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Efficient bacterial expression of recombinant potato mop-top virus non-structural triple gene block protein 1 modified by progressive deletion of its N-terminus

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Abstract

To obtain strong bacterial expression of proteins that seem to be hard to express in bacteria or are highly toxic for bacteria, it is possible to create a palette of similar constructs, differing only by several nucleotides, gradually deleted from the full-length clone by exonuclease III. When a construct is equipped with the 6×His tag, a simple colony-blot procedure can be performed and a colony giving strong and efficient expression can easily be selected for high range protein expression. We utilized this procedure to produce one of potato mop-top virus (PMTV) movement proteins, namely triple gene block protein 1 (TGBp1) which was very hard to express in bacteria in its original length. The TGBp1 gene was digested with exonuclease III and nuclease S1 from its 5′ terminus, leaving 6×His tag intact. The clone that showed the strongest signal with anti-His antibodies in colony-blot procedure was found to have 44 amino acids (of total 463) deleted. The SDS–PAGE and Western blot of high range bacterial culture lysate confirmed the efficient expression of this deleted 6×His tagged TGBp1 fragment.
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There are many reasons that make some proteins hard to express in the bacterial expression systems. The proteins could be toxic for bacteria, they can be improperly folded, thus being more unstable and degraded. Very often, on the N- or C-ends are sequences enabling targeting of proteins in different compartments of eukaryotic cells, or sequence signals for glycosylation, myrystylation, etc. that could also affect the stability of the protein, and when the modifications are missing the protein could be less stable.

The good example of hard-to-express proteins is triple gene block proteins (TGBp) of plant viruses, which enable the spread of plant virus infection from infected cell to a neighboring non-infected cell [1]. In the case of rod-shaped, fungus transmitted viruses, proteins taking part in this process are coded by three overlapping genes. The first one, coding for TGBp1, contains RNA helicase consensus sequence motifs and displays in vitro nucleic acid binding activity [2]. The second one, TGBp2, has two potentially membrane-spanning hydrophobic domains flanking a highly conserved sequence and the third protein, TGBp3, has the sequence more variable but rather hydrophobic [3].

The potato mop-top virus (PMTV) is fungus transmitted soil-borne virus and the type member of the genus *Pomovirus* [4]. PMTV occurs in potato growing regions in Europe, North and South America, and Asia in cool wet climate causing a wide range of symptoms in haulms and tubers which vary depending on the potato cultivar and environmental conditions, thus complicating the identification of the virus disease [5].

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PMTV has tubular and rigid particles, measuring 18–22 × 100–150 or 250–300 nm [6], encapsidating three RNA components, namely RNA 1, RNA 2, and RNA 3 [7–9]. The third RNA, 2.9 kb long, contains a triple gene block (TGB) encoding three proteins involved in cell-to-cell movement, and an additional open reading frame (ORF) for a predicted 'cystein-rich' protein (CRP) with unknown function [9].

Previous analysis of coat proteins sequence for different PMTV isolates showed that PMTV isolates from different parts of the world are highly conserved in this part of genome [10–12]. Extended sequence analysis of several Danish isolates revealed several amino acid changes, only two of them corresponding to symptom grouping of isolate, the meaning of which remains to be tested by mutation analysis [13,14].

The methods for sensitive and specific detection to produce PMTV-free seeds are still being developed. In recent years, monoclonal and polyclonal antisera were raised, using purified PMTV and recombinant coat protein expressed in *Escherichia coli* [15–19]. However, the PMTV is present at low concentrations in infected tissue and unevenly distributed. Moreover, as several recent works showed, the coat protein is dispensable for the virus movement. When CP-transgenic *Nicotiana benthamiana* plants were infected by mechanical inoculation of leaves, no RNA CP was detected, but RNA TGB was readily detected in leaves and roots of several plants. When the roots were inoculated with its fungal vector—the viruliferous *Spongospora subterranea*, all three RNAs were detected. However, no systemic movement of PMTV from roots to the above-ground parts was observed [20]. Additionally, when different combinations of full-length viral cDNA clones were used to inoculate *N. benthamiana* plants, it was shown that the multipartite virus PMTV is capable of establishing infection without the CP-encoding RNA, and also without the putative CRP [21]. Thus, detection based on some other non-structural protein could be advantageous when combined with CP detection methods and PCR methods [22–24]. Antisera raised against TGBp1, for example, could be used for such a purpose as well as for studying the fate of this protein in infected plant and its role in the viral movement through plant tissue.

