Journal of Insect Biotechnology and Sericology 82, 00-00 (2013)

Residue-specific incorporation of phenylalanine analogues into protein biosynthesis in silkworm cultured cells

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(Received August 1, 2013; Accepted October 21, 2013)

Here we describe the residue-specific incorporation of *para*-substituted L-phenylalanine (Phe) analogues into protein biosynthesis in *Bombyx mori* cultured cells, BmN, which express *B. mori* phenylalanyl-tRNA synthetase (BmPheRS) mutants with relaxed amino acid specificity. Aminoacylation capabilities of BmPheRS mutants (Ala450 to Gly and Thr407 to Ala or Gly mutants in the α-subunit) were first investigated *in vitro* and then the incorporation of Phe analogues into a reporter protein, EGFP, was investigated using BmN cells expressing one of the above mutants. We also evaluated the effects of lowering the Phe concentration in culture media, which competes with Phe analogues in aminoacylation reactions inside cells, and found that lowering Phe concentration was effective in increasing the substitution rate from Phe to its analogues in EGFP. We conducted electrophoretic and mass spectrometric analyses of synthesized EGFP to clarify the incorporation of Phe analogues. We obtained direct mass spectrometric data demonstrating the incorporation of *p*-bromo- and *p*-cyano-L-phenylalanine and indirect electrophoretic data, implying the incorporation of *p*-azido- and *p*-iodo-L-phenylalanine into protein biosynthesis in BmN cells.

Key words: unnatural amino acids, phenylalanine analogues, aminoacyl-tRNA synsthetase, BmN cells, silkworm, *Bombyx mori*

INTRODUCTION

In vivo incorporation of unnatural amino acids (UAAs) into proteins has become a powerful tool, especially in probing protein functions in living systems and in creating novel proteins with novel biological functions (Davis and Chin, 2012; Neumann, 2012; Zheng and Kwon, 2012). *In vivo* UAAs incorporation has been achieved with two major methods: site-specific (Neumann, 2012) and residue-specific (Singh-Blom *et al.*, 2013). The site-specific method employs orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pairs specific for target UAAs, which are incorporated into proteins at specific sites in response to unique codons such as UAG. In contrast, the residue-specific method can be achieved in a relatively simple way: it requires only the engineering of aaRSs to expand their recognition capabilities. To date, various organisms from unicellular (Chin *et al.*, 2003; Link and Tirrell, 2005; Wang *et al.*, 2001; Wiltschi *et al.*, 2008; Ye *et al.*, 2013) to multicellular (Bianco *et al.*, 2012; Greiss and Chin, 2011; Parrish *et al.*, 2012) have been engineered to incorporate UAAs bearing a wide variety of functional groups into proteins *in vivo* by using one of the two methods above. We recently reported on the residue-specific incorporation of *p*-chloro-L-phenylalanine (ClPhe) into protein biosynthesis in BmN cells, the ovary-derived cultured cells of the domesticated silkworm, *Bombyx mori* (Teramoto *et al.*, 2012). Here we report further expansion of the repertoire of *para*-substituted L-phenylalanine (Phe) analogues which can be residue-specifically incorporated as building blocks in protein biosynthesis in BmN cells.

B. mori has been one of the most important animals in industrial applications because of its incredible ability in protein biosynthesis: they secrete hundreds of milligrams of silk proteins per larva to make cocoons before pupation. Since ancient times, silk fiber has been attractive for its brilliant texture, toughness, and many other excellent characteristics. Silk has also been used as a surgical suture and can be easily transformed into various shapes, including transparent film and three-dimensional porous materials (Rockwood *et al.*, 2011; Tamada, 2005). These properties of silk have prompted many researchers to develop silk-based materials equipped with excellent biocompatibility and mechanical strength for use in biomedical fields (Kawakami *et al.*, 2011; Leal-Egaña and Scheibel, 2010). In addition, *B. mori* is useful as a bio-factory to produce recombinant proteins by baculovirus infection and transgenic techniques (Hong *et al.*, 2010; Lee *et al.*, 2012; Tomita, 2011). To expand the repertoire of amino acid building blocks in *B. mori* protein biosynthesis would therefore largely increase the utility of *B. mori* as an industrial animal.

We have previously cloned two genes encoding two subunits (α and β) of *B. mori* phenylalanyl-tRNA synthetase (BmPheRS), where its amino acid recognition site is located in the α-subunit, and have succeeded in relaxing the amino acid specificity of BmPheRS by amino acid substitution from Ala450 to Gly in its α-subunit to recognize

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ClPhe and *p*-bromo-L-phenylalanine (BrPhe) (Teramoto and Kojima, 2010). We have subsequently demonstrated that ClPhe can be incorporated into protein biosynthesis in BmN cells expressing the αA450G BmPheRS mutant (Teramoto *et al.*, 2012). However, incorporation of BrPhe was not observed, possibly due to its relatively slow recognition by the mutant under competitive conditions with the natural substrate, Phe.

On the other hand, the αT415A *Saccharomyces cerevisiae* PheRS (ScPheRS) mutant was previously reported to be effective for the incorporation of BrPhe into proteins in *Escherichia coli* (Kwon *et al.*, 2006). A sequence alignment shows that the αT415 residue of ScPheRS corresponds to the αT407 residue of BmPheRS (Teramoto and Kojima, 2010). Therefore, this amino acid residue could be another target for introducing novel mutations to relax the amino acid specificity of BmPheRS. In addition, removal of natural substrates which compete against UAAs from culture media is a quite effective method to maximize the incorporation efficiency of UAAs (Singh-Blom *et al.*, 2013). Although protein biosynthesis in BmN cells slows down extremely in culture media completely lacking Phe, which is one of the essential amino acids of *B. mori*, just lowering Phe concentration would be feasible. Such a decrease of Phe concentration would accelerate the aminoacylation reaction of $tRNA^{Phe}$ with Phe analogues bearing larger functionalities than Cl and might accomplish the incorporation of BrPhe and other Phe analogues into protein biosynthesis in BmN cells.

