System for Stable β-Estradiol-Inducible Gene Expression in the Moss Physcomitrella patens

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Abstract

Inducible transgene expression provides a useful tool to analyze gene function. The moss Physcomitrella patens is a model basal land plant with well-developed research tools, including a high efficiency of gene targeting and substantial genomics resources. However, current systems for controlled transgene expression remain limited. Here we report the development of an estrogen receptor mediated inducible gene expression system, based on the system used in flowering plants. After identifying the appropriate promoters to drive the chimeric transducer, we succeeded in inducing transcription over 1,000-fold after 24 h incubation with β-estradiol. The P. patens system was also effective for high-level long-term induction of gene expression; transcript levels of the activated gene were maintained for at least seven days on medium containing β-estradiol. We also established two potentially neutral targeting sites and a set of vectors for reproducible expression of two transgenes. This β-estradiol-dependent system will be useful to test genes individually or in combination, allowing stable, inducible transgenic expression in P. patens.

Citation: Kubo M, Imai A, Nishiyama T, Ishikawa M, Sato Y, et al. (2013) System for Stable β-Estradiol-Inducible Gene Expression in the Moss Physcomitrella patens. PLoS ONE 8(9): e77356. doi:10.1371/journal.pone.0077356

Introduction

The moss Physcomitrella patens lineage diverged from the flowering plant lineage approximately 450 million years ago [1,2] and comparisons of its physiology and development to those in flowering plants are useful to elucidate generality, diversity, and evolution in land plants. Various genomics resources including the genome sequence [3,4] and full-length cDNA clones [5] as well as efficient gene targeting via homologous recombination [6] enable analysis of gene functions in P. patens [7,8]. Transgenic gain-of-function and loss-of-function experiments are useful to investigate gene function. For example, systems to induce transgene expression by addition of external triggers are effective tools to investigate gene functions at specific stages [9–11]. Moreover, the availability of inducible and continuously strong promoters would facilitate the production of complex biopharmaceuticals in the moss bioreactor [12]. In P. patens, a heat shock promoter derived from the upstream sequence of Gmhsap17.3B can be used to conditionally induce transgene expression in a short time and in all examined tissues [13,14]. However, continuous induction is difficult because long exposure to heat stress results in eventual attenuation of promoter activity [15] and seriously affects growth. The tetracycline repression system can also be used as an inducible expression system in P. patens [16], but maintaining repression of transgene expression requires cultivating the moss on medium supplemented with tetracycline, which causes growth.
retardation [13]. Another chemically inducible expression system is the GVG system [13,17], which uses the DNA binding domain of the yeast GAL4 transcription factor, the transcriptional activation domain of the herpes viral protein VP16, and the mammalian glucocorticoid receptor. Activation of GVG with dexamethazone elicits expression of a set of defense related genes and growth retardation in flowering plants [18–20], although its effects on P. patens have not been well examined. Based on mammalian expression technologies several inducible and autoregulated promoters have been introduced into P. patens protoplasts [21] but have not been examined yet in stably transformed moss lines during the life cycle.

To establish a more feasible gene induction system in P. patens, we employed the XVE chimeric transcription activator, which has been effectively used in flowering plants and is composed of the DNA-binding domain of the bacterial repressor LexA, the transcriptional activation domain VP16, and the carboxyl region of the human estrogen receptor [22]. When activated by exogenous β-estradiol, XVE protein binds to the LexA operator and recruits RNA polymerase II to the cauliflower mosaic virus (CaMV) minimal 35S promoter (Pm35S) [23] to induce expression of the downstream gene. This system has no apparent toxic physiological effects in A. thaliana [22]. To adapt this system for P. patens, we screened for promoters to drive XVE expression at an appropriate level for transactivation of the target gene in protonema and gametophore cells. We also examined induction and duration of transactivation of genes in P. patens.

Genetic and epigenetic effects of the insertion site and surrounding regions affect transgene expression, which can thus vary among transgenic lines even if the same construct is transformed [24,25]. To avoid this problem, we established gene targeting sites that are located at putative neutral genomic positions. Furthermore, establishment of more than one site enabled simultaneous induction of two transactivated genes. This system has been successfully used in recent publications without detailed description [26,27] and here we fully describe the useful gene induction system in P. patens.