Unfortunately, the full-length TGBp1 (463 aa, 51 kDa) was hard to express in its full-length in bacterial system. Therefore, we decided to examine bacterial expression of deletion variants of TGBp1. We prepared a mixture of expression plasmids carrying TGBp1 gene, unidirectionally and gradually deleted from 5′ end by exonuclease III. The transformed bacteria were plated on the agar plate and screening of the transformants was performed with colony blot procedure using anti-His antibodies after induction of protein expression with IPTG from the inducible promotor. The screening revealed not only transformants with the correct reading frame of TGBp1, but also indicated expression levels of individual variants. Colonies giving the strongest signals were then selected for large culture expression.

Materials and methods

Virus source and IC-RT-PCR

The PMTV isolate 54-10 was kindly provided by Dr. Steen Nielsen from the Danish Institute of Agricultural Sciences, Flakkebjerg, Denmark.

cDNA of PMTV RNAs was obtained by immuno-capture reverse transcription polymerase chain reaction (IC-RT-PCR) as follows. The tubes were coated with 100 μl of anti-PMTV IgG (1 μg/ml) (Adgen) in coating buffer for 3 h at 37 °C. The wells were then washed (3 × 150 μl PBS + T) and 100 μl of the homogenate of PMTV infected leaves in conjugate buffer (1:10) was added. The samples were incubated overnight at 4 °C and washed again three times with PBS + T. After the last wash, the reverse transcription and amplification with Superscript II (Gibco) and *Taq* polymerase following manufacturer's recommendations were performed. The reverse transcription and subsequent polymerase chain reaction (RT-PCR) was done using PMTV specific primers (aaccatggaaagcggattcaacggaagt as a sense primer and atagatcttccggaccatacctgtctgttt as an anti-sense primer), based on the sequence of Sw isolate available in GenBank database under the Accession No. AJ277556. The sense and antisense primers have introduced restrictions sites that will be relevant for further recloning, *Nco*I and *Bgl*II, respectively (underlined). The PCR was carried out in 30 cycles: 30 s denaturation at 94 °C, 30 s annealing at 55 °C, and 1 min elongation at 72 °C.

Cloning procedure

The 1.4 kb fragment of interest was cloned to pTZR7T/A (Fermentas) using 3′-A overhangs generated by *Taq* polymerase. Then, it was recloned into plasmid pQE32 using *Bam*HI and *Bgl*II sites, thus equipping the construct with 6×His tag and into plasmids pQE30 and pQE31 (Qiagen GmbH, Germany). The sense primer introduced *Nco*I site to the start position of TGBp1; to make this site unique for further experiments, we destroyed pQE30/31 *Nco*I site prior to cloning by treatment with *Nco*I, Klenow fragment, and T4 ligase (MBI Fermentas). The *Nco*I⁻ plasmids were named pQE30/31NK. After inserting the fragment into *Sac*I site of pQE30/31NK, the *Nco*I site at 5′ end of amplified gene was unique. All molecular cloning procedures were performed according to Sambrook et al. [25].

Exonuclease digestion

The plasmids were then digested with *Sac*I making ends resistant to exonuclease III and *Nco*I giving ends sensitive to exonuclease III. The digestion with exonuclease III and nuclease S1 was performed according to the manual obtained with ExoIII/S1 Deletion Kit (MBI Fermentas). To obtain deletion variants differing by only a few nucleotides, we used frequent aliquoting and tried different temperatures and salt concentration to slow down the reaction. The typical course of reaction is shown in Fig. 2. Then, all aliquotes were mixed in one tube and the volume containing approximately 1 µg of DNA was loaded on the 1% SeaPlaque agarose gel (Cambrex Bio Science Rockland). Fragments in the range of 4.7–4.2 kb were isolated, religated, and used to transform competent cells.

