ELSEVIER

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Journal of Microbiological Methods 59 (2004) 181–188

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Construction and use of GFP reporter vectors for analysis of cell-type-specific gene expression in *Nostoc punctiforme*

Claudia Argueta, Kamile Yuksek, Michael Summers*

California State University Northridge, Department of Biology, 18111 Nordhoff St., Northridge, CA 91330-8303, United States

Received 6 June 2004; received in revised form 25 June 2004; accepted 25 June 2004
Available online 12 August 2004

Abstract

Two transcriptional reporter shuttle vectors were constructed for the filamentous cyanobacterium *Nostoc punctiforme* using the green fluorescence protein (GFP) reporter. Both the ampicillin- and kanamycin-resistant versions of the plasmid allow promoters to be directionally cloned into a multiple cloning site preceding a promoterless *gfp* gene using an *Escherichia coli* host. The ability of the self-replicating shuttle plasmids to report cell-type-specific gene expression in *N. punctiforme* was tested by cloning promoters expressed in normal vegetative cells, nitrogen-fixing heterocysts and spore-like akinetes. A P_{psaC} reporter gene fusion was expressed in vegetative cells and not in heterocysts, whereas GFP driven from P_{hetR} was found highly expressed in heterocysts. GFP expression driven by the promoter for the *N. punctiforme* homologue of the akinete-specific gene *avaK* was expressed in developing akinetes. Decreased expression of GFP from the P_{psaC} reporter in hormogonia was also observed. The results demonstrate the utility of these GFP vectors to study cell-type-specific gene expression in differentiating filamentous cyanobacteria.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Filamentous cyanobacteria; GFP; Transcriptional reporter vectors; Akinetes; Hormogonia; Heterocysts; pSUN119; pSUN202

1. Introduction

Cyanobacteria are an ancient and diverse class of bacteria that perform photosynthesis much like green plants. Some filamentous members, like *Nostoc punctiforme*, are capable of differentiating vegetative cells into three cellular types. In the absence of combined nitrogen, 5–10% of a filament's vegetative cells differentiate into N_2-fixing heterocysts equally spaced along the filament. Terminally differentiated heterocysts provide reduced nitrogen to neighboring vegetative cells and can be identified by their lack of light harvesting photosynthetic pigments and thicker external cell layers. Akinetes are produced from vegetative cells under conditions of energy limitation such as low light or limiting phosphate. They can withstand long periods of desiccation or cold, and germinate back into

* Corresponding author. Tel.: +1 818 677 7146; fax: +1 818 677 2034.
E-mail address: michael.l.summers@csun.edu (M. Summers).

0167-7012/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2004.06.009

vegetative cells when conditions improve. The small-celled motile hormogonia filaments move by gliding motility and are formed from vegetative cells through several rounds of division without cell growth. This transient cell type allows a filament to escape undesirable conditions before reverting back to vegetative cells, and is involved in colonization of its plant partner in symbiotic relationships (Meeks et al., 2002).

Due to its recently sequenced genome and the wide range of genetic tools available for use in *N. punctiforme*, there is increasing interest in the use of this organism to identify genetic regulation involved with simple cellular differentiation (Meeks et al., 2001). Because only a subset of cells in a culture typically differentiates into heterocysts or akinetes, methods of studying differential gene regulation such as Northern blotting, differential display, quantitative RT-PCR, or array analysis rely on RNA harvested from a mixed population of cell types. Proof that a gene is uniquely expressed in a particular cell type is usually required. Linking of the transcriptional reporters to the promoters of specific genes and visualization with microscopy is the usual method employed to provide proof of cell-type-specific gene expression.

Past work using transcriptional reporters to visualize cell-type-specific gene expression relied heavily on bacterial luciferase encoded by the *luxAB* genes (Thiel, 1994). This reporter requires sensitive digital cameras using long exposures to gather the faint light emission, and digital enhancement to eliminate electronic noise from the resulting images. Improvements to amplify luciferase production by placing the promoter under study in front of T7 polymerase that would in turn drive transcription of *luxAB* under regulation of the T7 promoter (Wolk et al., 1993), and to increase substrate availability by inclusion of genes required for synthesis of the aldehyde luciferase substrate on a plasmid within the cell (Fernandez-Pinas and Wolk, 1994) have increased the sensitivity of this reporter.