Results

Screening of promoters to induce the XVE gene in P. patens

We modified the XVE construct, pER8 ([22]: Figure 1A) containing the following three regions: (1) the XVE chimeric gene encoding a DNA-binding domain of the bacterial repressor LexA (X [28,29]); the transcriptional activation domain of VP16 (V [30]); and the regulatory region of the human estrogen receptor (E [31]), which is driven by the synthetic G10-90 promoter [32] and connected to Pisum sativum rbcS E9 terminator [33], (2) the HYGROMYCIN PHOSPHOTRANSFERASE (HPT [17]) gene cassette composed of a nopaline synthase promoter (Pnos [17]) and a nopaline synthase terminator (Tnos [17]) for selection of transformants, and (3) eight copies of a bacterial LexA operator [22] connected to Pm35S [23], a multiple cloning site to introduce a transgene, and a Pisum sativum pea 3A terminator[34]. For use in the moss Physcomitrella patens, we modified pER8 as follows (Figure 1B). First, we replaced the selection cassette with a marker that is effective in P. patens. The HPT cassette was replaced with the aphIV cassette, which uses a modified CaMV35S promoter and CaMV35S terminator that are efficient in P. patens [35,36]. Second, to facilitate cloning, the multiple cloning site was replaced by the Gateway cassette (Life Technologies). Third, to prevent position effects of insertion sites and to obtain stable expression, we used the P. patens inter-genic 1 (PIG1) site as a potential neutral site for site-specific insertion. In PIG1, no putative transcripts, loci, or repeat sequences have been assigned and no effects of gene insertion have been observed [14]. When we introduced the modified construct to P. patens, we could not detect induction of a transgene. Seeing that CaMV 35S promoter activity is different between seed plants and P. patens [37], we suspected the G10-90 promoter activity in P. patens and replaced the promoter used to express XVE with a promoter that works in P. patens. The G10-90 promoter induces the XVE gene in A. thaliana and tobacco [22] but we could not detect induction in P. patens protonemata. To find appropriate promoters, we searched previously published transcriptome data [38] for genes constitutively expressed in both protonemata and gametophores at similar levels and selected four promoter regions of genes that showed different expression levels. The two genes encode an RNA-binding domain protein (XM_001758956) and a kinesin protein KINID1a (AB434497 [36]), whose promoter and 5′ untranslated regions 976 and 1517 bp upstream from their start codons were designated as GX6 and GX8, respectively. The G10-90 promoter driving XVE was replaced by each of these promoter fragments. The resulting constructs were designated pPGX6 (AB537481) and pPGX8 (AB537482).

To monitor the activity of each GX promoter driving XVE, we examined the expression of the marker NLS-GFP-GUS (NGG), a fusion gene composed of a nuclear localization signal (NLS [39]), the green fluorescent protein (sGFP [40]) gene, and the uidA (GUS [41]) gene. The NGG marker gene was introduced with the Gateway system into each of the pPGX constructs, downstream of the LexA: Pm35S promoter (Figure 1B). These constructs were used to transform P. patens protoplasts with a conventional PEG-mediated method; insertion into the PIG1 site was confirmed by DNA gel blot analyses (Figures S1 and S2). We selected three single-insertion lines for each GX-NGG construct and examined expression of NGG in each line after 24 h incubation with or without β-estradiol (Figures 2 and S3). The ideal promoter for driving XVE would produce no NGG signal in the absence of β-estradiol, but would produce sustained, high-level NGG signal in protonema and gametophore cells following induction. Without β-estradiol, fluorescent NGG signals were not detected in any protonema or gametophore cells of GX6-NGG and GX8-NGG lines. With β-estradiol, spatial patterns of NGG signals were not distinguishable between GX-NGG lines with the same GX promoters but differed among different GX promoters (Figures 2 and S3). In GX6-NGG lines, NGG signals were detected in most of protonema cells (Figure S3A). Most chloronema cells retained NGG signals except a part of chloronema apical cells,
although we cannot detect morphological differences between chloronema apical cells with or without NGG signals. We could not detect NGG signals in caulonema cells. The first to approximately third leaf from a gametophore tip and younger leaves did not show NGG signals while the fourth and older leaves retained NGG signals. In GX8-NGG lines, NGG signals were detected in all cells in protonemata and gametophores.

