J. Biol. Macromol., **14**(3), 125- 132 (2014)

Regular article

Characterization of the *palF* gene from *Aspergillus oryzae*

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Received August 29, 2014; Accepted September 18, 2014

Here, we analyzed the role of PalF in the ambient pH signal transduction pathway of *Aspergillus oryzae* by disrupting the *palF* gene. *palF* disruption resulted in significantly decreased *pacC* expression. Based on this result, we concluded that PalF played an important role in the ambient pH signal transduction pathway of *A. oryzae*.

Keywords: *Aspergillus oryzae*, *palF*, *pacC*, alkaline protease

Introduction

Alkaline protease (Alp) is one of most important enzymes required for the production of soy sauce and is used to hydrolyze the raw materials—an important step that determines the flavor of soy sauce (1).

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Alp is a serine protease with an alkaline pH optimum. Under alkaline conditions, a signal transferred via the *pal* ambient pH signal transduction pathway (comprising *palA, palB, palC, palF, palH,* and *palI* genes) induces the activation of the transcription factor PacC (2). The zinc finger transcription factor PacC is synthesized as a 674-residue precursor. At alkaline

ambient pH, conformational changes lead to an open conformation in which PacC is accessible to a processing protease, and this protease then removes 400 residues from the C terminus, including a negative-acting domain. The resulting product is fully competent in transcriptional regulation through 5`-GCCARG-3′ sites in the promoters of both alkaline (activated by PacC) and acidic (repressed by PacC) genes (3). This activation of PacC is assumed to be related to the activation of the *alp* gene.

The alkaline pH-sensing module in the plasma membrane is composed of a 7-transmembrane-domain receptor (PalH), a 3-transmembrane-domain protein (PalI), and an arrestin-related protein (PalF) (4). PalH is an ambient pH sensor, and PalI promotes the plasma membrane localization of PalH. Arrestin-like PalF, which contains arrestin N-terminal and C-terminal domains, interacts strongly with PalH. PalF is ubiquitylated in a receptor (PalH)- and signal (alkaline pH)-dependent manner, and ubiquitylation of arrestin-like PalF leads to constitutive signaling (5). However, the direct relationship between PalF and PacC in *A. oryzae* remains unclear.

In a previous study, we analyzed the functions of PalI (6) and PalH (7) in *A. oryzae*. Therefore, in this study, we reported the effects of *palF* disruption on *pacC* and *alp* expression in *A. oryzae*.

Materials and Methods

Strains, media, and culture conditions

A. oryzae ΔLigD, Δ*niaD*, and *ΔpryG* were used as hosts for transformation. The control strain was transformed using only the *pyrG* gene. The *palF* disruption strain and control strains were grown on yeast-peptone-dextrose (YPD) medium at 30°C for 24 h. DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Construction of transformant strains

The *palF* disruption strain was created using previously described procedures (8). The *palF* gene disruption cassette was generated by fusion

polymerase chain reaction (PCR) using an Expand High Fidelity PCR System (Roche, Diagnostics, Mannheim, Germany). The 5′- and 3′-arms of the *palF* gene were amplified from genomic DNA with the primers LU/LL (5′-arm) and RU/RL (3′-arm). The *pyrG* gene was amplified with the primer pair PU/PL. Amplified fragments were purified using a Wizard Gel Extraction kit (Promega). The PCR products were used for a second round of PCR with the primer pair LU/RL to fuse the 5′ and 3′ regions of the target gene at each end of the *pyrG* gene. The primer sequences (5′→3′) were as follows: ATCCGACTCGCCGTTTGTACAT (LU), CACAGGGTACGTCTGTTGTCAGTG GATACGCACACTGATGCTAC (LL), GTGCGTATCCACTGACAACAGACG TACCCTGTGATGTTC (PU), GTTGCTCATCCGACTAACTGCACCT CAGAAGAAAAGGATG (PL), TTCTTCTGAGGTGCAGTTAGTCGG ATGAGCAACAGCAGTCAAA (RU), and ACGCTTGTGGCACTGTATTCTC (RL). The amplified fragment was purified using a Wizard Gel Extraction kit and was subsequently used for transformation.

Fungal transformation was carried out essentially as previously described (9).

