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# Re-engineering the two-component systems as light-regulated in *Escherichia coli*

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Bacteria live in environments with dynamic changes. To sense and respond to different external stimuli, bacteria make use of various sensor-response circuits, called two-component systems (TCSs). A TCS comprises a histidine protein kinase (HK) sensing environmental stimuli and a response regulator protein (RR) regulating downstream genes. The two components are coupled via a phosphorylation control mechanism. In a recent study, we adopted an optogenetics approach to re-engineer the sensor HKs in *Escherichia coli* as a light-sensing fusion protein. We constructed a light-controllable HK by replacing the original signal-specific sensing domain of HK with the light-sensing domain of Cph1 from *Cyanobacteria Synechocystis*, so that HK can be investigated by red light. Here, we extended the study to other 16 HK-RR TCSs and constructed a library of light-responsible HK-Cph1 chimeras. By taking the NarX-NarL system as an example, we demonstrated the light responsiveness of the constructed chimera and investigated the frequency response of the NarX-NarL system. The constructed library serves as a toolkit for future TCS study using optogenetics approach.

**Keywords.** Cph1; histidine kinase; two component systems (TCSs)

## 1. Introduction

Phosphotransfering schemes are widespread in most prokaryotic and a few eukaryotic signal transduction systems, which involve two conserved components, a sensory histidine kinase (HK) and a response regulator (RR). The HK, which is regulated by environmental stimuli, autophosphorylates at a histidine residue and creates a high-energy phosphoryl group that is subsequently transferred to an aspartate residue in the response regulator (RR). Then, the phosphorylation induces a conformational change in the regulatory domain of RR and triggers the downstream response (Bourret *et al*. 1991; Parkinson and Kofoid 1992; Stock *et al*. 2000). In bacteria, numerous signal pathways operate by making use of this mechanism and are referred to as two-component systems (TCSs) (Caren and Richard 1998).

In prototypical TCSs, diverse HKs share the same basic building blocks, namely a sensing domain and a conserved kinase core. The N-terminal sensing domains are responsible for detecting environmental stimuli. Despite that the sensing domains share little primary sequence similarity, the kinase core domains for TCS coupling are extremely conserved. The RRs function as phosphorylation-activated switches of the adaptive response. Most RRs consist of two domains: a conserved N-terminal regulatory domain and a variable C-terminal effector domain. Many RRs are transcription factors with DNA-binding domains.

A number of literatures (Stock *et al*. 1989; Bourret *et al*. 1991; Parkinson and Kofoid 1992) have been published detailing the biology and chemistry of well-characterized TCSs, such as chemotaxis (Falke *et al*. 1997; Alon *et al*. 1999), aerobic/anaerobic regulation (Iuchi and Weiner 1996), osmoregulation (Forst and Roberts 1994; Cai and Inouye 2002), nitrate/nitrite regulation (Nohno *et al*. 1989; Stewart *et al*. 1989; Moir and Wood 2001; Stewart 2003) and phosphate regulon genes expression (Tommassen *et al*. 1982; Makino *et al*. 1989; Baek and Lee 2007) of *Escherichia coli*, the sporulation system of *Bacillus subtilis* (Hoch 1993; Perego 1998), and differentiation in *Caulobacter crescentus* (Domian *et al*. 1996; Shapiro and Losick 1997; Wu and Newton 1997) and *Myxococcus Xanthus* (Kaplan

and Plamann 1996; Ward and Zusman 1997). The cumulative data have provided a picture of how these systems transduce extracellular signals and elicit appropriate cellular responses. However, few systematic investigations have been done partly due to the complication brought by the diversity of the signals. Recently, an optogenetics strategy has been adopted by some labs to investigate the TCSs (Levskaya *et al*. 2005; Olson and Tabor 2014; Schmidl *et al*. 2014; Toettcher *et al*. 2013). Optogenetics is a technology that allows targeted control of precisely defined events in biological systems by using light-responsive control elements (Deisseroth 2011). Here, we follow the same approach to build a series of light-responsive HKs in *Escherichia coli* by fusing the conserved HK core domains with a photoreceptor derived from *Synechocystis* (Wu *et al*. 2017), so that various signals can be simulated by light and the TCSs can be explored in a unified framework (figure 1). The constructed light-responsive HKs serve as a toolkit for detailed analysis and manipulation of TCSs through precise temporal control via light.

