Engineering an Enhanced, Thermostable, Monomeric Bacterial Luciferase Gene As a Reporter in Plant Protoplasts

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Abstract

The application of the luxCDABE operon of the bioluminescent bacterium Photorhabdus luminescens as a reporter has been published for bacteria, yeast and mammalian cells. We report here the optimization of fused luxAB (the bacterial luciferase heterodimeric enzyme) expression, quantum yield and its application as a reporter gene in plant protoplasts. The fused luxAB gene was mutated by error prone PCR or chemical mutagenesis and screened for enhanced luciferase activity utilizing decanal as substrate. Positive luxAB mutants with superior quantum yield were subsequently shuffled by DNase I digestion and PCR assembly for generation of recombinants with additional increases in luciferase activity in bacteria. The coding sequence of the best recombinant, called eluxAB, was then optimized further to conform to Arabidopsis (Arabidopsis thaliana) codon usage. A plant expression vector of the final, optimized eluxAB gene (opt-eluxAB) was constructed and transformed into protoplasts of Arabidopsis and maize (Zea mays). Luciferase activity was dramatically increased for opt-eluxAB compared to the original luxAB in Arabidopsis and maize cells. The opt-eluxAB driven by two copies of the 35S promoter expresses significantly higher than that driven by a single copy. These results indicate that the eluxAB gene can be used as a reporter in plant protoplasts. To our knowledge, this is the first report to engineer the bacterium Photorhabdus luminescens luciferase luxAB as a reporter by directed evolution which paved the way for further improving the luxAB reporter in the future.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its supporting information files, and the nucleic acid sequence of opt-eluxAB is available from the GenBank database (accession number: KJ957766).

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Introduction

Reporter genes are valuable tools for promoter analysis [1], imaging of gene expression [2,3], detecting xenobiotic compounds [4], protein subcellular localization [5], protein-protein interactions [6], and discovery of genes as potential targets for disease [7]. Although the current reporter gene assay systems, such as GFP, firefly luciferase (Fluc), LacZ, CAT and GUS have greatly advanced molecular biology research, all except the bacterial luciferase-based reporter has been published for bacteria, yeast and mammalian cells. We report here the optimization of fused luxAB (the bacterial luciferase heterodimeric enzyme) expression, quantum yield and its application as a reporter gene in plant protoplasts. The fused luxAB gene was mutated by error prone PCR or chemical mutagenesis and screened for enhanced luciferase activity utilizing decanal as substrate. Positive luxAB mutants with superior quantum yield were subsequently shuffled by DNase I digestion and PCR assembly for generation of recombinants with additional increases in luciferase activity in bacteria. The coding sequence of the best recombinant, called eluxAB, was then optimized further to conform to Arabidopsis (Arabidopsis thaliana) codon usage. A plant expression vector of the final, optimized eluxAB gene (opt-eluxAB) was constructed and transformed into protoplasts of Arabidopsis and maize (Zea mays). Luciferase activity was dramatically increased for opt-eluxAB compared to the original luxAB in Arabidopsis and maize cells. Theopt-eluxAB driven by two copies of the 35S promoter expresses significantly higher than that driven by a single copy. These results indicate that the eluxAB gene can be used as a reporter in plant protoplasts. To our knowledge, this is the first report to engineer the bacterium Photorhabdus luminescens luciferase luxAB as a reporter by directed evolution which paved the way for further improving the luxAB reporter in the future.
NADPH-FMN oxidoreductase [13], but substantial light production by the lux reaction was demonstrated to occur only in the lower eukaryote Saccharomyces cerevisiae. Recently, Close et al. [14] have reported a major advance in developing an autonomous mammalian light production system by expression of all five genes (luxCDABE) of the lux operon simultaneously in a mammalian background. However, it should be noted that the bioluminescent signal from the human-optimized lux cassette was also relatively weak (several orders of magnitude lower than that of the Fluc reporter). Thus, it is clear that the bacterial luciferase could potentially benefit from further optimization to reach its full potential as a eukaryotic reporter.

In this research, to simplify the usage of the bacterial luciferase as a reporter protein in eukaryotic cells, we have constructed a new fused luxAB gene encoding a fusion protein as a reporter. Furthermore, we show that the activity of the fused bacterial luciferase was significantly increased by several steps of directed evolution, compared to the wild-type fused luxAB enzyme. The enhanced luxAB fusion gene has been successfully expressed in protoplasts of Arabidopsis and maize. These results show promise toward the potential development of a eukaryotic reporter system allowing in vivo real-time detection in the future.

