Biochemical and Biophysical Research Communications 391 (2010) 598–603

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of a fast maturating red fluorescent protein by a combined approach of elongation mutagenesis and functional salvage screening

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ARTICLE INFO

Article history:
Received 2 November 2009
Available online 20 November 2009

Keywords:
Red fluorescence
DsRed
FmRed
Elongation mutagenesis
Maturation time

ABSTRACT

Fluorescent proteins that can be useful as indicators or reporters must have rapid maturation time, high quantum yield and photobleaching stability. A red fluorescent protein DsRed that has a high quantum yield and photostability has an innately slow maturation time when compared to other fluorescence proteins. In this study, we combined a functional salvage screen (FSS) and elongation mutagenesis to obtain a DsRed variant that maintained structural features closely linked with a high quantum yield and photostability and evolved to have a rapid maturation time. It is expected that the variant generated here, FmRed (fast maturating red fluorescent protein), will be widely used as an indicator or reporter because it maintained traits superior to that of the wild-type protein and also matured rapidly.

Introduction

The potential application of proteins as indicators or reporters for cell physiology-related gene expression, interactions between proteins and tracking localization under non-invasive conditions has steadily increased. Proteins related to this purpose include several chromogenic enzymes (peroxidase, alkaline phosphatase and glycosidase), luminescent luciferase and fluorescent proteins [1–4]. In particular, the range of applications of fluorescent proteins is widening because they do not require cofactors or substrates to function and can be excited at a broad range of wavelengths [5].

Fluorescent proteins that are used in widespread applications include green fluorescence protein (GFP) and its variants CFP, BFP and YFP [4,6]. These proteins can be used either independently as reporters or in combination for fluorescence resonance energy transfer (FRET) [7,8]. However, they require high energy level UV to excite them for detection and occasionally have inferior quantum yields when compared to wild-type GFP. Conversely, the red fluorescent protein, DsRed, can use visible wavelengths for excitation. In addition, DsRed has a unique fluorophore surrounded by four identical subunits, which results in its being stable against photobleaching and having a high quantum yield [9,10]. Thus, it is preferred due to its low cytotoxicity and reproducible fluorescence, although it has a slow maturation time due to its tetrameric structure [10,11].

Many attempts to improve the slow maturation time of DsRed have been made, and it is believed that this deficiency is closely linked to its tetrameric structure [12]. Although DsRed variants with reduced maturation times have been generated [13], these variants have had a low quantum yield and tolerance for photobleaching when compared with the wild-type [14,15]. These changes likely occurred because the structures of DsRed variants were changed into monomers or dimers to reduce the maturation time [16]. Recently, more improved variants have been developed to restore the photobleaching sensitivity of monomeric variants; however, their quantum yields are still lower than those of wild-type protein DsRed [16,17].

In this study, we attempted to construct a new DsRed variant that is expected to have innate structural features (closely linked with high quantum yield and photostability) and a rapid maturation time. This was accomplished by elongating the terminus of the defeat template through the use of synthetic oligonucleotides on the basis of the principle of a functional salvage screen (FSS) [18].

Materials and methods

Hosts, plasmids and culture conditions. *Escherichia coli* XL1-Blue was used as the host for gene cloning and library construction. The DsRed gene from pDsRed-N1 (Clontech) was employed as the template for generation of the mutant pools. The pTrc99A plasmid was used for library construction and expression of the DsRed variants. The recombinant cells were routinely cultured in typical LB medium supplemented with ampicillin (50 μg/ml) and no inducer at 37 °C.

Construction and screening of the mutant library. The library pool for the screening of fast maturating mutant was constructed as fol-

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doi:10.1016/j.bbrc.2009.11.105

lows. The mutant DsRed template without the start and stop codon was constructed by PCR using a pair of specific primers, F1 and R1 (Table 1). To induce a functional defect, the F1 primer had a base deletion from the N-terminus of wild-type DsRed. The resulting construct was digested with EcoRI and HindIII and then cloned into the same sites of the pTrc99A plasmid. This defective gene was tested to determine if it was able to express the functional protein with red fluorescence under normal or induction conditions using a fluorescence photometer (RF-5301PC, Shimadzu), after which the DNA sequence was confirmed.