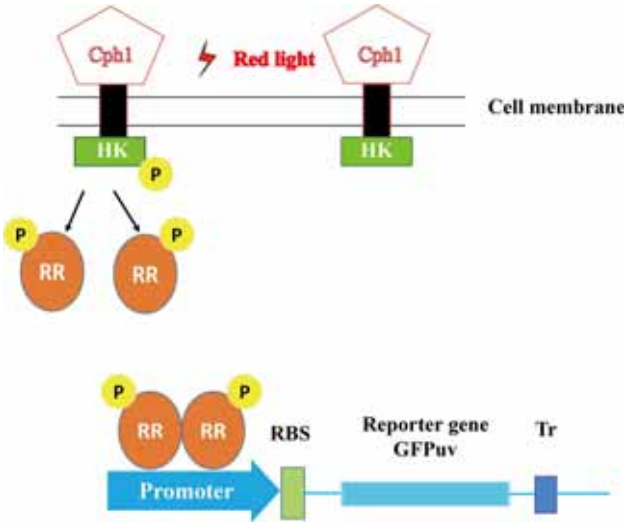


**Figure 1.** Gene expressions regulated by light-responsive HKs in *Escherichia coli.* The native signal-sensing domain of the HK is replaced with the light-sensing domain of Cph1, so that the re-engineered HK can be regulated by the red light. As a result, red light serves as a substitution for the original chemical/physical signal to trigger the original downstream actions from HK autophosphorylation to RR activation and target gene expression.

## 2. Materials and methods

### 2.1 *Sequences*

The sequences of HKs were downloaded from Uniprot and the sequence of Cph1 was from literature (Gambetta and Lagarias 2001). The primers used in this study were designed using Oligo 7 and synthesized at Shanghai Sangon Biotech.

### 2.2 *Construction of plasmids*

The plasmid pPLPCB was constructed by inserting ho1 and pcyA cloned from *Synechocystis* PCC 6803 into pSTV28 (figure 2a). The phytochrome domain of Cph1 was amplified from *Cyanobacteria Synechocystis* (PCC 6803) genomic DNA, and digested with *Eco*RI and *Spe*I before being inserted into the vector pSP73 to produce the pCph1-pSP73 plasmid. *Escherichia coli* strain DH5α was used as the template to amplify the HK kinase core domains (table 1). In order to determine a suitable length for the linker sequence between the HK kinase core and the photoreceptor domain of Cph1, we generated five linker sequences with different lengths for each HK by extending the 5′ and 3′ ends of the HK kinase core to its flanking sequence with 0, 10, 20, 30 and 40 amino acids, respectively. The amplified fragments were then digested with *Eco*RI and *BamH*I and ligated to the plasmid pCph1-pSP73 yielding the recombinant plasmid pHK-Cph1-pSP73 (figure 2b). The segment that contains the native promoter $PRO_{RR}$ of the conjugated RR, RBS, GFPuv and the terminator was synthesized through PCR amplification reaction. Then the segment was digested with *Kpn*I and *Nhe*I and ligated with pHK-Cph1-pSP73 to generate the plasmid $pPRO_{RR}$-GFPuv-HK-Cph1-pSP73 (figure 2c). For each HK, a blank control plasmid $pPRO_{RR}$-GFPuv-Cph1-pSP73 was built, in which the conserved HK core domain is absent.

### 2.3 *Screening for light-responsive HKs*

In order to determine the light-inducible construct with the most appropriate linker sequence for an HK, we co-transformed pPLPCB and each HK-Cph1 construct into an HK-null *Escherichia coli* strain ΔHK using the heat shock method. The bacteria were cultivated on the plate containing ampicillin and chloramphenicol with final concentration of ampicillin (100 μg/mL) and chloramphenicol (170 μg/mL) at 37°C for 10–12 h. Positive clones with green fluorescence were randomly selected from the plate into 5 ml fresh double resistant medium shaking overnight at 37°C with 250 rpm until the optical density (OD) at 600 nm ($OD_{600}$) reaching 2.5 ± 0.3. The overnight culture was diluted into ten tubes of 2 mL fresh LB medium containing ampicillin (100 μg/mL), chloramphenicol (170 μg/mL) and 0.5 mM IPTG with $OD_{600}$ reaching about 0.01. All the tubes were cultured in the thermostatic mixing device shaking at 37°C with 650 rpm, of which five were exposed to red light (620–650 nm) constantly and the other five were kept in the dark. After 10 h' culturing, samples were taken every 30 min until the 12th hour. The samples were immediately