Materials and Methods

Bacterial strains and vectors

Bacterial strains and plasmids used in this study are listed in Table S1. Escherichia coli and Corynebacterium glutamicum strains were cultured in Luria-Bertani (LB) broth or on LB plates at 37°C and 30°C, respectively. Yersinia pseudotuberculosis strains were cultured in Yersinia-Luria-Bertani (YLB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or on YLB plates at 26°C [15]. These bacterial strains represent a wide spectrum of possible prokaryotic organisms in which a lux reporter system might be useful. When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml for E. coli and Y. pseudotuberculosis; chloramphenicol, 20 μg/ml for E. coli and 10 μg/ml for C. glutamicum; kanamycin, 50 μg/ml for E. coli and Y. pseudotuberculosis, and 25 μg/ml for C. glutamicum; nalidixic acid, 30 μg/ml for C. glutamicum.

The enhanced luxAB reporter was also tested in eukaryotic cells represented by the common baker’s yeast Saccharomyces cerevisiae and in leaf protoplasts of Arabidopsis thaliana and Zea mays (maize). YPD liquid medium (1% yeast extract, 2% peptone, 2% glucose) was used for routine growth of yeast. Plasmid p425GPD used in this study contained the LEU2 selectable marker and a glyceraldehyde 3-phosphate dehydrogenase (GPD) promoter which controls the expression of exogenous proteins. S. cerevisiae strains harboring plasmids p425GPD were propagated in synthetic complete (SC) minimal media lacking leucine and containing 0.67% yeast nitrogen base (Invitrogen, Carlsbad, CA, USA) [16].

The method of protoplast preparation was optimized from previously published methodology [17]. Fully expanded leaves from 4 weeks old Arabidopsis (ecotype Columbia), growing under neutral or short photoperiods (12–13 h light or less) of 50–150 μE, were used to prepare protoplasts. Leaf strips (0.5 mm) were cut length-wise and vacuum infiltrated with digestion solution (1% cellulase, 0.2% macerozyme, 0.1% BSA, 0.035% β-mercaptoethanol, 10 mM CaCl2·2H2O, 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7) for 30 min at −80 kPa pressure. The cells were then incubated on a rotary shaker (40 rpm) at 23°C for 1.5 h in the dark, followed by swirling at 80 rpm for 1 min. The digested cells were filtered through 75 μm mesh filters. The protoplasts were collected by centrifugation at 300 rpm for 5 min, washed once with digestion buffer, before being re-suspended with MMG solution (0.4 M mannitol; 15 mM MgCl2·6 mM MES, pH 5.8).

For maize protoplast preparation as published [18], three day old, light-grown B5 seedlings were moved to the dark at 25°C until the second leaf was about 10–15 cm long. The central 6–8 cm of the second leaf were cut into 0.5 mm strips length-wise and vacuum infiltrated with digestion solution (1% cellulase, 0.3% macerozyme, 0.1% BSA, 0.05% β-mercaptoethanol, 1 mM CaCl2·2H2O, 0.6 M mannitol, 10 mM KCl, 20 mM MES, pH 5.7) for 50 min with a pressure of −80 kPa. The digested cells were then incubated on a rotary shaker (40 rpm) at 25°C for 2 h in the dark, followed by swirling at 80 rpm for 5 min. Cells were then filtered through 45 μm mesh filters. Protoplast collection, washing, and resuspension were as described above for Arabidopsis.

Construction of plasmids

Primers used in this study are listed in Table S2. The entire coding region of the cloned pDM4-luxCDABE was used as a template to amplify a fused luxAB gene by overlap PCR generated using the gene SOEing method described by Horton et al. [19]. In the first round of PCR, the 1083 bp luxA gene and the 984-bp luxB gene PCR products were amplified using primer pairs luxA-XbaI-F/luxA-L15-R and luxB-L15-F/luxB-BglII-R. The resulting PCR products were used as template in the second round of PCR with luxA-XbaI-F and luxB-BglII-R as primers. The 2.1 kb PCR product was recovered from an agarose gel and digested with XbaI/BglIII and then ligated into XbaI/BamHI digested pBluescript II KS(+) to create the luxAB vector. The lac promoter in plasmid pUT18 and T6SS4 promoter in the enteric pathogen Y. pseudotuberculosis [20] were amplified with primer pairs pLac-SacI-F/pLac-XbaI-R and pT6SS4-SacI-F/pT6SS4-XbaI-R, respectively, and digested with SacI/XbaI and ligated into the same restriction enzyme digested pBS-luxAB and pBluescript II KS(+)vector, resulting in plasmids pBS-pLac-luxAB, pBS-pLac, pBS-pT6SS4-luxAB and pBS-pT6SS4. To produce the expression plasmid pET28a-luxAB and pET28a-luxAB, the bicistronic luxA+B gene amplified from pDM4-luxCDABE, and the luxAB gene fusion amplified from pBS-pLac-luxAB using the primers luxA-BamHI/P-luxB-Sall-R were cloned into compatible sites within the pET28a vector downstream of the T7 promoter, respectively.

