

# Transcripts of a heterologous gene encoding mite allergen Der f 7 are stabilized by codon optimization in *Aspergillus oryzae*

Mizuki Tanaka · Masafumi Tokuoka ·  
Takahiro Shintani · Katsuya Gomi

Received: 14 February 2012 / Revised: 8 May 2012 / Accepted: 10 May 2012  
© Springer-Verlag 2012

**Abstract** We have previously demonstrated that transcripts of an AT-biased heterologous gene encoding mite allergen Der f 7 from *Dermatophagoides farinae* were polyadenylated prematurely within the coding region when native cDNA was expressed in *Aspergillus oryzae*, and that this premature polyadenylation was prevented by the codon optimization of the *Der f 7* gene, resulting in increased steady-state mRNA levels. In this study, we tested the stability of transcription products derived from expression constructs of the native and codon-optimized *Der f 7* gene in *A. oryzae* using 1,10-phenanthroline as a transcription inhibitor. Transcription products of native *Der f 7* cDNA fused to the *A. oryzae* glucoamylase gene (*glaA*) were rapidly degraded; the half-life of the mRNA was approximately 13 min. However, the half-life of codon-optimized *Der f 7* mRNA fused to *glaA* was approximately 43 min, which was highly similar to that of endogenous *glaA* mRNA. These results indicate that *Der f 7* mRNA is significantly stabilized by codon optimization. In addition, *Der f 7*

mRNA was stabilized by the codon optimization of only the 3'-half region, where premature polyadenylation sites were exclusively situated; the half-life of the chimeric *Der f 7* mRNA was approximately 39 min. This suggested that destabilization of native *Der f 7* mRNA is mainly triggered by premature polyadenylation within the coding region. To the best of our knowledge, this is the first report to provide experimental evidence that heterologous mRNA is significantly stabilized by codon optimization in eukaryotic cells.

**Keywords** mRNA stability · *Aspergillus oryzae* · Codon optimization · Heterologous gene expression · 1,10-Phenanthroline · Nonstop mRNA decay

## Introduction

The filamentous fungus *Aspergillus oryzae* has been used in Japan for the production of traditional fermented foods such as sake, soy sauce, and miso (soybean paste) for over a thousand years (Machida et al. 2008). *A. oryzae* can also secrete large amounts of proteins, and it has recently become attractive as a host organism for recombinant protein production (Christensen et al. 1988; Fleissner and Dersch 2010; Lubertozzi and Keasling 2009; Tsuchiya et al. 1992, 1993). However, the secretory yields of heterologous proteins are generally low compared with those of homologous proteins or proteins from closely related fungal species (Gouka et al. 1996, 1997a). In a previous study, we reported that transcripts of a heterologous gene encoding house dust mite allergen (Der f 7) derived from *Dermatophagoides farinae* were prematurely polyadenylated within the coding region when native cDNA was expressed in *A. oryzae*, probably

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-012-4169-y) contains supplementary material, which is available to authorized users.

M. Tanaka · M. Tokuoka · T. Shintani · K. Gomi (✉)  
Laboratory of Bioindustrial Genomics,  
Department of Bioindustrial Informatics and Genomics,  
Graduate School of Agricultural Science, Tohoku University,  
1-1 Tsutsumidori-Amamiyamachi, Aoba-ku,  
Sendai 981-8555, Japan  
e-mail: gomi@biochem.tohoku.ac.jp

### Present Address:

M. Tokuoka  
National Research Institute of Brewing,  
3-7-1 Kagamiyama,  
Higashi-Hiroshima, Hiroshima 739-0046, Japan

because of the presence of AT-biased codons (Tokuoka et al. 2008). Alteration of AT-biased codons of *Der f 7* cDNA that better suit *Aspergillus* codon usage might eliminate the *A. oryzae* 3'-end processing signal, which constituted of AU-rich sequence elements (Tanaka et al. 2011), thereby resulting in an increase in the steady-state *Der f 7* mRNA level in *A. oryzae* (Tokuoka et al. 2008). In addition, insertion of a termination codon upstream of the polyadenylation sites elevated the steady-state mRNA level (Tokuoka et al. 2008). These results suggest that prematurely polyadenylated mRNA lacking the translational termination codon is rapidly degraded by the non-stop mRNA degradation pathway, which is an mRNA quality control mechanism (Frischmeyer et al. 2002; van Hoof et al. 2002), and that codon optimization prevents premature polyadenylation of the heterologous gene. However, there are no experimental data on the stability of heterologous gene transcripts from filamentous fungi.

The measurement of mRNA half-lives after the inhibition of total mRNA synthesis is a most popular method to examine the stability of target transcripts, and various chemical compounds have been used as transcription inhibitors (Passos and Parker 2008). Transcription inhibitors used with filamentous fungi include 15–32 µg/ml of proflavin with *Aspergillus nidulans* (Platt et al. 1996; Caddick et al. 2006), 12 µg/ml of thiolutin with *Neurospora crassa* (Guo et al. 2009), and 500 µg/ml of 1,10-phenanthroline with *Trichoderma reesei* (Pakula et al. 2003).

In this study, we established an experimental method for monitoring mRNA stability in *A. oryzae* using 1,10-phenanthroline as a transcription inhibitor. We also examined whether the increase in the steady-state *Der f 7* mRNA levels resulted from an elevated mRNA stability by codon optimization of the target heterologous gene expressed in *A. oryzae*.

## Materials and methods

### Strains and media

*A. oryzae* NS4 (*niaD*, *sC*), which is derived from the wild-type strain RIB40 (Yamada et al. 1997), was used as the recipient strain for transformation. *Escherichia coli* DH5α was used for the construction and propagation of plasmid DNAs. The minimal medium for *A. oryzae* cultivation was Czapek–Dox (CD) medium, which contained 0.6 % NaNO<sub>3</sub>, 0.05 % KCl, 0.2 % KH<sub>2</sub>PO<sub>4</sub>, 0.05 % MgSO<sub>4</sub>, and trace amounts of FeSO<sub>4</sub>, ZnSO<sub>4</sub>, CuSO<sub>4</sub>, MnSO<sub>4</sub>, Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, and (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, and 1 % sugar, supplemented with 0.0003 % (0.02 mM) methionine. For the cultivation of

strain NS4, 0.6 % NaNO<sub>3</sub> was replaced with 0.9 % (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> as the nitrogen source.