More recently, the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has become the reporter of choice for in vivo analysis (Yoon and Golden, 1998; Kunert et al., 2000; Wong and Meeks, 2001). This inert reporter is excited by UV light (395 nm) and emits green light (509 nm) making it more easily visualized using epifluorescence microscopy employing commonly available filter sets. GFP has the additional advantage in that it does not require a substrate, eliminating associated solubility, toxicity, or permeability problems.

This work describes the construction and testing of two GFP reporter shuttle plasmids enabling a single-step cloning process to be done in *E. coli*, and direct transfer of the resulting plasmid for testing in filamentous cyanobacteria. The aim of this work was to create vectors useful for quick confirmation of cyanobacterial cell-type-specific gene expression identified by other RNA-based approaches such as differential display or array analysis.

2. Materials and methods

2.1. Strains and culture conditions

All cloning was done using *Escherichia coli* strain DH5α MCR grown at 37 °C in Luria broth (Sambrook et al., 1989) liquid media supplemented with 100 μg/ml ampicillin or 25 μg/ml kanamycin, and on plates solidified with 1.5% agar. *N. punctiforme* was cultured in AA/4 liquid medium (Allen and Arnon, 1955) supplemented with 5 mM MOPS buffer (pH 7.8) with or without 2.5 mM NH_4Cl added as a combined nitrogen source, and on AA plates solidified with 1% noble agar with similar buffer and nitrogen additions. For *N. punctiforme*, 5 μg/ml ampicillin or 10 μg/ml neomycin was used for plasmid selection. The phosphate component of the medium was not added when preparing liquid medium for akinete induction. Plates, or shaking liquid cultures, were grown in air at 25 °C under 19 μmol photons/m^2/s. Incubation for akinete induction was performed at 11 μmol photons/m^2/s in non-shaking flasks.

2.2. Molecular biology methods

All routine DNA techniques such as plasmid isolation and digestion, PCR amplification using *Taq* polymerase, preparation and transformation of $CaCl_2$ competent *E. coli*, phenol/chloroform extractions, and ligations were performed using standard methods (Sambrook et al., 1989). Isolation of total genomic DNA and electrotransformation of plasmids into *N. punctiforme* was performed as previously described (Summers et al., 1995).

2.3. Details of vector construction

The ampicillin-resistant promoter reporter vector pSUN202 was constructed by using PCR to amplify a 1078-bp fragment of pIGA (Kunert et al., 2000) containing the promoterless *gfp* gene using primer set Psak1 and Psak2 (Table 1). On this fragment, the *gfp* gene is preceded by aT7terminator sequence, simple multiple cloning site, and an associated ribosome binding site. The resulting PCR fragment was digested with *Sph*I, and cloned into the *Eco*RI (destroyed by blunting with T4 DNA polymerase)/*Sph*I sites of the *E. coli*/*N. punctiforme* shuttle vector pSCR202 (Summers et al., 1995). The resulting pSUN202 plasmid contains a multiple cloning site in the order T7 terminator–*Pst*I–*Cla*I–*Sma*I(*Xma*I)–*Kpn*I–*Eco*RI–*gfp* (Fig. 1).

The kanamycin-resistant promoter reporter vector pSUN119 was constructed by ligating a similarly prepared *gfp* fragment into the *Sac*I (destroyed by blunting with T4 DNA polymerase)/*Sph*I sites of the *E. coli*/*N. punctiforme* shuttle vector pSCR119 (Summers et al., 1995). The resulting pSUN119 plasmid contains a multiple cloning site in the order T7 terminator–*Pst*I–*Cla*I–*Sma*I(*Xma*I)–*Kpn*I–*gfp* (Fig. 1). Correct cloning junctions for both plasmids were confirmed by sequencing using the M13 forward and reverse primer (Table 1) sites present within the original shuttle vectors.

Table 1
Primers used to amplify DNA fragments by PCR for vector construction, confirmation, and generation of cloned promoter fragments

Primer name	Sequence
Psak1–*Sph*I	AGCTGCATGCGCTGCTGCCACCGCTGAGCA
Psak2	TATTTGTAGAGCTCATCCA
PhetR1–*Pst*I	CGGCTGCAGTTGAGATTACTCCCAACGAT
PhetR2–*Eco*RI	GTTGAATTCATTACAGACAATTGAATAGC
PhetR2–*Kpn*I	GGGGTACCATTACAGACAATTGAATAGC
PpsaC1–*Pst*I	GTTCTGCAGTGCGCTTATTAAGAGTTATG
PpsaC2–*Eco*RI	TGAGAATTCCGCTCCTTTCGAGTGTTCTT
PpsaC2–*Kpn*I	GGGGTACCGCTCCTTTCGAGTGTTCTT
PavaK1–*Pst*I	AAACTGCAGTGTTACAGTGCCTATTCAA
PavaK2–*Pst*I	CAACTGCAGCATCAGTACCTTGTGTATAA
M13 forward	CGCCAGGGTTTTCCCAGTCACGAC
M13 reverse	TCACACAGGAAACAGCTATGAC
GFP forward	TATAGCGCTAGAGTCGACCT
GFP reverse	GAGTCTCCAGTTTGTTTCACT

Restriction enzyme sites added to primer 5′-ends are underlined.

