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Cloning and sequencing of *Toxoplasma gondii* major surface antigen (SAG۱) gene

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ABSTRACT

Genetic typing methods of *T. gondii* strains have been extensively perfected in recent years. From a technical point of view, many tools usable for genetic studied on single-copy loci have been used: RFLP, PCR-RFLP, sequencing, RAPD-PCR and isoenzyme analysis. We described the cloning and sequence analysis of the gene which encodes the major surface antigen (SAG۱ or P۳۰) of *T. gondii*. SAG۱ is the immunodominant antigen of *Toxoplasma gondii* tachyzoites being considered as the most promising molecule for a recombinant vaccine or such as DNA vaccine against toxoplasmosis. In the present work, first, genomic DNA of *Toxoplasma gondii* was extracted and used for amplifying of SAG۱ gene as a template. Then PCR product was cloned into pTZ۵۷R/T vector and plasmid containing SAG۱ gene (pT-SAG۱) was extracted from transformed bacteria and SAG۱ gene cloned into pTZ۵۷R/T was sequenced. Results showed that the P۳۰ gene contains no introns and can extract it from genomic DNA of tachyzoite stage. Results showed also that SAG۱ gene is cloned in pTZ۵۷R/T plasmid, forming pT-SAG۱ recombinant plasmid and *E. coli* TG۱ strain is the best host for pT-SAG۱ transformation. Sequence analysis of SAG۱ gene cloned into pTZ۵۷R/T vector showed that SAG۱ gene sequence from a high virulent strain of *T. gondii* (Known as RH strain) has ۱۰۰٪ sequence identity with P-Br strain, P strain and C strain and high homology of ۹۸٪ with RH strain and ZS۱ strain.

***Keywords*:** Cloning, Sequencing, *Toxoplasma gondii*, SAG۱, P۳۰

INTRODUCTION

Toxoplasmosis, caused by an intracellular protozoan parasite, *Toxoplasma gondii*, is widespread throughout the world (Bhopale ۲۰۰۳). The disease is of major medical and veterinary importance, being a cause of congenital disease and abortion in humans and in domestic animals (Bhopale ۲۰۰۳). There are marked biological differences among *Toxoplasma gondii* stocks concerning their pathogenicity to mice: most of the stocks are avirulant in mice producing asymptomatic chronic infections, while few which are highly virulent in mice stocks produce acute

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toxoplasmosis killing all mice with less than ۱۰ tachyzoites. Isoenzyme analysis using six different enzyme systems allowed the identification of ۱۲ zymodemes (Z۱-Z۱۲) among a population of ۸۶ stocks (Ajzenberg *et al* ۲۰۰۲a). For biologic and epidemiologic studies, three main genotypes are generally recognized in the *T. gondii* population: type I, II, and III (Ajzenberg *et al* ۲۰۰۲b). Lekutis *et al* (۲۰۰۱) believed that in addition to developmentally regulated differences in SAG expression, there is measurable allelic variation between the three prototypic strains of *T. gondii*. Interestingly, just to alleles were identify at the SAG۱ and SAG۲A luci when Type I, II and III strains were compared. In fact, most SAGs are dimorphic. SAG۱ or P۳۰ protein has an apparent M.W. ۳۰ kDa (Kasper *et al* ۱۹۸۵) and is stage specific, being detected only in the tachyzoite stage, but absent in the sporozoite and bradyzoite stages (Burg *et al* ۱۹۸۸, Hunter *et al* ۱۹۹۹, Kimbita *et al* ۲۰۰۱).This Antigen is abundant and homogeneously distributed on the surface of both extracellular and intracellular tachyzoites (Burg *et al* ۱۹۸۸). SAG۱ has two glycoforms (Zienker *et al* ۲۰۰۱) and is a highly conformational antigen (Chen *et al* ۲۰۰۱). The gene encoding SAG۱ occurs as a single copy, without introns (Kimbita *et al* ۲۰۰۱, Biemans *et al* ۱۹۹۸) and is highly conserved in *T.gondii* strains (Letscher-Bru *et al* ۱۹۹۸, Burg *et al* ۱۹۸۸). We are interested in the role of P۳۰ in the parasite's life cycle. Because of this and its importance in the immune response to *T. gondii* infection (and therefore its potential as a diagnostic tool and/or subunit or DNA vaccine), we have studied further molecular characterization of this protein through cloning and sequencing of the P۳۰ gene.