In this study, we aimed to further expand the amino acid repertoire of *B. mori* protein biosynthesis by means of (1) employment of novel BmPheRS mutants bearing different amino acid mutations (αT407A and αT407G) and (2) lowering Phe concentration in culture media. We investigated the incorporation of six *para*-substituted Phe analogues, ClPhe, BrPhe, *p*-iodo-L-phenylalanine (IPhe), *p*-amino-L-phenylalanine (AmPhe), *p*-cyano-L-phenylalanine (CyPhe), and *p*-azido-L-phenylalanine (AzPhe) (Fig. 1), and revealed that BrPhe, CyPhe, and presumably AzPhe and IPhe were incorporated into protein biosynthesis in BmN cells in place of Phe.

MATERIALS AND METHODS

Materials

All chemicals used in this study were of reagent grade and used as received. Phe analogues, ClPhe, BrPhe, IPhe, and AzPhe, were from Bachem AG (Bubendorf, Switzerland), and AmPhe • HCl and CyPhe were from Watanabe Chemical Industries (Hiroshima). An ovary-derived cell line of *B. mori*, BmN, was provided by the RIKEN BioResource Center and adapted to Grace's insect medium (Life Technologies; Carlsbad, CA, USA) supplemented with a 10% fetal bovine serum (FBS) (Life Technologies). Grace's insect medium with lowered Phe concentration was prepared by adding a requisite amount of Phe to the specially-prepared Grace's insect medium completely lacking Phe (Nacalai tesque; Kyoto). The temperature for the BmN culture was set to 26°C throughout the experiments.

Plasmids construction

A commercially available expression vector pQE-30 (QIAGEN; Hilden, Germany) was used for recombinant protein expression in *E. coli* strain M15[pREP4] (QIAGEN). The pQE-30 plasmids encoding the wild-type BmPheRS or one of the αA450G, αT407A, and αT407G BmPheRS mutants were constructed, as reported previously (Teramoto and Kojima, 2010). For introducing amino acid substitutions, that is, Thr407 to Ala and Gly in the α-subunit of BmPheRS, the following primer sets were used: 5'-CAACCCCTAC**GCC**GAACCCAGCATGG AAA-3' and 5'-ATTTCCATGCTGGGTTC**GGC**GTAGGG GTTG-3' for the T407A mutation and 5'-CAACCCCTAC **GGC**GAACCCAGCATGGAAA-3' and 5'-ATTTCCATGC TGGGTTC**GCC**GTAGGGGTTG-3' for the T407G mutation, where the original Thr codon and the mutated nucleotides are respectively underlined and in bold. An expression plasmid bearing an improved *ie1* promoter derived from the *B. mori* nuclear polyhedrosis virus (Kojima, 2002) was used to express the wild-type or one of the mutants of the α-subunit of BmPheRS, the wild-type β-subunit of BmPheRS, and an enhanced green fluorescent protein (EGFP) as a reporter protein (bearing a Strep tag on its N-terminal) in BmN cells, as with the previous study (Teramoto *et al.*, 2012).

In vitro aminoacylation assay

In vitro-transcribed *B. mori* $tRNA^{Phe}$ and recombinant BmPheRSs (wild-type or one of αA450G, αT407A, and αT407G mutants) were prepared, as reported previously (Teramoto and Kojima, 2010). The capability of each recombinant BmPheRS to aminoacylate *B. mori* $tRNA^{Phe}$ with Phe and its analogues was evaluated *in vitro* based on the previous report (Teramoto and Kojima, 2010). Briefly, aminoacylation reactions were performed for 30 min at 30°C in 10 μL of reaction mix (0.1 M Na-HEPES pH 7.5, 30 mM KCl, 10 mM $MgCl_2$, 2 mM Mg-ATP, and 2.5 μM *in vitro*-transcribed *B. mori* $tRNA^{Phe}$) with 25 μM amino acids and 0.01 A_{280} units of recombinant BmPheRSs, where one A_{280} unit is defined as the protein amount that gives an A_{280} value of 1 when dissolved in 80 μL of 6 M guanidine hydrochloride and is measured lengthwise in a 1-cm path. Reactions were stopped by adding 16.7 μL of a quenching solution, which was a 4:1 mixture of acid-urea PAGE sample buffer (0.1 M NaOAc pH 5.0, 8 M urea, 0.05% xylene cyanol, and 0.05% bromophenolblue)

and 80% glycerol, to each 10 μL reaction solution followed by vortexing. Unreacted and aminoacylated *B. mori* $tRNA^{Phe}$ were separated by electrophoresis on a 10% acid-urea polyacrylamide gel in 0.1 M NaOAc (pH 5.0) at 4°C at 9 V/cm until xylene cyanol migrated about 12 cm. Separated tRNAs were visualized with GelRed (Biotium Inc.; Hayward, CA, USA).