2.4. Insertion of cell-type-specific promoter fragments

The *hetR* gene promoter was amplified from genomic DNA by PCR using primer set PhetR1–*Pst*I and PhetR2–*Kpn*I (Table 1). The resulting product containing 502 bp upstream of the *N. punctiforme hetR* coding region was digested with *Pst*I/*Kpn*I, and ligated into the *Pst*I/*Kpn*I sites present within the multiple cloning site of pSUN119 to produce the heterocyst reporting plasmid pSUN5. The ampicillin-resistant version of the promoter reporter was made by PCR amplification using primer set PhetR1–*Pst*I and PhetR2–*Eco*RI (Table 1). The resulting product was digested with *Pst*I/*Eco*RI, and ligated into the *Pst*I/*Eco*RI sites present within the multiple cloning site of pSUN202 to produce plasmid pSUN4.

The *psaC* gene promoter was amplified by PCR using primer set PpsaC1–*Pst*I and PpsaC2–*Kpn*I (Table 1). The resulting product containing 400 bp upstream of the *N. punctiforme psaC* coding region was digested with *Pst*I/*Kpn*I, and ligated into the *Pst*I/*Kpn*I sites of pSUN119 to produce the vegetative cell reporting plasmid pSUN6. The ampicillin-resistant version of the promoter reporter was made by PCR amplification using primer set PpsaC1–*Pst*I and PpsaC2–*Eco*RI (Table 1). The resulting product was digested with *Pst*I/*Eco*RI, and ligated into the *Pst*I/*Eco*RI sites of pSUN202 to produce the vegetative cell reporting plasmid pSUN10.

The *avaK* gene promoter was PCR amplified using primer set PavaK1–*Pst*I and PavaK2–*Pst*I (Table 1). The resulting product containing 99 bp of the *N. punctiforme avaK* coding region and 686 bp of upstream DNA was digested with *Pst*I, and ligated into *Pst*I-digested and phosphatased-treated vectors pSUN119 and pSUN202 to produce the akinete reporting plasmid pSUN7 and pSUN8, respectively. Correct orientation of the plasmid inserts to drive *gfp* transcription was confirmed by PCR using PavaK1 and the GFP-R primer located near the translational start of the *gfp* gene. The sequence of all plasmid inserts was confirmed by sequencing using forward and reverse GFP primers (Table 1) located on either side of the MCS in pSUN119 and pSUN202.