The enhanced luxAB (euxAB) generated by DNA shuffling described below was amplified using luxA-XbaI-F and luxB-BglII-R primers and digested with restriction enzymes XbaI/BglIII and ligated into pBS-pLac digested with XbaI and BamHI. After transformation and selection on plates with ampicillin, the plasmid pBS-pLac-euxAB was obtained. Similarly, the plasmid pXMJ19-pTac::luxAB was constructed by inserting the luxAB gene downstream of the tac promoter in pXMJ19 plasmid with the unique restriction sites BamHI and SalI, and the plasmid pBS-pT6SS4::luxAB was constructed by inserting the luxAB gene downstream of the T6SS4 promoter in pBluescript II KS(+). Plasmids pBS-pLac-euxAB, pXMJ19-pTac::luxAB and pBS-pT6SS4::luxAB were electroporated into E. coli DH5α, C. glutamicum RES167 and Y. pseudotuberculosis YPPIII cells, respectively.

The luxAB, euxAB and codon optimized euxAB (opt-euxAB) described below were introduced into the plasmids p425GPD with the restriction sites BamHI at the 5’ end and SalI at the 3’ end to produce the plasmid p425GPD-luxAB, p425GPD-euxAB and p425GPD-opt-euxAB. These three plasmids were introduced into S. cerevisiae strains and selected on SC minimal selective media.

The plant expression vector was constructed as follows: For plasmid a1 construction, the euchB fragment was generated by
PCR from pBS-pLac:luxAB using a pair of primers luxB-NcoI-F and eluxB-R, the nos fragment was PCR-amplified using a pair of primers nos-F and nos-Sall-R from the pGL3-dual luciferase vector [20], then the eluxB-nos fragment was amplified by overlapping extension PCR using a pair of primers eluxB-NcoI-F and nos-Sall-R and cloned into the corresponding sites of the dual luciferase vector to replace the Fluc ORF. The designed primers luxA-NheI-F and eluxA-XbaI-R, were used to PCR-amplify the eluxA fragment from pBS-pLac:eluxAB, after digested by NheI and XbaI, the fragment was cloned into the dual luciferase vector to replace another Remilla luciferase (Rluc) ORF. For plasmid a2 construction, the opt-eluxB fragment was amplified using primers opt-eluxB-NcoI-F and opt-eluxB-R, from the pUC57-Simple plasmid which includes the optimized sequence of eluxB, the same strategy was applied to obtain the opt-eluxB-Nos fragment, the primers used for amplification of opt-eluxB-nos were eluxB-NcoI-F and nos-Sall-R. The opt-eluxA fragment was amplified from pUC57-Simple plasmid by PCR-amplify using primers opt-eluxA-NheI-F and opt-eluxA-XbaI-R. The opt-eluxA fragment was cloned into a1 vector to replace the eluxA fragment. For plasmid a3 construction, the luxAB fragment was amplified from pBS-pLac:luxAB using primers eluxA-NheI-F and eluxB-XbaI-R. The PCR products and the a1 vector were digested by NheI and XbaI, the fragment was cloned into the dual luciferase vector to replace another Remilla luciferase (Rluc) ORF. For plasmid a4 construction, the opt-eluxB fragment was amplified using primers opt-eluxB-NcoI-F and opt-eluxB-R, from the pUC57-Simple plasmid and then the amplified fragment was cloned into the dual luciferase vector to replace the Fluc ORF. The primers used for amplification of opt-eluxB-nos were eluxB-NcoI-F and nos-Sall-R. The opt-eluxA fragment was amplified from pUC57-Simple plasmid by PCR-amplify using primers opt-eluxA-NheI-F and eluxB-XbaI-R. The PCR products and the a1 vector were digested by NheI and XbaI, the fragment was cloned into the dual luciferase vector to replace another Remilla luciferase (Rluc) ORF. The PCR products and the a1 vector were digested by NheI and XbaI, the fragment was cloned into the dual luciferase vector to replace another Remilla luciferase (Rluc) ORF.