Cell culture assay

Incorporation of Phe analogues in EGFP synthesized in BmN cells in place of Phe by BmPheRS mutants was investigated based on our previous report (Teramoto *et al.*, 2012). BmN cells ($1.4\text{-}3.0 \times 10^6$) were seeded in 25 cm^2 culture flasks and transfected with the plasmid vectors expressing the wild-type or one of the mutants of the α-subunit of BmPheRS (1.5 μg/flask) and the wild-type β-subunit of BmPheRS (1.5 μg/flask) and EGFP (3 μg/flask) under a serum-free condition using the HilyMax transfection reagent (24 μL/flask) (Dojindo; Kumamoto) according to the manufacturer's instructions. After overnight transfection, the medium was changed to a fresh one supplemented with 10% FBS –with or without Phe analogues. Although gene expressions from transfected plasmids were generally expected to become maximal in two to three days after transfection, the amount of EGFP accumulated in cells was not sufficient for analyzing the incorporation of Phe analogues by mass spectrometry at that time. Hence, the transfected cells were cultured for 9 days at 26°C with two medium changes at a three-day interval. The cells were then harvested and lysed in Buffer W (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA) supplemented with 1% (v/v) Igepal CA-630 by a 10-min incubation on ice. The cleared lysates obtained after centrifugation (9500 × g, 4°C, 15 min) were purified under a native condition using Strep-Tactin spin columns (IBA; Göttingen, Germany) according to the manufacturer's instructions. Five micro liters of the affinity-purified EGFP eluted with 50 μL of Buffer BE (100 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 2 mM D-biotin) were subjected to SDS-PAGE. Electrophoresis was performed by standard techniques using Any kD TGX precast gels (Bio-Rad; Hercules, CA, USA) at a constant voltage of 200 V. Separated proteins were stained by EzStain AQua (ATTO; Tokyo).

In-gel digestion

The affinity-purified EGFP was separated on 12.5% e-PAGEL precast gels (ATTO) at a constant current of 20 mA and visualized by EzStain Aqua (ATTO). Slices containing EGFP were excised from the gels and cut into small pieces. The gel pieces were then destained in 50% acetonitrile/ 100 mM NH_4HCO_3 at 37°C with shaking, washed thoroughly in 50% methanol/ 10% acetic acid, equilibrated in 50 mM NH_4HCO_3, dehydrated in acetonitrile, and dried in a vacuum centrifuge. The minimum volume of the solution containing 0.02 μg/μL Trypsin Gold (Promega; Fitchburg, WI, USA) in 40 mM NH_4HCO_3/ 10% acetonitrile was infiltrated into the dried gel pieces and the extra solution was removed. The hydrated gel pieces were covered with a digestion buffer (40 mM NH_4HCO_3, 10% acetonitrile) and incubated at 37°C overnight. Digested peptide fragments were extracted with water followed by 50% acetonitrile/ 5% trifluoroacetic acid (TFA) and then dried in a vacuum centrifuge. The dried peptide fragments were stored at −20°C until analysis.

MALDI-TOF-MS

The trypsin-digested peptide fragments were dissolved in a small amount of TA (2:1 mixture of 0.1% TFA and acetonitrile) and mixed with α-cyano-4-hydroxycinnamic acid solution saturated in TA. The mixtures were spotted on a target plate and dried at room temperature. MALDI-TOF-MS spectra were collected in the positive ion reflector mode with an autoflex III mass spectrometer (Bruker Daltonics; Billerica, MA, USA). The mass spectrometric data was analyzed by *mMass* software (Niedermeyer and Strohalm, 2012).

RESULTS AND DISCUSSION

In vitro aminoacylation assay of the recombinant BmPheRS mutants

Either the wild-type BmPheRS or one of the αA450G, αT407A and αT407G BmPheRS mutants were expressed in *E. coli* and affinity-purified. Their capabilities to aminoacylate *B. mori* $tRNA^{Phe}$ with six *para*-substituted Phe analogues (Fig. 1) were assayed *in vitro*. Aminoacylation was detected by acid-urea polyacrylamide gel electrophoresis (acid-urea PAGE) (Fig. 2). The wild-type BmPheRS aminoacylated the tRNA only with Phe. As previously reported, *B. mori* $tRNA^{Phe}$ was aminoacylated with ClPhe and BrPhe by the αA450G mutant (Teramoto and Kojima, 2010). These two Phe analogues were also recognized by the αT407A and αT407G mutants. The tRNA was only slightly aminoacylated with IPhe by the αA450G mutant, as previously reported (Teramoto and Kojima, 2010). The αT407A mutant exhibited a similar result in aminoacylation with IPhe while the αT407G mutant showed a much improved recognition of IPhe. It was found that AmPhe was not a good substrate for all three mutants and that CyPhe was weakly recognized by all three mutants. AzPhe, which bears an azide functionality useful for chemoselective reactions (Lallana *et al.*, 2011; van Berkel *et al.*, 2011), was found to be well recognized by the αT407G mutant while the other two mutants showed a weaker recognition.