Expression analysis by real-time PCR

To analyze the expression of *pacC* and *alp* using real-time PCR, we isolated total RNA and mRNA from the control and disrupted strains. These strains were precultured at 30°C for 24 h in minimum medium (MM; 2% glucose, 1.2% $NaNO_3$, 0.3% KH_2PO_4, 0.1% KCl, and 0.1% $MgSO_4$) containing 0.025% yeast extract. The main culture was then established for 3 h at 30°C in MM containing 0.2% casamino acids, buffered to pH 4.0 using 0.1 M citrate-phosphate (pH 3.8) and to pH 8.0 using 0.1 M Tris-HCl (pH 9.5). Total RNA was extracted using RNAiso Plus (TaKaRa, Shiga, Japan), according to the manufacturer's instructions. mRNA was purified using an *Oligotex-dT30<super>* mRNA Purification Kit (TaKaRa), following the manufacturer's protocol. Reverse transcription and real-time PCR

were performed as described previously (10) using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA, USA). The sequences (5′→3′) of the *pacC* primers were GACTGCGGCTGCTCCTATTG (forward primer) and TGTCGTGGTAGCAGAGACTTGAG (reverse primer). The *alp* primers were synthesized according to previously described sequences (7). Histone H1 was used as an endogenous control.

Results and Discussion

Gene disruption and phenotype analysis

First, we identified the *palF* gene from *A. oryzae* (XM_003189575). *A. oryzae palF* was homologous to *palF* from other organisms. For example, *A. oryzae palF* showed 71% homology to *palF* from *A. nidulans*, 75% homology to *palF* from *A. fumigatus*, and 99% homology to *palF* from *A. flavus*. Therefore, these data demonstrated that the *palF* gene was highly conserved in *Aspergillus.*

To analyze PalF function in *A. oryzae*, a *palF* disruption strain of *A. oryzae* was constructed. The transformants constructed by homologous recombination with the *palF*-disruption gene cassette were confirmed by PCR (Fig. 1).

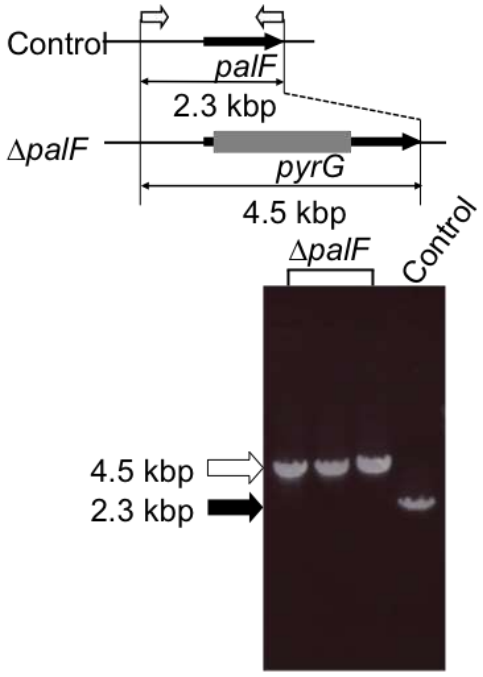

Fig. 1. Confirmation of *palF* disruption by PCR

Physical maps of the *palF* locus and the gene disruption cassette. The primers used for PCR amplification are indicated. The white arrow indicates amplified products from disruption constructs, and the black arrow indicates amplified products from the *palF* gene.

The band sizes of *palF* of the control strain and the *palF* disruption strain were 2.3 and 4.5 kbp, respectively. These results showed successful homologous recombination at the resident *palF* locus.

The *palF* disruption strain showed reduced growth on alkaline pH plates (Fig. 2), but normal growth on acid pH plates. Under alkaline pH conditions, growth of the *A. oryzae palH*-disrupted strain was completely inhibited (7), whereas the *palI*-disrupted strain showed moderate growth (6).

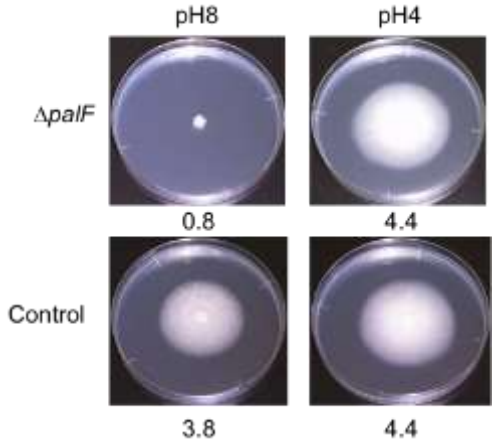

Fig. 2. Growth phenotype of the *palF* disruption strain

The control and *palF* disruption strains were grown at 30°C for 5 days on MM+0.6% casamino acid agar. The values are colony sizes (cm).

These data suggested that PalF played an important role in the pH signal transduction pathway of *A. oryzae*, but was not essential for this process.