### Construction of plasmids for 5'- or 3'-half codon-optimized *Der f 7* expression

DNA manipulation and propagation were performed according to standard DNA techniques (Sambrook et al. 1989). To generate the 5'- or 3'-half codon-optimized *Der f 7* constructs, native and codon-optimized *Der f 7* cDNA fragments were obtained from the expression plasmids pDer/ntv and pDer/opt (Tokuoka et al. 2008) by digestion with *Sal*I and *Xba*I, and inserted into the plasmid pBluescriptSK(+). The 3'-half fragment of native *Der f 7* cDNA was then replaced with the corresponding fragment of codon-optimized *Der f 7* cDNA at the *Eco*RV site in the middle of *Der f 7* cDNA, to yield the 3'-half codon-optimized *Der f 7* cDNA. Next, the chimeric *Der f 7* cDNA fragment prepared by PCR using the primers 5'-CCCCACGTGAAAAGAGATCCAATTCACATGATAA-3' (*Pma*CI site underlined) and 5'-CCCCATATGTTAATTCCTTTCCAGCTCAC-3' (*Nde*I site underlined) was digested by *Pma*CI and *Nde*I, and inserted into pNGL (Tokuoka et al. 2008), which was constructed by insertion of the glucoamylase A (*GlaA*) catalytic domain region into pNGA142 (Tamalampudi et al. 2007) harboring the improved *glaA* promoter, to yield pGlaDer/ntv-opt. Similarly, the 5'-half fragment of native *Der f 7* cDNA was replaced with the corresponding fragment of codon-optimized *Der f 7* cDNA, to yield pGlaDer/opt-ntv. The hexanucleotide encompassing the KexB cleavage site (Lys–Arg) was located between the *GlaA* catalytic domain region and *Der f 7* cDNA (Mizutani et al. 2009).

### Fungal transformation

Transformation of *A. oryzae* was performed using the method of Gomi et al. (1987).

### Southern blot analysis

Mycelia grown in YPD liquid medium were ground to fine powder in liquid nitrogen with a mortar and pestle. The powdered mycelium was suspended in DNA extraction buffer [50 mM Tris–HCl (pH 7.0), 10 mM EDTA, 0.05 M NaCl, and 1 % SDS] and incubated at room temperature for 15 min, followed by centrifugation at 20,400×g for 10 min at 4 °C. The supernatant was purified using phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1) extractions, followed by ethanol precipitation. The resultant DNA precipitate was dissolved in TE

buffer containing RNase A. The genomic DNA digested with *Pst*I was electrophoresed on a 0.8 % agarose gel and transferred onto a Biodyne® B membrane (Pall Corporation, Pensacola, FL) by alkaline transfer, using a vacuum blotter (Model 785; Bio-Rad, Hercules, CA). Probe labeling, hybridization, and detection were performed using the ECL Direct™ Nucleic Acid labeling and detection system (GE Healthcare Bio-Science, Piscataway, NJ), according to the manufacturer's instructions.

#### Northern blot analysis

Total RNA was extracted from mycelia using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. Twenty micrograms of total RNA was electrophoresed on a 1.0 % formaldehyde-agarose gel and transferred onto a Hybond™-N<sup>+</sup> membrane (GE Healthcare) using the capillary transfer method using 3 M NaCl and 0.3 M sodium citrate (SSC) as the transfer buffer. A digoxigenin (DIG)-labeled DNA fragment was used as a probe. Probe labeling, hybridization, and detection were performed according to the DIG luminescence kit protocol (Roche, Indianapolis, IN).

#### Transcript stability analysis

*A. oryzae* strains were grown in liquid minimal medium containing 1 % fructose as a sole carbon source at 30 °C with shaking for 36 h. The mycelia were harvested by filtration through Miracloth (Calbiochem, La Jolla, CA), washed with carbon source-free minimal medium, and transferred to 100 ml of minimal medium containing 1 % maltose as a sole carbon source. After shaking for 1 h at 30 °C to induce *glaA* or *Der f 7* gene expression, 1,10-phenanthroline was added at a final concentration of 500 µg/ml, followed by further shaking for 20 min at 30 °C. The mycelia were harvested at equal intervals, washed with distilled water, and frozen in liquid nitrogen. Finally, the mycelia were subjected to total RNA extraction and Northern blot analysis.

#### 3'-RACE analysis

3'-Rapid amplification of cDNA ends (3'-RACE) analysis was performed as described previously (Tokuoka et al. 2008).

#### SDS-PAGE and Western blot analysis

SDS-PAGE and Western blot analysis using anti-*Der f 7* polyclonal antibody were performed as described previously (Tokuoka et al. 2008). Detection employed a Chemi-Lumi

One L kit (Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions.

## Results

### Inhibition of total mRNA synthesis in *A. oryzae* with chemical compounds

To investigate the stability of transcripts derived from *Der f 7* cDNA, we attempted to identify chemical compounds that acted as a suitable transcription inhibitor in *A. oryzae*. There are no experimental data available on transcription inhibition in *A. oryzae*, hence we first examined the susceptibility of *A. oryzae* to chemical compounds used as transcription inhibitors in other filamentous fungi (Platt et al. 1996; Pakula et al. 2003; Caddick et al. 2006; Guo et al. 2009). The growth of *A. oryzae* was strongly inhibited by 1,10-phenanthroline supplemented at a concentration of 500 µg/ml, whereas *A. oryzae* growth was unaffected by proflavin or thiolutin at the test concentrations (data not shown). To determine whether 1,10-phenanthroline had acceptable performance as a transcription inhibitor, we examined the mRNA abundance of the intrinsic gene encoding glucoamylase A (*glaA*) in conditions with or without 1,10-phenanthroline after induction of *glaA* expression by maltose. The level of *glaA* mRNA decreased at a constant rate under conditions with 1,10-phenanthroline, whereas a high abundance of *glaA* mRNA was detected under conditions without 1,10-phenanthroline (Fig. 1a). This result indicated that transcription of the *glaA* gene was inhibited by the addition of 1,10-phenanthroline, and consequently, the level of mRNA was decreased by mRNA degradation. Thus, we concluded that 1,10-phenanthroline could be used as a transcription inhibitor to measure the stability of target transcripts in *A. oryzae*. The half-life of *glaA* mRNA was estimated by plotting the signal intensities as approximately 43 min (Fig. 1b).