Colony-blotting procedure

The colonies grown out on an agar plate were blotted onto nitrocellulose membrane. The protein expression was induced with IPTG and 6×His tag-containing proteins were detected by anti-His antibodies (Sigma) according to manufacturer's instructions. The colonies giving the strongest signal were selected for further experimenting. All steps in colony-blotting procedure were performed according to The QIAexpressionist handbook (Qiagen GmbH, Germany).

Sequencing

The cloned fragments were sequenced to check the integrity of open reading frame, the presence of 6×His tag on the N-end of expressed protein, and the extent of TGBp1 deletion. The sequencing was performed using an ALFexpressII Sequencer with the AutoRead Sequencing Kit (AP Life Science), using QIAexpress pQE sequencing primers (Qiagen GmbH, Germany). The sequence analyses were carried out using programs available on ExPASY server [26]. The deduced amino acid sequence was aligned with the TGBp1 sequence available in GenBank under Accession No. AY426745 by ClustalW [27]. The protein secondary structure was analyzed using the PredictProtein server [28].

Bacterial expression

The proteins were expressed and prepared in either standard (10 ml) or large (500 ml) culture of *E. coli* according to The QIAexpressionist handbook (Qiagen GmbH, Germany). The protein expression was induced by 0.5 mM IPTG for 3 h. Several *E. coli* strains were tested to obtain the optimal expression: BL21, M15, and TG1 which were found to be the most suitable for our experiments.

Preparation of bacterial cell fractions

Deleted variant of TGBp1 was partially purified from 500 ml of the bacterial culture of colony exo13 according to the procedure described by Lin and Cheng [29]. The insoluble fraction resuspended in PBS was further purified by high speed centrifugation through 30% sucrose cushion (2 h; 27,000 rpm; Beckman Ti 50.2 rotor). The soluble and insoluble fractions were subjected to SDS–PAGE and Western blot analysis.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS–PAGE)

The pellet from 10/500 ml bacterial culture was resuspended in 100 µl of the Laemmli buffer, boiled for 2 min and aliquots were loaded on 12% polyacrylamide gel containing SDS [30]. For visualization of the separated proteins, Coomassie brilliant blue R250 was employed.

Western blot analysis

The proteins separated by SDS–PAGE were electroblotted to a nitrocellulose membrane (0.45 µm, Sleicher & Schuell Protran) in semidry system (OMNI-TRANS apparatus, Omnibio Brno, Czech Republic) according to Hirano and Watanabe [31]. The membrane was then incubated for 1 h in 4% bovine serum albumin (BSA) in PBS and then washed four times in PBS. The recombinant tagged proteins were detected with anti-His antibodies. The bands of interest were visualized by reaction with a substrate BCIP/NBT (Sigma) according to Sambrook et al. [25].

Results

Cloning of TGB1p1 full-length gene into pTZR and pQE-32 plasmids

The RT-PCR product covering the gene for TGBp1 obtained from the PMTV viral RNA (isolate 54-10) was cloned into the pTZR vector. The sense primer introduced *Nco*I site, relevant for further experimenting. The fragment was then excised by enzymes *Bam*HI and *Bgl*II and reclone into expression plasmid pQE32. The construct was expected to express TGBp1 carrying 6×His tag on its 5′ end.