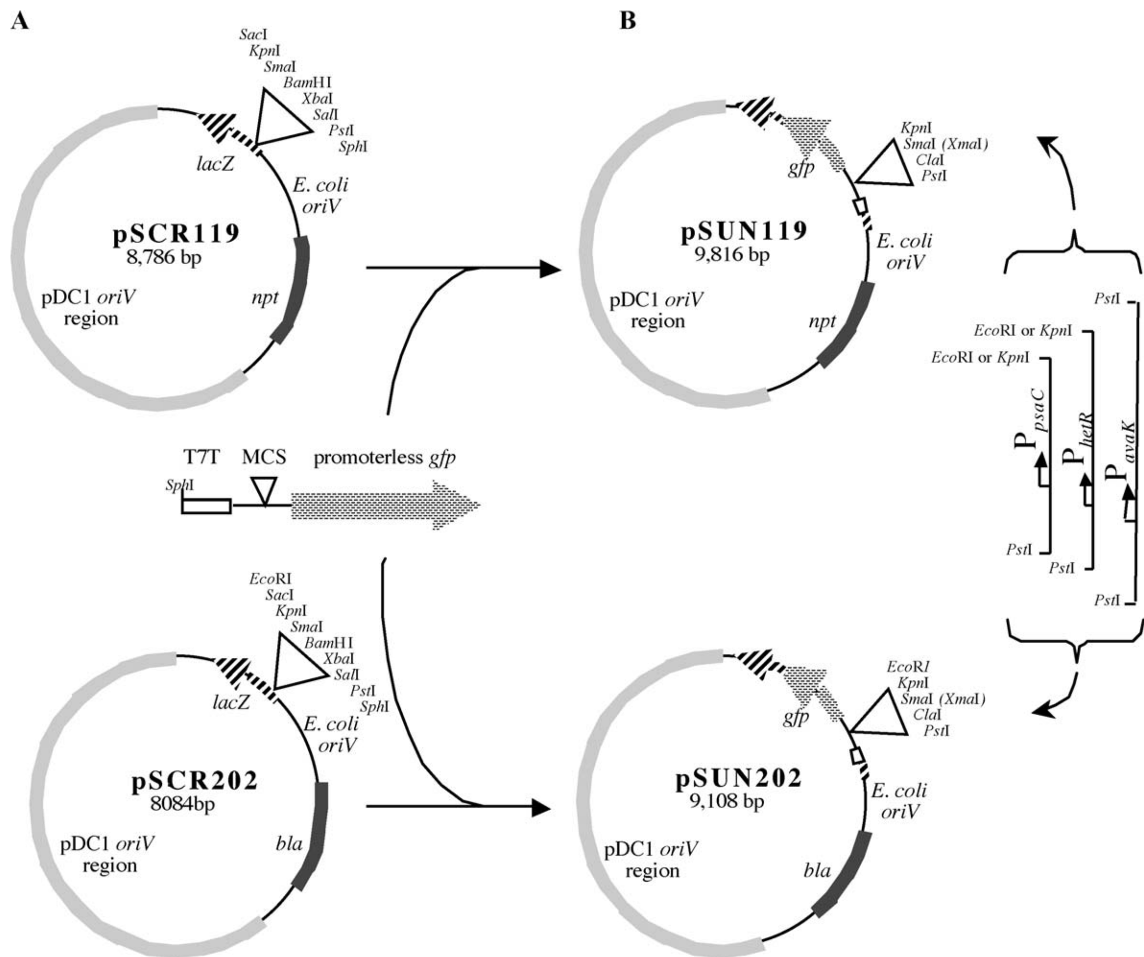

Fig. 1. Schematic diagram of plasmid construction. (A) pSUN199 and pSUN202 were formed by replacing the multiple cloning site (MCS) of the shuttle vectors pSCR119 and pSCR202, respectively, with a PCR fragment containing a promoterless green fluorescent protein gene (*gfp*) obtained from pIGA (Kunert et al., 2000). The PCR fragment contained a T7 terminator (T7T) and small MCS preceding *gfp*. pSUN119 and 202 confer resistance to kanamycin/neomycin and ampicillin, through the action of neomycin phosphotransferase (*npt* gene product) and beta-lactamase (*bla* gene product), respectively. (B) PCR fragments bearing promoter regions used to illustrate the usefulness of these vectors were directionally cloned into the MCS pSUN119 and pSUN202 using the restriction enzyme sites shown. Inserts not drawn to same scale as plasmids.

2.5. Visualization of cell-type-specific GFP expression

Images were obtained using a Zeiss Axiolab microscope containing a ×100 oil immersion objective for brightfield and epifluorescence microscopy. Fluorescence images were obtained using a longpass blue excitation filter (395 nm) and a green bandpass (509 nm) filter set (Omega Optical) and captured with a DVC 1312 high resolution digital camera. Uniform exposure times were used for all photos to allow comparisons of relative GFP expression between strains.

3. Results and discussion

The *gfp* gene used to form the pSUN119/202 plasmids originated from an improved version, having 45-fold increased fluorescence over the wild-type product, primarily due to increased solubility and

subsequent reduction of inclusion body formation (Crameri et al., 1996). This *gfp* gene is preceded by a strong ribosome binding site from the *E. coli atpE* gene (Suarez et al., 1997) to assure translation once transcribed, and a strong transcriptional terminator upstream of the multiple cloning site to remove reporter expression from promoters within the plasmid (Kunert et al., 2000; Milkowski et al., 1998). Minimal expression of GFP was observed in controls having no inserts, indicating that any transcription arising from the *lac* promoter present in the parent vector was not able to extend into the *gfp* gene and cause background fluorescence in any cell type of *N. punctiforme* (Fig. 2G and H).