### Comparison of native and codon-optimized *Der f 7* mRNA stabilities

In our previous study, we showed that transcripts of native *Der f 7* cDNA were polyadenylated prematurely within the coding region, and this premature polyadenylation was prevented by the codon optimization of *Der f 7* cDNA (Tokuoka et al. 2008). *Der f 7* mRNA containing a premature poly(A) tract lacks a translational termination codon, hence the prematurely polyadenylated transcription products of native *Der f 7* cDNA are presumed to be rapidly degraded by the nonstop mRNA degradation pathway. To

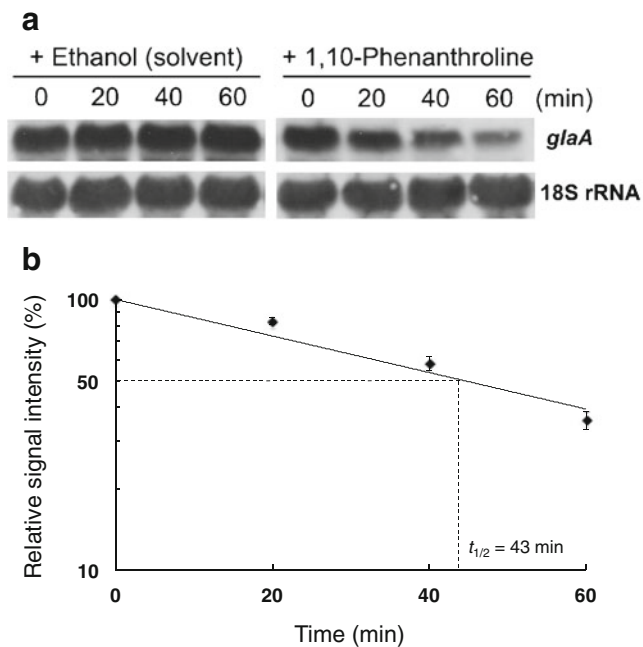


**Fig. 1** Inhibition of *glaA* mRNA synthesis by the addition of 1,10-phenanthroline. **a** Northern blot analysis of glucoamylase A (*glaA*) mRNA under conditions with or without 1,10-phenanthroline. NS4 strain was grown on minimal medium containing 1 % fructose, followed by transfer to minimal medium containing 1 % maltose. After incubation for 1 h to induce *glaA* expression, 500  $\mu\text{g/ml}$  of 1,10-phenanthroline or an equal volume of ethanol (solvent used for dissolving 1,10-phenanthroline) was added to the medium, and mycelia were harvested at 20-min intervals. Total RNA was extracted from the harvested mycelia, and 20  $\mu\text{g}$  of the total RNA was subjected to Northern blot analysis. A digoxigenin (DIG)-labeled *glaA* fragment was used as a probe. The loading control used was 18 S rRNA. **b** Determination of the half-life of *glaA* mRNA in cells treated with 1,10-phenanthroline. Signal intensities for *glaA* mRNA were determined by densitometric scanning using the Image J program. The percentage of the initial level of the transcripts at 0 min was plotted for each time point. Error bars indicate the standard errors from three independent experiments. The half-life ( $t_{1/2}$ ) of the transcripts was estimated as the time required for 50 % of the mRNA to decay, as shown by dotted lines

compare the stability of transcription products derived from native and codon-optimized *Der f 7* cDNAs, we measured the half-lives of each *Der f 7* mRNA. The transcription products of nonfused native *Der f 7* cDNA were difficult to detect clearly by Northern blot analysis because of its low transcription level; hence, a *glaA*-fused construct was used for this purpose. Northern blot analysis of *Der f 7* transcripts derived from *glaA*-fused native *Der f 7* (GlaDer/ntv) cDNA after inhibition of transcription showed that GlaDer/ntv cDNA transcripts were markedly unstable (Fig. 2a) and the half-life of GlaDer/ntv mRNA was estimated as approximately 13 min (Fig. 2c). By contrast, the transcripts of codon-optimized *Der f 7* cDNA fused to *glaA* (GlaDer/opt) were remarkably stable compared with GlaDer/ntv mRNA (Fig. 2b). The half-life of GlaDer/opt mRNA was estimated as approximately 43 min (Fig. 2d), which was

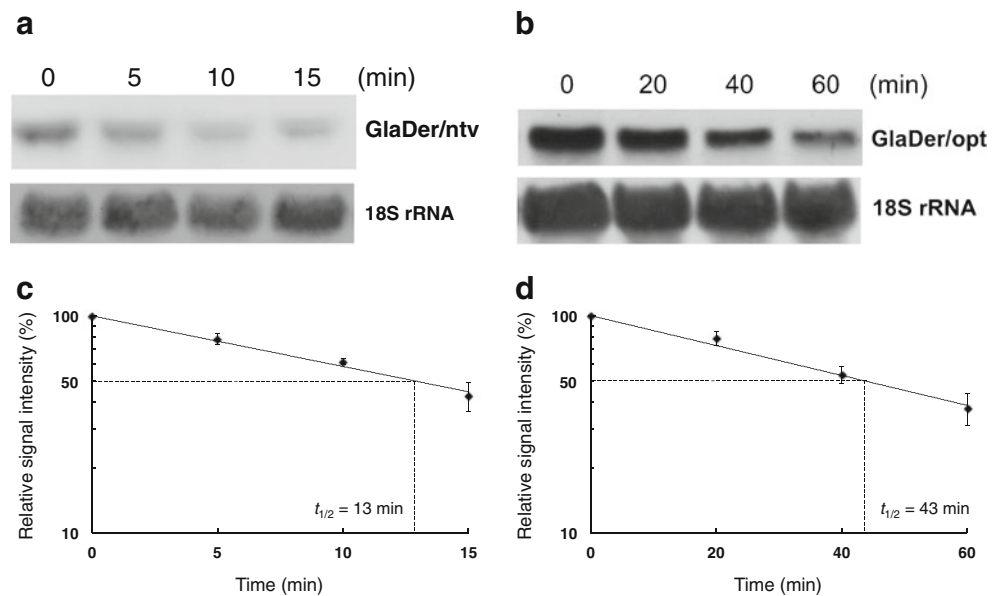
almost equal to that of the endogenous *glaA* mRNA. These results clearly suggested that *Der f 7* mRNA was stabilized by codon optimization. It has been proposed that nonstop mRNA degradation is triggered in budding yeast by stalling of the ribosome on nonstop mRNA (van Hoof et al. 2002; Inada and Aiba 2005; Ito-Harashima et al. 2007). The degradation of mRNA in the cytoplasm is coupled to translation in most cases. Hence, the inhibition of translation is known to stabilize many different mRNAs (Atwar et al. 1990). Thus, we examined the effect of cycloheximide treatment, which blocks translation elongation, on the degradation of GlaDer/ntv mRNA. As shown in Fig. 3, GlaDer/ntv mRNA was significantly stabilized by cycloheximide treatment, suggesting that GlaDer/ntv mRNA was degraded by the nonstop mRNA degradation pathway coupled to translation, as described previously (van Hoof et al. 2002; Inada and Aiba 2005).