Using the construct as a template, functionally salvaged mutant pools were prepared by elongated PCR using a set of synthetic primers (F2 and R2) containing 3–13 peptides with a base that corrected the frame shift (Fig. 1). The resulting genes were purified using a DNA clean up system (Promega) and digested with two restriction enzymes, NcoI and HindIII. To accomplish this, the innate NcoI site (**CC**ATGG) of DsRed was changed to C**AA**TGG by site-directed mutagenesis [18]. The resulting gene was cloned into the same sites on pTrc99A and transformed into *E. coli* XL1-Blue. The recombinant cells were grown on LB agar plates without inducer, and positive clones emitting red fluorescence were screened by direct observation under daylight conditions. As a control, the clone harboring the wild-type DsRed was grown under the same conditions. The elongated peptide from the functionally salvaged mutant showing fast maturation time was identified by DNA sequencing.

Analysis and comparison of protein expression in vivo. To compare the properties of protein expression *in vivo*, the wild-type DsRed and the mutant were subcloned into the NcoI and HindIII sites of the pTrc99A plasmid. The maturation time and fluorescence intensity of each protein from *E. coli* cells were primarily compared on the solid plate of LB agar. To provide a more clearer comparison, *E. coli* cells were then cultivated in LB liquid media supplemented with or without inducer (1 mM IPTG) at 37 °C and 200 rpm. During cultivation, an aliquot of the culture broth (0.5 ml) was withdrawn and subjected to analysis by SDS–PAGE and fluorescence activated cell sorter (FACS, BD Biosciences).

Protein purification. Each *E. coli* strain harboring the plasmid with a gene of DsRed or FmRed was cultured in LB broth at 37 °C for 16 h. An aliquot (5 ml) of each culture was then inoculated into 500 ml of the same medium containing 50 μg/ml ampicillin and 0.1 mM IPTG. After the cultivation at 37 °C for 20 h, the cells were harvested by centrifugation at 5000*g* for 10 min. The resulting pellets were resuspended in 50 ml of sodium phosphate (0.3 M NaCl and pH 8.0) and lysed by sonication. The samples were centrifuged at 10,000*g* for 30 min. The supernatant was withdrawn and treated with cell debris remover (Sigma), followed by centrifugation at 18,000*g* for 30 min. The resulting supernatant was analyzed by SDS–PAGE to determine the expression level and solubility of the protein, and then further purified to apparent homogeneity using a metal affinity chromatograph as previously described, with slight modifications [19,20]. To prepare the metal affinity column, bare resin (chelating Sepharose, GE Healthcare) was used and the flow rate of the mobile phase was adjusted to 1 ml/min. The homogeneity of the purified protein was analyzed on a 10% acrylamide gel. The protein concentration was determined using the Bradford dye binding method with BSA solution as a standard (BioRad).

Table 1
The primer sequences used in this work.

Name	Sequence
F1	5′-ATGAATTCAGCCTCCTCCGAG AACGTCATCACC (deletion of start codon)
R1	5′-ATAAGCTTCAGGAACAGGTGGTGGCGGCCCTC (deletion of stop codon)
F2	5′-ATCCATGGNNN(NNN)$_{3-13}$AGCCTCCTCCG
R2	5′-ATAAGCTTTAA(NNN)$_{3-13}$CAGGAACAGG
FmRed F	5′-ATAGGATCCATGGGCTCCACAGC
FmRed R	5′-ATAGGATCCCCCGGGGGAGCC
Esterase F	5′-ATAGGATCCGTGCAGATTCAGGGTCATTAC
Esterase R	5′-ATAGGATCCTTACAGACAACCGGCCAAT