Random mutagenesis

To increase the luciferase activity of luxAB, random mutations were introduced with either three rounds of error-prone PCR or two rounds of chemical mutagenesis. For error-prone PCR, random mutations were introduced into the amplification product during PCR by using the plasmid pBS-pLac:luxAB as template and oligos luxA-XbaI-F and luxB-BamHI-R as primers (Table S2). The mutagenesis frequency was controlled to the desired level (two to four amino acid substitutions per kb) by altering the concentration of the template and the cycle numbers. For a typical reaction, 0.2 ng template DNA were added to 100 μl of the error-prone PCR system (0.2 mM of each dATP and dTTP, 1.0 mM of each dCTP and dGTP, 7 mM MgCl2, 50 mM KCl, 1 mM MnCl2, 10 mM Tris-HCl (pH 8.3), 0.3 μM of primer luxA-XbaI-F and luxB-BamHI-R, 5 U Taq DNA polymerase) and program subjected to 25 PCR cycles [22,23]. Chemical mutagenesis was performed by treating the pBS-pLac:luxAB plasmid DNA with hydroxylamine. In this instance, 5 μg of plasmid DNA was incubated in a 100 μl reaction mixture containing 0.5 M hydroxylamine, 0.5 mM Na2EDTA, and 5 mM Tris-HCl (pH 6.0) for 10 h at 37°C. The DNA was precipitated and washed thoroughly with 70% ethanol, and redissolved in ddH2O [24,25]. The mutated luxAB produced in both error-prone PCR and chemical mutagenesis were recovered by digestion with XbaI/BamHI, and ligated into pBS-pLac digested with the same enzymes, resulting in the pBS-pLac:luxAB* mutant library and was transformed into the E. coli strain DH5α for storage and further screening.

DNA shuffling and screen assay

The substrates for the shuffling reaction were nine mutant plasmids (pBS-pLac:luxAB*) generated from the prior error prone PCR or chemical mutagenesis that showed enhanced lux quantum yield. These nine templates were each used to amplify a 2.1-kb PCR product using primers luxA-XbaI-F and luxB-BamHI-R. After the amplicons were purified, about 4 μg was digested with 0.15 unit of Dnase I (Promega) in 100 μl of 50 mM Tris-HCl (pH 7.4), 1 mM MgCl2, for 10–20 min at room temperature. The digestion was loaded on a 1% agarose gel and the smear from 50 to 100 bp was purified by electrophoresis onto DE81 ion-exchange paper (Whatman) and eluted with 1 M NaCl, then precipitated by ethanol. The purified fragments were redissolved in a PCR reaction mixture at a concentration of 30 ng/μl and no primers were added at this point. Taq DNA polymerase (TaKaRa, Dalian, China) was used at 2.5 units, and ddH2O added to a total volume of 100 μl and subjected to 40–45 PCR cycles. The PCR products were purified by gel extraction. After 1:40 dilution of these primerless PCR products into a second PCR reaction mixture, 0.3 μM of each primer luxA-XbaI-F and luxB-BamHI-R were added and 15 additional PCR cycles were applied to typically obtain a single amplicon of the expected, 2.1 kb size [26,27].

Plasmid pBS-pLac:luxAB constructs from the random mutagenesis or subsequent Dnase I recombination library, were transformed into E. coli DH5α cells and grown at 37°C to an OD600 of 0.8 in LB medium with ampicillin (100 μg/ml). 200 μl of each culture was resuspended and placed into a well of a 96-well black plate, before the rapid addition of 2 μl decanal in each well [28]. The lux activity of these assays was estimated with an Infinite M200 PRO (TECAN). Strains which had the greatest lumines-

exhaustively dialyzed against TBS to remove imidazole. The molecular weight and concentrations of the purified proteins were also analyzed using 12% SDS-PAGE gels [21].
cence activity, relative to the original construct, were called *eluxAB* (enhanced *luxAB*) and served as templates for the next experiment.

**Effects of pH and temperature on enhanced luciferase activity**

The optimum pH of the best *eluxAB* enzyme’s activity was determined within a pH range of 4–12. The buffers were as follows: acetate (pH 4–5); phosphate (pH 6–8); glycine-NaOH (pH 9–12) [29]. The pH optimum was measured by incubating the cells in different buffers in the pH range of 4–12 at 37°C using decanal as substrate. The effect of temperature on enzyme activity was measured by incubating the enzyme from 20 to 60°C for 30 min at the previously determined pH optimum of 10. The activities of *eluxAB* were measured under standard assay conditions.

**Complete codon optimization of *eluxAB***

The *eluxAB* sequence was codon optimized according to *Arabidopsis thaliana* codon usage data from the information tabulated in GenBank. The overall ratio for usage of each codon within the *eluxAB* gene was altered to more closely match *Arabidopsis* usage. The OptimumGene™ algorithm optimized a variety of parameters critical to the efficiency of gene expression, including but not limited to codon usage bias adjustment; GC content adjustment; restriction enzymes and CIS-acting elements; removal of repeat sequences and mRNA secondary structure mitigation. All efforts focused on defining a single gene sequence that would attain the highest possible level of expression in the plant cell. Once optimized, the genes were synthesized by GenScript Co., Ltd (Nanjing, China) and polyacrylamide gel electrophoresis (PAGE) purified to ensure full-length products. These optimized DNA fragments were ligated using TA cloning and the sequence was verified in each case [14,30]. The codon optimized *eluxAB* was named opt-*eluxAB* and the optimized bicistronic *eluxA*+*B* was named opt-*eluxA*+*B*, respectively.