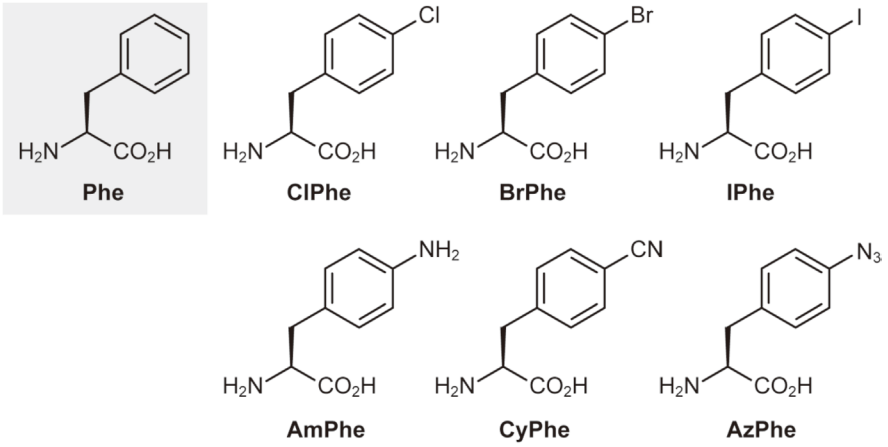

Fig. 1. Structures of the natural substrate, L-phenylalanine (Phe), and its *para*-substituted analogues investigated in this study. ClPhe: *p*-chloro-L-phenylalanine, BrPhe: *p*-bromo-L-phenylalanine, IPhe: *p*-iodo-L-phenylalanine, AmPhe: *p*-amino-L-phenylalanine, CyPhe: *p*-cyano-L-phenylalanine, AzPhe: *p*-azido-L-phenylalanine.

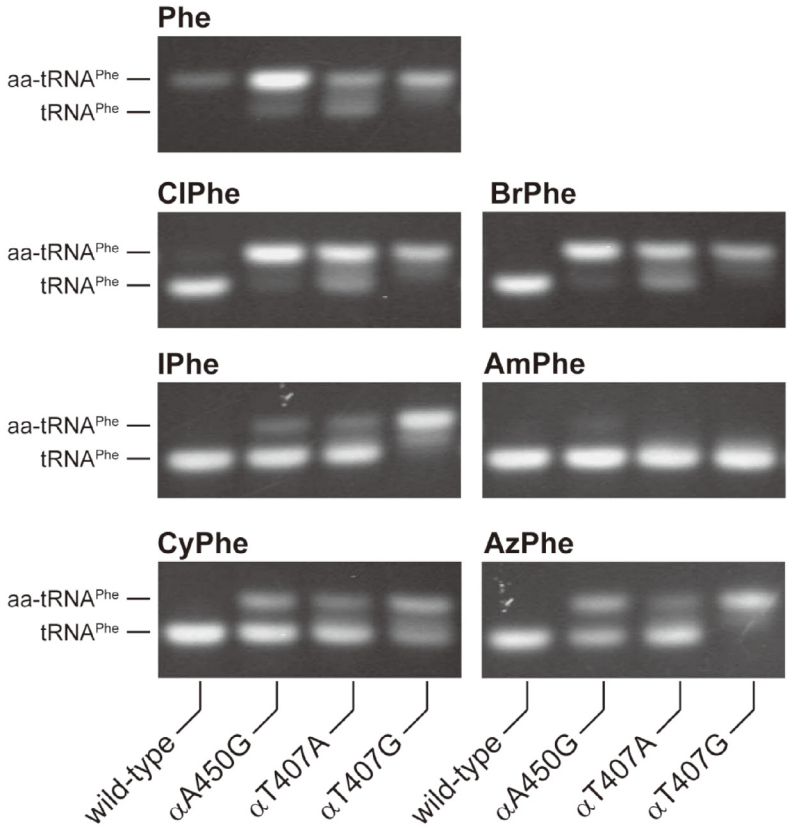

Fig. 2. *In vitro* aminoacylation of *B. mori* $tRNA^{Phe}$ with Phe and six *para*-substituted Phe analogues by the wild-type BmPheRS or one of the αA450G, αT407A, or αT407G BmPheRS mutants. After 30 min aminoacylation reactions at 30°C, the unreacted and aminoacylated tRNAs were separated by acid-urea PAGE where the aminoacylated tRNAs (aa-$tRNA^{Phe}$) exhibited slower mobility than the unreacted tRNA.

Effects of expressing BmPheRS mutants on protein biosynthesis in BmN cells under normal and low Phe conditions

Expression of BmPheRS mutants with relaxed amino acid specificity might be toxic for living systems because they might recognize inappropriate amino acids as a substrate. Such adverse effects arising from the expression in BmPheRS mutants can be speculated to be more serious when the concentration of the native substrate, Phe, in the culture media is lowered. We then tested the effects of expressing BmPheRS mutants in BmN cells on their protein biosynthesis activity under normal and low Phe conditions using EGFP as a reporter protein.