Several attempts had to be made to obtain a few clones carrying apparently full-length TGBp1 gene. However, none of them showed detectable expression of TGBp1 after IPTG induction, though it was tested in different *E. coli* strains—BL21, M15, and TG1, and two growth temperatures (28 and 37 °C). We assume that even leaky expression interferes with the viability of bacteria, therefore only clones with some expression-abol-
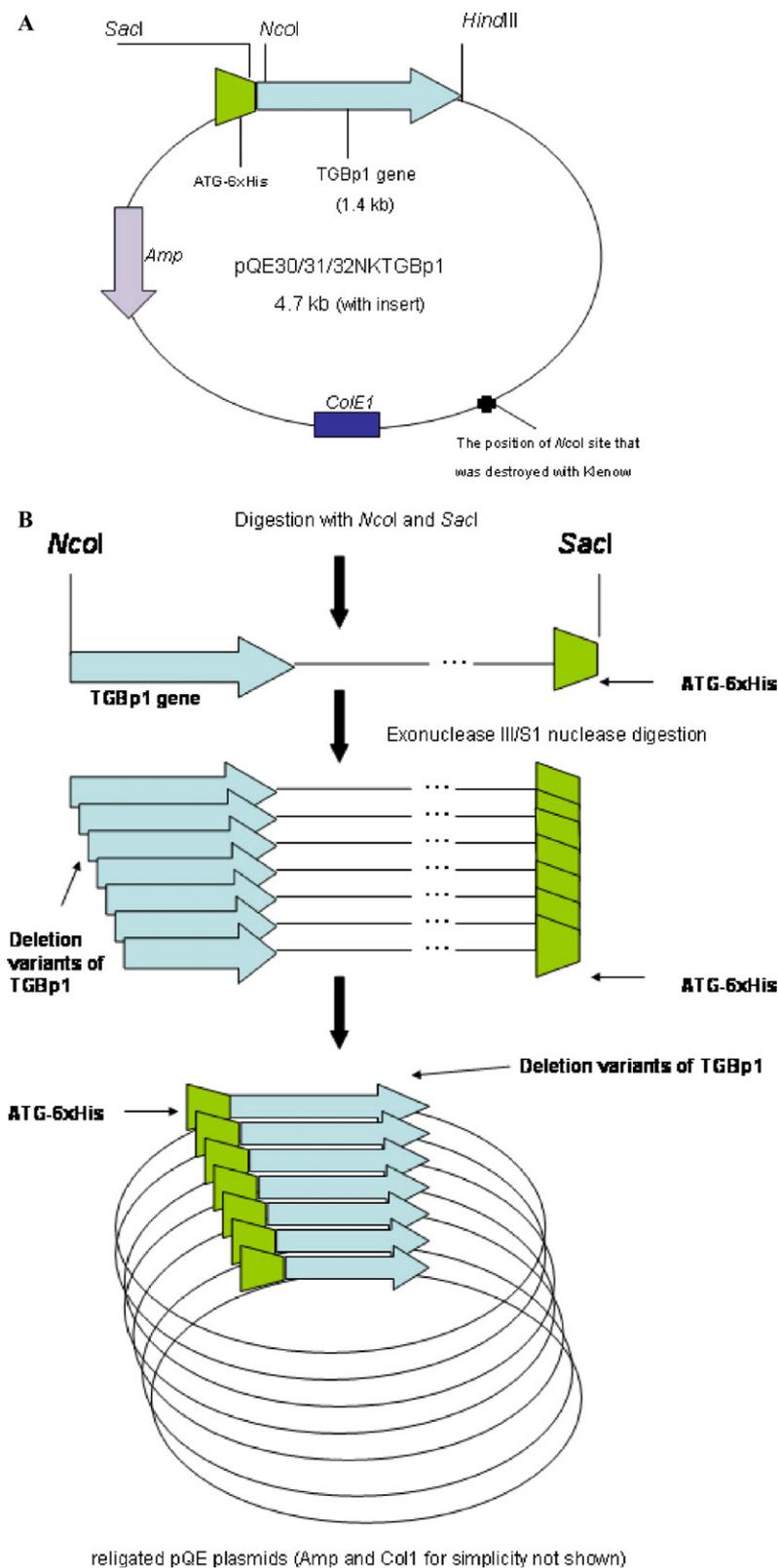
Fig. 1. (A) The schematic drawing of TGBp1 gene cloned into pQE30/31/NK vectors (pQE30/31 previously treated with *Nco*I and Klenow fragment) with marked restriction sites relevant for cloning and exonuclease III digestion. The *Sac*I and *Hin*dIII sites were used for recloning of TGBp1 gene from pTZR into pQE30/31NK plasmids. (B) The schematic drawing of construction of TGBp1 deletion variants. The *Sac*I site was used to create a DNA end resistant to exonuclease III digestion while the digestion with the *Nco*I produced ends sensitive to exonuclease III digestion.

ishing mutation could be obtained (e.g., frame-shift mutation).