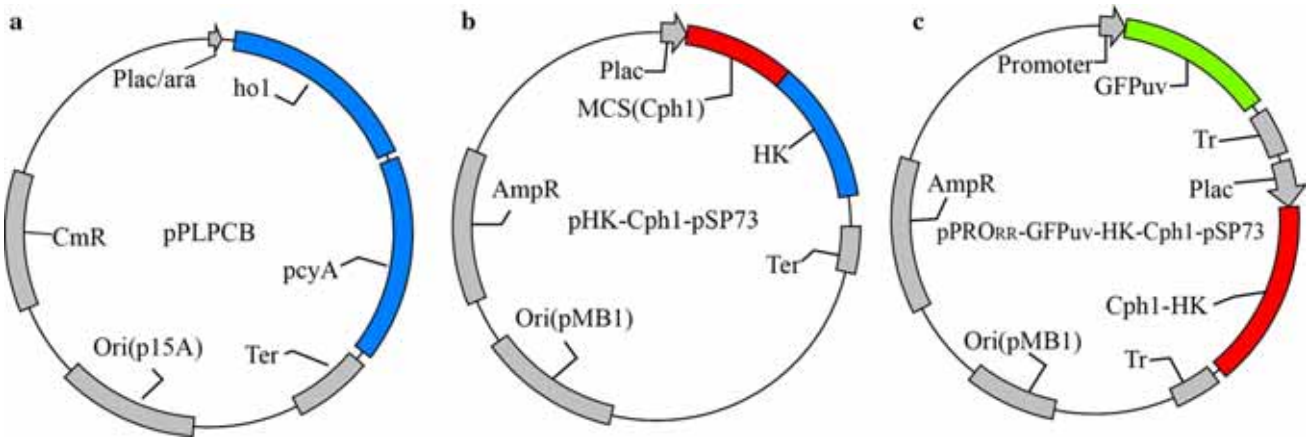


**Figure 2.** The physical maps of constructed plasmids. (**a**) The plasmid pPLPCB was constructed by inserting the ho1 and pcyA genes into pSTV28. It is responsible for converting heam into phycocyanobilin the natural chromophore of Cph1. (**b**) The plasmid pHK-Cph1-pSP73 was constructed by inserting the light-sensing domain of Cph1 and the HK core domain from different TCSs into the backbone vector pSP73. It serves as a bridge to construct $pPRO_{RR}$-GFPuv-HK-Cph1-pSP73. (**c**) In addition to the chimeric HK-Cph1, the plasmid $pPRO_{RR}$-GFPuv-HK-Cph1-pSP73 carries the promoter $PRO_{RR}$ recognized by the conjugated RR and the reporter gene GFPuv under the control of $PRO_{RR}$.

**Table 1.** Strains and plasmids used in this study

| Strains or plasmid | Relevant characteristic(s) | References or source |
|---|---|---|
| *Escherichia coli* strains | | |
| DH5α | Derived from K-12, *R*-, *M*-, *AMP*- | Hanahan (1983); this study |
| JW1213 | ΔNarX | General Electric Company |
| *Cyanobacteria Synechocystis* | Chromophore Cph1 contained | PCC 6803/*Kazusa* |
| Plasmids | | |
| pSP73 | Amplicillin resistance | Promega |
| pSTV28 | Chloramphenicol resistance | TaKaRa |
| Plam-RBS-GFPuv-Tr-pSP73 | Amplicillin resistance | Our laboratory |
| pCph1-pSP73 | | This study |
| pHK-Cph1-pSP73 | | This study |
| pPLPCB | | Levskaya *et al.* (2005) |
| $pPRO_{RR}$-GFPuv-HK-Cph1-pSP73 | | This study |

transferred into ice-water for 5 min to stop the reactions. 200 μL bacterial liquid were transferred into a 1.5 mL EP tube and centrifuged for 4 min at 4°C with 12000 rpm. Then 200 μL double distilled water was added to clean the cells, centrifuging for 4 min at 4°C with 12000 rpm to remove the supernatant. Finally, the bacteria were re-suspended in 100 μL double distilled water for measuring $OD_{600}$ and GFPuv fluorescence intensity. The receiving wavelength is from 500 nm to 550 nm and the exciting wavelength is 365 nm.

