Mol Biol Rep (2010) 37:2157–2162
DOI 10.1007/s11033-009-9688-y

The reversed terminator of octopine synthase gene on the Agrobacterium Ti plasmid has a weak promoter activity in prokaryotes

Jun-Li Shao · Yue-Sheng Long · Gu Chen · Jun Xie · Zeng-Fu Xu

Received: 24 January 2009 / Accepted: 28 July 2009 / Published online: 9 August 2009
© Springer Science+Business Media B.V. 2009

Abstract *Agrobacterium tumefaciens* transfers DNA from its Ti plasmid to plant host cells. The genes located within the transferred DNA of Ti plasmid including the octopine synthase gene (*OCS*) are expressed in plant host cells. The 3′-flanking region of *OCS* gene, known as *OCS* terminator, is widely used as a transcriptional terminator of the transgenes in plant expression vectors. In this study, we found the reversed *OCS* terminator (*3′-OCS-r*) could drive expression of hygromycin phosphotransferase II gene (hpt II) and beta-glucuronidase gene in *Escherichia coli*, and expression of *hpt II* in *A. tumefaciens*. Furthermore, reverse transcription-polymerase chain reaction analysis revealed that an open reading frame (ORF12) that is located downstream to the *3′-OCS-r* was transcribed in *A. tumefaciens*, which overlaps in reverse with the coding region of the *OCS* gene in octopine Ti plasmid.

Jun-Li Shao and Yue-Sheng Long contributed equally to this work.

J.-L. Shao · Y.-S. Long · J. Xie · Z.-F. Xu
Key Laboratory of Gene Engineering of the Ministry of Education and State Key Laboratory of Biocontrol, College of Life Sciences, Sun Yat-sen University, 510275 Guangzhou, China

Z.-F. Xu (✉)
Laboratory of Molecular Breeding of Energy Plants, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, 650223 Kunming, Yunnan, China
e-mail: zengfu.xu@gmail.com

Y.-S. Long
Institute of Neuroscience and the Second Affiliated Hospital of Guangzhou Medical College, 510260 Guangzhou, China

G. Chen
College of Light Industry and Food Sciences, South China University of Technology, 510641 Guangzhou, China

Keywords *Agrobacterium tumefaciens* · Antisense overlapping transcripts · *OCS* terminator · Promoter activity · Hygromycin phosphotransferase II gene (*hpt II*) · Beta-glucuronidase (*GUS*)

Introduction

The soil phytopathogen *Agrobacterium tumefaciens* can cause crown gall disease in dicotyledonous plants by infecting wound tissues and transferring a piece of tumor-inducing (Ti) plasmid [1], the transferred DNA (T-DNA), to the plant cells where the T-DNA becomes integrated into the plant nuclear genome [2–4]. The T-DNA carries genes that are expressed within plant host cells and encode proteins responsible for crown gall tumorigenesis [5–8] and production of the bacterial nutrients (opines), which define crown galls and Ti plasmids as octopine, nopaline or agropine type [9]. Among the T-DNA genes, the octopine synthase gene (*OCS*) encodes octopine synthase, which reductively condenses pyruvate with either arginine, lysine, histidine, or ornithine to produce octopine, lysopine, histopine, or octopinic acid, respectively, all of which can be detected in crown gall tumors [10, 11].

The 3′-flanking region of *OCS* gene, known as *OCS* terminator, has the typical character of eukaryotic genes, e.g., a hexanucleotide 5′-AATAAT-3′ occurred 17 bp in front of the poly(A) addition site [11], and is widely used as a transcriptional terminator of the transgenes in plant expression vectors [12–14]. Here, we found that the reversed *OCS* terminator (i.e., when placed in reverse with respect to its native orientation, designated *3′-OCS-r*) could drive expression of hygromycin phosphotransferase II gene (*hpt II*) and beta-glucuronidase (GUS) gene in *Escherichia coli*, and expression of *hpt II* in *A. tumefaciens*. We also

demonstrated that an open reading frame (ORF12), which is located downstream to the *3′-OCS-r* and overlaps in reverse with the *OCS* gene coding sequence in the octopine Ti plasmid, was transcribed in octopine *A. tumefaciens* A348 and R10 strains.