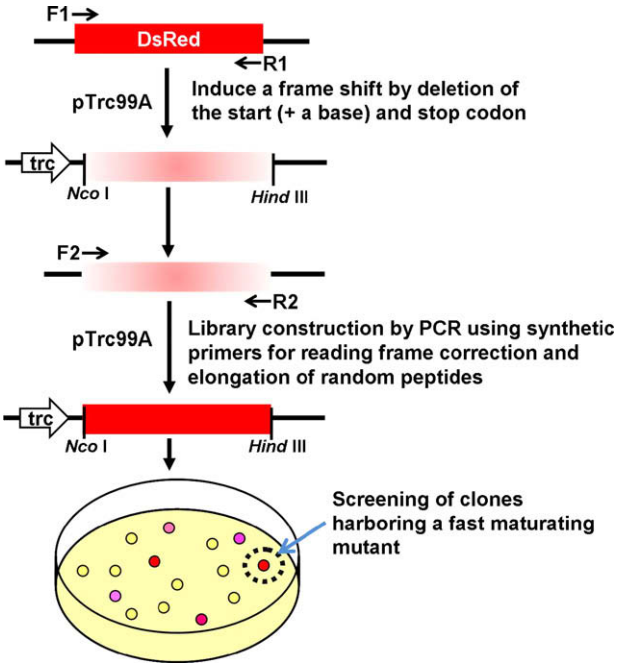

Fig. 1. Schematic representation of a typical procedure for random elongation mutagenesis on a functionally defective template. The defective DsRed gene was constructed by deletion of the start (plus a base) and stop codon as a starting template. For the functional salvage process, PCR was conducted as described in method section. The functionally salvaged clones emitting red fluorescence faster than the clone harboring intact DsRed gene were then screened from the library. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Characterization of the mutant protein FmRed. The oligomeric structure of the protein was determined on a fast protein liquid chromatography system with a gel filtration column (Superose TM12, GE Healthcare). The purified protein was loaded onto the column at a flow rate of 0.5 ml/min after complete equilibrium of the column with buffer (20 mM Tris, 150 mM NaCl, pH 8.0).

Emission and excitation maxima of the fluorescence proteins were determined by analyzing the purified enzymes using a fluorescence photometer. The quantum yield and photostability were calculated as described elsewhere [21,22].

Fusion ability and evaluation of FmRed as a reporter protein. The fusion ability of the mutant protein as a reporter was compared with that of the wild-type for functional fusion with other proteins. The fusion partner tested was maltose binding protein (MBP) from pMAL-c2X plasmid and an esterase previously reported [23]. To fuse the gene with MBP, each wild-type or mutant gene was subcloned into the EcoRI and HindIII sites of pMAL-c2X. Alternatively, the fused gene encoding both fluorescent protein and esterase was amplified by PCR using specific primers without linker (Table 1) and expressed according to a procedure described in a previous work [24]. The activity and fluorescence of the resulting constructs were analyzed in liquid and solid media and by native PAGE.

To verify its function as an indicator, the mutant FmRed was incorporated into the promoter trap vector pBGRI [25]. For this purpose, reporter DsRed facing the opposite direction to another reporter GFPuv without promoter, was substituted for FmRed by subcloning it into the EcoRI and HindIII site. The resulting plasmid was designated pBGRI-RF and its performance was compared with that of the pBGRI system.

Results and discussion

Library construction for screening of DsRed variants with rapid maturation times

The distinct properties of DsRed, including high quantum yield and photostability, are known to be closely related to its tetrameric structure surrounding the fluorophores [11]. This assumption can be explained by the finding that mutants with fast maturation times lose the above-mentioned features by converting their structure into monomer or dimer [12,14,16]. This indicates a need for a new method that can maintain the intrinsic oligomeric structure but introduce mutations related to the expression level and/or folding of DsRed for fast maturation, thereby adding an advantage to its innate superior features over the wild-type. In general, the sequence of amino acids that exist at the terminus is an important factor related to the expression, localization or folding landscape of a protein. Indeed, these N-terminus sequences are known to play an important role in interaction with chaperones and elongation of ribosome during the initial stage of expression [26,27]. Therefore, we devised a strategy to induce the elongation of a random oligopeptide at the terminus to construct protein variants that enable fast maturation. The system was arbitrary designed to randomly add only 3–10 amino acids without forming a secondary structure to avoid deformation of the tetrameric structure of DsRed due to amino acids at the elongated terminus. In addition, the functional expression of wild-type DsRed was suppressed by inducing a frame shift into the ORF of DsRed (Fig. 1).