**PEG mediated transformation of *Arabidopsis* and maize protoplasts**

PEG-Ca mediated transformation followed a previously published protocol [17] with some modifications. The protoplasts of *Arabidopsis* were suspended in 100 μl of MMG solution at a density of 10^7 (and the maize protoplasts at 5 x 10^6) cells/ml in a 2 ml round-bottom centrifuge tube containing 15 μg of DNA and mixed well. An equal volume of 40% PEG solution (40% PEG4000, 0.2 M mannitol and 100 mM CaCl_2·2H_2O) was added to the protoplast-DNA mix drop-wise with gentle shaking for 25 min. The mixture was diluted with 440 μl of cell culture solution which, depending on the species, was: (*Arabidopsis*: 134 mM NaCl, 125 mM CaCl_2·2H_2O, 5 mM KCl, 2 mM MES pH 5.7) or (maize: 0.6 M mannitol, 4 mM KCl, 4 mM MES, pH 5.7). The protoplasts were then centrifuged at 112 g for 5 min, the supernatant removed, the protoplasts re-suspended in 1 ml cell culture solution, and plated in the wells of 24-well tissue culture plates (NEST Biotechnology, China). The protoplasts were incubated at 23°C (*Arabidopsis*) or 25°C (maize) for 18 h prior to harvest for luciferase activity determination.

**Luciferase activity detection**

*E. coli* cells transformed with pBS-pLuc::luxAB or pBS-pLuc::eluxAB constructs were grown at 37°C to an OD600 of 1.8 in LB broth with chloramphenicol (20 μg/ml). *Y. pseudotuberculosis* cells transformed with pBS-pT6SS::luxAB or pBS-pT6SS::eluxAB were grown at 26°C to an OD600 of 0.8 in YLB medium with ampicillin (100 μg/ml). Yeast cells containing plasmids p425GPD::luxAB and p425GPD::eluxAB were grown at 30°C to an OD600 of 1.0 in synthetic complete (SC) minimal medium. 100 μl of each resuspended culture was transferred into a 1.5 ml microcentrifuge tube and luminescence reactions were initiated by the addition of decanal. The activity determinations of the fused *luxAB* and enhanced mutants were performed on the GloMax 20/20 Luminometer (Promega) as described [31]. Protoplasts from one well of a 24 well plate were recovered by aspiration, placed in an Eppendorf tube and harvested by centrifugation at 1300 rpm for 5 min before being resuspended in 20 μl of lysis buffer (100 mM potassium phosphate, 1 mM dithiothreitol, pH 7.8). The cells were subjected to three rounds of freezing in liquid nitrogen for 30 sec, followed by thawing in a 37°C water bath for 3 min as described [14]. 200 μl detection solution (0.2 mM NADPH, 90 μM FMN, 1% (v/v) decanal) was added, vortexed, and the solution assessed for luciferase activity. After the substrate was added, the luminescence activity was measured.

**Statistical analysis**

Statistical analysis was conducted using GraphPad Prism 5.01 software.

**Results**

**Expression of the fused and non-fused *luxA* and *luxB* gene in bacteria**

Cell extracts from *E. coli* BL21(DE3) harboring pET28a-*luxAB* possessed a 78-kDa *luxAB* fusion protein clearly present in IPTG induced bacteria when compared with the non-induced lane (Figs. 1A&B). Extracts from induced BL21(DE3) cells possessing the non-fused pET28a-*luxA*+*B* construct show a 40 kDa subunit and a 36 kD β subunit polypeptide observed in the SDS-PAGE gel (Figs. 1A&B). The *luxAB* and *luxA*+*B* genes expressed under the T6SS4 promoter in *Y. pseudotuberculosis* cells demonstrated that the luminescence intensity of *luxAB* is about 0.002% that of the *luxA*+*B* (Figs. 1C&D). These data indicate a potential of improving the *luxAB* activity by random mutagenesis and directed evolution.

**Error-prone PCR, chemical mutagenesis and DNA shuffling**

Following mutagenesis, nine *luxAB* mutants were picked that had been individually verified to result in higher luciferase activity than the original *luxAB* (Figs. 2A&B). These genes, carrying positive mutations, were selected as a pool to initiate DNA shuffling. After DNase I digestion, 50 to 100 bp fragments were recovered to ensure the best recombination frequency. The *luxAB* mutants were then re-assembled by PCR (Fig. 3). After two rounds of DNA shuffling, about two thousand mutant colonies were screened. One mutant was identified (*eluxAB*, enhanced *luxAB*) with remarkably greater luciferase activity than all others tested. Compared to the sequence of wild type *luxAB*, there were six mutation sites in the *eluxAB* gene, five of which resulted in amino acid changes (Table 1). Of these five, four were in the *luxA* subunit.
Protein modeling