Materials and methods

Bacterial strains and growth conditions

E. coli DH5α and TOP10, two *endA*- strains in common use for cloning, were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). Three strains of *A. tumefaciens*, EHA105 harbouring L, L-succinamopine Ti plasmid pTiBo542ΔT [15], A348 harbouring octopine Ti plasmid pTiA6Nc [16], and R10 harbouring octopine Ti plasmid pTiR10 [17] were grown in YEB medium (0.5% peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose, 0.05% $MgSO_4 \cdot 7H_2O$).

Vector construction

A cassette containing the cauliflower mosaic virus (CaMV) 35S promoter and the *OCS* terminator from pHANNIBAL (GenBank accession no. AJ311872) [14] was inserted into the binary vector pART27 [12] at *Sac*I and *Spe*I sites, resulting an intermediate plasmid pYSLXU4. The pYSLXU4 was digested with *Eco*RI to remove the pdk intron from pHANNIBAL, and was self-ligated to form the pYS1, in which the *3′-OCS-r* corresponds to the sequence from 10,930 to 11,636 in Ti plasmid (GenBank accession no. AF242881). The *hpt II* coding region excised from pCAMBIA1300 (Canberra, Australia) was then cloned into pYS1 at the *Xho*I site, which resulted in two constructs, pYSLXU6 in which *hpt II* is in antisense orientation with respect to the CaMV 35S promoter, and pYSLXU7 in which *hpt II* is in sense orientation with respect to the CaMV 35S promoter. pYS7 was obtained by replacing the CaMV 35S promoter of pBI221 (Clontech) with the *3′-OCS-r* excised from pYS1 with *Pst*I and *Xba*I. pYS8 was obtained by removing the CaMV 35S promoter from pBI221. Schematic diagrams of the constructs used in this study are shown in Fig. 1.

Hygromycin resistance analysis of transformed *E. coli* and *A. tumefaciens*

For *E. coli* resistance analysis, a single clone of *E. coli* DH5α or Top10, each harbouring the indicated plasmid, was inoculated in liquid LB medium with or without spectinomycin (Spec, 100 μg ml^{-1}) or hygromycin (Hyg,

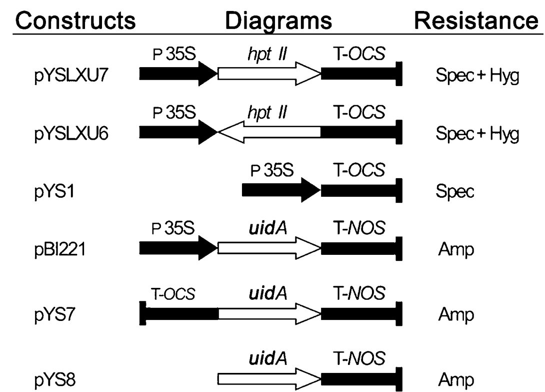

Fig. 1 Schematic diagrams of constructs used in this study. P35S, CaMV 35S promoter; *hpt II*, hygromycin phosphotransferase II gene; T-*OCS*, octopine synthase terminator; *uidA*, beta-glucuronidase (GUS) gene; T-*NOS*, nopaline synthase terminator; Spec, spectinomycin; Hyg, hygromycin; Amp, ampicillin. The Spec, Hyg and Amp resistance are from the backbone of the constructs

50 μg ml^{-1}) and was cultured at 37°C, 200 rpm. The overnight cultures were adjusted to $OD_{600} = 0.2$. One milliliter of each culture ($OD_{600} = 0.2$) was added to 20 ml of liquid LB with Hyg at the indicated concentration (50–500 μg ml^{-1}) and grown at 37°C, 200 rpm. The OD_{600} of all cultures were determined with a spectrophotometer after 8 h culture.