Various known cell-type-specific gene promoters were placed in pSUN119 and pSUN202 to test the utility of these plasmids. Results obtained for pSUN202 promoter reporter and control plasmids

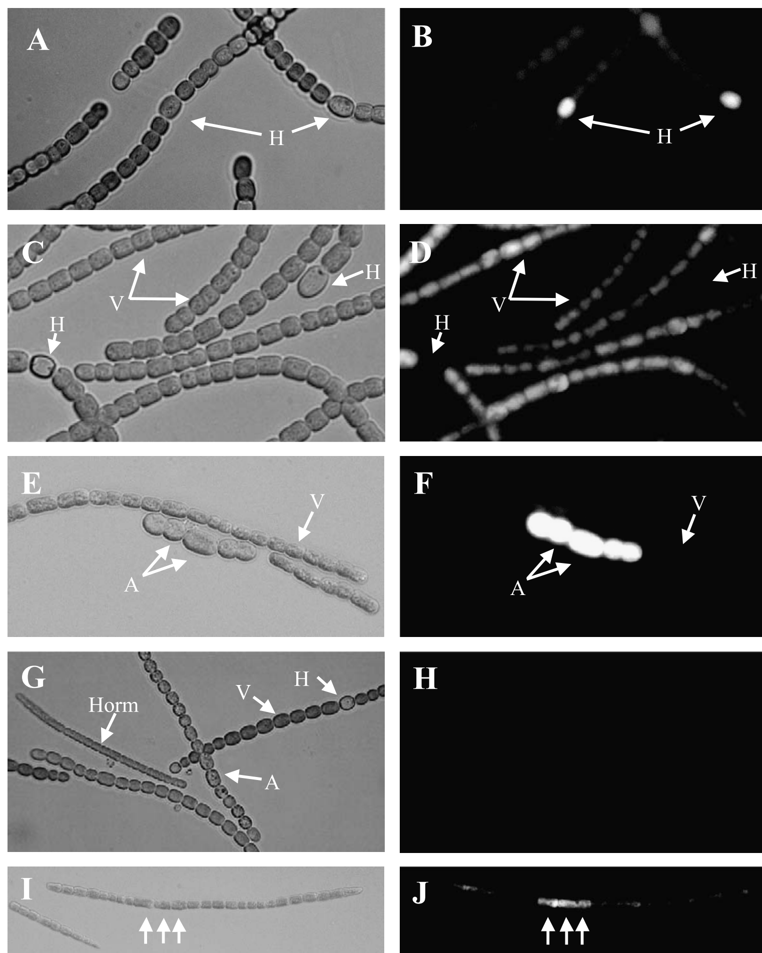

Fig. 2. Confirmation of cell type-specific gene expression. Brightfield (A, C, E, G, I) and epifluorescence (B, D, F, H, J) micrographs of plasmid bearing strains of *N. punctiforme*. (A, B) pSUN5 bearing strain containing the *hetR*-GFP reporter plasmid gown in medium lacking combined nitrogen illustrating heterocyst (H) specific gene expression. (C, D) pSUN6 bearing strain containing the *psaC*-GFP reporter plasmid gown in medium lacking combined nitrogen illustrating vegetative cell (V) gene expression. (E, F) pSUN7 bearing strain containing the *avaK*-GFP reporter plasmid after 14 days of phosphate starvation illustrating akinete (A) specific gene expression. (G, H) Strain bearing pSUN119 vector alone demonstrating low background fluorescence in vegetative cells, heterocysts, akinetes, and in small cells of a hormogonia filament (Horm). (I, J) pSUN6 bearing strain illustrating reduced *psaC*-GFP reporter expression in developing hormigonial cells. Larger and brighter cells (arrows) have not yet divided into smaller type cells found in a mature hormogonia filament. Similar results were obtained for strains bearing the ampicillin version of identical constructs using plasmid pSUN202 (data not shown).

(pSUN4, pSUN10, pSUN8) were identical to those constructed in pSUN119 (pSUN5, pSUN6, pSUN7, respectively), therefore only the results from the pSUN119 plasmid series are presented in to avoid redundancy. The nucleotide sequence of the pSCR119, pSUN119, pSUN202 and pSCR202 vectors have been assigned GenBank accession numbers AY622810 through AY622813, respectively.

HetR has been shown to be essential for heterocyst development in both *Anabaena* PCC 7120 and *N. punctiforme* (Buikema and Haselkorn, 1991; Wong and Meeks, 2001). The *hetR* gene encodes a serine-type protease that has recently been shown to bind DNA when dimerized (Huang et al., 2004). HetR positively autoregulates its own expression and is transcribed early in developing heterocysts following nitrogen starvation (Black et al., 1993). The *hetR* promoter region of *N. punctiforme* was used to test our vectors for heterocyst-specific gene expression. Following transfer to medium lacking combined nitrogen, heterocysts exhibited high levels of GFP expression as expected, demonstrating the utility of these vectors to report transcription in this cell type.