Fig. 3A shows EGFP fluorescence in BmN cells ex-

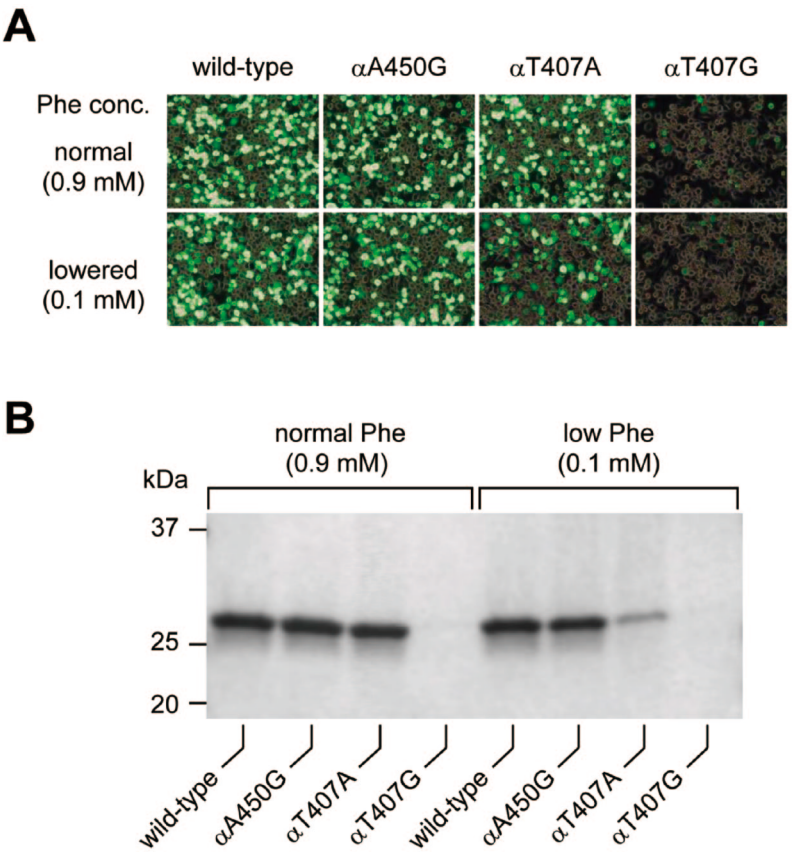

Fig. 3. Effects of expressing the BmPheRS mutants on protein biosynthesis in BmN cells. (A) Images showing EGFP fluorescence in BmN cells expressing the wild-type BmPheRS or one of the αA450G, αT407A, or αT407G BmPheRS mutants. The cells were cultured for 9 days under normal (0.9 mM) and low (0.1 mM) Phe concentrations. The dark-field images detecting fluorescence of EGFP were superimposed on the bright-field images. (B) The SDS-PAGE of affinity-purified EGFP obtained from BmN cells cultured under each condition shown in Panel A. The constant volume of the affinity-purified EGFP solution was loaded on SDS-PAGE gels to compare the relative amount of synthesized EGFP among different culture conditions.

pressing the wild-type BmPheRS or one of the αA450G, αT407A, and αT407G BmPheRS mutants. The cells were cultured for 9 days under normal (0.9 mM) and low (0.1 mM) Phe conditions. The synthesized EGFP was affinity-purified from cell lysates and subjected to SDS-PAGE (Fig. 3B). Under both normal and low Phe conditions, a drastic decrease of EGFP synthesis was observed when the αT407G mutant was expressed, suggesting a misincorporation of inappropriate amino acids in place of Phe. This observation is consistent with the *in vitro* assay results where the αT407G mutant showed mostly relaxed substrate specificity (Fig. 2). On the other hand, the other two mutants exhibited little effects on EGFP synthesis under normal Phe concentration. However, EGFP synthesis largely decreased when the αT407A mutant was expressed under low Phe conditions. This observation shows that the misincorporation of inappropriate amino acids by the αT407A mutant was negligible with sufficient amounts of Phe but became noticeable when Phe was lowered. In contrast, expression of the αA450G mutant continued to have little effect on EGFP synthesis even under low Phe conditions. From these experiments, it was found that the αT407G mutant is inappropriate for assays using BmN cells due to its excessive relaxation of its amino acid specificity and that the αT407A mutant could be only employed under normal Phe conditions.

It was previously reported that L-lysine (Lys) and L-tryptophan (Trp) was misincorporated in place of Phe when the αT415G ScPheRS mutant was expressed in *E. coli* (Kwon *et al.*, 2006), where the αT415 residue in ScPheRS corresponds to the αT407 residue in BmPheRS according to sequence alignment (Teramoto and Kojima, 2010). It is thus likely that Lys and/or Trp were misincorporated in place of Phe in protein biosynthesis in BmN cells when EGFP synthesis decreased.

Incorporation of BrPhe in EGFP synthesized in BmN cells expressing the αT407A BmPheRS mutant

Fig. 2 shows that the αT407A BmPheRS mutant recognizes BrPhe *in vitro* as well as the αA450G mutant. We then investigated whether BrPhe can be incorporated into

protein biosynthesis in BmN cells expressing the αT407A mutant under normal Phe conditions. The synthesized EGFP was affinity-purified and subjected to SDS-PAGE (Fig. 4A). The SDS-PAGE data exhibited that the amount of EGFP gradually decreased with the increase in BrPhe concentration. Such a decrease of EGFP was not observed in the cells expressing the wild-type BmPheRS even in the presence of 1 mM BrPhe. When BmPheRS mutants successfully aminoacylate *B. mori* $tRNA^{Phe}$ with Phe analogues in BmN cells, the analogues will be equally incorporated into all newly-synthesized proteins, including essential enzymes for protein biosynthesis machinery, which would slow down the biosynthesis of proteins, including EGFP. In fact, a marked decrease of EGFP was previously observed for the incorporation of ClPhe with the αA450G mutant (Teramoto *et al.*, 2012). Hence, a decrease of EGFP synthesis could be indicative of the incorporation of Phe analogues into protein biosynthesis. MALDI-TOF-MS analysis of the trypsin-digested fragments of EGFP showed that BrPhe was partially incorporated in place of Phe (Fig. 4B). This result implies that the αT407A mutant is more appropriate for incorporating BrPhe into proteins in BmN cells than the αA450G mutant which did not show detectable incorporation of BrPhe under the same assay conditions (Teramoto *et al.*, 2012).