For *A. tumefaciens* resistance analysis, a single clone of *A. tumefaciens* A348, R10 or EHA105, each harbouring the indicated plasmid, was inoculated in liquid YEB medium with Spec (100 μg ml^{-1}) and with or without Hyg (20 μg ml^{-1}) and was cultured at 28°C, 200 rpm. The 36 h cultures of *A. tumefaciens* A348, R10 and EHA105 were adjusted to $OD_{600} = 0.2$. One milliliter of each culture ($OD_{600} = 0.2$) was added to 20 ml of liquid YEB with Spec (100 μg ml^{-1}) and Hyg (20 μg ml^{-1}) and grown at 28°C, 200 rpm. The OD_{600} of all strains were determined at 5, 7, 9, 11, and 13 h after culture. For plate culture, suspensions ($OD_{600} = 0.2$) were inoculated and grown on YEB agar with Spec (100 μg ml^{-1}) and with or without Hyg (20 μg ml^{-1}) at 28°C for 36 h.

GUS activity assay

The method for quantifying GUS activity was modified from Jefferson et al. [18]. Briefly, 1 mg of bacteria (wet weight) was suspended in 100 μl of PBS and incubated with 100 μl MUG reaction solution (2 mM MUG, 100 mM NaHPO4 (pH 7.0), 2 mM Na_2EDTA, 0.2% Sarcosyl, 0.2% Triton X-100, 10 mM β-mercaptoethanol) at 37°C. Ten microliters of reaction mixture were taken out every 5 min for fluorescence determination with excitation at 365 nm, emission at 455 nm on a DyNA Quant® 200 (Amersham, Piscataway, NJ, USA).

Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of *A. tumefaciens* A348 and R10 was extracted using Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Primers for RT-PCR were designed according to the sequence of T-DNA region of the octopine-type Ti plasmid (GenBank Accession number: AF241881). Two micrograms of total RNA treated with DNase I were used to synthesize cDNA by using reverse transcriptase M-MLV with primer P1 or primer P2 at 42°C for 1 h, followed by the inactivation of reverse transcriptase at 70°C for 10 min. Then PCR was performed using the different primer pairs P1/P2, P3/P2, P4/P2, or P5/P2 with the indicated template. The positions and sequences of the primers are shown in Fig. 4a, b. The PCR program consisted of 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 58°C, and 45–100 s (P1/P2: 45 s, P3/P2: 60 s, P4/P2: 75 s, P5/P2: 100 s) at 72°C, and one final extension step of 10 min at 72°C. The RT-PCR products were cloned into pGEM-T easy vector (Promega) and were analyzed by sequencing.

Results

Promoter activity of the reversed *OCS* terminator (*3′-OCS-r*) in recombinant *E. coli*

During construction of a plant expression vector pYSLXU7 containing Hyg resistant gene *hpt II* under control of the CaMV 35S promoter and *OCS* terminator (Fig. 1), we found, unexpectedly, that the *E. coli* DH5α containing another vector pYSLXU6 (Fig. 1), in which *hpt II* was in antisense orientation with respect to the CaMV 35S promoter, also grew well in LB liquid containing 50 μg ml^{-1} Hyg (Fig. 2a). The bacteria without plasmid or containing pYS1 lacking *hpt II*, however, could not grow in the same medium (Fig. 2a). This observation was further confirmed by growing another *E. coli* strain Top10 harbouring these constructs (Fig. 2a). These results suggested that the reversed *OCS* terminator (*3′-OCS-r*) had promoter activity in *E. coli*.

To compare the strength of the promoter activity between the CaMV 35S promoter and *3′-OCS-r*, *E. coli* strains harbouring different plasmids were inoculated into liquid LB with different concentrations of Hyg, and the OD_{600} was determined after 8 h culture. As shown in Fig. 2b, Top10/pYSLXU6, DH5α/pYSLXU6 grew slower than Top10/pYSLXU7 and DH5α/pYSLXU7 at 100 μg ml^{-1} Hyg, although they had the similar growth rate at 50 μg ml^{-1} Hyg. The promoter activity of *3′-OCS-r* was weaker than of CaMV 35S in *E. coli*.