### 2.4 *Light frequency response of the light-sensing NarX-NarL system*

Two frequencies of the red light stimuli were investigated, a low frequency of 1/3600 Hz and a high frequency of 1/600 Hz. The corresponding period is 60 and 10 min, respectively. The experimental procedure is almost the same as described in section 2.3. One exception is that all the tubes were constant cultured under the red light for 10 h after IPTG induction, before being exposed to three on-and-off cycles of the red light. In each cycle, the red light is off in the first half of the cycle and on in the last half. That is, the red light is on and off for 30 and 5 min for the case of 1/3600 Hz and 1/600 Hz, respectively. Samples were taken periodically and the GFPuv levels were measured until the third cycle was finished. In addition to the GFPuv fluorescence intensity, real-time fluorescence quantitative PCR was also performed to measure the mRNA level of *yeaR*, the downstream target gene of NarL. Samples were prepared from the tubes of the last time point of every period and sent to Shanghai Sangon Biotech for measurement.

**Table 2.** TCSs re-engineered in this study

| TCS | Annotated function |
|---|---|
| NarX-NarL | Nitrate/nitrite regulation |
| EnvZ-OmpR | Osmoregulation |
| AtoS-AtoC | Acetoacetate metabolism |
| BarA-UvrY | Carbon metabolism |
| BasS-BasR | Resistance to polymyxin |
| CpxA-CpxR | Envelope stress response |
| CreC-CreB | Catabolic regulation |
| DcuS-DcuR | Involved in the anaerobic fumarate respiratory system |
| EvgS-EvgA | Regulates the expression of emrKY operon and yfdX |
| GlnL-GlnK | Nitrogen regulation |
| GlrK-GlrR | Upregulates transcription of the glmY sRNA when cells enter the stationary growth phase |
| NarQ-NarP | Nitrate/nitrite regulation |
| QseC-QseB | Activates the flagella regulon |
| RcsC-RcsB | Involved in colanic acid capsule synthesis, biofilm formation and cell division etc. |
| YpdA-YpdB | Involved in carbon control network |
| ZraS-ZraR | In response to high concentrations of zinc or lead in the medium |

## 3. Results

### 3.1 *Construction of a two-plasmids system*

Photoreceptors, also known as phytochromes, are used in plants and some bacteria to control phototaxis, photosynthesis and production of protective pigments (Yeh *et al.* 1997; Davis *et al.* 1999; Schmitz *et al.* 2000). However, photoreceptors are not found in enterobacteria, such as *Escherichia coli*. In order to obtain a light-responsive version of the native HK in *Escherichia coli*, we re-engineered the wild-type HK to fuse with the phytochrome of Cyanobacterial phytochrome 1 (Cph1) in *Synechocystis* (Essen *et al.* 2008), so that the HK-Cph1 chimera is able to respond to light (figure 1).

Cph1 is a light-regulated histidine kinase (Yeh *et al.* 1997) with similar composition of bacterial HKs. It has an N-terminal chromophore-binding sensor module and a C-terminal histidine autokinase-phosphotransferase transmitter module. The structural similarity between Cph1 and HK makes it possible to construct a light-controllable HK by replacing the signal-specific sensing domain of HK with the light-sensing domain of Cph1, so that the HK-Cph1 chimera can be investigated using red light. However, phycocyanobilin (PCB), the natural chromophore of Cph1 (Davis *et al.* 1999), is not naturally produced in *Escherichia coli*. Therefore, we constructed a plasmid pPLPCB that carries the two phycocyanobilin-biosynthesis genes (ho1 and pcyA) from *Synechocystis* and converts heam into phycocyanobilin (Gambetta and Lagarias 2001) (figure 2a).

To facilitate the screening of a light-sensing HK-Cph1 construct, we set up a second plasmid pPRO$_{RR}$-GFPuv-HK-Cph1-pSP73 (figure 2c). This plasmid encompasses an HK-Cph1 chimera by integrating the conserved kinase core domain of the corresponding native HK with the phytochrome fragment of Cph1. To check whether a chimera is able to sense light, the reporter gene GFPuv is put under the control of the promoter sequence PRO$_{RR}$ recognized by the cognate RR. Therefore, if an HK-Cph1fusion protein is responsive to light, the binding of the downstream RR to the RR-specific promoter will be regulated by light, which in turn will impact the expression of GFPuv. Briefly, the level of GFPuv serves as an indicator of whether a constructed HK-Cph1 chimera is capable of responding to light.