To compare the induction ability of the P. patens XVE system to other systems, each Pm35S [23] lacking the transcriptional enhancer region as a negative control, the rice Actin1 promoter (Pact1 [16,42]), and the soybean heat-shock protein Gmhsp17.3B promoter (HSP [13,14]) were fused with NGG and introduced at the PIG1 site to obtain singly inserted transgenic lines (Figure S4, S5, S6). We quantified NGG transcripts in protonemata for each line by quantitative real time PCR (RT-qPCR). ALPHA TUBULIN 1 (TUA1) was used as a control because it has been reported to be expressed in several tissues and conditions in P. patens at similar levels [26,36]. NGG transcripts accumulated at 0.3 ± 0.1 and 4.8 ± 0.3 copies per pg total RNA in Pm35S:NGG and Pact1:NGG lines, respectively (mean ± SD calculated based on biological replicates) (Figure 3A). By contrast, in HSP:NGG lines, NGG transcripts accumulated at 91.1 ± 3.0 and 0.9 ± 0.0 copies per pg total RNA with or without heat shock for 1 h at 38°C, respectively. Transcripts of NGG induced by heat shock attenuated to approximately one tenth of maximal levels after 24 h of incubation at 38°C (Figure S7) [13]. Transcripts of TUA1 in Pm35S:NGG and Pact1:NGG accumulated at 91.1 ± 3.0 and 0.9 ± 0.0 copies per pg total RNA, respectively. In HSP:NGG lines, transcripts of TUA1 were 30.1 ± 0.6 and 31.6
Figure 2. Spatial expression patterns of NGG induced by P. patens XVE system. Fluorescence images of protonemata (A) and gametophores (B) of GX6-NGG#63 and GX8-NGG#4 lines. Fluorescent signals were observed after 24 h inoculation in water with (+) or without (-) 1 µM β-estradiol. Yellowish color of leaf veins in GX8-NGG#4 is caused by reflection but not by NGG signals. (C) Magnified views of apexes of gametophores in GX6-NGG#63 and GX8-NGG#4 lines. Pictures in A, B, and C panels are at the same magnification. Bars = 100 µm.

doi: 10.1371/journal.pone.0077356.g002

Figure 3. Amounts of NGG and TUA1 transcripts in Pm35S:NGG, Pact1:NGG, and HSP:NGG lines. (A, B) Transcript amounts of NGG (A) and TUA1 (B) in protonemata incubated for 8 days after propagation. Three independently transformed lines harboring a single copy of each DNA fragment at the PIG1 targeting site were analyzed (Figures S4 to S6). Copy numbers of the NGG and TUA1 transcripts per pg total RNA were estimated by RT-qPCR. In HSP:NGG lines, protonemata were cultured for 8 days at 25°C and collected after 1 h incubation at 25°C (crosses) or 38°C (squares).

doi: 10.1371/journal.pone.0077356.g003

± 1.2 copies per pg total RNA with and without heat shock for 1 h at 38°C, respectively (Figure 3B).

We next quantified transcript amounts of NGG and TUA1 in protonemata incubated with β-estradiol for 24 h for each of GX-NGG line by RT-qPCR (Figure 4). Each protonema sample was incubated with or without β-estradiol for 24 h. Without β-estradiol, 3.0 ± 0.1 and 0.4 ± 0.0 copies per pg total RNA of NGG transcripts accumulated in GX6-NGG and GX8-NGG lines, respectively. By contrast, with β-estradiol, 180.2 ± 13.2 and 72.7 ± 2.4 copies per pg total RNA of NGG transcripts accumulated in GX6-NGG and GX8-NGG lines, respectively. In the case of TUA1 transcripts, we could not detect significant differences in any line with or without β-estradiol. Independent transgenic lines harboring the same single GX-NGG construct showed similar NGG transcript amounts with or without β-
Estradiol. These indicate that relative transcript levels of NGG increased 54.8 ± 15.1 and 171.6 ± 80.7 times in GX6-NGG and GX8-NGG lines, respectively.