As with the previous results observed for ClPhe incorporation (Teramoto *et al.*, 2012), the mobility of EGFP in SDS-PAGE did not change by BrPhe incorporation (Fig. 4A). From a rough estimation of relative peak intensities in MALDI-TOF-MS spectra (Fig. 4B) by assuming that ionization efficiencies of the analyzed peptides and its counterparts containing BrPhe are identical, the replacement ratio from Phe to BrPhe was estimated to be at most *ca.* 5%. Since EGFP contains 14 Phe residues, the 5% replacement denotes that, at most, one Phe residue was replaced by BrPhe in EGFP. This replacement leads to a ~ 79 Da increase of the molecular weight of EGFP which is *ca.* 30 kDa including purification tags. Such a faint increase of molecular weight will have practically no effects on the mobility of EGFP. For this reason, we did not observe any changes in the EGFP mobility in SDS-PAGE concerning the incorporation of different Phe analogues, as described later.

Increased substitution rate by reducing Phe concentration in culture medium

As previously reported, ClPhe can be incorporated into protein biosynthesis in BmN cells expressing the αA450G BmPheRS mutant by simply adding it to the culture medium which contain standard concentrations of Phe (0.9 mM) (Teramoto *et al.*, 2012). It will be important to know whether the replacement ratio from Phe to ClPhe increases when the Phe concentration in the medium is lowered. Fig. 5A shows the SDS-PAGE pattern of the affinity-purified EGFP, expressed in normal (0.9 mM) and low (0.1 mM) Phe conditions in the presence of ClPhe. A drastic decrease in the EGFP amount was observed when the cells expressing the αA450G mutant were cultured under low Phe conditions. The observed, relatively larger decrease of EGFP synthesis compared to those under normal Phe conditions could be attributable to an increased incorporation of ClPhe into protein biosynthesis. As expected, MALDI-TOF-MS analysis of the trypsin-digested peptide fragments demonstrated that lowering the Phe concentration in the culture medium led to a relatively increased replacement ratio from Phe to ClPhe (Fig. 5B).

Incorporation of BrPhe into EGFP synthesized in BmN cells expressing the *α*A450G BmPheRS mutant under low Phe conditions

An increased replacement ratio from Phe to ClPhe under low Phe conditions prompted us to investigate the incorporation of BrPhe into EGFP synthesized in BmN cells expressing the αA450G BmPheRS mutant under low Phe conditions (0.1 mM). SDS-PAGE of the affinity-purified EGFP showed that its amount gradually decreased with the increase of BrPhe concentration when the αA450G mutant was expressed (Fig. 6A). This behavior is similar to the case of the αT407A mutant shown in Fig. 4A, suggesting that BrPhe was incorporated into protein biosynthesis. MALDI-TOF-MS analysis of the trypsin-digested fragments of EGFP confirmed that a small portion of Phe was replaced with BrPhe (Fig. 6B). This observation demonstrated that lowering Phe concentration in the culture medium is effective for incorporating Phe analogues which are not normally incorporated into protein biosynthesis in BmN cells.

Incorporation of CyPhe, AzPhe, and IPhe in EGFP synthesized in BmN cells expressing the *α*A450G BmPheRS mutant under low Phe conditions

Incorporation of CyPhe, AzPhe, and IPhe, which were relatively weakly recognized with the αA450G BmPheRS mutant (Fig. 2), was investigated. It was previously reported that these three Phe analogues were all incorporated into protein biosynthesis in *E. coli* expressing the αA294G *E. coli* PheRS mutant (Kirshenbaum *et al.*, 2002). This mutant has a homologous amino acid substitution with the αA450G BmPheRS mutant.

Incorporation of CyPhe was first investigated under low Phe conditions. EGFP synthesis in BmN cells cultured under a 0.1 mM Phe condition was not impeded (Fig. 6A), implying no remarkable incorporation of CyPhe. This observation might reflect the *in vitro* assay results (Fig. 2), that is, the recognition of CyPhe by the αA450G mutant was weaker than that of BrPhe, which was incorporated in

