA
QsrpkFF	CCTGAATTAATTCCAGAGAGC
QsrpkFR	TGAGTAAATCTTCCATGCTG

Figure Legends

Fig. 1. Identification of the cellulose-responsive induction-deficient mutant. qRT-PCR analysis of *cbhI* and *xynIb* expression at 9 h postinduction with 1% (w/v) Avicel® was performed for control (NCP2) and Q3 strains. The relative expression corresponds to the ratio of the mean expression levels of *cbhI* divided by those of *gpdA*, the reference gene. The relative expression levels are the means of three independent experiments, and the error bars indicate the standard deviations. Letters indicate significant differences between groups ($p < 0.05$, Student's *t*-test).

Fig. 2. Determination of T-DNA integration pattern and identification of the disrupted gene. Deduced T-DNA integration pattern via Southern blotting analysis (a) and a schematic representation of the *SrpK* locus (b).

Fig. 3. qRT-PCR analysis of the *srpK* expression in wild-type *A. aculeatus* under the following conditions: 1% D-glucose for 3 h (G), 1% D-xylose for 3 h (X), 1% Avicel® for 9 h (A), 0.1% Bacto™ Tryptone for 3h (B), and no carbon source for 3 h (N). The relative expression corresponds to the ratio of the mean expression levels of *srpK* divided by those of *gpdA*. The relative expression levels are the means of three independent experiments, and the error bars indicate the standard deviations. Letters indicate significant differences between groups ($p < 0.05$, one-way ANOVA).

Fig. 4. Effect of *srpK* deletion on expression of cellulase and hemicellulase genes. qRT-PCR results of each gene in MR12 (M, black bars), Δ *srpK* (Δ , white bars), and *srpK*⁺ (+, gray bars) incubated for 6 and 9 h under the noninduced condition (–) and the 1% Avicel®-inducing condition (Avi). The relative expression corresponds to the ratio of the mean expression levels of each gene divided by that of *gpdA*. The relative expression levels are the means of three independent experiments, and the error bars indicate the standard deviations. Letters indicate significant differences between groups ($p < 0.05$, one-way ANOVA).

Fig. 5. Effect of *srpK* overexpression on expression of cellulase and hemicellulase genes. qRT-PCR results of each gene in MR12 (M, black bars) and the *srpK*-overexpressing strain OEs*srpK* (O, striped bars). **a, b:** RNA was prepared from strains grown for 2–4 h in the presence of 0.1% cellobiose with 50 μ g/L DNJ. The expression level of each gene was normalized by that of the *gpdA* (a). The fold induction of each test gene reflects the gene expression level under the inducing condition divided by that under the noninducing condition (b). **c, d:** RNA was prepared from strains grown for 1.5–3.0 h in the presence of 1% (w/v) D-xylose. The expression level of each gene was normalized by that of the *gpdA* (c). **d.** The fold induction of each test gene reflects the gene expression level under the inducing condition divided by that under the noninducing condition (d). For all panels, the results shown are the means of three

independent experiments, and the error bars indicate the standard deviations. An asterisk indicates a significant difference between the expression of test genes in MR12 and in OE*srpkF* ($p < 0.05$, Student's *t*-test).

Fig. 6. Phylogenetic analysis of serine–arginine protein kinase-like genes. The phylogenetic relationship of *srpk* orthologs is shown as a consensus neighbor-joining tree based on sequences orthologous to *srpkF* in *A. aculeatus*. The alignment was performed using ClustalW. The individual nodes of the resulting trees were examined with 1000 bootstrap replicates; only values below 1000 are shown.

Supplementary Fig. S1. Construction of the Δ *srpkF* and *srpkF*⁺ strains. The upper portion of the figure shows restriction enzyme maps of the *srpkF* loci in the MR12, Δ *srpkF*, and *srpkF*⁺ strains. The underlined DNA regions were used as DNA probes for Southern blot analysis to detect DNA fragments digested with *Bam*HI (lower portion of figure).

Supplementary Fig. S2. Construction of the Δ *CsrpkF* and complement strains (*CsrpkF*⁺). The upper portion of the figure shows restriction enzyme maps of the *srpkF* loci in the MR12, Δ *CsrpkF*, and complement strains. An asterisk indicates the position of a stop codon. The underlined DNA regions were used as DNA probes for Southern blot analysis to detect DNA fragments digested with *Dra*I (lower portion of figure).

Supplementary Fig. S3. Construction of the OE*srpkF* strain. The upper portion of the figure shows restriction enzyme maps of the *srpkF* loci in the MR12 and OE*srpkF* strains. The underlined DNA regions were used as DNA probes for Southern blot analysis to detect DNA fragments digested with *Pst*II (lower portion of figure).

Supplementary Fig. S4. Effect of *srpkF* deletion on expression of *cbhl* and *xynIb*. qRT-PCR results of each gene in MR12 (M), Δ *srpkF* (Δ), *srpkF*⁺ (+), Δ *CsrpkF* (Δ C), and the complement strain of Δ *CsrpkF* (C+) incubated for 9 h under the noninduced condition and the 1% Avicel®-inducing condition. The expression level of each gene was normalized by that of the *gpdA*. The fold induction of each test gene (the gene expression level under the inducing condition divided by that under the noninducing condition) is the mean of three independent experiments, and the error bars indicate the standard deviations. Letters indicate significant differences between groups ($p < 0.05$, one-way ANOVA).

Supplementary Fig. S5. Expression of *srpkF* in the overexpression strain. qRT-PCR analysis of *srpkF* in MR12 (M) and the *srpkF*-overexpressing strain (OE*srpkF*, OE). RNA was prepared from strains grown from 24 h in the presence of 0.1% cellobiose with 50 μ g/L DNJ (a) or 1% D-xylose (b). The expression

level of each gene was normalized by that of the *gpdA*. The relative expression corresponds to the ratio of the mean expression levels of *srpkF* divided by that of *gpdA*. The relative expression levels are the means of three independent experiments, and the error bars indicate the standard deviations. M: MR12, filled bars; OE: OE*srpkF*, striped bars.

Fig. 1.

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Fig. 2.

a

b

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Fig. 3.

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Fig. 4.

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Fig. 5.

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Fig. 6.

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Supplementary Fig. S1.

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Supplementary Fig. S2.

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Supplementary Fig. S3.

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Supplementary Fig. S4.

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Supplementary Fig. S5.

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