Dose and time responsiveness to β-estradiol

We selected GX6 and GX8 promoters in the following experiments because they showed higher induction rates than the other two promoters. To analyze the dose responsiveness of GX6-NGG and GX8-NGG lines to β-estradiol, transcript levels of NGG were measured with RT-qPCR using protonemata incubated in different β-estradiol concentrations (Figure 5). NGG transcripts of both lines were induced at 0.001 µM and higher concentrations. Transcript levels were maximal at around 1 µM β-estradiol in all lines.

The temporal change in NGG transcript levels was monitored in GX6-NGG#63 and GX8-NGG#4 protonemata after β-estradiol supplementation (Figure 6). NGG transcripts increased approximately 10- and 20-fold after 1 h with β-estradiol in GX6-NGG#63 and GX8-NGG#4 lines, respectively, and mostly plateaued until 24 h. We also monitored the maintenance of transcript levels of NGG in protonemata cultivated with β-estradiol. When we cultivated protonemata for 7 days in water with 1 µM β-estradiol, NGG transcripts were maintained for 7 days at the maximal level (Figure S8) and we did not detect visible changes in the morphology and growth of protonemata and gametophores during the induction period.
Transactivation in multiple loci

Simultaneous induction of multiple genes in a single line is useful to analyze complex biological pathways involving several genes. We examined whether the XVE system can be used to induce multiple genes introduced in the \textit{P. patens} genome. In addition to the pPGX vectors (Figure 1B), we constructed another vector pLGZ2 (AB602443) containing the LexA operator and the Pm35S promoter connected to the Gateway cassette to introduce a transactivated gene and the bleomycin expression cassette [36] as a selection marker for \textit{P. patens} transformants (Figure 1C). The \textit{monomeric red fluorescent protein1 (mRFP1) [43] gene} was fused with a nuclear localization signal and then the NLS-mRFP1 (NmRFP1) was integrated into pLGZ2 with the GATEWAY system to form LGZ2-NmRFP1 DNA fragment (Figure S9A). This fragment was introduced into the potential neutral site PTA2. This site consists of one of two sister ribosomal L31-like genes, both of which are similarly expressed in protonema and gametophores [38]. Transgenes showed similar expression levels in gametophore and protonema of the GX6-NGG#63 line, and singly-inserted transgenic lines were selected (Figure S9B). Protonemata and gametophores of these lines were not distinguished from those of wild type, indicating the PTA2 site is neutral at least in these tissues under regular cultivated conditions. When we observed these transgenic lines, NGG and NmRFP1 signals co-localized in the nuclei in 82.8\% (n = 64) protonemal cells and 91.6\% (n = 237) gametophore leaf cells (Figure 7). Next, we measured the transcript amounts of NGG and NmRFP1 in protonemata with RT-qPCR in six independent transgenic lines (Figure 8). Transcripts of NGG and NmRFP1 accumulated at similar levels in all lines in the presence of β-estradiol.

**Discussion**

Establishment of an estrogen-inducible gene expression system in \textit{P. patens}

We modified the \textit{A. thaliana} XVE system by changing the original promoter driving XVE to a promoter effective in \textit{P. patens} (Figure 1) and obtained reproducible amounts of transcript from a transactivated gene by β-estradiol application. Transcript levels were generally similar among independent transgenic lines for each construct (Figure 4), indicating that targeting to a specific site in the genome was effective in excluding position effects and allowed reproducible induction for each construct. Spatial patterns and transcript amounts of a transactivated gene depended on the specific GX promoter regulating XVE (Figures 2 and 4); therefore a specific GX promoter may be chosen depending on the desired levels of transcript and tissue specificity. For example, the transactivated gene was more highly induced in GX6-NGG lines than in GX8-NGG lines (Figure 4), although we need to be careful with the leaky expression of GX6 (Figure 4A), whose level is similar to that induced by the rice actin and endogenous tubulin promoters (Figures 3A and 4B). In GX6-NGG lines, the NGG marker protein was detected in a part of protonema cells (Figures 2 and S3). By contrast, in GX8-NGG lines, NGG was detected in all cells examined (Figures 2 and S3B). These constructs will be useful for induction of specific transgenes in a wide range of cells. Cell or tissue specific induction is a useful tool for functional analyses of genes [44] and further development of the \textit{P. patens} XVE system with additional promoters will enable future analysis of cell or tissue specific gene functions.