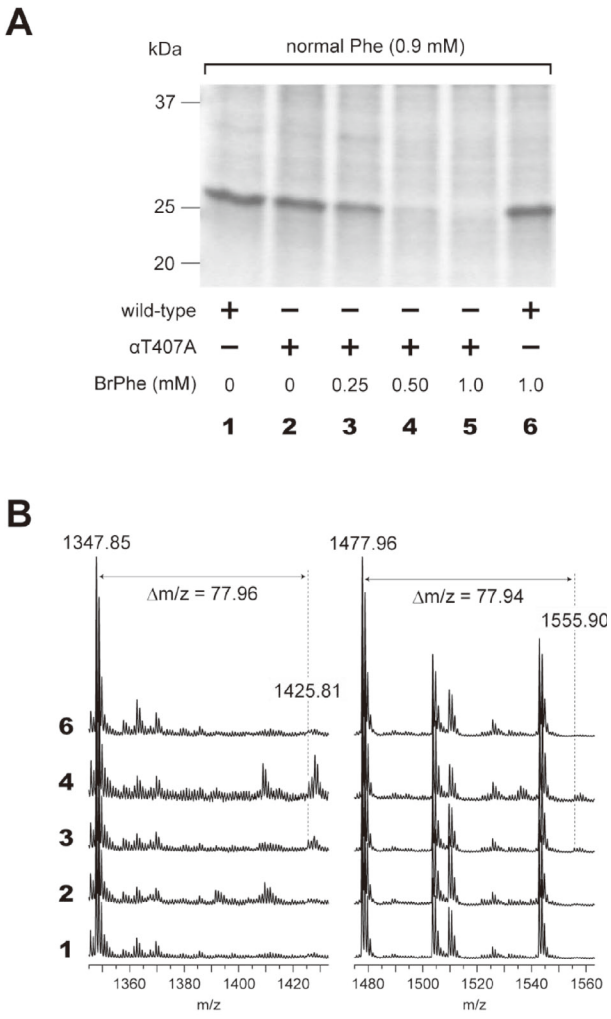

Fig. 4. Analysis of EGFP produced in BmN cells expressing the αT407A BmPheRS mutant cultured in the presence of BrPhe. (A) The SDS-PAGE of the affinity-purified EGFP produced under varied conditions. The constant volume of the affinity-purified EGFP solution was loaded on SDS-PAGE gels to compare the relative amount of synthesized EGFP among different culture conditions. (B) MALDI-TOF-MS spectra of the trypsin-digested peptide fragments, TI**FF**KDDGNYK ($[M + H]^+$ = 1347.66 Da) and AEVK**F**EGDTLVNR ($[M + H]^+$ = 1477.76 Da), from the affinity-purified EGFP. The numbering at the left of the spectra corresponds to Panel A. Replacement of one Phe residue in the fragment peptides by BrPhe leads to a theoretical mass increase of 77.91 Da (^{79}Br-^{1}H). The sample from Condition 5 did not give good quality spectra due to a low quantity of EGFP.

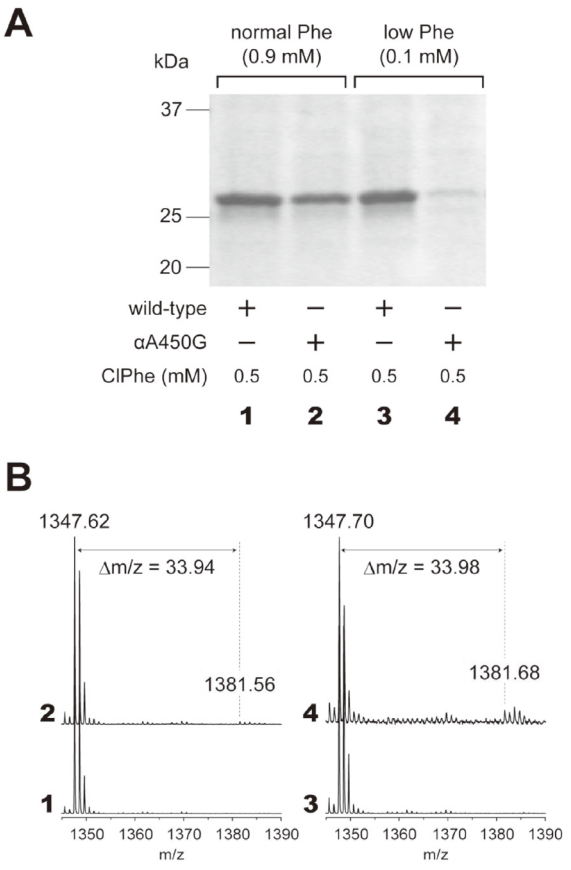

Fig. 5. Analysis of EGFP produced in BmN cells expressing the αA450G BmPheRS mutant cultured in the presence of ClPhe under different Phe concentrations. (A) The SDS-PAGE of the affinity-purified EGFP expressed under normal (0.9 mM) and low (0.1 mM) Phe conditions. The constant volume of the affinity-purified EGFP solution was loaded on SDS-PAGE gels to compare the relative amount of synthesized EGFP among different culture conditions. (B) MALDI-TOF-MS spectra of the trypsin-digested peptide fragment, TI**FF**KDDGNYK ($[M + H]^+$ = 1347.66 Da), from the affinity-purified EGFP. The numbering on the left of the spectra corresponds to Panel A. The replacement of one Phe residue in the fragment peptide to ClPhe leads to a theoretical mass increase of 33.96 Da (^{35}Cl-^{1}H).

EGFP under the 0.1 mM Phe condition. When the concentration of Phe was further lowered to 0.02 mM, a significant decrease of EGFP synthesis was observed (Fig. 6A), indicating the incorporation of CyPhe into protein biosynthesis. MALDI-TOF-MS analysis confirmed that a part of Phe was substituted with CyPhe in EGFP (Fig. 6B).

We next investigated the incorporation of AzPhe, which was moderately recognized with the αA450G mutant *in vitro* (Fig. 2). Similar to CyPhe, EGFP synthesis was not disrupted under the 0.1 mM Phe condition (Fig. 6A), implying no remarkable incorporation of AzPhe. When Phe is lowered to 0.02 mM, EGFP synthesis significantly decreased, as with the case of CyPhe (Fig. 6A). This result strongly implies that AzPhe was incorporated into protein biosynthesis. However, MALDI-TOF-MS analysis of the affinity-purified EGFP did not give any detectable peaks demonstrating the incorporation of AzPhe (data not shown). Trials to detect the incorporation of AzPhe by a chemoselective reaction to azide groups (Lallana *et al.*, 2011) also failed (data not shown). We supposed that the substitution rate from Phe to AzPhe was too small to be detected regardless of the observed adverse effects on EGFP synthesis. Alternatively, unwanted photolysis of aromatic azide groups during cell culture, cell lysis, and/or EGFP purification might have made AzPhe undetectable.