The elongated mutant library, which consisted of about 2500 functional clones, was generated from a defective template by PCR using a randomly synthetic forward (also with a base for reading frame correction) and reverse primer. The constructed library was cultured for 24 h on LB plates, after which red fluorescence clones that showed a faster maturation time than that of the wild-type were selected as candidates. These clones were then sub-cultured on the same media and their maturation time and fluorescence intensity was analyzed repeatedly. Consequently, the clone (Red217) that showed the fastest maturation time was selected as the final candidate. The sequence analysis showed that the Red217 was a mutant with 3 extended amino acids (MGS) at the N-terminus and 5 extended amino acids (CGSPG) at the C-terminus. These sequences were found to be responsible for the fast maturation time because they resulted in a rapid maturation time of the wild-type DsRed when introduced into that protein, while protein variants from which these sequences were removed showed fluorescent features similar to those of the wild-type. Additionally, when an isolated recombinant plasmid from Red217 was retransformed into the freshly made host, it showed a distinct fluorescence after cultivation for 10–12 h. This maturation time was found to be similar to that of GFPuv cultured in the same plate as a control. Wild-type DsRed showed a fluctuation time of maturation, exhibiting weak read fluorescence after at least 20–24 h (40 h in some cases) (Fig. 2A). Based on these results, the selected variant was designated fast maturating red fluorescent protein (FmRed) and subjected to further analyses.

Analysis of expression patterns of FmRed in E. coli

The recombinant cells were grown in liquid medium without inducer for 24 h and with inducer for 2 h after induction with 1 mM IPTG. These cells were harvested and lysed to compare the expression levels and fluorescence between FmRed and DsRed. When both supernatants and precipitates were evaluated by SDS–PAGE, the expression levels of both proteins were quite similar under induced or non-induced conditions. These results provided clear evidence that the rapid maturation time of FmRed was primarily due to facilitation of the maturation process and not the increase in the expression level. When the same amounts of proteins were loaded onto the 10% native gel, FmRed exhibited a more distinct red band than DsRed (data not shown).

For an accurate comparison, each clone was cultured in liquid medium and analyzed using FACS for cells that were collected at different times. As shown in Fig. 2B, FmRed showed a remarkable increase in maturation time when compared with the wild-type under non-induced conditions. For FmRed clones, most of the cells exhibited fluorescence at about 20 h, but few cells of the DsRed clones exhibited a comparable fluorescence. When inducers were added, both clones exhibited measurable fluorescence at 7 h. However, the fluorescent potential of FmRed cells was shown to be superior to that of DsRed cells. Taken together, these findings confirmed that FmRed could be used to detect the expression in a relatively short time when compared with DsRed. This could indicate that FmRed has a wider range of applicability as indicators or reporters.

Purification and preliminary characterization of FmRed

The eight His and one Cys found in the primary structure of DsRed can form a weak ionic bond with metal ions by exposing themselves on the surface of the quaternary structure consisting of four subunits [19,20]. The basic structure of FmRed was expected to be similar to that of DsRed because the fundamental amino acid sequence of both proteins was identical except for the terminal regions, even though one additional Cys was incorporated into the N-terminus of FmRed. Based on this, the protein was isolated using a copper affinity column [20]. Both FmRed and DsRed with a purity of over 90% were isolated in a single step using this method (Fig. 3A) and further purified to apparent homogeneity using a gel filtration column.

Comparison of the same amount of purified DsRed with FmRed on a 10% native gel revealed that the migration rates of both proteins were slower than that of GFPuv (mainly monomer) loaded as a control. The resulting fluorescent bands of both proteins were exhibited at the same position. The difference in fluorescence of both proteins was insignificant, but FmRed exhibited red color[1] more distinctly than DsRed at the same concentration (Fig. 3B). From the gel filtration column chromatography, it was found that the elution times of DsRed and FmRed were identical corresponding to the size of a tetrameric structure.

The excitation and emission wavelengths of DsRed and FmRed were compared using a spectrofluorophotometer. For DsRed, the maximum excitation and emission wavelength was detected at 562 and 582 nm, respectively. Under the same conditions, the maximum wavelength for excitation and emission of FmRed was the same (Fig. 3C and D). As observed in a native gel, the fluorescence intensity of FmRed was about 1.2-fold higher than that of DsRed. The calculated value also showed that the quantum yield of FmRed (0.89) was higher than that of DsRed (0.78).

The photostability of both proteins was compared using two light sources, a spectrofluorophotometer and FACS. Light at a wavelength of 558–560 nm was applied to proteins, and the resulting fluorescence was then measured after a certain period of time. When the same wavelength was applied for up to 100 min, both the excitation (DsRed, 96.9%; FmRed, 92.9%) and emission intensity (DsRed, 89.1%; FmRed, 88.7%) decreased slightly when compared with the initial intensity. However, the difference between the two proteins was negligible. In addition, both proteins maintained

[1] For interpretation of color mentioned in this figure the reader is referred to the web version of the article.