In \textit{P. patens}, the XVE system offers several advantages over current systems; specifically, it offers substantial, long-term...
induction of gene expression in the absence of detrimental side effects. The induction of NGG in GX6-NGG and GX8-NGG lines was more than ten times higher than that of Pact1 [16,42] and was similar to that of HSP [13,14] (Figures 3 and 4). Transactivated gene expression was induced by 0.001 µM β-estradiol and mostly saturated around 1 µM (Figure 5). Therefore we can regulate induction levels with β-estradiol concentrations between 0.001 µM and 1 µM. The *P. patens* XVE system also has a longer duration of maximal gene expression, at least 7 days (Figure S8), without detectable morphological or growth defects. For the heat-shock inducible system, transcript amounts peaked at 1 h after heat shock, but started to decrease at 2 h (Figure S7). Therefore the *P. patens* XVE system is useful for experiments that need long-term continuous induction like the production of biopharmaceuticals in moss bioreactors [45]. However, the XVE system needs 24 h for full induction (Figure 6), but the heat shock inducible system needs 1 h for maximum induction, indicating that the heat shock system is more suitable for experiments requiring immediate induction.

**Figure 7.** Spatial expression patterns of dually induced NGG and NmRFP1. Fluorescence images of protonemata and a gametophore leaf of the GX6-NGG#63/LGZ2-NmRFP1#11 line. They were immersed in water containing 1 µM β-estradiol for 24 h before microscopy. Fluorescence images of NGG and chlorophyll autofluorescence (top row), NGG (second row), NmRFP1 (third row), and a merged image of NGG and NmRFP1 (bottom row) are indicated. Each picture is at the same magnification. Bar = 200 µm.

doi: 10.1371/journal.pone.0077356.g007
Dual gene induction with the *P. patens* XVE system

We successfully induced two genes in each cell when GX6-NGG and LGZ2-NmRFP1 were introduced to independent targeting loci in a single line (Figures 1 and S9). When LGZ2-NmRFP1 was introduced into transgenic lines with GX6-NGG we had to be careful as LGZ2-NmRFP1 may be targeted by homologous recombination to the sequences shared with GX6-NGG. In our experiments, we obtained appropriate integration in 16.3% of transformants (n = 49). We could not find differences in frequency to other gene targeting experiments with single copy insertion at a targeting site [46]. Therefore, dual gene induction is feasible with this system.

It is noteworthy that GX-NGG and LGZ2-NmRFP1 DNA fragments integrated in different targeting sites were similarly regulated by XVE proteins and NGG and NmRFP1 transcripts were induced in similar amounts (Figure 8, Student’s t-test: p=0.3014). In addition, both proteins showed similar spatial expression patterns (Figure 7). By contrast, we compared relative transcript levels of NGG in GX6-NGG#63 lines to those in GX6-NGG#63/LGZ2-NmRFP1 lines with β-estradiol (Figures 4 and 8), the former was significantly higher than the latter (nonparametric ANOVA: p=0.0351, *<0.05). This difference appears to be caused by the number of targeted loci by XVE: GX6-NGG#63 lines harboring one targeted locus (NGG), on the other hand, GX6-NGG#63/LGZ2-NmRFP1 lines harboring two targeted loci (NGG and NmRFP1). This system will be useful to induce genes that encode proteins that function as heterodimers or as sequentially reacting enzymes. Furthermore, when a transgene is introduced to the PTA2 site of a GX6-NGG or GX8-NGG line, the NGG signal marks the cells in which the transgene should be induced. Moreover, this XVE system with dual gene induction is also applicable for loss-of-function analyses by inducing artificial microRNA [47] or inverted repeat dsRNAs with stem-loop structures [27].