### 3.2 *Construction of chimeric HK-Cph1 library*

Sixteen pairs of HK-RR TCSs in *Escherichia coli* were selected in this study, which span a variety of functions (table 2). Since the length of the linker sequence between the light-sensing domain Cph1 and the conserved HK core domain has impact on the transfer of light signal, for each HK we prepared five constructs with different linker length by constructing corresponding pPRO$_{RR}$-GFPuv-HKn-Cph1-pSP73 plasmids. In addition, a blank control plasmid pPRO$_{RR}$-GFPuv-Cph1-pSP73 was also built for each HK, in which the conserved HK core domain is absent.

### 3.3 *Screening for light-sensing HKs*

In order to determine the most appropriate light-inducible construct for an HK, we evaluated the light responsiveness of each HK-Cph1 chimera by measuring and comparing the GFPuv expression when the cells were exposed to red light or kept in the dark, respectively. Here, we took NarX-NarL, an HK-RR pair involving in nitrate/nitrite regulation, as an example to demonstrate the screening procedure. NarX is a sensor for nitrate and it activates NarL by phosphorylation in

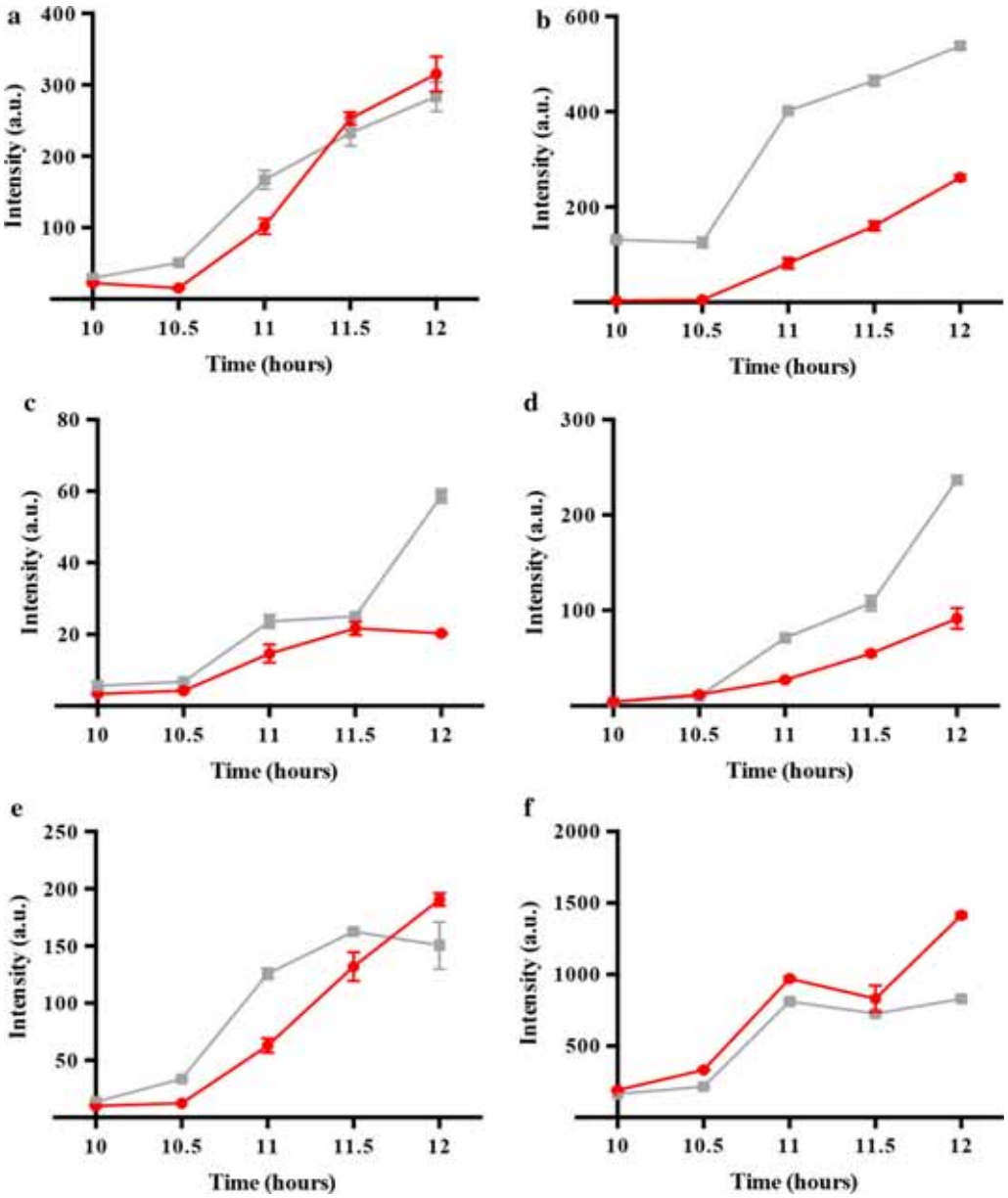


**Figure 3.** Screening for light-responsive NarX-Cph1 constructs. The horizontal axis is the duration of IPTG induction. The vertical axis represents the fluorescence intensity of GFPuv. (**a**–**f**) give the time courses of the five NarX-Cph1 constructs with different linker lengths and the blank control. The red and dark lines show the fluorescence intensity of GFPuv under the conditions of being exposed to red light or kept in dark, respectively. Given data represents three similar independent experiments.

the presence of nitrate (Nohno *et al*. 1989; Stewart *et al*. 1989; Stewart 2003).