The crystal structure of the Vibrio harveyi luciferase luxA and luxB holoenzyme has been resolved to 1.5 Å (1 Å = 0.1 nm) under low salt conditions [32]. Comparison of sequences showed that LuxA and LuxB from P. luminescens shared 84% and 59% amino acid identities with LuxA and LuxB from V. harveyi, respectively (Fig. S1). To investigate why the observed mutations of eluxAB enhanced luciferase activity, homology-based model structures of luxA and luxB from P. luminescens were obtained using structures of V. harveyi luciferase using the Swiss protein-modeling server [33]. Based on the model structures, none of the mutations directly affect the active site (FMN binding sites) or substrate binding residues [residues 166–233] [23]. However, the substitution of H234Y (Fig. 4) was in close proximity to the substrate-binding pockets and might indirectly affect the binding of the substrate. While the other mutation sites (Table 1) probably had no effect or only subtly changed the conformation of the active site and/or substrate specificity.

Characterization of the eluxAB

The optimal pH for eluxAB activity was distinctly centered around 10 using decanal as a substrate, with a rapid decline on either side of this value (the enzyme exhibited only 40% activity at pH 9.0 and 11.0; Fig. 5A). The temperature optimum of eluxAB at pH 10 was 40°C (Fig. 5B). Considerable activity was retained also at 30°C and 50°C after an incubation time of 30 min after which time residual activities were reduced to approximately 70% and 50%, respectively. Studies on the effect of pH and thermostability on the eluxAB clearly indicated that this enzyme is the best candidate for a wide range of applications where alkaline conditions prevail.

Use of eluxAB as a reporter in E. coli, C. glutamicum, Y. pseudotuberculosis and Yeast cells

The luxAB and eluxAB were constructed in pXMJ19-pTac, pBS-pLac, pBS-pT6SS4, and p425GPD vectors under the control of their respective promoters. The plasmids pXMJ19-pTac::luxAB and pXMJ19-pTac::eluxAB, when expressed in the Gram-positive bacterium C. glutamicum resulted in an luxAB luminescence approximately 4-fold greater than that of luxAB (Fig. 6). Similarly, the plasmids pBS-pLac::luxAB and pBS-pLac::eluxAB were transformed into E. coli cells and the luminescence was detected. Enzyme activity of eluxAB is about five-fold higher than luxAB (Fig. 6). The greatest increase in luminescence was for Y. pseudotuberculosis cells where pBS-pT6SS4::eluxAB registered a quantum yield 40 fold greater than that of pBS-pT6SS4::luxAB (Fig. 6). Saccharomyces cerevisiae cells harboring p425GPD-luxAB or p425GPD-eluxAB had an eluxAB activity approaching 10-fold that of luxAB (Figs. 7A&B). Thus we conclude that eluxAB can be used as a robust reporter in a variety of prokaryotic and simple eukaryotic species.

Codon optimization of the eluxAB gene for Arabidopsis expression and use of opt-eluxAB as a reporter in Arabidopsis and maize protoplasts

In order to maximize the expression of eluxAB in Arabidopsis and maize protoplasts, the codons of eluxAB were optimized according to Arabidopsis codon bias (Fig. S2). The optimized eluxAB gene opt-eluxAB (Genbank accession number: KJ957766) was also transformed into S. cerevisiae cells in vector p425GPD. Surprisingly, compared to the luciferase activity of eluxAB and luxAB in yeast, the quantum yield of opt-eluxAB was least (Fig. 7B). The possible reason was that Arabidopsis codon biased
opt-eluXAB wasn’t suitable for expression in yeast cells. The monocistronic eluXAB, opt-eluXAB and the bicistronic eluXAB+B, opt-eluXAB+B were constructed in the pGL3 basic vector under the control of two copies of the CaMV35S promoter (Fig. 8A). The monocistronic opt-eluXAB cassette was also placed under the control of a single copy of the CaMV35S promoter. The plant expression vectors were transformed into Arabidopsis and maize protoplasts using PEG and, following incubation, the luminescence was detected. Compared to the non-codon-optimized eluXAB+B or eluXAB genes, the opt-eluXAB+B or opt-eluXAB showed dramatically increased luminescence activity in Arabidopsis cells (Fig. 8B). When these mono- or bi-cistronic eluXAB+B or opt-eluXAB vectors were tested in maize protoplasts, opt-eluXAB luminescence was approximately 3 fold greater than that of the other three constructs (Fig. 8C) although it was 6 times less than the same construct in Arabidopsis protoplasts (compare Figs. 8B&C for which it was designed. To further determine if opt-eluXAB could be applied as a reporter gene in Arabidopsis cells, the opt-eluXAB+B gene was driven by one copy or two copies of 35S promoter and transformed into Arabidopsis protoplasts. The luminescence of opt-eluXAB+B under the regulation of two copies of 35S promoter was significantly greater than that produced when under the control of one copy of the 35S promoter (Fig. 8D).