Analysis of pacC gene expression

Figure 3 shows the relative expression levels of *pacC* in the control and disrupted strains (n = 3).

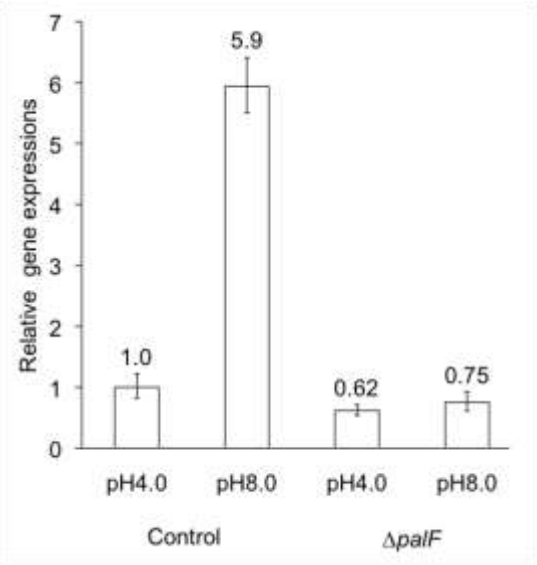

Fig. 3. Comparison of *pacC* gene expression by real-time PCR

Gene expression in the control strain at pH 4.0 was set at 1.0. The error bars indicate standard deviations.

At pH 4.0, the expression level of *pacC* differed slightly between the control and *palF* disruption strains.

However, at pH 8.0, the expression level of *pacC* in the *palF* disruption strain was about 7.9-fold less than that of the control strain. The transcription factor PacC was strongly expressed in the control strains under alkaline conditions. In *A. nidulans*, *pacC* is expressed under alkaline conditions and is subjected to autogenous transcriptional activation (11). *pacC* transcript levels are low in the absence of pal signal transduction. Therefore, the pal signal transduction pathway could not function in the *palF* disruption strain, and the expression level of *pacC* decreased. This finding suggested that PalF was important for the expression of *pacC* under alkaline conditions.

Analysis of alp gene expression

Next, we investigated the influence of *palF* disruption on *alp* gene expression at pH 8.0. The relative expression levels of *alp* in the control strain were approximately 2.6-fold higher than those in the *palF* disruption strain (Fig. 4). Expression of the *alp* gene decreased in the *palF* disruption strain because the pal signal transduction pathway did not function in this strain.

The Alp activity in the *palF* disruption strain decreased to 8.7% that of the control (data not shown).

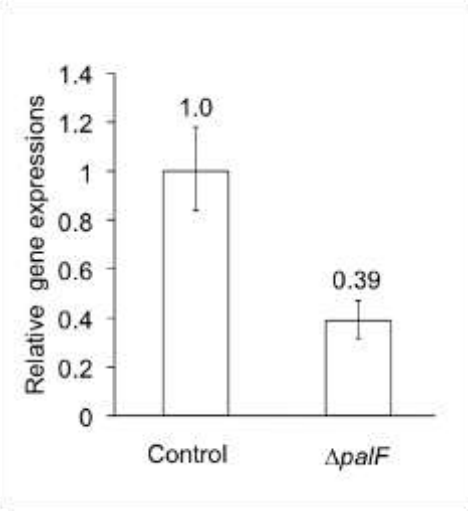

Fig. 4. Comparison of *alp* gene expression in the various *A. oryzae* strains by real-time PCR

Gene expression in the control strain was set at 1.0. The error bars indicate standard deviations.

Thus, our data demonstrated that *palF* disruption had significant effects on *alp* gene expression. We concluded that pH signal transduction was interrupted by the disruption of *palF*. On the other hand, Alp activity in the *palH*-disrupted strain decreased to 6.3% that of the control (7).

These results indicated that PalH was more important than PalF in the pH signal transduction pathway.

In conclusion, we analyzed the role of PalF in the ambient pH signal transduction pathway in *A. oryzae*. We found that *palF* disruption affected PacC expression. PalF had an important role in the ambient pH signal transduction pathway of *A. oryzae*. The *palF* disruption strain exhibited decreased expression of *pacC* and reduced growth on alkaline pH plates. Therefore, PalF did not play an essential role in the pH signal transduction pathway. We intend to analyze this ambient pH signal transduction pathway in future studies.

Acknowledgement

This work was supported by MEXT (Ministry of Education, Culture, Sports, Science and Technology) -Supported Program for the Strategic Research Foundation at Private Universities, 2009-2013. We thank Harue Kitagawa for technical assistance.

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Communicated by Ishijima Sumio