In *Nostoc ellipsosporum* a *hetR*::*lux AB* fusion was used to demonstrate expression of *hetR* in akinetes, and to support the hypothesis that *hetR* is required for differentiation of both heterocyst and akinete differentiation (Leganes et al., 1994). However, in *N. punctiforme*, a *hetR* mutant was able to form cold-tolerant akinetes that lacked the granulation associated with these cell types (Wong and Meeks, 2002). To test if *hetR* is expressed in akinete of *N. punctiforme*, we observed our *hetR* reporter strain following phosphorous starvation. The *hetR* gene reporter was not induced in akinetes relative to non-differentiating vegetative cells (data not shown).

The *psaC* gene encodes one subunit of photosystem I (PSI) containing two iron–sulfur centers that are the terminal electron acceptors of photosystem I, and is essential for the stable association of the PsaD and PsaE proteins into the cyanobacterial PSI complex (Mannan et al., 1994). This gene is expected to be expressed in actively growing photoautotrophic cells and was used as our vegetative cell control. Although heterocysts contain an active photosystem I to produce ATP via cyclic photophosphorylation for use in nitrogen fixation (Tel-Or and Stewart, 1976), these terminally differentiated cells do not grow. Thus, we hypothesized only limited *psaC* transcription would occur in heterocysts. High levels of GFP expression by the *N. punctiforme psaC* promoter was observed in vegetative cells and not in terminally differentiated heterocysts (Fig. 2C and D) as expected, indicating the vectors correctly report transcription in vegetative cells. PsaC is similarly expressed in phosphate starvation induced akinetes and vegetative cells, but expression declines in older mature akinetes.

In hormogonia, GFP expression from the *psaC* promoter is reduced relative to vegetative cells (Fig. 2I and J). This observation correlates with decreased photosynthetic rate in hormogonia found by Campbell and Meeks (1989). Reduced transcription of this photosystem I protein in developing hormogonia might also be expected, since it correlates with transient transcriptional repression of phycobiliprotein genes required for synthesis of photosystem associated phycobilisome complexes (Damerval et al., 1991).

The *avaK* gene encodes a protein of unknown function that is present in filamentous cyanobacteria, but not in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. This gene is expressed primarily in akinetes as demonstrated by akinete-specific fluorescence in an *avaK*::*gfp* chromosomal insertion strain of *Anabaena variabilis* (Zhou and Wolk, 2002). When the promoter region of the *N. punctiforme avaK* gene homologue was placed in pSUN119 to form pSUN7, high *avaK* driven GFP expression was observed in akinetes (Fig. 2E and F). GFP expression was not observed in heterocysts or photoautotrophicaly grown vegetative cells containing pSUN7. In summary, the results from *avaK* combined with the *hetR* and *psaC* controls indicate the utility of these vectors to accurately report cell-specific gene expression in *N. punctiforme*.

Both pSUN119 and 202 contain the ColE1 origin of replication originating from pUC19, and routine high plasmid yields from *E. coli* indicates that the plasmid copy number for these plasmids remains high. When the plasmids are in *N. punctiforme,* the pDC1 origin of replication used by both vectors was determined by DNA hybridization to provide approximately 14 copies per chromosome for pSCR119 (Summers et al., 1995). This value changed little when inserts of up to 10 kb in length were inserted, so it is unlikely that the copy number for pSUN119 and pSUN202 would be affected by the addition of the 1.1 kb *gfp* fragment or added

promoter fragments. The copy number of these plasmids would increase the reporter sensitivity for genes expressed at low levels when compared to a reporter in single copy on the chromosome. Since the plasmid copy number variation between different cell types has not yet been determined, quantitative analysis of relative expression levels between cell types using GFP fluorescence is not possible. However, as our controls indicate, these plasmids can effectively indicate cell-type-specific gene expression where large differences are usually observed.