Comparison of XVE systems between *P. patens* and *A. thaliana*

In both *A. thaliana* and *P. patens* XVE systems, the transactivated gene was induced 1 h after β-estradiol application, and the transcript levels reached a maximum around 24 h [22] (Figure 6). This suggests that the activation of XVE proteins by β-estradiol proceeds with similar kinetics in both plants. By contrast, the induced transcript amount in *A. thaliana* was attenuated at 48 h after β-estradiol addition [22], but transcript levels in *P. patens* were maintained for at least 7 days on medium containing β-estradiol (Figure S8). Differences were also observed in dose-responsiveness to β-estradiol. Induction of the transactivated gene in *P. patens* (Figure 5) and *A. thaliana* [22] was detected with 0.001 µM and 0.008 µM β-estradiol, respectively, and reached a maximum at around 1 µM and 5 µM, respectively. Although the β-estradiol treatment regimen differs for these plants, these differences may also be related to diverged metabolic pathways of steroid biosynthesis and degradation between *A. thaliana* and *P. patens* [48].

Materials and Methods

Plant materials and growth conditions

The moss *Physcomitrella patens* Gransden 2004 strain [3] was used as a wild type and cultured on BCDAT agar medium under continuous white light at 25°C [5].

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**Figure 8. Transcript amounts for dually induced NGG and NmRFP1 in transgenic *P. patens* lines.** (A, B, C) Transcript amounts of NGG (A), NmRFP1 (B), and TUA1 (C) in protonemata of GX6-NGG#63/LGZ2-NmRFP1 lines. Protonemata were immersed in water with (+: circles) or without (-: crosses) 1 µM β-estradiol for 24 h before sampling. Copy numbers of NGG, NmRFP1, and TUA1 genes per pg total RNA were estimated by RT-qPCR.

doi: 10.1371/journal.pone.0077356.g008
**Plasmid construction**

To construct the Pm35S DNA fragment, we annealed the oligonucleotides 5'-GACCCCTCTCTATATAAGGAAGTTCATTCTTATGGGAGGAG and 5'-GACTAGCTTACGTGTTCTCCTACAGATAAAGTAACTTCTTATAGAGGAAGGGTC-3'. To construct the PAct1 DNA fragment, the OsACT1 promoter region [16,42] was PCR-amplified from the pTKM1 plasmid [16,42] with primers 5'-CTTCAGGTCTGTTCTCATTATAGGTGAG-3' and 5'-ATCTTCTACTTACAAAAAGCTCAG-3'. To construct the HSP DNA fragment, the Gmhsp17.3B promoter region was PCR-amplified from pPTA2-HSP with primers 5'-TCTAGATAGTCAGCTTTAAAGAGATAAG-3' and 5'-P4345 HSP-blunt-r-3'. Pm35S, Pact1, and HSP DNA fragments were inserted into pPIG1b-LGXH at the XbaI site to construct Pm35S:NGG, Pact1:NGG, and HSP:NGG plasmids, respectively. Direction and sequences of the inserts in the constructs were confirmed by PCR and sequencing.

To construct the pPGX vectors, DNA fragments including the LexA:Pm35S and the Tpea3A terminator region were PCR-amplified from the pPIG1b-NGGII plasmid (AB537478) [26,36] at the Smal site to construct Pm35S:NGG, Pact1:NGG, and HSP:NGG plasmids, respectively. Direction and sequences of the inserts in all constructs were confirmed by PCR and sequencing.

To construct the pPGX vectors, DNA fragments including the LexA:Pm35S and the Tpea3A terminator region were PCR-amplified from the pPIG1b-NGGII plasmid (AB537478) [26,36] at the Smal site to construct Pm35S:NGG, Pact1:NGG, and HSP:NGG plasmids, respectively. Direction and sequences of the inserts in all constructs were confirmed by PCR and sequencing.

**Transformation**

Transformation of *P. patens* with the polyethylene glycol method was carried out as described previously [50]. Appropriate targeting was confirmed by PCR and DNA gel blot analyses [26,36].

**β-estradiol treatment**

β-estradiol (Wako) was dissolved to 10 mM in dimethyl sulfoxide (DMSO) (Wako) and stored at -30°C. To apply β-estradiol to GX-NGG lines, the stock solution was diluted to an appropriate concentration with sterile deionized water and then, 20 µl of β-estradiol solution was poured into a 9 cm plastic dish in which GX-NGG lines were cultivated. They were incubated at 25°C under continuous light until used for microscopy and sampling for RNA extraction.