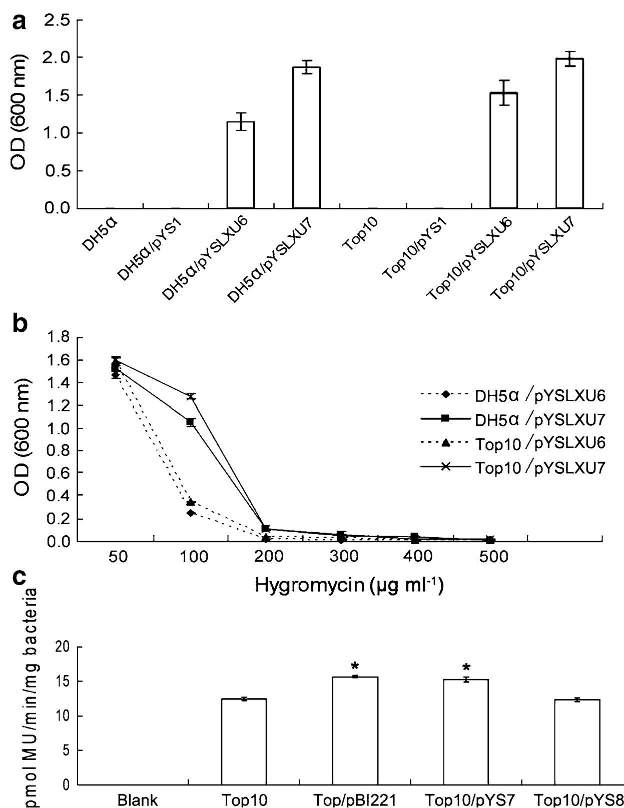

Fig. 2 The 3′-*OCS-r* drives *hpt II* and *uidA* expression in *E. coli*. (**a**), Growth of the *E. coli* strains harbouring the indicated constructs grown in liquid LB with 50 μg ml^{-1} Hyg at 37°C for 8 h. $n = 6$. (**b**), Growth of the *E. coli* strains harbouring pYSLXU6 and pYSLXU7 grown in liquid LB with Hyg of different concentrations at 37°C for 8 h. $n = 6$. (**c**), Quantitative assay of GUS activity in the *E. coli* strains harbouring the indicated constructs. The means of three experiments ±SE are shown. *, $P < 0.05$ (compared to Top10/pYS8) in *t*-test

In order to further confirm the promoter activity of the *3′-OCS-r* in *E. coli*, the construct pYS7 containing the report gene *uidA* encoding beta-glucuronidase (GUS) under the control of the *3′-OCS-r* and nopaline synthase (*NOS*) terminator, and the promoterless pYS8 construct (as a negative control) were generated (Fig. 1). As shown in Fig. 2c, the GUS activity of the bacteria containing pYS7 and pBI221 (positive control plasmid from Clontech, in which the P35S drives GUS expression) was significantly higher than the endogenous GUS activity of the host bacteria Top10 and the bacteria containing the promoterless plasmid pYS8. This result suggested that the *3′-OCS-r* could also drive the reporter gene *uidA* expression in *E. coli*.

Promoter activity of the *3′-OCS-r* in recombinant *A. tumefaciens*

To determine the promoter activity of the *3′-OCS-r* in *A. tumefaciens*, the constructs pYS1, pYSLXU6 and