Transfer of plasmids to *N. punctiforme* can be affected by triparental conjugation from an *E. coli* host using the IncP type plasmid transfer system (Cohen et al., 1998), as has been developed for *Anabaena* sp. strain PCC 7120 (Wolk et al., 1984). In addition, electrotransformation has also been used routinely in *N. punctiforme* for transfer of plasmids lacking an *oriT* site required for conjugation. The *E. coli* replicon portion of pSUN119 is derived from pARO191 (Parke, 1990), but has lost the *oriT* site from RP4 enabling conjugal transfer in the cloning process. Similarly, the *E. coli* replicon component of pSUN202 was derived from the non-mobilizable plasmid pUC19 (Yanisch-Perron et al., 1985). Therefore, both plasmids require transfer by artificial transformation. The method of electroporation is especially useful due to the large size of many cyanobacterial vectors and the efficient transfer of plasmids irregardless of the plasmid size (Sambrook et al., 1989). Electrotransformation of non-conjugatable shuttle vectors does not require extensive liquid growth following transfer to remove contaminating *E. coli* following triparental conjugation, and therefore requires less time between cloning and testing in cyanobacteria. Electrotransformation may not be amenable to other filamentous cyanobacteria unless the plasmid is obtained from an appropriate *E. coli* host that provides methylation patterns required for protection from endogenous cyanobacterial restriction enzymes (Elhai and Wolk, 1988).

Translational fusions to GFP that retain their fluorescence properties have been used to identify protein localization in bacteria when fused to either the N- or C-terminus of a protein (Margolin, 2000). Recently, a HetR–GFP fusion has been demonstrated in *N. punctiforme* to retain HetR functionality and reporter activity (Wong and Meeks, 2001). Careful choice of restriction fragment cloning or PCR primers for generating promoter fragments may allow the user a choice of a GFP transcriptional reporter or GFP fusion protein using these vectors. This is possible since the MCS in pSUN119 and 202 is located 68–99 bp upstream from the translational start of the promoterless *gfp* gene. Two reading frames within this region contain multiple stop codons, ensuring a true transcriptional reporter. Lack of stop codons between the MCS and *gfp* gene in the third reading frame may allow convenient expression of protein–GFP fusion proteins. The resulting protein fusions would contain the intervening 22–33 amino acids present on the vector as a linker attached to the C-terminus of the cloned protein. Such C-terminal GFP fusions make it convenient to express the protein from its own promoter as required for studies involving cell-type-specific protein expression. The possibility of generating functional protein–GFP fusion proteins was not addressed experimentally in this study, but should be considered in experimental design using these vectors.

In conclusion, our system allows easy generation of reporter plasmids by a single step cloning process performed in *E. coli*, followed by direct transformation into *N. punctiforme* for determination of cell-type-specific gene expression. This reporter system should increase the efficiency of testing putative cell-type-specific gene expression identified by other molecular genetic approaches such as array analysis or differential display. These vectors also have additional usefulness for identification of genes regulated in response to environmental changes in vegetative cells. For this use, a random genomic library made in these promoter probe vectors, could be transformed into *N. punctiforme*, and plasmid-bearing transformants selected on nitrocellulose filters. The filters containing colonies could be sterilely transferred to a different medium or stress condition, and, by comparing images recording GFP expression from colonies before and after the change, promoters responsive to an environmental change could be identified.

Acknowledgments

We thank Anja Kunert and Elsie Campbell for plasmids, and J.C. Meeks for the sequence of the

pDC1 origin region. This work was supported by NSF grant MCB93327, and NIH grants GM48680 and GM63787.

References

Allen, M., Arnon, D.I., 1955. Studies on nitrogen-fixing blue-green algae: I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. 30, 366–372.

Black, T.A., Cai, Y., Wolk, C.P., 1993. Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. Mol. Microbiol. 9, 77–84.

Buikema, W.J., Haselkorn, R., 1991. Characterization of a gene controlling heterocyst differentiation in the cyanobacterium *Anabaena* 7120. Genes Dev. 5, 321–330.

Campbell, E.L., Meeks, J.C., 1989. Characteristics of homogonia formation by symbiotic *Nostoc* spp. in response to the presence of *Anthosceros punctatus* or its extracellular products. Appl. Environ. Microbiol. 55, 125–131.

Cohen, M.F., Meeks, J.C., Cai, Y.A., Wolk, C.P., 1998. Transposon mutagenesis of heterocyst-forming filamentous cyanobacteria. Meth. Enzymol. 297, 3–17.

Crameri, A., Whitehorn, E.A., Tate, E., Stemmer, W.P.C., 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nat. Biotechnol. 14, 315–319.

Damerval, T., Guglielmi, G., Houmard, J., Tandeau de Marsac, N., 1991. Hormogonium differentiation in the cyanobacterium *Calothrix* sp.: a photoregulated developemental process. Plant Cell 3, 191–201.