We prepared five amplicons (denoted as NarX1 to NarX5) of the NarX core domain with different length of the flanking sequences. We constructed a plasmid pP*yeaR*-GFPuv-NarXn-Cph1-pSP73 for each amplicon, which puts GFPuv under the control of P*yeaR*, the native *yeaR* promoter bound by NarL. The blank control plasmid pP*yeaR*-GFPuv-Cph1-pSP73 plasmid was also constructed, which lacks the HK core domain of NarX. We co-transformed pPLPCB and each pP*yeaR*-GFPuv-NarXn-Cph1-pSP73 into JW1213 competent cells, the *Escherichia coli* strain without NarX. The transformants were cultivated in separate tubes exposed to red light and kept in the dark, respectively. After 10 h culturing, the GFPuv fluorescence intensity was measured every 30 min until the 12th hour.

As shown in figure 3F, the blank control showed a similar GFPuv expression behavior no matter it was in the light or in the dark, although the GFPuv expression was lower in the dark. The observation was consistent with the fact that the

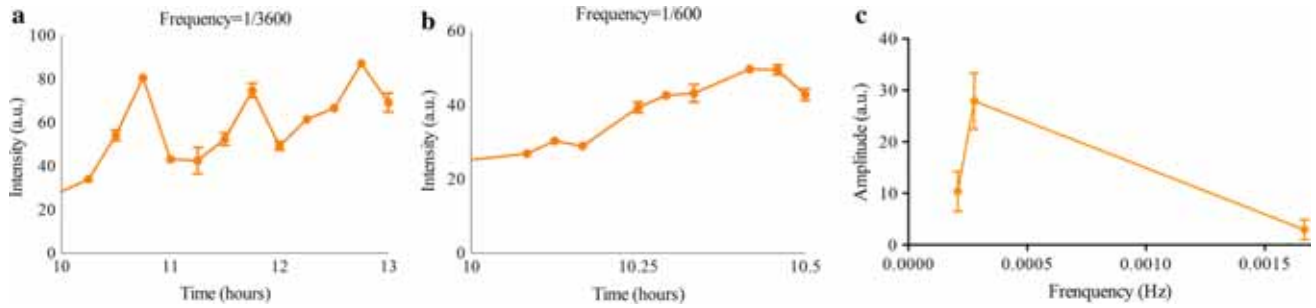


**Figure 4.** The response of NarX2-Cph1 to red light with high and low frequency. The horizontal axis is the duration of IPTG induction. The bacteria were exposed to on-and-off cycles of the red light after 10th hour, the starting point of the horizontal axis. The vertical axis represents the fluorescence intensity of GFPuv. (**a**) The NarX-Cph1 system was able to respond to red light with a low frequency of 1/3600 Hz, as indicated by the oscillation of GFPuv level with the on-and-off cycles of the red light. (**b**) However, the system was unresponsive to red light with a high frequency of 1/600 Hz. (**c**) The reflected feature of NarX2-Cph1 system with different frequency stimulation. Results are representation of three similar experiments.