In addition, we had previously reported that the steady-state expression levels of native and codon-optimized *Der f 7* mRNAs were significantly increased by fusion to *glaA* (Tokuoka et al. 2008). Thus, we hypothesized that *Der f 7* transcripts would be stabilized by *glaA* fusion. To test this hypothesis, we monitored the degradation rate of codon-optimized *Der f 7* mRNA which was not fused to *glaA* (Der/opt). As shown in Fig. 4, the half-life of Der/opt mRNA was approximately 39 min (Fig. 4b), which was similar to that of GlaDer/opt mRNA (43 min). This result suggested that fusion of *glaA* to *Der f 7* cDNA had no apparent effect on *Der f 7* mRNA stability.

#### Codon optimization of the 3'-half region of *Der f 7* cDNA containing premature polyadenylation sites

Stabilization of *Der f 7* mRNA by codon optimization suggested that prevention of premature polyadenylation would stabilize *Der f 7* mRNA efficiently. However, it was possible that *A. oryzae* possessed rare codons in its native *Der f 7* cDNA that affected mRNA stability independently of premature polyadenylation. Interestingly, premature polyadenylation sites in the coding region of native *Der f 7* cDNA were localized exclusively in its 3'-half region, whereas both 5'- and 3'-half regions contained AT-biased codons. This suggested that the 3'-half region of native *Der f 7* cDNA was involved in premature polyadenylation. To investigate the effect of the 3'-half region of native *Der f 7* cDNA on mRNA stability, we generated chimeric *Der f 7* cDNA where the 3'-half region was codon optimized, i.e., GlaDer/ntv-opt (Fig. 5a). The expression plasmid for GlaDer/ntv-opt was introduced into the NS4 strain and a transformant containing a single copy of the expression plasmid at the *niaD* locus was selected by Southern blot analysis (data not shown). To investigate the effects of the codon optimization of the 3'-half region on the *Der f 7*

**Fig. 2** Stability of native and codon-optimized *Der f* 7 mRNAs. Northern blot analysis of *glaA*-fused native *Der f* 7 (a) and codon-optimized *Der f* 7 (b) mRNAs after the inhibition of transcription. Mycelia were harvested at the time points indicated, as described in Fig. 1. DIG-labeled fragments of 5'-half native *Der f* 7 cDNA and full-length codon-optimized *Der f* 7 cDNA were used as probes in a and b, respectively. The half-lives of *glaA*-fused native *Der f* 7 (c) and codon-optimized *Der f* 7 (d) mRNAs were determined, as described in Fig. 1



mRNA level, transformants expressing GlaDer/ntv or GlaDer/ntv-opt were cultured in YPM medium, and total RNA was extracted from each mycelium and subjected to Northern blot analysis. The steady-state mRNA level of GlaDer/ntv-opt was significantly increased compared with that of GlaDer/ntv (Fig. 5b). We did not detect any *Der f* 7 cDNA clone that was prematurely polyadenylated within the coding region in 3'-RACE analysis using the transcription products of GlaDer/ntv-opt (data not shown). These results suggested that the codon optimization of the 3'-half region of native *Der f* 7 cDNA is sufficient to eliminate the *A. oryzae* 3'-end processing signal from *Der f* 7 cDNA, resulting in an increase in the steady-state mRNA levels. Furthermore, SDS-PAGE analysis of the culture supernatants showed that the codon optimization of the full-length or 3'-half regions of *Der f* 7 cDNA had a similar effect on improving the *Der f* 7 protein production level (Fig. 6). Finally, we monitored the stability of GlaDer/ntv-opt mRNA (Fig. 5c) and estimated the half-life of GlaDer/ntv-opt mRNA as approximately 39 min (Fig. 5d). This half-life

was similar to that of GlaDer/opt mRNA. By contrast, the codon optimization of the 5'-half region of *Der f* 7 cDNA had no effect on the mRNA stability or protein production level (data not shown).