To compare the luminescence activity of opt-eluXAB with Rluc or Fluc reporters, luminescence activities were compared in Arabidopsis and maize protoplast cells transformed with Rluc and Fluc reporter vector (pGL3-dual luciferase) or opt-eluXAB reporter vector (pGL3-opt-eluXAB). The luminescence activity of opt-eluXAB is comparable to Rluc, though it is significantly lower than Fluc (Table S3). This result indicates that the bacterial luciferase has the potential to be applied as a new reporter in plant cells. Since the toxicity of decanal to Arabidopsis protoplast cells was negligible at the detection concentration (1%, Fig. S8), the opt-eluXAB reporter can be used for real-time detection in viable plant protoplast cells.

**Discussion**

Engineering of an enhanced, monomeric bacterial luciferase eluXAB gene from *P. luminescens* as a reporter in prokaryotes and eukaryotes provides researchers a unique tool allowing real-time monitoring of exogenous gene expression from whole cells without lysis. A fused luxAB gene from *Photorhabdus luminescens* has been cloned and successfully expressed in yeast [16] and mammalian cells [14,30] previously, but the luminescence intensity was measured without the need to generate, and yet exclude, excitation wavelengths, avoiding some problems such as photonic bleaching and high background [36,37]. Third, previous work with lux genes isolated from *P. luminescens* has demonstrated that the luciferase is thermostable at temperatures as high as 45°C [38], which is a greater thermal stability than that of the *V. harveyi* or *V. fischeri* luciferase enzymes. Therefore, the bacterial luciferase system seems more suitable to the study of environmental or developmental changes in gene expression. High activity at alkaline pH is normally regarded as an appreciable character for the industrial production [29].

Directed evolution has become a powerful strategy for improving the activity of enzymes in a targeted manner coupling various methods such as error-prone PCR and chemical mutagenesis to generate large variant libraries [39]. In this study random mutagenesis and DNA shuffling of luxAB provides a directed approach to improve the activity of the fused bacterial luciferase. As a result, the luxAB strain was produced, which shows considerably improved luciferase activity in a variety of organisms. There are five mutations resulting in amino acid substitutions and one silent mutation that occurred to result in eluXAB. Previous work on the luciferase enzyme determined that the lux catalytic properties were primarily determined by the luxA subunit [40]. In this paper, of the five missense mutations, four appeared in luxA. None of the mutations occurs in the catalytic active site or appears to directly affect substrate binding. Because the amino acid sequences of luxA and luxB are highly conserved according to the result of alignments of amino acid sequences of luxA and luxB from *P. luminescens* and *V. harveyi* (Fig. S1), it is likely that if mutations occurred in the active site, they would inactivate the luciferase. Therefore, although it is speculative, it is most probable that the mechanism resulting in enhanced lux

| Position | Base substitution | Position in codon | Amino acid | Amino acid substitution |
|----------|------------------|------------------|------------|------------------------|
| 432      | G→A              | 3                | 144        | M→I                     |
| 700      | C→T              | 1                | 234        | H→Y                     |
| 967      | A→G              | 1                | 323        | I→V                     |
| 1009     | A→G              | 1                | 337        | I→V                     |
| 1318     | T→C              | 1                | 440        | E→K                     |
| 1450     | G→A              | 1                | 484        | L→L                     |

Table 1. DNA and amino acid substitutions in eluXAB gene.
activity involves mutations that exert their effects by indirectly changing the conformation of the aldehyde binding sites rather than by interacting with substrates directly.

The bacterial luciferase eLuxAB can be assayed with high sensitivity compared to the LuxAB both in intact bacterial- and yeast-cells. In addition, the opt-eLuxAB or opt-eLuxA+B has a greater luciferase activity than the eLuxAB and eLuxA+B in Arabidopsis cells. Evidently, codon optimization of eLuxAB led to an increased luminescence. Greater luminescence may be caused by greater lux expression levels, greater luciferase activity due to superior folding of the tethered LuxA and LuxB subunits, higher
quantum yield per catalytic round, or any of a plethora of different reasons or combinations thereof. The luciferase activity of monocistronic opt-e<sub>luxAB</sub> is greater than that of opt-e<sub>luxA</sub> and opt-e<sub>luxB</sub> expressed in two separate cistrons when expressed from the same promoter (Figs. 8B&C). It is tempting to speculate that the separate subunits cannot be assembled efficiently into a functional holoenzyme in <i>Arabidopsis</i> cells. What’s more, under the regulation of the 2×35S promoter, the luminescence of opt-e<sub>luxAB</sub> is about two times greater than that from one copy of the 35S promoter. This is compelling evidence that the opt-e<sub>luxAB</sub> can eventually be developed as a useful marker gene for the quantitative assessment and detection of different activities from promoters in transgenic plants.