NarX core domain was absent in the blank control. Since the transduction of light signal through NarX to NarL was broken, expression of the downstream reporter GFPuv was independent of light conditions. It could be seen that the light-responsiveness of NarX1-Cph1 (figure 3a) and NarX5-Cph1 (figure 3e) were not stable. Although they showed higher GFPuv expression levels in the dark than in the light from 10th to 11.5th hour, the behavior flipped from 11.5th to 12th hour. In contrast, NarX2-Cph1 (figure 3b), NarX3-Cph1 (figure 3c) and NarX4-Cph1 (figure 3d) exhibited stable GFPuv expression differences between the light and the dark conditions. That is the GFPuv level was higher for the cells kept in the dark than those exposed to red light. The observation indicated that GFPuv expression was inhibited by red light. Among the three constructs, NarX2-Cph1 showed the most significant GFPuv expression difference during the whole time course (figure 3b), which indicated that it was the most effective light-sensing construct for NarX. The stable and consistent light-responsive behavior of the NarX2-Cph1 fusion protein makes it a good candidate for subsequent light-mediated studies.

### 3.4 *Response of the NarX-NarL system to different light frequency*

The frequency response of a system to its input is a fundamental property of the system. It reflects the working range of the system in frequency domain. Due to the good performance of NarX2-Cph1 fusion protein, we investigated the frequency response of the NarX-NarL system by using the pP*yeaR*-GFPuv-NarX2-Cph1-pSP73 system as a tool. As shown in figure 4, the GFPuv level oscillated with the on-and-off cycles of the low frequency stimuli, suggesting the NarX-NarL system is responsive to the low frequency stimuli (figure 4a). In contrast, the insensitivity of the system to the high frequency stimuli indicated that the stimuli were filtered out (figure 4b). Similar phenomena had been reported for the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway in the budding yeast (Hersen *et al.* 2008). These frequency-regulating results revealed this light-regulated NarX-Cph1 chimera is a low-pass filter that prefers to stimulation of low frequency (figure 4c). In our previous study, we observed consistent results for the light-sensing UphB-Cph1 chimera (Wu *et al.* 2017), in which case red light promoted the expression of target gene.

### 3.5 *Red light-mediated downregulation of yeaR at the transcriptional level*

The results of GFPuv fluorescence intensity indicated that the *yeaR* promoter is repressed by red light. To confirm the effect of red light, real-time fluorescence quantitative PCR (figure 5) was applied for measuring the downstream target gene *yeaR*. Consistent with the above results, red light treatment induced decreased expression of *yeaR* at the mRNA level. Taken together, these data clearly demonstrated that red light mediated the induction of *yeaR* expression in the light-controllable NarX-NarL system.

## 4. Discussion

Here we extended our recent study (Wu *et al.* 2017) to other TCSs in *Escherichia coli*. We constructed a series of light-regulated HK-RR TCSs by fusing the HK core domain from the TCSs in *Escherichia coli* with the phytochrome of Cph1 from *Synechocystis*. Due to the variety of extracellular signals, systematical study of TCSs requires operation on many different signals, which complicates experiment design and is time consuming and labour intensive. Re-engineering the original sensor proteins in TCSs by constructing light-