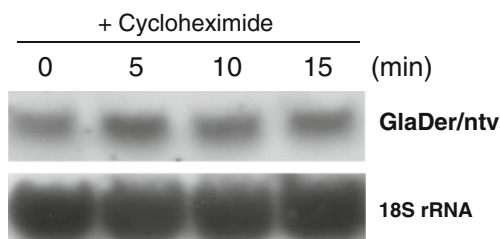
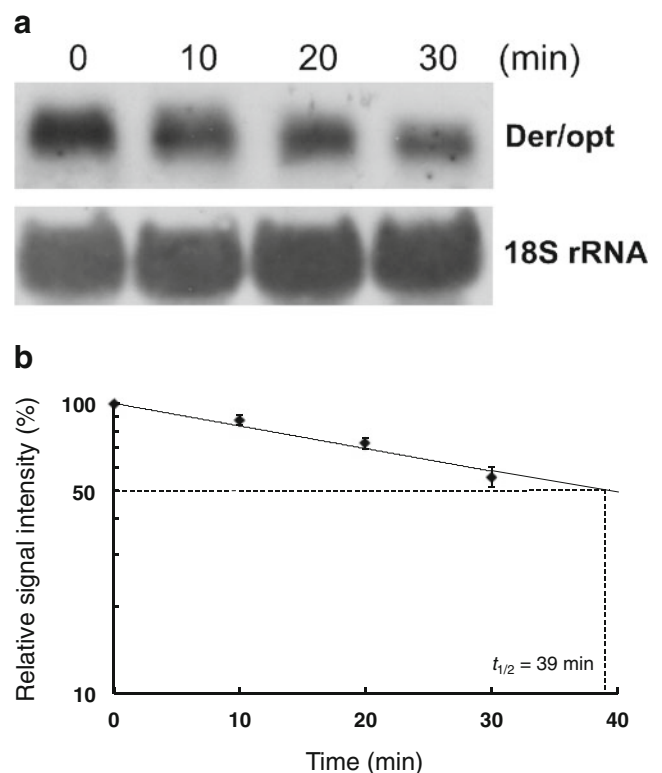


**Fig. 3** Effect of cycloheximide on the native *Der f* 7 mRNA level. After induction of *Der f* 7 gene expression by maltose, 100  $\mu$ g/ml of cycloheximide was added with 1,10-phenanthroline (500  $\mu$ g/ml)

**Fig. 4** Stability of nonfused *Der f* 7 mRNA. Northern blot analysis (a) and determination of the half-life (b) of nonfused *Der f* 7 mRNA after transcription inhibition

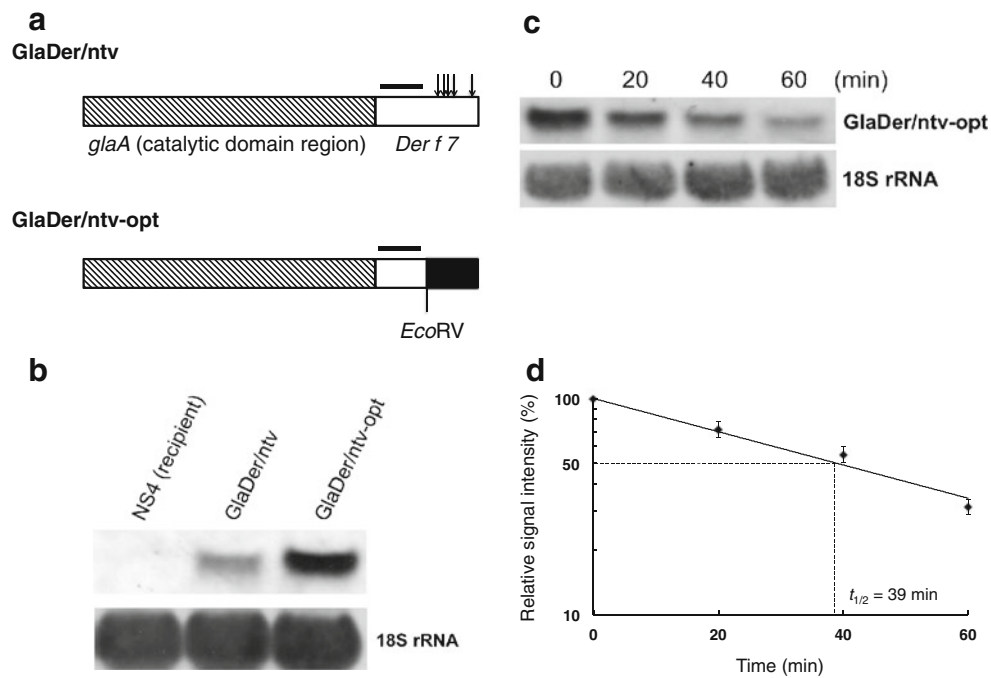


**Fig. 5** Codon optimization of the 3'-half region of *Der f 7* cDNA. **a** Schematic representation of the chimeric *Der f 7* cDNA expression construct. Chimeric *Der f 7* cDNA was generated using the *EcoRV* site in the middle of *Der f 7* cDNA. The codon-optimized region is represented as a black box. The arrows indicate the premature polyadenylation sites within the coding region of native *Der f 7* mRNA (Tokuoka et al. 2008). The probe used for the Northern blot analysis is

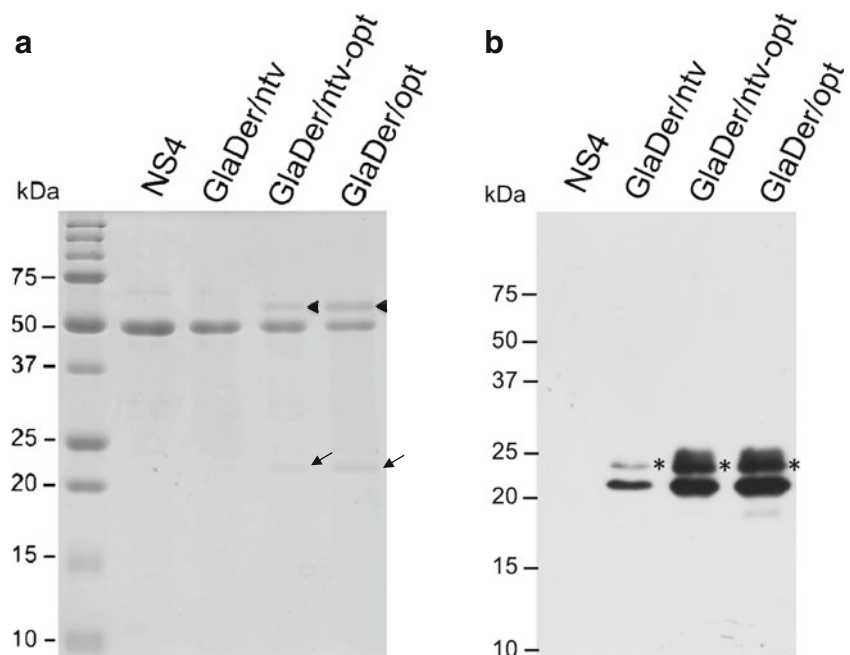
represented as a black bar. **b** Northern blot analysis of the steady-state *Der f 7* mRNA levels. Total RNA was extracted from mycelia grown in YPM medium for 24 h at 30 °C, and 20 µg of total RNA was used for Northern blot analysis. Determination of the half-life (**d**) of chimeric *Der f 7* mRNA after inhibition of transcription based on the Northern blot analysis (**c**)

