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The nitrate reductase gene-switch: A system for regulated expression in transformed cells of *Dunaliella salina*

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Abstract

The control of promoter activity by nitrogen source has recently emerged as an intriguing system for regulated expression of the heterologous genes. The purpose of this work was to investigate whether heterologous gene expression in transgenic *Dunaliella salina* would be controlled by an inducible promoter. Here we identify that the nitrate reductase (*NR*) transcripts of *D. salina* are induced by nitrate but repressed by ammonium. The *bar* gene integrated into the genome of *D. salina* is transcribed by a promoter of the *NR* gene from *D. salina* and the *bar* transcripts are induced by nitrate but repressed by ammonium. PPT-resistance of transformants disappears when they are transferred from nitrate-containing medium to ammonium-containing medium. The findings of this study demonstrate that the promoter of the *D. salina NR* gene can be used to control expression of the heterologous genes in transgenic *D. salina*.

Keywords: *bar* gene; *Dunaliella salina*; Expression; Nitrate reductase; Promoter

1. Introduction

Dunaliella salina, one of the most extremely halotolerant eukaryotes, has great potential in bioengineering for producing valuable polypeptides and proteins (Geng et al., 2003). However, the lack of efficient expression systems has been a major limitation in the genetic manipulation of this microalga.

An availability of reliable regulation elements (e.g. promoter and terminator) is a prerequisite for the development of a practical transformation system. In this connection, promoters are critical cis-regulation elements in transcription regulation (Smith and Hager, 1997). Recently, a series of promoters have been developed and used in studies on transgenic algae but activities of the promoters are comparatively low (Geng et al., 2002; Li et al., 2005). Additionally, previous studies from our laboratory have shown that expression of a heterologous gene driven by a constitutive promoter may affect growth of the transformants of *D. salina* (Jiang et al., 2005; Li et al., 2007). In a separate study, we have demonstrated that expression of the *bar* gene was induced, but not controlled by an inducible promoter of the duplicated carbonic anhydrase 1 gene in the transformants of *D. salina* (Lu et al., 2004). Obviously, an inducible expression system, in which expression of the heterologous genes can be regulated by a controllable promoter-terminator cassette, is worth further studying and developing.

Nitrate reductase (NR) is a key enzyme in nitrogen metabolism and transcription of the *NR* gene is markedly influenced by the environmental factors, e.g., induced by nitrate and repressed by ammonium, in higher plants (Warning and Hachtel, 2000), fungi (Muro-Pastor et al., 1999) and green algae (Dawson et al., 1996; Loppes and Radoux, 2002). The property of *NR* transcription with a natural switch provides a good idea for isolating controllable regulation elements. By using cDNA of green fluorescent protein as a reporter gene, Poulsen and Kroger (2005) identified that gene expression under the control of the Pnr/Tnr cassette was switched off when cells were grown in the presence of ammonium ions and became switched on when cells were transferred to nitrate-containing medium. The promoters of the

Abbreviations: NR, nitrate reductase; PPT, phosphinothricin; NRE, nitrogen response element; Pnr, promoter of *D. salina* nitrate reductase gene; Tnr, terminator of *D. salina* nitrate reductase gene; ITS, internal transcribed space; SOEing, splicing by overlap extension; PAT, phosphinothricin acetyltransferase.

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NR genes from *Chlamydomanas* (Koblenz and Lechtreck, 2005) and *Chlorella* (Wang et al., 2004) have also been employed as inducible promoters to drive expressions of the heterologous genes. It has been reported that NR of *D. salina* is located in the pyrenoid (Lopez-Ruiz et al., 1985). He et al. (2004) also showed that nitrate transporter transcripts of *D. salina* were induced by nitrate but repressed by ammonium. Those studies have encouraged us to explore whether the *NR* gene promoter of *D. salina* is an inducible one by means of a thorough analysis of gene expression driven by the *NR* gene promoter.

In the present study, expression pattern of the *D. salina NR* gene in response to different nitrogen sources was analyzed. The promoter (Pnr) and terminator (Tnr) of the *D. salina NR* gene were isolated and an expression plasmid containing a Pnr-bar-Tnr expression cassette was transformed into cells of *D. salina*, and the expression and integration patterns of the heterologous gene (*bar* gene) were then investigated in the stable transformants of *D. salina*.