Construction of 5′-deletion variants of TGBp1

In attempts to obtain TGBp1 variants sustainable in bacteria, we utilized progressive exonuclease III digestion from 5′ end of TGBp1 gene. TGBp1 fragment was recloned from pTZR vector into the plasmids pQE30NK and pQE31NK (pQE30/pQE31 plasmids devoid of their original unique *Nco*I site) using *Sac*I and *Hin*dIII restriction sites. The obtained constructs had additional several amino acids encoded within the part of pQE30/31NK polylinker (GSACELG or TOPHASS, respectively). The constructs were then digested with enzymes *Nco*I and *Sac*I yielding linearized plasmids with *Sac*I end resistant to exonuclease III digestion and *Nco*I sensitive to exonuclease III digestion (Fig. 1B). The several parallel exonuclease III/S1 nuclease digestion were performed under different conditions (NaCl concentration varying from 50 to 150 mM; temperature: 5, 10, 25, and 37 °C) and samples were taken at intervals (from 30 s to 5 min). The progress of the reaction was controlled by the gel electrophoresis (an example is shown in Fig. 2). Samples forming proper array (such as in Fig. 2) were mixed and separated on agarose gel. The products ranging from 4.7 kb (original length) to approximately 4.2 kb were excised and purified. Plasmids obtained by recircularization of the purified product were used to transform bacteria. Obtained colonies were blotted on a nitrocellulose membrane and after IPTG induction several colonies giving strong signal with anti-His antibodies were selected (Fig. 3).

Expression levels of deletion variants of TGBp1

Expression levels of different deletion variants of TGBp1 in suspension culture after IPTG induction were compared using Coomassie stained gel and Western blot as depicted for eight chosen colonies in Figs. 4A and B. Fig. 4B shows bands revealed by anti-His antibodies appearing after induction and migrating with an apparent molecular weight of approximately 50 kDa in the case of colonies named exo1, 5, 11, 13, 15, and 16, and approximately 40 kDa in the case of exo3 and 14
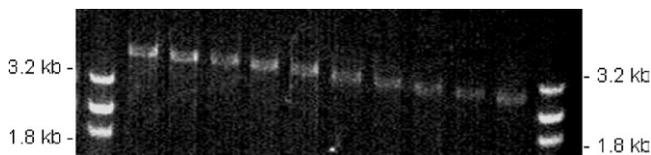

Fig. 2. The example of progressive exonuclease III/S1 nuclease digestion of pQE30/31NK carrying TGBp1 gene linearized with *Sac*I and *Nco*I restriction enzymes and subjected to exonuclease III/S1 nuclease digestion at 37 °C, with 100 mM NaCl. Samples were taken in intervals of 35 s.

Fig. 3. Detection of clones with high level expression of TGBp1 deletion variants by anti-His antibodies after colony-blotting.
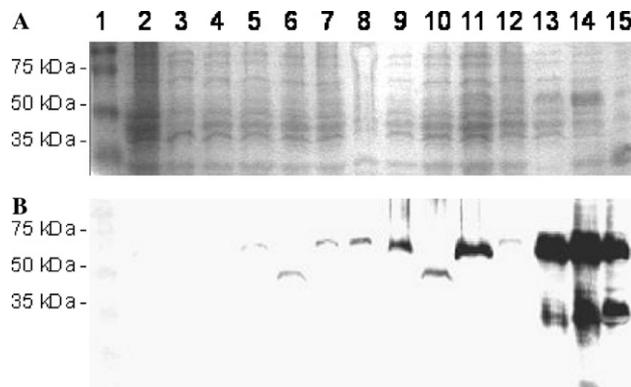

Fig. 4. Comparison of expression levels in selected clones carrying TGBp1 deletion variants. (A) Coomassie stained SDS–PAGE gel of expressed full-length TGBp1 and its deletion variants. Lane 1: pre-stained protein molecular weight marker (Amersham Life Science); lane 2: whole cell extract from a induced culture of *E. coli* TG1 transformed with empty pQE32; lane 3: whole cell extract of a non-induced apparently full-length TGBp1 in pQE32 cell culture; lane 4: whole cell extract of a induced apparently full-length TGBp1 in pQE32; lanes 5–12: whole cell extract of the induced TGBp1 deletion variants of colonies exo1, exo3, exo5, exo11, exo13, exo14, exo15, and exo16, respectively; lane 13: soluble fraction of whole cell extract of colony exo13; lane 14: insoluble fraction of whole cell extract of colony exo13; and lane 15: insoluble fraction after centrifugation through 30% sucrose cushion. (B) Western blot analysis. The arrangement of samples in lanes is as in (A).