## Discussion

In a previous study, we had demonstrated that premature polyadenylation in the coding region of the *Der f 7* gene

containing AT-biased codons was prevented by codon optimization (Tokuoka et al. 2008). In this study we provided direct evidence that heterologous gene transcripts were significantly stabilized by codon optimization, which was

**Fig. 6** SDS-PAGE analysis of transformants that expressed different *glaA-Der f 7* fusion genes. The culture supernatants (250 µl) of each transformant grown in YPM medium for 24 h were concentrated with trichloroacetic acid and subjected to SDS-PAGE. **a** CBB staining. Arrows and arrowheads indicate the *Der f 7* and carrier *GlaA* proteins, respectively, which resulted from the cleavage of the *GlaA-Der f 7* fusion protein by KexB protease. **b** Western blot analysis using the anti-*Der f 7* antibody. Asterisks indicate the glycosylated form of *Der f 7* protein



probably due to the prevention of premature polyadenylation in filamentous fungi.

The steady-state mRNA levels can be regulated by transcription and several other processes, including the rates of processing, transport, and degradation. To investigate the stability of heterologous gene transcripts, we screened several chemical compounds that have been used as transcription inhibitors in other filamentous fungi and obtained successful transcription inhibition in *A. oryzae* by the addition of 1,10-phenanthroline (Fig. 1a). 1,10-Phenanthroline acts as a metal chelator that probably inhibits RNA polymerase II by trapping magnesium ions, and the effect of 1,10-phenanthroline on mRNA stability profiles is similar to an RNA polymerase II mutation in budding yeast (Grigull et al. 2004). Although the transcription of *GlaDer/ntv* mRNA appeared to be inhibited incompletely when cells were treated with 1,10-phenanthroline and cycloheximide (Fig. 3), our results suggested that 1,10-phenanthroline is a suitable transcription inhibitor to examine mRNA stability under physiological conditions in *A. oryzae*.

Determination of the half-lives of *glaA*-fused *Der f 7* mRNAs showed that the stability of codon-optimized *Der f 7* mRNA is equivalent to that of endogenous *glaA* mRNA (Figs. 1b and 2d), whereas native *Der f 7* mRNA is rapidly degraded (Fig. 2c). To the best of our knowledge, this is the first experimental evidence of the stabilization of heterologous mRNA by codon optimization in eukaryotic cells. Unexpectedly, smear signals derived from the premature polyadenylated mRNA could not be detected by Northern blot analysis, whereas 3'-RACE analysis of the mRNA sample used in the Northern blot analysis confirmed that prematurely polyadenylated transcripts of *GlaDer/ntv* actually persisted for 20 min after treatment with 1,10-phenanthroline (Supplemental Fig. 1). This suggested that the full-length and prematurely polyadenylated transcripts of *GlaDer/ntv* ranging from 2.2 to 2.5 kb in length could not be clearly separated by agarose gel electrophoresis during Northern blot analysis. In addition, we showed that *Der f 7* mRNA was stabilized by the codon optimization of only the 3'-half region (Fig. 4), suggesting that the mRNA stability was not affected by possible rare codons present in the 5'-half region of *Der f 7* cDNA. Thus, the destabilization of *Der f 7* mRNA resulted mainly by premature polyadenylation in the coding region. The stabilization of *GlaDer/ntv* mRNA after the addition of cycloheximide also suggested that prematurely polyadenylated mRNA of *GlaDer/ntv* without a stop codon was degraded by the nonstop mRNA degradation pathway. However, we could not exclude the possibility that rare codons in the 3'-half region of native *Der f 7* cDNA might have lead to the destabilization and degradation of the *Der f 7* mRNA. Further studies are required to understand the effect on mRNA stability of rare codons present in the heterologous gene.

Improvement of the production level of heterologous proteins from filamentous fungi has been achieved using a carrier protein fusion strategy (Tsuchiya et al. 1994; Gouka et al. 1997b; Tokuoka et al. 2008; Ohno et al. 2011), which could contribute to increased steady-state mRNA levels in some cases, including *Der f 7* (Gouka et al. 1997b; Tokuoka et al. 2008). However, the current study showed that there was no apparent difference in stability between *glaA*-fused and nonfused *Der f 7* mRNA (Figs. 2d and 3). This suggested that improvement of the steady-state *Der f 7* mRNA level in the *glaA*-fused construct was independent of mRNA stability. It should be noted that four introns exist within the catalytic domain region of *glaA* fused to *Der f 7* cDNA. In eukaryotes, especially mammalian cells, it is known that splicing of introns is coupled to transcription and mRNA transport from the nucleus to the cytoplasm, which results in increased steady-state mRNA levels (Furger et al. 2002; Le Hir et al. 2003; Nott et al. 2003; Köhler and Hurt 2007). Therefore, we hypothesized that *glaA* introns fused to *Der f 7* cDNA would have an effect on the improved steady-state *Der f 7* mRNA levels independently of mRNA stability.

In conclusion, codon optimization of the 3'-half region of the heterologous *Der f 7* cDNA, where premature polyadenylation sites were present, resulted in a significant stabilization of mRNA transcripts, whereas the native *Der f 7* mRNA was rapidly degraded. The prematurely polyadenylated transcripts of native *Der f 7* cDNA were presumed to be degraded by the nonstop mRNA degradation pathway. In eukaryotic cells, normal and abnormal mRNAs including nonstop mRNA are degraded by two degradation pathways, 5' to 3' decay by Xrn1 and 3' to 5' decay by the exosome-Ski complex (van Hoof et al. 2002; Inada and Aiba 2005; Doma and Parker 2007; Garneau et al. 2007; Isken and Maquat 2007). We are currently studying the degradation mechanism of mRNA derived from heterologous genes in filamentous fungi including *A. oryzae*.