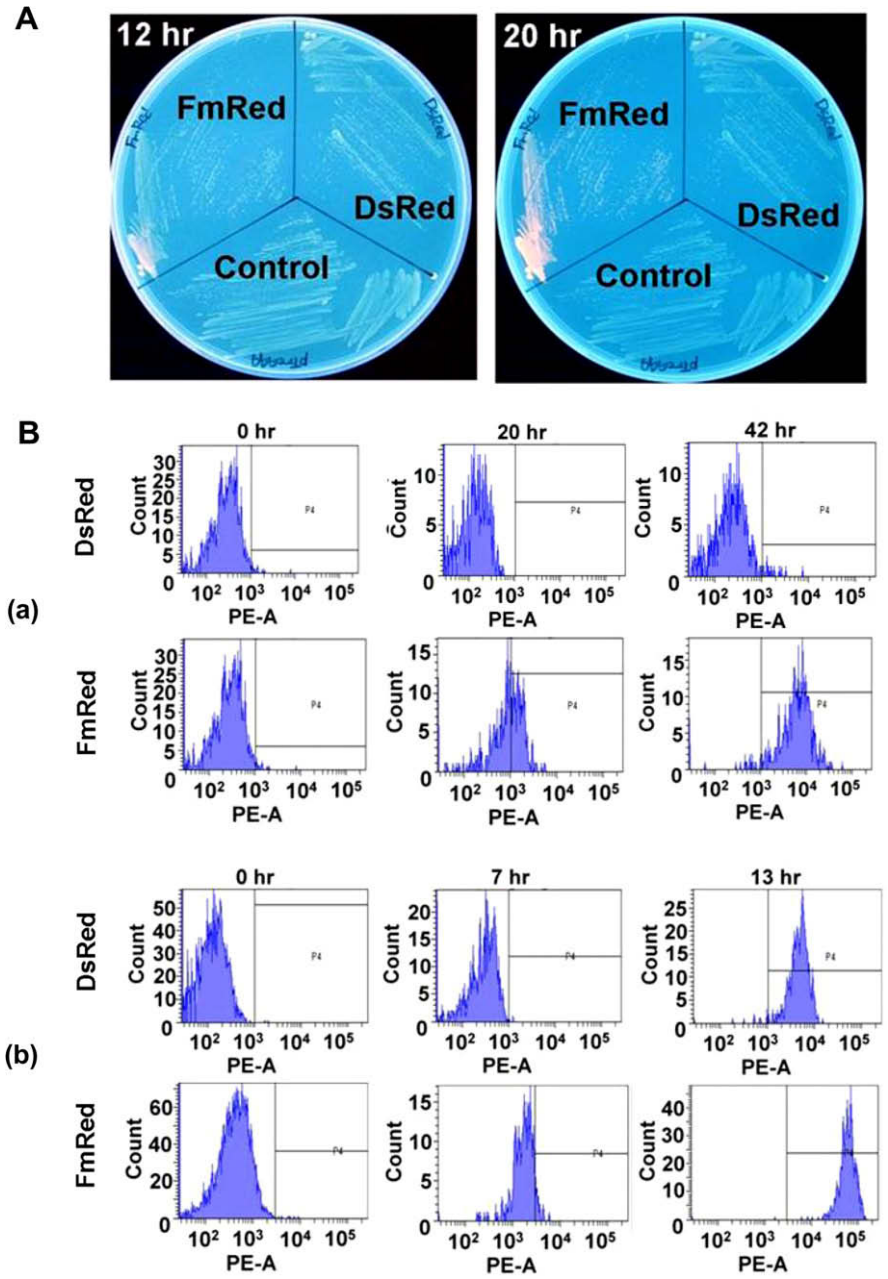

Fig. 2. Comparison of maturation time of FmRed and DsRed on solid LB media (A) or using a fluorescence activated cell sorter (B). Both mutant and wild-type clones were cultured on solid plate without inducer at 37 °C. Cells grown for FACS analyses were cultured in LB liquid media without (a) and with inducer (b) at 37 °C, then subjected to analysis. The inducer, IPTG (1 mM), was added to the culture medium when the optical density at 600 nm was about 0.4.

over 85% of their initial fluorescence after they were stored for more than 1 month at room temperature under daylight conditions.