Elhai, J., Wolk, C.P., 1988. Conjugal transfer of DNA to cyanobacteria. Meth. Enzymol. 167, 747–754.

Fernandez-Pinas, F., Wolk, C.P., 1994. Expression of *luxCD-E* in *Anabaena* sp. can replace the use of exogenous aldehyde for in vivo localization of transcription by *luxAB*. Gene 150, 169–174.

Huang, X., Dong, Y., Zhao, J., 2004. HetR homodimer is a DNA-binding protein required for heterocyst differentiation, and the DNA-binding activity is inhibited by PatS. Proc. Natl. Acad. Sci. U. S. A. 101, 4848–4853.

Kunert, A., Hagemann, M., Erdmann, N., 2000. Construction of promoter probe vectors for *Synechocystis* sp. PCC 6803 using the light-emitting reporter systems Gfp and LuxAB. J. Microbiol. Meth. 41, 185–194.

Leganes, F., Fernandez-Pinas, F., Wolk, C.P., 1994. Two mutations that block heterocyst differentiation have different effects on akinete differentiation in *Nostoc ellipsosporum*. Mol. Microbiol. 12, 679–684.

Mannan, R.M., Pakrasi, H.B., Sonoike, K., 1994. The PsaC protein is necessary for the stable association of the PsaD, PsaE, and PsaL proteins in the photosystem I complex: analysis of a cyanobacterial mutant strain. Arch. Biochem. Biophys. 315, 68–73.

Margolin, W., 2000. Green fluorescent protein as a reporter for macromolecular localization in bacterial cells. Methods 20, 62–72.

Meeks, J.C., Elhai, J., Thiel, T., Potts, M., Larimer, F., Lamerdin, J., Predki, P., Atlas, R., 2001. An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosyth. Res. 70, 85–106.

Meeks, J.C., Campbell, E.L., Summers, M.L., Wong, F.C., 2002. Cellular differentiation in the cyanobacterium *Nostoc punctiforme*. Arch. Microbiol. 178, 395–403.

Milkowski, C., Quinones, A., Hagemann, M., 1998. A DNA fragment from the cyanobacterium *Syncehocystis* sp. PCC 6803 mediates gene expression inducible by osmotic stress in *E. coli*. Curr. Microbiol. 37, 108–116.

Parke, D., 1990. Construction of mobilizable vectors derived from plasmids RP4, pUC18 and pUC19. Gene 93, 135–137.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, USA.

Suarez, A., Guttler, A., Stratz, M., Staender, L.H., Timmis, K.N., Guzman, C.A., 1997. Green fluorescent protein-based reporter systems for genetic analysis of bacteria including monocopy applications. Gene 196, 69–74.

Summers, M.L., Wallis, J.G., Campbell, E.L., Meeks, J.C., 1995. Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium Nostoc sp. strain ATCC 29133. J. Bacteriol. 177, 6184–6194.

Tel-Or, E., Stewart, W.D., 1976. Photosynthetic electron transport, ATP synthesis and nitrogenase activity in isolated heterocysts of *Anabaena cylindrica*. Biochim. Biophys. Acta. 423, 189–195.

Thiel, T., 1994. Genetic analysis of cyanobacteria. In: Bryant, D.A. (Ed.), The Molecular Biology of Cyanobacteria. Kluwer Academic Publishing, The Netherlands, pp. 581–611.

Wolk, C.P., Vonshank, A., Kehoe, P., Elhai, J., 1984. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen fixing filamentous cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. 81, 1561–1565.

Wolk, C.P., Elhai, J., Kuritz, T., Holland, D., 1993. Amplified expression of a transcriptional pattern formed during development of *Anabaena*. Mol. Micrbiol. 7, 441–445.

Wong, F.C., Meeks, J.C., 2001. The *hetF* gene product is essential to heterocyst differentiation and affects HetR function in the cyanobacterium *Nostoc punctiforme*. J. Bacteriol. 183, 2654–2661.

Wong, F.C., Meeks, J.C., 2002. Establishment of a functional symbiosis between the cyanobacterium *Nostoc punctiforme* and the bryophyte *Anthoceros punctatus* requires genes involved in nitrogen control and initiation of heterocyst differentiation. Microbiol. 148, 315–323.

Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103–119.

Yoon, H.S., Golden, J.W., 1998. Heterocyst pattern formation controlled by a diffusible peptide. Science 282, 935–938.

Zhou, R., Wolk, C.P., 2002. Identification of an akinete marker gene in *Anabaena variabilis*. J. Bacteriol. 184, 2529–2532.