**Microscopy**

Fluorescent signals were observed by a fluorescence stereo microscope (SZX16; Olympus) and an inverted fluorescence microscope (IX-70; Olympus). Images were recorded by digital camera (DP-71 and E-PL1s; Olympus). Image intensities of cDNAs, 1 µg of total RNA was dissolved in 11 µl of nuclease-sulfoxide (DMSO) (Wako) and stored at -30°C. To apply β-estradiol to GX-NGG lines, the stock solution was diluted to an appropriate concentration with sterile deionized water and then, 20 µl of β-estradiol solution was poured into a 9 cm plastic dish in which GX-NGG lines were cultivated. They were incubated at 25°C under continuous light until used for microscopy and sampling for RNA extraction.

**RNA extraction and RT-qPCR**

Total RNA from protonemata cultured on BCDAT medium covered with cellophane for 8 days was extracted by RNeasy plant mini kit (Qiagen) with DNase treatment on columns according to the manufacturer's instructions. To synthesize cDNAs, 1 µg of total RNA was dissolved in 11 µl of nuclease-free water and 1 µl of 0.5 µg/µl oligo-(dT)12-18 primer (Life Technologies) was added.
DNA Gel-Blot Analyses

Approximately 3 µg of genomic DNA was digested with restriction enzymes, run on 0.7% (w/v) SeaKem GTG agarose (Lonza), and transferred to a Hybond-N+ nylon membrane (GE Healthcare). Probe labeling, hybridization, and detection were performed using the AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare) according to the manufacturer’s instructions [26,36].

Statistical analysis

Statistical analysis of qPCR data was performed using R [52]. Relative transcript levels of NGG in GX6-NGG#63 and GX6-NGG#63/LGZ2-NmRFP1 lines deviated significantly from the normal distribution (Shapiro.test [53]). Hence, data were analyzed using nonparametric ANOVA as implemented in the nparcomp package [54].

Accession Numbers

Sequences and information of the vectors in this work have been deposited to DDBJ/GenBank/EMBL data libraries as follows: pPIG1b-LGXH (AB602444), pPGX6 (AB537481), pPGX8 (AB537482), and pLGZ2 (AB602443).

Supporting Information

Figure S1. DNA gel blot analyses of P. patens the GX6-NGG transgenic line. (A) Schematic representation of a genomic locus and the construct. LexA:Pm35S: eight copies of LexA operators [22] connected to CaMV minimal 35S promoter [23]. NGG: the NLS-GFP-GUS (NGG) fusion gene composed of a nuclear localization signal (NLS [39]), the green fluorescent protein (sgFP [40]) gene, and the uidA (GUS [41]) gene, Tpea3A: a Tpea3A terminator [34], PGX8: the hygromycin phosphotransferase expression cassette [35,36]. Gray bars indicate probe regions for DNA gel blot analyses. (B) DNA gel blot analyses of transgenic P. patens lines GX6-NGG. Each genomic DNA was digested with EcoT22I. DNA gel blot analyses of GX6-NGG#63 and GX6-NGG#129 were previously reported [26]. (TIF)

Figure S2. DNA gel blot analyses of P. patens GX8-NGG transgenic lines. (A) Schematic representation of a genomic locus and the construct. LexA:Pm35S: eight copies of LexA operators [22] connected to CaMV minimal 35S promoter [23]. NGG: the NLS-GFP-GUS (NGG) fusion gene composed of a nuclear localization signal (NLS [39]), the green fluorescent protein (sgFP [40]) gene, and the uidA (GUS [41]) gene, Tpea3A: a Tpea3A terminator [34], PGX8: GX8 promoter, XVE: the chimeric gene with a LexA-binding [28,29], a VP16 activator [30], and an estrogen receptor domain [31], TrbcS: a TrbcS terminator [34], aphIV: the hygromycin phosphotransferase expression cassette [35,36]. Gray bars indicate probe regions for DNA gel blot analyses. (B) DNA gel blot analyses of transgenic P. patens lines GX8-NGG. Each genomic DNA was digested with BglII. (TIF)