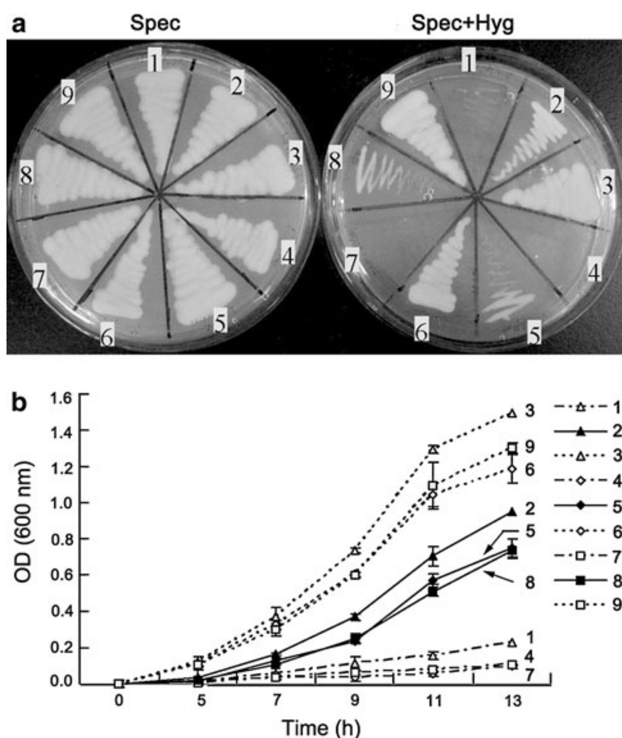

Fig. 3 The 3′-*OCS-r* drives *hpt II* expression in *A. tumefaciens*. (**a**), *A. tumefaciens* harbouring different vectors grown on YEB agar with the indicated antibiotics at 28°C for 36 h. (**b**), Growth curves of the *A. tumefaciens* grown in liquid YEB with Spec and Hyg. The OD_{600} of all strains were determined at 5, 7, 9, 11 and 13 h after culture. The means of three experiments ±SE are shown. *A. tumefaciens* strains in (**a**) and (**b**) are: 1, A348/pYS1; 4, R10/pYS1; 7, EHA105/pYS1; 2, A348/pYSLXU6; 5, R10/pYSLXU6; 8, EHA105/pYSLXU6; 3, A348/pYSLXU7; 6, R10/pYSLXU7; 9, EHA105/pYSLXU7

pYSLXU7 described above were transformed into *A. tumefaciens* octopine strains A348, R10 and L, L-succinamopine strain EHA105, and then the transformed *A. tumefaciens* were tested for the Hyg resistance. As shown in Fig. 3a, the strains containing pYSLXU6 (Fig. 3a, right panel, sectors 2, 5 and 8) could grow on YEB agar containing Spec and Hyg, as the case for the strains containing pYSLXU7 (Fig. 3a, right panel, sectors 3, 6 and 9). However, the strains with pYS1 lacking *hpt II* gene (Fig. 3a, right panel, sectors 1, 4 and 7) could not grow on the same plate. As positive controls, all the strains could grow on YEB agar containing Spec only (Fig. 3a, left panel) because of the presence of the spectinomycin resistance gene carried on the backbone of the plasmids. Additionally, the growth curves of these strains in liquid YEB with Spec and Hyg also showed the similar results, as depicted in Fig. 3b. These data suggested that the *3′-OCS-r* also had promoter activity in *A. tumefaciens*, although its activity was weaker than that of the CaMV 35S promoter.

Transcription of an open reading frame (ORF12) located downstream to the *3′-OCS-r* in native *A. tumefaciens*

Since the *3′-OCS-r* showed promoter activity to drive the expression of the reporter genes in recombinant plasmids in *E. coli* and *A. tumefaciens*, we speculated that it was possible that the *3′-OCS-r* may drive the transcription of some genes downstream of it in octopine Ti plasmid of native *A. tumefaciens*. Open reading frame (ORF) analysis of the DNA sequence downstream of the *3′-OCS-r* in octopine Ti plasmid (GenBank accession no. AF242881) revealed the presence of several ORFs, including an ORF12 that was the twelfth ORF in the T-DNA region of the Ti plasmid pTi15955 (GenBank accession no. X00493) [19]. To detect the transcription of ORF12 in *A. tumefaciens*, we initially examined ORF12 expression by northern blot analysis, but found that ORF12 transcript was not detectable (data not shown). We therefore performed a more sensitive RT-PCR analysis. RNA extracted from *A. tumefaciens* strain A348 (harbouring the octopine Ti plasmid pTiA6Nc) and strain R10 (harbouring the octopine Ti plasmid pTiR10) were treated with DNase I to eliminate the contaminated plasmid DNA and were used to synthesize cDNAs using the primer P2 (Fig. 4a, b). The cDNA products, which were incubated at 70°C for 10 min to inactivate the reverse transcriptase, were used for PCR with the primer pair P1/P2 designed according to the ORF12 DNA sequence (Fig. 4a, b). PCR products of the predicted size (480 bp) were obtained and conformed to be the ORF12 fragment by DNA sequencing analysis. This result indicated that *ORF12* was transcribed in *A. tumefaciens*.