**Acknowledgments** We thank Natsumi Okada for the DNA sequencing. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, Applied Genomics (no. 17019001), from the Ministry of Education, Culture, Sports, Science and Technology of Japan. M. Tanaka was the recipient of a JSPS Fellowship for Japanese Young Scientists.

## References

- Atwar JA, Wisdom R, Verma IM (1990) Regulated mRNA stability. *Ann Rev Genet* 24:519–541
- Caddick MX, Jones MG, van Tonder JM, Cordier HL, Narendja F, Strauss J, Morozov IY (2006) Opposing signals differentially regulate transcript stability in *Aspergillus nidulans*. *Mol Microbiol* 62:509–519

- Christensen T, Woeldike H, Boel F, Mortensen SB, Hjortshøj K, Thim L, Hansen M (1988) High level expression of recombinant genes in *Aspergillus oryzae*. *Bio/Technology* 6:1419–1422
- Doma MK, Parker R (2007) RNA quality control in eukaryotes. *Cell* 131:660–668
- Fleissner A, Dersch P (2010) Expression and export: recombinant protein production systems for *Aspergillus*. *Appl Microbiol Biotechnol* 87:1255–1270
- Frischmeyer PA, van Hoof A, O'Donnell K, Guerrero AL, Parker R, Dietz HC (2002) An mRNA surveillance mechanism that eliminates transcripts lacking termination codon. *Science* 295:2258–2261
- Furger A, O'Sullivan JM, Binnie A, Lee BA, Proudfoot NJ (2002) Promoter proximal splice sites enhance transcription. *Genes Dev* 16:2792–2799
- Garneau NL, Wilusz J, Wilusz CJ (2007) The highways and byways of mRNA decay. *Nat Rev Mol Cell Biol* 8:113–126
- Gomi K, Iimura Y, Hara S (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. *Agric Biol Chem* 51:2549–2555
- Gouka RJ, Punt PJ, Hessing JGM, van den Hondel CAMJJ (1996) Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. *Appl Environ Microbiol* 62:1951–1957
- Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997a) Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Appl Microbiol Biotechnol* 47:1–11
- Gouka RJ, Punt PJ, van den Hondel CAMJJ (1997b) Glucoamylase gene fusions alleviate limitations for protein production in *Aspergillus awamori* at the transcriptional and (post)transcriptional levels. *Appl Environ Microbiol* 63:488–497
- Grigull J, Mnaimneh S, Pootoolal J, Robinson MD, Hughes TR (2004) Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. *Mol Cell Biol* 24:5534–5547
- Guo J, Cheng P, Yuan H, Kiu Y (2009) The exosome regulates circadian gene expression in a posttranscriptional negative feedback loop. *Cell* 138:1236–1246
- Inada T, Aiba H (2005) Translation of aberrant mRNAs lacking a termination codon or with a shortened 3'-UTR is repressed after initiation in yeast. *EMBO J* 24:1584–1595
- Isken O, Maquat LE (2007) Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function. *Genes Dev* 21:1833–1856
- Ito-Harashima S, Kuroha K, Tatematsu T, Inada T (2007) Translation of the poly(A) tail plays crucial roles in nonstop mRNA surveillance via translation repression and protein destabilization by proteasome in yeast. *Genes Dev* 21:519–524
- Köhler A, Hurt E (2007) Exporting RNA from the nucleus to the cytoplasm. *Nat Rev Mol Cell Biol* 8:761–773
- Le Hir H, Nott A, Moore MJ (2003) How introns influence and enhance eukaryotic gene expression. *Trends Biochem Sci* 28:215–220
- Lubertozzi D, Keasling JD (2009) Developing *Aspergillus* as a host for heterologous expression. *Biotechnol Adv* 27:53–75
- Machida M, Yamada O, Gomi K (2008) Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future. *DNA Res* 15:173–183
- Mizutani O, Furukawa K, Ichianagi S, Matsuda Y, Tokuoka M, Fujioka T, Yamagata Y, Gomi K, Abe K (2009) Alternative processing of proproteins in *Aspergilli kexB* gene disruptants under hyperosmotic conditions. *Biosci Biotechnol Biochem* 73:40–46
- Nott A, Meislin SH, Moore MJ (2003) A quantitative analysis of intron effects on mammalian gene expression. *RNA* 9:607–617
- Ohno A, Maruyama J, Nemoto T, Arioka M, Kitamoto K (2011) A carrier fusion significantly induces unfolded protein response in heterologous protein production by *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 92:1197–1206
- Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttilä M (2003) The effects of drugs inhibiting protein secretion in the filamentous fungus *Trichoderma reesei*. Evidence for down-regulation of genes that encode secreted proteins in the stressed cells. *J Biol Chem* 278:45011–45020
- Passos DO, Parker R (2008) Analysis of cytoplasmic mRNA decay in *Saccharomyces cerevisiae*. *Methods Enzymol* 448:409–427
- Platt A, Langdon T, Arst HN, Kirk D, Tollervey D, Sanchez JM, Caddick MX (1996) Nitrogen metabolite signaling involves the C-terminus and the GATA domain of the *Aspergillus* transcription factor AreA and the 3' untranslated region of its mRNA. *EMBO J* 15:2791–2801
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor
- Tamalampudi S, Rahman MMT, Hama S, Suzuki Y, Kondo A, Fukuda H (2007) Development of recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing lipase-encoding gene from *Candida antarctica*. *Appl Microbiol Biotechnol* 75:387–395
- Tanaka M, Sakai Y, Yamada O, Shintani T, Gomi K (2011) *In silico* analysis of 3'-end-processing signals in *Aspergillus oryzae* using expressed sequence tags and genomic sequencing data. *DNA Res* 18:189–200
- Tokuoka M, Tanaka M, Ono K, Takagi S, Shintani T, Gomi K (2008) Codon optimization increases steady-state mRNA levels in *Aspergillus oryzae* heterologous gene expression. *Appl Environ Microbiol* 74:6538–6546
- Tsuchiya K, Tada S, Gomi K, Kitamoto K, Kumagai C, Jigami Y, Tamura G (1992) High level expression of the synthetic human lysozyme gene in *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 38:109–114
- Tsuchiya K, Gomi K, Kitamoto K, Kumagai C, Tamura G (1993) Secretion of calf chymosin from the filamentous fungus *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 40:327–332
- Tsuchiya K, Nagashima T, Yamamoto Y, Gomi K, Kitamoto K, Kumagai C, Tamura G (1994) High level secretion of calf chymosin using a glucoamylase-prochymosin fusion gene in *Aspergillus oryzae*. *Biosci Biotechnol Biochem* 58:895–899
- van Hoof A, Frischmeyer PA, Dietz HC, Parker R (2002) Exosome-mediated recognition and degradation of mRNAs lacking a termination codon. *Science* 295:2262–2264
- Yamada O, Lee BR, Gomi K (1997) Transformation system for *Aspergillus oryzae* with double auxotrophic mutations, *niaD* and *sC*. *Biosci Biotechnol Biochem* 61:1367–1369