We finally investigated the incorporation of IPhe, which

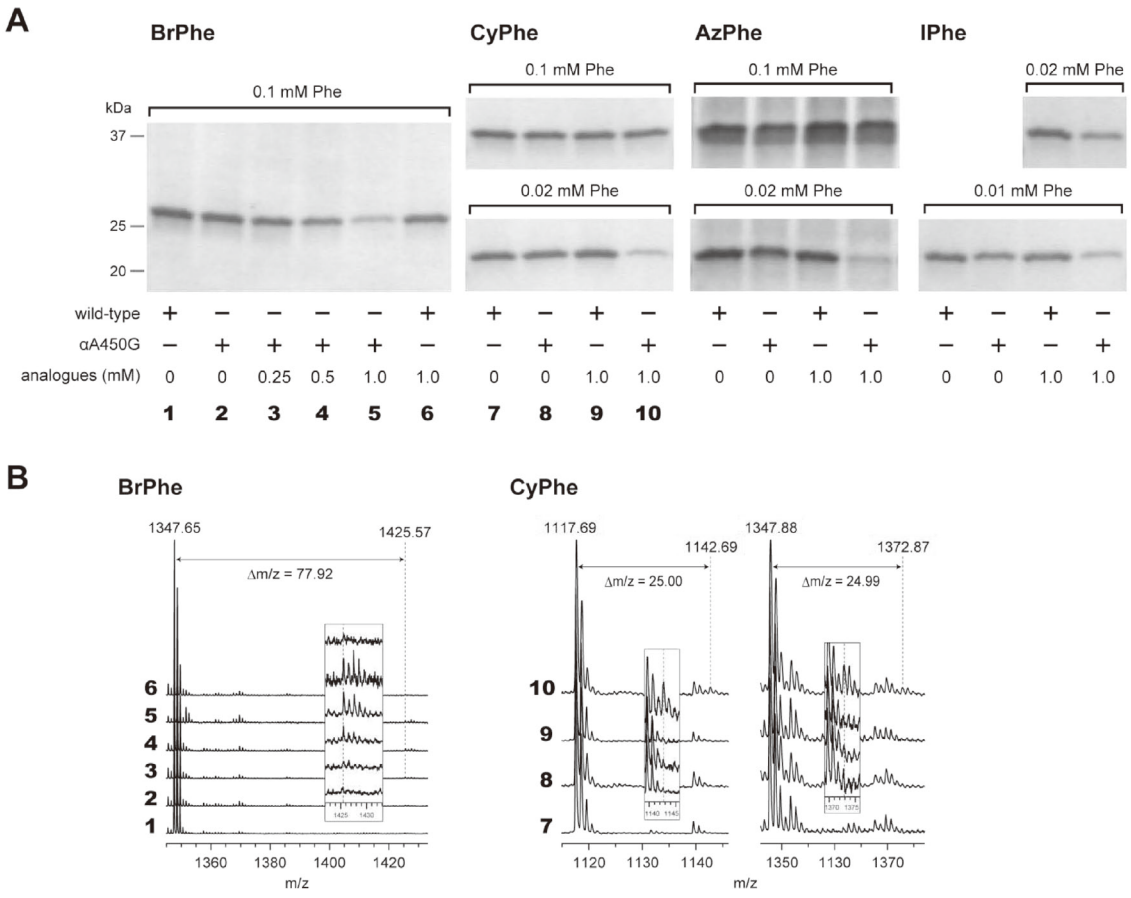

Fig. 6. Analysis of EGFP produced in BmN cells expressing the *α*A450G BmPheRS mutant cultured in the presence of BrPhe, CyPhe, AzPhe, or IPhe under varied Phe concentrations. (A) SDS-PAGE of the affinity-purified EGFP. A constant volume of the affinity-purified EGFP solution was loaded on SDS-PAGE gels to compare the relative amount of synthesized EGFP among different culture conditions. (B) MALDI-TOF-MS spectra of the trypsin-digested peptide fragments, E**F**HHHHHH ($[M + H]^+$ = 1117.48 Da) and/or TI**FF**KDDGNYK ($[M + H]^+$ = 1347.66 Da), from the affinity-purified EGFP. The numbering on the left of the spectra corresponds to Panel A. The replacement of one Phe residue in the fragment peptides to BrPhe and CyPhe leads to theoretical mass increases of 77.91 Da (^{79}Br-^{1}H) and 25.00 Da (^{12}C^{14}N-^{1}H), respectively.

was only slightly recognized with the αA450G mutant *in vitro* (Fig. 2). EGFP synthesis in BmN cells cultured under the 0.02 mM Phe condition seemed to be slightly decreased (Fig. 6A). A more apparent decrease of EGFP was observed when the Phe concentration was further lowered to 0.01 mM. This observation strongly suggests the incorporation of IPhe into protein biosynthesis in BmN cells under severely restricted Phe concentration. However, similar to the case of AzPhe, a direct observation of the replacement from Phe to IPhe by MALDI-TOF-MS analysis failed (data not shown). We speculated that the incorporation of IPhe strongly impaired protein biosynthesis activity in BmN cells, most likely due to the presence of a bulky iodo group even when the replacement ratio from Phe to IPhe was too low to be detected.

In this study, we succeeded in expanding the repertoire of amino acids used in protein biosynthesis in BmN, the ovary-derived *B. mori* cultured cells. We found that the αT407A BmPheRS mutant is effective for the incorporation of BrPhe in protein biosynthesis in BmN cells. Moreover, we found that BrPhe, CyPhe, and most likely AzPhe and IPhe can be incorporated into protein biosynthesis in BmN cells expressing the αA450G BmPheRS mutant when concentration of the competing amino acid, Phe, in the culture media was adequately lowered. These findings would contribute to create novel proteins containing UAAs in *B. mori* cultured cells and even in *B. mori* larvae in the near future.

ACKNOWLEDGEMENTS

The authors thank Dr. Jun Ishibashi (NIAS) for his support in MALDI-TOF-MS analysis and Dr. Qiang Zhang for her technical assistance. This work was supported by JSPS KAKENHI Grant Numbers 20780039, 22658019, and 24688008.

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