In order to determine the transcriptional initiation region of the *ORF12*, The primers P3, P4 and P5 upstream to primer P1 were designed (Fig. 4a, b) and used for RT-PCR analysis of the RNA extracted from *A. tumefaciens* A348. As shown in Fig. 4c, when the template was the transcriptional product of primer P2, prime pairs P1/P2, P3/P2, P4/P2 could amplify out the products of the predicted size (Fig. 4c, lane 3, 5 and 7), while primer pair P5/P2 did not yield any PCR product (Fig. 4c, lane 9). As positive controls, all the primer pairs P1/P2, P3/P2, P4/P2, P5/P2 could amplify out the products of the predicted size when the templates were total DNA from *A. tumefaciens* A348 (Fig. 4c, lane 4, 6, 8 and 10). As a negative control, no product was amplified when the template was the DNase I-treated RNA (Fig. 4, lane 1), which indicated that the RNA samples were not contaminated with plasmid DNA. In addition, there was also no RT-PCR product when the template was the reverse transcriptional product of primer P1 (Fig. 4, lane 2), which showed that *OCS* gene didn't express in *A. tumefaciens* and therefore didn't account for the amplification of fragments in the region. These results

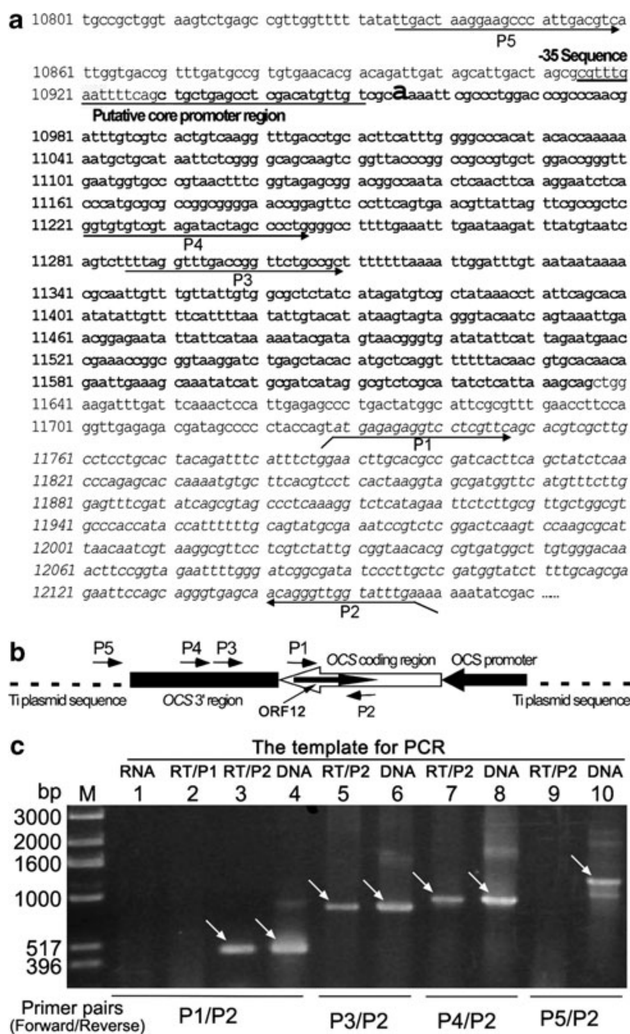

Fig. 4 Determination of the transcriptional initiation region of *3′-OCS-r* in octopine Ti plasmid. (**a**) The sequence of the *3′-OCS-r* and *ORF12* in the Ti plasmid (GenBank accession no. AF242881). The bold and italic letters indicate the sequence of the *3′-OCS-r* and *ORF12* respectively. The region underlined shows the putative minimal core promoter with one putative "−35 sequence" (*shaded box*). The larger *bold* "*a*" indicates the putative transcription start site. The primers used for RT-PCR are indicated with *arrows*. (**b**) Schematic drawing of (**a**). *ORF12* overlaps in reverse with the *OCS* coding sequence. (**c**) Transcriptional initiation region analysis of *ORF12* in octopine *A. tumefaciens* A348 by RT-PCR. *Lane 1*, Total RNA treated with DNase I as template. *Lane 2*, Primer P1-directed reverse transcriptional products as template; *Lane 3, 5, 7* and *9*, Primer P2-directed reverse transcriptional products as template; *Lane 4, 6, 8* and *10*, DNA from *A. tumefaciens* A348 as template. The bands with the predicted size are indicated by *arrows*

suggest that the transcriptional initiation region of the *ORF12* is between the primer P5 and the primer P4.

Discussion

The *OCS* terminator is a widely used transcriptional terminator of transgenes in plant expression constructs [13, 20]. In this study, we found, unexpectedly, the reversed *OCS* terminator (*3′-OCS-r*) has a weak promoter activity in prokaryotes, by showing its ability to drive the expression of reporter genes in recombinant *E. coli* and *A. tumefaciens*.