(Fig. 4B). The level of production of recombinant protein was estimated densiometrically using Coomassie staining. The highest value was measured for exo13—5% of the total bacterial protein, as determined by densiometric analysis (GelDoc 2000, Bio-Rad). The protein was present mainly in insoluble fraction (lane 14), making 10% of proteins in insoluble fraction. Attempts to concentrate this protein product through sucrose cushion were not effective (lane 15).

Protein sequence analysis

The exo13 construct that was the most effective in recombinant protein production was sequenced. As shown in Fig. 5, the deduced protein product is 44 amino acids shorter than original TGBp1 gene. The molecular weight of this construct (6×His tag including) is 48 kDa. The isoelectrical point (p*I*) is 6.88, while the p*I* of the full-length TGBp1 is 8.45.

The prediction of secondary structure for exo13 deletion variant and full-length TGBp1 was done using PredictProtein and NORSp server [32]. The first about 110 amino acids of the full-length TGBp1 protein show highly flexible structure belonging to the category of so-called "no regular secondary structure" regions which are presumed to have important biological function. It is possible that the abolishing of this function, achieved by deleting the part of this sequence including the basic region NNNKK, makes the expression of this construct tolerable for bacteria.

Discussion

Very often there is a need to produce a protein in bacterial system that could be further used, e.g., for antibody production. However, sometimes the effort to obtain bacterial clone producing full-length protein is unsuccessful. The reason might be either degradation of the protein in bacterial environment due to its wrong processing and/or folding or the full-length protein is interacting with bacterial cell in a way that impairs cell viability. For such cases, we propose using of exonuclease III digestion to produce a set of deletion variants, some of which might not show the problems mentioned above and could be readily expressed in bacteria.

The TGBp1 is a typical protein for which many unsuccessful attempts were made to express it in bacteria. During our experiments, we could not obtain a single bacterial clone producing full-length protein TGBp1, apparently because the protein was incompatible with cell viability.

Fig. 5. The alignment of the sequence of the original TGBp1 gene with the sequence obtained for deletion variant exo13. The first several amino acids of original TGBp1 were introduced by the primer (see Materials and methods).

In this work, we describe a method of producing collection of TGBp1 clones successively deleted on its 5′ end. The vector with cloned TGBp1 gene was digested with the pair of enzymes, one of them giving end resistant to exonuclease III digestion, just immediately after the 6×His tag and the other enzyme leaving end sensitive to exonuclease digestion, being located immediately at the beginning of TGBp1 gene. The exonuclease III then digests one strand of DNA progressively from the sensitive end, while the S1 nuclease blunts this DNA with long single-stranded overhangs left by exonuclease III as well as the short overhangs left by the second restriction enzyme digestion. To obtain deletion variants differing by only a few nucleotides, we used frequent aliquoting and tried different temperatures and salt concentrations to slow down the reaction. When the products of exonuclease III/S1 nuclease digestion were mixed in one tube and loaded on the preparative gel electrophoresis, a continuum of deletion variants was obtained, ranging from the original length (approximately 4.7 kb) to fragments smaller than 1 kb. We restricted our attention to fragments between the original length and approximately 500 bp shorter, which we excised, purified, and recircularized.

This way, in very short time, we obtained the sets of recombinant molecules which code for proteins differing only in several amino acids on the N-terminus of expressed protein, just behind the 6×His tag. The exonuclease III reactions under different conditions could be performed in half-a-day. If the standard cloning procedures go smoothly (one prior to exonuclease III digestion and the other one after exonuclease III digestion), it is possible to obtain the collection of clones in one week with the expectation that some of them will give the high yield protein expression. Additional one to two days are needed for the screening of the whole collection of clones by colony-blotting procedure.

The same approach could be used to delete gradually sequences from the C-terminus of the recombinant protein. We have indications in the case of TGBp1 that the C-terminus deletion also ensures high yield protein expression (in progress).

There are many steps to be made in order to obtain purified protein. It is also possible to proceed in working with different constructs even with weaker expression to obtain a variant that will be more abundant in the soluble cell fraction, if there is a special need to produce a protein in its native form.

Acknowledgments

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