2. Materials and methods

2.1. Alga strain and culture

D. salina UTEX-LB-1644 was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA (Xie et al., 2007) and grown at 26 °C, 60 μmol photon m^{-2} s^{-1} illumination with a 12 h-light/day in medium (Ben-Amotz and Avron, 1990) comprising NaCl 2 M, $NaHCO_3$ 50 mM, $NaNO_3$ 2.5 mM, $MgSO_4$ 5 mM, KH_2PO_4 0.2 mM, EDTA 6 μM, $FeCl_3$ 2 μM, $CaCl_2$ 0.2 mM, $MnCl_2$ 7 μM, $ZnSO_4$ 1 μM, $Co(NO_3)_2$ 1 μM, $CuSO_4$ 1 μM. *D. salina* cells at logarithmic phase were spread on a solid medium containing agar (0.8%) and cultured for ~3 weeks. Subsequently, individual colonies were picked out and transferred to liquid medium for further propagation. For selection and maintenance, the transformants of *D. salina* were grown in medium containing phosphinothricin (PPT).

2.2. Expression pattern of D. salina NR gene

To investigate the effects of different nitrogen sources on the production of *NR* mRNA of *D. salina*, cells grown for 48 h in media containing different nitrogen sources were harvested and Northern blots were carried out. Briefly, total RNAs were isolated with TRIzol from *D. salina* cells, the concentrations and qualities of total RNAs were determined spectrophotometrically at 260 nm and by agar electrophoresis, respectively. A 10 μg of total RNAs separated on 1% formaldehyde agarose gels was transferred to positively-charged nylon membranes using the capillary transfer system. The cDNA fragments of the *NR* gene were amplified with a *NR* cDNA-containing plasmid as template using the primers 5′-CCGCACTCGCCAACAACACAG-3′ and 5′-CCCTCCTTCGTGGCAATCT-3′, and then amplified fragments (987 bp) were labeled with DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The labeled *NR* cDNA fragments were hybridized with RNAs on the membranes according to the manufacturer's instructions.

2.3. NR activity assay

Cells grown in media containing different nitrogen sources were washed with the nitrogen-free medium and resuspended in 0.5 ml of extraction buffer containing 25 mM potassium phosphate (pH 8.8) and 1 mM EDTA and 10 mM cysteine. The cells ruptured by grinding were centrifuged at 4 °C at 4000 g for 20 min, and then the supernatant (enzyme extract) was reacted with 0.5 ml of 5 mM potassium nitrate in 100 mM potassium phosphate buffer (pH 7.4) containing either 0.3 ml of 2 mg/ml NADH or 0.3 ml of distilled water as controls at 25 °C for 30 min. The reaction was terminated by adding 0.5 ml of 1% sulfanilamide and 0.5 ml of 1% N-(1-naphthyl) ethylenediamine hydrochloride. Optical density was measured with a spectrophotometer at 540 nm and NR activity in the extracts was expressed as μmol nitrite/10^8 cells/h.

2.4. Promoter and terminator of the NR gene from D. salina

Based on the mRNA sequences of the *NR* gene of *D. salina* cloned in our laboratory (GenBank: AY312143), the gene specific outer (S1) and inner (S2) primers were respectively designed (Table 1, Fig. 1), and LA PCR-based genomic walking (GW), as described by Price-Schiavi et al. (2000), was employed to obtain

Table 1
Primers used in construction of recombinant plasmids

Template	Primers	Enzymes	PCR products (bp)
Genome	S1: 5′-agccttcagcttagacagcatcatctcg-3′		
	S2: 5′-gtgttgttggcgagtgcgggcat-3′		
	TnrF1: 5′-agcttctgagcggggtcagcagga-3′		
	TnrR1: 5′-tcgatcagcctttgcaatccctcc-3′		
pMD-Pnr	BSOE1: 5′-ccc*aagcttctcgag*agcttgccattattgtgt-3′	*Hin*dIII, *Xho*I	1176
	BSOE2: 5′-**gcgtcgttctgggctcat***ctgctgctgcggacagg*-3′		
pMDDC-B	BSOE3: 5′-*cctgtccgcagcagcagc*a**atgagcccagaacgacgc**-3′		
	BSOE4: 5′-caat*atcgat*tcatcagatctcggtgac-3′	*Cla*I	550
pMD-Tnr	Tnr-Fs: 5′-caat*cccggg*ttctgagcggggtca-3′	*Sma*I	880
	Tnr-Rx: 5′-ccc*gctcgag*tcgatcagcctttgcaat-3′	*Xho*I	

Note: enzyme sites were underlined; BSOE2 and BSOE3 are two hybrid primers. Complement sequences of the *bar* cDNA are in bold and Pnr complement sequences are in italic. Complement translation initiator codons of *bar* cDNA are boxed.