And we also demonstrated that downstream to the *3′-OCS-r* there is an open reading frame *ORF12*, which overlaps in reverse with the coding region of the *OCS* gene and is weakly expressed in native *A. tumefaciens*. Consistently, a putative prokaryotic promoter (with minimal core promoter score = 0.91, Fig. 4a) was found between the primer P4 and P5 by the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). This putative prokaryotic core promoter is untypical, because only one putative "−35 sequence", but no "−10 sequence", was found (Fig. 4a). This could result in the low transcription of *ORF12* in *A. tumefaciens*. Further studies should be performed by primer extension or by S1 mapping to determine the transcription start site.

Antisense overlapping ORFs occur frequently in various prokaryotic and eukaryotic genomes [21–25]. All these antisense overlapping ORFs have been found to be transcribed in the same cells, which represents an important mechanism for regulating gene expression [24, 25]. In this paper, however, we report the first case of a pair of antisense overlapping transcripts, *OCS* and *ORF12*, which are expressed in different host cells. The *OCS* gene is abundantly expressed in plant cells [26, 27], whereas the *ORF12* is expressed at very low levels in *Agrobacterium*, as shown in this study. This novel pattern of antisense overlapping ORFs can provide a means to increase the coding capacity of the DNA sequence. The putative product of ORF12 is an unnamed protein (GenBank accession no. CAA25175). Further studies are required to determine whether the *ORF12* transcript is translated into a protein in *A. tumefaciens* and what's the function of the *ORF12* transcript and/or the corresponding protein.

Acknowledgments We would like to thank an anonymous reviewer for her/his critical and constructive comments that led to the improvement of this article. We thank Dr. Stephen C. Winans (Cornell University, USA) for the *A. tumefaciens* strains of A348 and R10, and Dr. Peter M. Waterhouse (CSIRO Plant Industry, Australia) for the pHANNIBAL and pART27 plasmids. This work was supported by the National Natural Science Foundation of China (grant nos. 30570943 and 30771111), and the Knowledge Innovation Program of the Chinese Academy of Sciences (grant no. KSCX2-YW-Z-0723) to Z.-F. Xu.

References

1. Zaenen I, Van Larebeke N, Van Montagu M et al (1974) Supercoiled circular DNA in crown-gall inducing Agrobacterium strains. J Mol Biol 86:109–127
2. Chilton MD, Drummond MH, Merio DJ et al (1977) Stable incorporation of plasmid DNA into higher plant cells: the

molecular basis of crown gall tumorigenesis. Cell 11:263–271. doi:10.1016/0092-8674(77)90043-5