Evaluation of potential as a reporter

In many cases, fluorescent proteins are expressed as fused with other proteins required for folding, localization or interacting reporters. Therefore, we attempted to fuse FmRed with other proteins to determine if it functioned in a fusion state as a reporter. To accomplish this, ORF of FmRed or DsRed was inserted into the EcoRI and HindIII sites of pMAL-c2X for fusion with MBP. As expected, FmRed in the fused state was functional and revealed a similar expression level (95–120%) to that of DsRed. When the same amount of protein was loaded onto a native gel, their fluorescence intensities were shown to be quite similar (Fig. 4A). When they were fused with esterase, the fused proteins were expressed functionally in fusion states with only slight differences in the amount of expression, even though this esterase was known to be poorly expressed due to the low solubility [23,24]. When the crude extracts of both proteins were loaded onto the native gel under the same conditions, FmRed more clearly exhibited red fluorescence (data not shown). Therefore, no decrease in the fusion ability of FmRed was observed in response to the addition of amino acids to both terminuses.

When DsRed was used as a trap reporter for the functional promoter, the maturation time was remarkably longer than that of other reporter (GFPuv), thus the screening time was also longer than expected [25]. In fact, for pBGRI plasmid (a trap vector that was constructed to face each other using two fluorescent proteins,

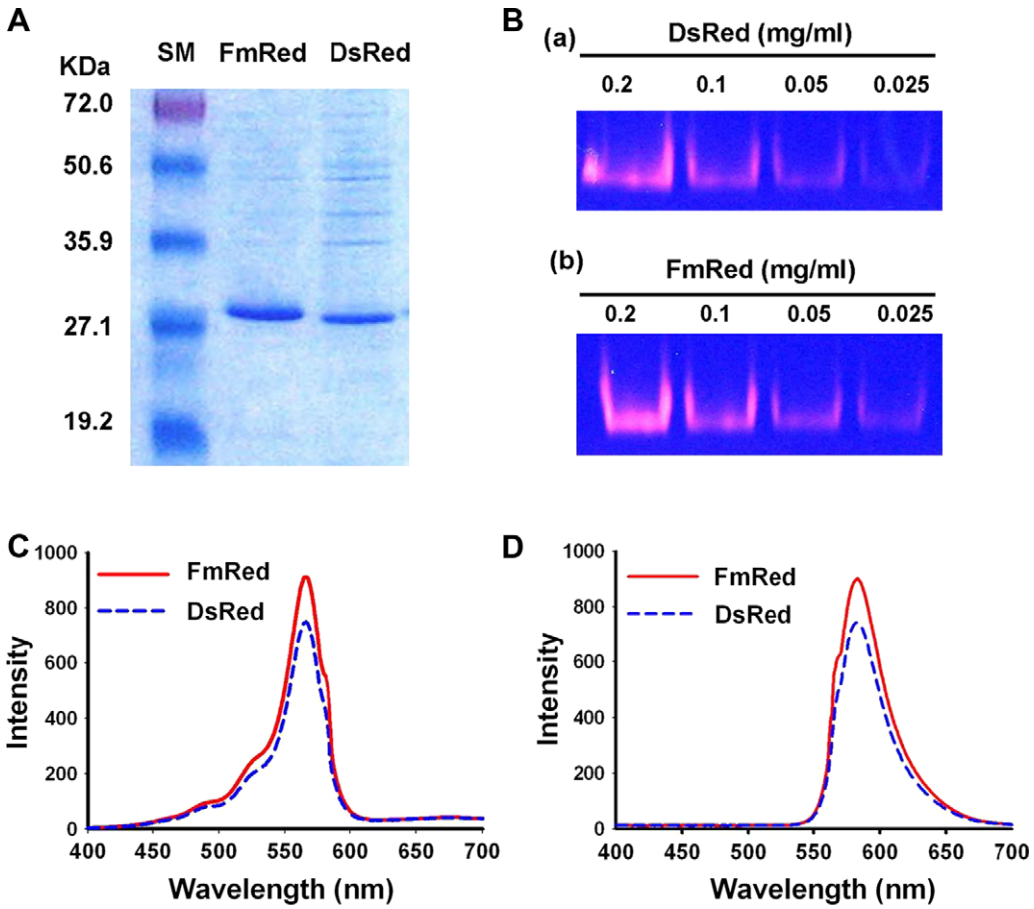

Fig. 3. PAGE analysis and fluorescence spectra of FmRed and DsRed. SDS–PAGE analysis (A) and comparison of the fluorescence intensity (B) on a 10% native gel. The concentration of each protein was adjusted to be approximately 0.2 mg/ml and the indicated amount was then loaded onto a gel. After gel electrophoresis, two protein bands were excited at 558 nm. Comparison of excitation (C) and emission (D) wavelengths of DsRed and FmRed. Each protein was subjected to scanning at the same concentration under standard conditions.

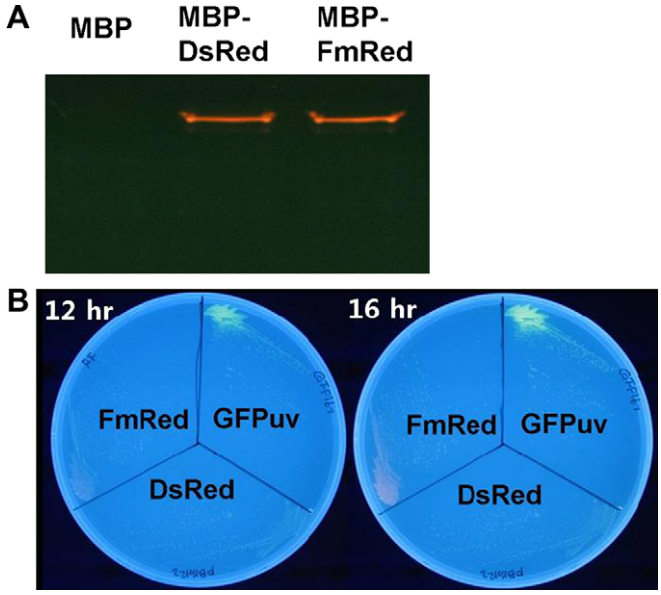

Fig. 4. The fusion ability and rapid maturation time of FmRed as a reporter. (A) Typical fusion ability of FmRed with monomeric MBP. The fusion proteins were visualized by excitation after gel electrophoresis on a 10% native gel under non-reducing conditions. (B) Rapid maturation time of FmRed as a reporter for functional promoter *in vivo*. The same promoter was inserted into the upstream region of FmRed, GFPuv and DsRed under promoter trap conditions generally employed. GFPuv and FmRed reported a promoter function simultaneously, whereas DsRed did not report promoter function in this time.