## Transcripts of a heterologous gene encoding mite allergen Der f 7 are stabilized by codon optimization in *Aspergillus oryzae*

Mizuki Tanaka, Masafumi Tokuoka\*, Takahiro Shintani, and Katsuya Gomi<sup>†</sup>

*Laboratory of Bioindustrial Genomics, Department of Bioindustrial Informatics and Genomics, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555, Japan*

<sup>†</sup>To whom correspondence should be addressed.

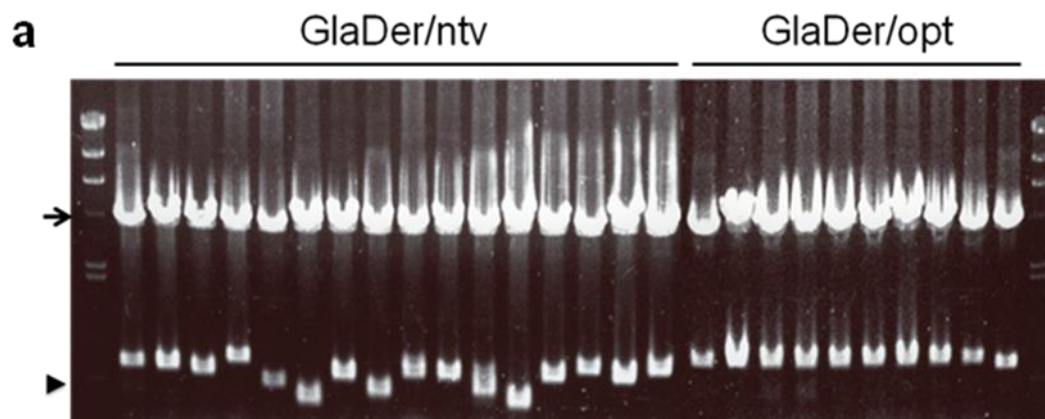
Tel.: +81-22-717-8901; Fax: +81-22-717-8902; E-mail: gomi@biochem.tohoku.ac.jp

*\*Present address: National Research Institute of Brewing, 3-7-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-0046, Japan*

### Supplemental Fig. 1:

3'-RACE analysis of *Der f 7* cDNA using total RNA prepared from mycelia treated with 1,10-phenanthroline for 20 min

**(a)** Analysis of the inserted fragments of 3'-RACE clones. First-strand cDNA was synthesized from the total RNA that was used for Northern blot analysis in Fig. 2 (time 0). Amplified PCR fragments of *Der f 7* cDNA were cloned into the pCRII TA-cloning vector, as described previously (Tokuoka et al. 2008). The resulting plasmid DNAs were digested with *EcoRI*, followed by agarose gel electrophoresis. The arrow indicates the bands of the TA-cloning vector used for cloning of the RT-PCR products, while the arrowhead indicates the molecular marker of 500 bp. **(b)** Polyadenylation sites of native *Der f 7* mRNA determined by the sequencing of 3'-RACE clones. The nucleotide sequence of the coding region is indicated in uppercase letters, whereas the terminator sequence is indicated in lowercase letters. Arrows indicate the polyadenylation sites, while the numbers beside the arrows indicate the number of clones containing a poly(A) tract at the indicated position.



**b**

GlaDer/ntv

```

1695 CCAGGCGCGCCAGACAGCCCCTGCCAGGTGCCGACGACTCACGTGAAAAGAGATCCAATT
1755 CACTATGATAAAATCACCGAAGAAATCAACAAAGCTATTGATGATGCCATTGCTGCTATT
1815 GAACAATCCGAAACAATAGATCCAATGAAAGTACCTGATCATGCCGATAAATTCGAACGT
1875 CATGTTGGTATTGTGGATTTCAAAGGTGAATTAGCCATGCGAAACATTGAGGCTCGAGGA
1935 TTGAAACAAATGAAACGTCAAGGTGATGCTAATGTCAAAGGTGAAGAGGGTATTGTTAAA
1995 GTCATTTGTTGATCGGTGTTACGATGATATCGTCTCGATGGAATATGATTTAGCATAC

2055 AAATTGGGTGATCTTCATCCAACCACTCATGTCATTTTCGGATATTCAAGATTTTGTTGTT
                                     ↑1
2115 GCCTTGTCCTTGAAATTTCTGATGAAGGTAACATAACAATGACATCTTTTGAAGTACGA
                                     ↑1
2175 CAATTCGCTAATGTTGTCAACCATATTGGTGGTCTTTCAATCTTGGATCCAATTTTTGGC
                                     ↑1                                     ↑2
2235 GTTTTATCTGATGTATTGACCGCTATTTTCCAAGACACCGTACGTAAGGAAATGACCAAA

2295 GTATTGGCACCAGCATTTAACGTGAATTGGAAAAAATTAACatattggcatgctctaga
                                     ↑2                                     ↑1↑4
2355 agcgtaacaggatagcctagacccacatactatctgtatacaactccgcaatatgaagtg

2415 atgaatgcaaactagcagcgaatcggatatcagtag
      ↑1  ↑1  ↑1  ↑1
  
```