The e<sub>luxAB</sub> is detectable using currently available technologies and offers prolonged expression without cell lysis in both prokaryotes and yeast [11]. The <i>lux</i> system derived from bacteria is particularly useful as a prokaryotic reporter because the <i>luxC</i>, <i>luxD</i>, and <i>luxE</i> genes can provide continuous supplies of the aldehyde substrate of <i>lux</i> within the cells without any external manipulations [14]. Of course, endogenous <i>luxA</i> and <i>luxB</i> genes within this operon may provide background <i>lux</i> readings precluding its use in these systems. However, modifications of the reporter system to include the other genes within the bacterial <i>lux</i> operon in the plant of interest may produce greater <i>lux</i> activity.

The plant-adapted bacterial luciferase gene opt-e<sub>luxAB</sub> also has some disadvantages. For example plant cells do not contain sufficient FMN<sub>H2</sub> to drive the opt-e<sub>luxAB</sub> catalyzed reaction at <i>V<sub>max</sub></i>, because FMN is enzyme-bound or enclosed in cell compartments in plant cells [34]. However, this is not a serious concern because, upon exogenous application of FMN<sub>H2</sub>, plant cells expressing <i>luxAB</i> may produce greater quantum yields [34,41]. However, our ultimate goal is to have efficient expression
and activity without the need of cell lysis or exogenous substrate application. One possible means to circumvent the poor availability of FMNH₂ in the cytoplasm will be to target the opt-eLuxAB into an organelle containing this cofactor using a target peptide on the N-terminus of opt-eLuxAB. Further studies are needed to determine the best means by which to express the frp gene in vivo to produce enough FMNH₂ in plant cells. Additionally, the basic pH optimum (10) of the eLuxAB enzyme, and the abrupt attenuation of eLuxAB activity to either side of this optimum is a major hurdle for the current use of this system in eukaryotic cells. Reducing the pH optimum of the eLuxAB enzyme closer to that of the neutral pH of the cell is the current major focus of our synthetic biology approach using similar techniques to those described here. Alterations of pH optima and/or a broadening of the pH optimum, of several pH units, using directed evolution has been possible for a number of commercial applications [42,43].

In a word, the eLuxAB and opt-eLuxAB demonstrate that, using directed evolution, synthetic biology, and by paying attention to codon bias, we can greatly increase the luminescence of a bacterial luciferase in plant cells, thus making it more suitable and convenient to use in these eukaryotes. This demonstration provides the foundation for further alterations in the opt-eLuxAB gene to produce a reporter suitable as a practical bioprocess monitor, as a high-throughput promoter expression screening technique and in applications for medical diagnosis.

Figure 8. The application of the bacteria luciferase luxAB gene as a reporter in plant protoplasts. A. Schematic representation of expression vectors for eLuxAB, or opt-eLuxAB gene fusions in plant protoplasts driven by one or two copies of the CaMV35S promoter. B. Comparison of luciferase activity of eLuxA+B, eLuxAB, opt-eLuxA+B and opt-eLuxAB in Arabidopsis. C. Comparison of luciferase activity of eLuxA+B, eLuxAB, opt-eLuxA+B and opt-eLuxAB in maize. D. Comparison of opt-eLuxAB luminescence under different promoter strengths.

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Supporting Information

Figure S1 Pairwise sequence alignment of the amino acid sequences between luxA and luxB genes from P. luminescens and V. harveyi. (TIF)

Figure S2 Alignment of eluxAB gene (Un-optimized) and codon-optimized eluxAB gene (Optimized) sequences. Base changes are indicated in orange. The six mutation sites in the eluxAB gene are indicated with the black hollow box. (TIF)

Figure S3 The tolerance of Arabidopsis protoplasts to decanal treatment. Protoplasts treated with different concentrations of decanal were photographed under optical microscope at time 0, 1 and 4 hours, respectively. Row 5 shows the protoplast cells tested with 200 μg/ml kanamycin as a control. Kanamycin but not decanal treatment leads to quick lysis of protoplast cells. (TIF)

Table S1 Bacterial strains and plasmids used in this study. (DOCX)

Table S2 Primers used in this study. (DOCX)

Table S3 Comparison of luminescence activities between bacterial, Renilla and firefly luciferases in plant protoplasts. Luminescence activities were measured in Arabidopsis and maize protoplast cells transformed with Rluc and Fluc reporter vector (pGLO3-dual-luciferase) or with opt-eluxAB reporter vector (pGLO3-opt-eluxAB). The number of Arabidopsis protoplasts used was about 5×10⁶ and the maize protoplasts was about 3×10⁷ in 100 μl volume culture. (DOCX)

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Author Contributions

Conceived and designed the experiments: XS TZ YW. Performed the experiments: BC TZ JW CL TW. Analyzed the data: XS TZ BC. Contributed reagents/materials/analysis tools: XS TZ YW. Contributed to the writing of the manuscript: XS TZ YW BC.

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