GFPuv and DsRed, as reporters), functional promoters trapped in the direction of the GFP reporter could be discriminated within 12–14 h. However, promoters functioning by being trapped in the opposite direction needed more than 24–48 h for the expression of the reporter DsRed. In addition, when the colony size was small or the trapped promoter had weak function, more than 48 h was necessary to detect the fluorescence. To solve this problem, the functionality of the reporter was compared by introducing promoter sequences after replacing DsRed with FmRed. The results revealed that the expression time of both reporters (GFPuv and FmRed) was quite similar, when inserted the same promoter into the upstream of both reporters (Fig. 4B). These findings indicate that rapid promoter trapping was possible using this system when screening for a new promoter.

We confirmed that a DsRed variant, FmRed, with rapid maturation time could be constructed using a relatively simple principle. Additionally, the FmRed and DsRed were shown to exhibit the same excitation and emission wavelengths, as well as similar photostability. FmRed also showed superior features when compared to the wild-type with respect to quantum yield and fusion ability. In these contexts, it is believed that this mutant has advantageous protein folding due to the addition of several extended amino acids or the reduction in the degree of quenching against the environment. We analyzed the structure of proteins to the level of 1.5 Å to trace this structural change, but almost all of the structures were found to overlap. Unfortunately, exact structural differences could not be determined in the FmRed or wild-type due to non-fixed structures in both terminuses [28]. However, the binding

of the proteins to the metal affinity column, FACS data, protein expression and deletion and swapping of both extended terminuses confirmed that the elongated amino acids may facilitate protein folding or association of the quaternary structure, thus resulting in a fast maturation time.

Acknowledgments

This work was partly supported by a research Grant (M10866020003-08N6602-00311) and the second stage BK21 project, Republic of Korea.

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