3. Lemmers M, De Beuckeleer M, Holsters M et al (1980) Internal organization, boundaries and integration of Ti-plasmid DNA in nopaline grown gall tumours. J Mol Biol 144:353–376
4. Zambryski P, Holsters M, Kruger K et al (1980) Tumor DNA structure in plant cells transformed by *A. tumefaciens*. Science 209:1385–1391. doi:10.1126/science.6251546
5. Klee H, Montoya A, Horodyski F et al (1984) Nucleotide sequence of the tms genes of the pTiA6NC octopine Ti plasmid: two gene products involved in plant tumorigenesis. Proc Natl Acad Sci USA 81:1728–1732
6. Korber H, Strizhov N, Staiger D et al (1991) T-DNA gene 5 of Agrobacterium modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants. EMBO J 10: 3983–3991
7. Lichtenstein C, Klee H, Montoya A et al (1984) Nucleotide sequence and transcript mapping of the tmr gene of the pTiA6NC octopine Ti-plasmid: a bacterial gene involved in plant tumorigenesis. J Mol Appl Genet 2:354–362
8. Tinland B, Fournier P, Heckel T et al (1992) Expression of a chimaeric heat-shock-inducible Agrobacterium 6b oncogene in Nicotiana rustica. Plant Mol Biol 18:921–930. doi:10.1007/BF00019206
9. Guyon P, Chilton MD, Petit A et al (1980) Agropine in "null-type" crown gall tumors: evidence for generality of the opine concept. Proc Natl Acad Sci USA 77:2693–2697
10. Zhu J, Oger PM, Schrammeijer B et al (2000) The bases of crown gall tumorigenesis. J Bacteriol 182:3885–3895
11. De Greve H, Dhaese P, Seurinck J et al (1982) Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J Mol Appl Genet 1: 499–511
12. Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol 20:1203–1207. doi:10.1007/BF00028910
13. Jones JD, Shlumukov L, Carland F et al (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. Transgenic Res 1:285–297. doi:10.1007/BF02525170
14. Wesley SV, Helliwell CA, Smith NA et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590. doi:10.1046/j.1365-313X.2001.01105.x
15. Hood EE, Gelvin SB, Melchers LS et al (1993) New Agrobacterium helper plasmids for gene transfer to plants. Transgenic Res 2:208–218. doi:10.1007/BF01977351
16. Sciaky D, Montoya AL, Chilton MD (1978) Fingerprints of Agrobacterium Ti plasmids. Plasmid 1:238–253
17. Lyi SM, Jafri S, Winans SC (1999) Mannopinic acid and agropinic acid catabolism region of the octopine-type Ti plasmid pTi15955. Mol Microbiol 31:339–347. doi:10.1046/j.1365-2958.1999.01178.x
18. Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907
19. Barker RF, Idler KB, Thompson DV et al (1983) Nucleotide sequence of the T-DNA region from the *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol Biol 2:335–350. doi:10.1007/BF01578595
20. Chung SM, Frankman EL, Tzfira T (2005) A versatile vector system for multiple gene expression in plants. Trends Plant Sci 10:357–361. doi:10.1016/j.tplants.2005.06.001
21. Boldogkoi Z (2000) Coding in the noncoding DNA strand: a novel mechanism of gene evolution? Journal of Molecular Evolution 51:600–606
22. Williams T, Fried M (1986) A mouse locus at which transcription from both DNA strands produces mRNAs complementary at their 3′ ends. Nature 322:275–279
23. Merino E, Balbas P, Puente JL et al (1994) Antisense overlapping open reading frames in genes from bacteria to humans. Nucleic Acids Res 22:1903–1908
24. Borsani O, Zhu J, Verslues PE et al (2005) Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in arabidopsis. Cell 123:1279–1291
25. Cock JM, Swarup R, Dumas C (1997) Natural antisense transcripts of the S locus receptor kinase gene and related sequences in *Brassica oleracea*. Mol Genet Genomics 255:514–524
26. Gelvin SB, Gordon MP, Nester EW et al (1981) Transcription of the agrobacterium Ti plasmid in the bacterium and in crown gall tumors. Plasmid 6:17–29
27. Murai N, Kemp JD (1982) Octopine synthase mRNA isolated from sunflower crown gall callus is homologous to the Ti plasmid of *Agrobacterium tumefaciens*. Proc Natl Acad Sci USA 79: 86–90