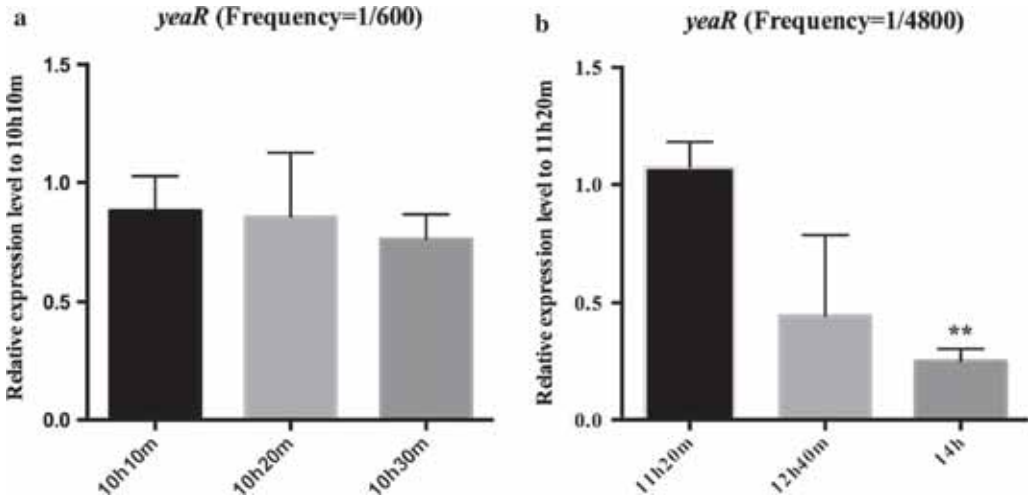


**Figure 5.** Red-light-mediated downregulation of *yeaR* in light-regulated NarX2. (**a**, **b**) Decreased *yeaR* expression at the mRNA level in light-controllable JW1213 cells. (**a**) 1/600 optical frequency, (**b**) 1/4800 optical frequency. All data are presented as the mean ± SD of 3 independent experiments. $^{**}p < 0.01$. The 16S rRNA is used for reference gene.

controllable fusion proteins provides an alternative option to investigate TCSs. In this unified framework, various native signals can be replaced with light, which is simple and easy to control. As demonstrated in this study and our previous study, properly constructed chimeric HK-Cph1 fusion protein could serve as a light-controllable substitute for the wild-type HK.

To construct an effective light-sensing HK-Cph1 chimera, it is essential to obtain a suitable linker sequence between the HK kinase core domain and the photoreceptor domain of Cph1. In a native HK, there is a HAMP domain between the signal-sensing domain and the kinase core domain. The HAMP module is a ubiquitous intracellular module connecting input to output domains, which relays the regulatory signal from periplasmic ligand-binding domains to cytoplasmic signaling kinases via conformational changes (Heinz *et al*. 1998; Aravind and Ponting 1999; Williams and Stewart 1999). During HK-Cph1 construction, the phytochrome domain of Cph1 replaced the original signal-sensing domain of the HK, which also disrupted the original linker module. Therefore, finding a suitable linker sequence is critical to rescue the signal transduction. In this study, we used a simple approach to generate linker sequences by extending different lengths to the 5′ and 3′ ends of an HK kinase core to its flanking sequence. However, we did not observe simple relationship between the linker length and the light responsiveness. For instance, despite of the superior of NarX2-Cph1 (figure 3b), NarX1-Cph1 (figure 3a) and NarX3-Cph1 (figure 3c), which were more close to NarX2-Cph1 in their linker lengths, were in fact inferior to NarX4-Cph1 (figure 3d). In addition, different HKs might have different proper linker lengths. For instance, the best linker sequence we obtained is 10 for NarX and 30 for UhpB (Wu *et al*. 2017). For the other TCSs investigated in this study, different suitable linker lengths were also observed. Although usable HK-Cph1 chimeras were obtained using the simple strategy, it explored a very limited number of possibilities and could not guarantee the obtained linkers are optimal. For example, for some of the TCSs we explored, the constructed HK-Cph1 chimera did not show as large GFPuv expression difference between the light and dark conditions as NarX2-Cph1 did, which indicated further optimization might be needed.

Equipped with light-sensing HK-Cph1 chimeras, we are able to investigate various TCSs using the same input, the light, which simplifies the study procedures. Light can be easily controlled by adjusting its intensity, frequency and waveform. In this study, adjusting frequency enables us to explore the response of the NarX-NarL system under different light frequency (or equivalently chemical turn-over rate), which would be more difficult to accomplish by adjusting nitrate concentration directly. This improvement also provides us an opportunity to study biological signal transduction systems from new perspectives. For example, as shown in the case of NarX-NarL system we could examine their signal processing properties by referring to artificial communication systems. The low-pass behavior of NarX-NarL reveals the similarity between biological and artificial signaling processing systems. In fact, the unique frequency response property of a light-sensing TCS makes it a potential part for synthetic biology. For instance, to control a mixture of cells that carry different target genes, we can put the target genes under the control of different HK-RR pairs

responding to different light frequencies. By using different light frequencies, we can implement precise control the specific cells carrying the corresponding frequency-responsive HK-RR pair.

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