Figure S3. 8-day old protonemata of GX6-NGG and GX8-NGG lines and high-resolution images of chloronema and cauronema cells in GX6-NGG lines with β-estradiol. (A,B) Fluorescence images of 8-day old protonemata of GX6-NGG#63 (A) and GX8-NGG#4 (B) lines. The field (C, E, G) and fluorescence (D, F, H) images of chloronema (C, D, E, F) and cauronema (G, H) cells of GX6-NGG#63 lines. Arrows in D and F indicate nuclei with GFP signals. Bars: B = 1 mm, D, F, and H = 200 µm. (TIF)

Figure S4. DNA gel blot analyses of transgenic P. patens Pm35S:NGG lines. (A) Schematic representation of the genomic locus and the construct. Pm35S: CaMV minimal 35S promoter [23]. NGG: the NLS-GFP-GUS (NGG) fusion gene composed of a nuclear localization signal (NLS [39]), the green fluorescent protein (sgFP [40]) gene, and the uidA.
gene cassette [26]. Other abbreviations are indicated in the protein

Figure S5. DNA gel blot analysis of transgenic P. patens Pact1:NGG lines. (A) Schematic representation of a genomic locus and the construct Pact1: the rice actin1 promoter [16,42], NGG: the NLS-GFP-GUS (NGG) fusion gene composed of a nuclear localization signal (NLS [39]:), the green fluorescent protein (sGFP [40]:) gene, and the uidA (GUS [41]:) gene, Tnos: a nos terminator [26], BSD: the blasticidin S deaminase gene cassette [26]. Gray bars indicate probe regions for DNA gel blot analyses. (B) DNA gel blot analyses of GX6-NGG transgenic P. patens lines. Each genomic DNA was digested with EcoT22I.

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Figure S6. DNA gel blot analysis of transgenic P. patens HSP:NGG lines. (A) Schematic representation of a genomic locus and the construct. HSP: the soybean Gmhsps17.3B promoter [13,14], NGG: the NLS-GFP-GUS (NGG) fusion gene composed of a nuclear localization signal (NLS [39]:), the green fluorescent protein (sGFP [40]:) gene, and the uidA (GUS [41]:) gene, Tnos: a nos terminator [26], BSD: the blasticidin S deaminase gene cassette [26]. Gray bars indicate probe regions for DNA gel blot analyses. (B) DNA gel blot analyses of HSP:NGG transgenic P. patens lines. Each genomic DNA was digested with Hincll.

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Figure S7. Time-course responsiveness to heat shock treatment of the HSP:NGG#5 line. Protonemata were incubated at 25°C (crosses) and 38°C (squares) for 0, 1, 2, 6, 12, and 24 h and then immediately collected. Relative transcript levels of NGG are normalized to TUA1 and then standardized to a normalized transcript level of protonemata at 0 h. Error bars indicate SD of the mean (n = 3).

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Figure S8. Time course responsiveness to β-estradiol in the GX6-NGG and GX8-NGG lines. (A, B) Relative transcript levels of NGG in protonemata of GX6-NGG#63 (A) and GX8-NGG#4 (B) lines. Relative transcript levels of NGG are normalized to TUA1 and then standardized to a normalized transcript level of protonemata at 0 h. Protonemata were immersed in water with (+: circles) or without (−: crosses) 1 µM β-estradiol and collected after 1, 2, 4, and 7 d. Error bars indicate SD of the mean (n = 3).

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Table S1. Primers for cloning of GX promoter regions.

(PDF)

Table S2. Primers for qPCR.

(PDF)

Acknowledgements

We would like to thank N.-H. Chua for providing the pER8 vector; T. Murata and Y. Tamada for experimental suggestions; D. Lang for helping statistical analyses; and Y. Oguri, M. Obara, T. Nishi, A. Akita, N. Misawa, C. Honda, S. Wakazuki, and members of the ERATO Reprogramming Evolution Project for technical assistance. We are also grateful to the Functional Genomics Facility, Model Plant Research Facility and Data Integration and Analysis Facility of National Institute for Basic Biology.

Author Contributions

Conceived and designed the experiments: MK TN MH. Performed the experiments: MK AI MI YS TK YH. Analyzed the data: MK TN TK MH. Wrote the manuscript: MK TN RR MH.

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