MATERIALS AND METHODS

Production of *T. gondii* tachyzoites. A high virulent strain of *T. gondii* (presented in experimental laboratory of Parasitology Department of Medical sciences Faculty of Tarbiat Modarres University, Known as RH strain), maintained in BALB/c mice by serial intraperitoneal inoculation of about ۱×۱۰^{۶} tachyzoites, was used for production of tachyzoites.

Genomic DNA extraction. About ۵×۱۰^{۷} *T. gondii* tachyzoites (۱۰۰µl) were concentrated by centrifugation, washed with phosphate buffer saline (PBS), then lysed in ۹۰۰µl lysis buffer (۰,۱M Tris-HCl pH ۸,۰ containing ۱٪ sodium dodecyl sulphate, ۰,۱M NaCl and l۰mM EDTA) and then treated with ۱۰µl proteinase K (۱۰۰µg/ml) at ۵۵ °C for ۲hr (Kimbita *et al* ۲۰۰۱). The lysate was then added to an equal volume of phenol/chloroform (۲۵:۲۵) to remove proteins. This mixture was centrifuged at ۱۳۰۰۰rpm for ۱۵min and an equal volume of chloroform was added to the supernatant which was then re-centrifuged. The supernatant was mixed with ۱/۱۰ volume of ۳M sodium acetate and two volumes of ۱۰۰٪ ethanol to precipitate DNA by centrifugation at ۱۳۰۰۰rpm for ۱۰min. The DNA pellet was washed with ۷۰٪ ethanol, dissolved in sterile distilled water and stored at -۲۰°C until use (Sambrook *et al* ۱۹۸۹). DNA extraction products were detected in ۰,۸٪ agarose gel and photographed.

PCR amplification and gel electrophoresis. Genomic DNA isolated from tachyzoites was used as a template to amplify the SAG۱ gene by PCR performed in ۲۵ µl of solution containing ۳µl of template DNA, ۰,۵ µl dNTP, ۰,۵ µl Taq DNA polymerase, ۲,۵ µl ۱۰X PCR buffer, ۰,۷۵ µl $MgCl_{۲}$, ۱۵,۷۵ µl distilled water and ۱ µl each of primers [Forward primer, ۲۷ nt: introduced Hind III recognition site, underlined: ۵′-ATT AAG CTT ATG TTT CCG AAG GCA GTG-۳′ (۱-۱۸ nt); Reverse primer, ۲۶ nt; introduced EcoRI recognition site, underlined: ۵′-ATT GAA TTC TCA CGC GAC ACA AGC TG-۳′(۹۶۰-۹۴۳ nt)] under the following conditions: After an initial ۵min denaturation at ۹۴ °C, each cycle consisted of ۶۰ s at ۹۴ °C, ۳۰ s at ۵۴ °C and ۴۵ s at ۷۲ °C at the end of

the ۳۰ cycles of amplification, a final extension was continued for ۵ min at ۷۲ °C.

The PCR products analyzed by electrophoresis on a ۱٪ agarose gel and photographed. The size markers used to estimate PCR products were the ۱۰۰bp and ۱kbp DNA ladders (Fermentas). The DNA sequence of gene encoding the surface antigen P۳۰ (SAG۱) of *T. gondii* was obtained from the GenBank database (http//www.ncbi.com) with accession No. AY۲۱۷۷۸۴,۱ and ۹۶۰bp. The forward and reverse primers were designed according to the nucleotide sequence in GenBank database and GenRuner Software.

Extraction of PCR products (SAG۱ gene band) from agarose gel. PCR products were purified using a DNA Extraction Kit from agarose gel (Ferments), following the manufacturer's recommendations.

Ligation of SAG۱ gene to pTZ۵۷R/T Cloning vector. The purified PCR products were ligated to pTZ۵۷R/T cloning vector (InsT/Aclone™ PCR product cloning kit, Fermentas), following the manufacturer's protocol.

First ligation reaction was prepared ۳۰ µl of solution, containing ۵ µl of pTZ۵۷R/T plasmid, ۱۵ µl purified PCR products (SAG۱ gene), ۱ µl T۴ DNA ligase, ۳ µl l۰X buffer, ۳ µl PEG and ۳ µl Nuclease free D.W. After vortex and spin, this mixture was incubated at ۲۲ °C for overnight, and then ligation reaction product was stored at – ۲۰ °C until use.

Transformation and Screening. Preparation of competent cells from *Escherichia coli* TG۱ strain was performed by calcium chloride method (Sambrook *et al* ۱۹۸۹).

For transformation, ۱۰ µl of ligation reaction product was added to ۱۵۰ µl competent cells, after vortex and spin the mixture was incubated at ۴۲ °C for ۹۰ s, and immediately was placed on ice for ۲-۳min. The transformed cells were allowed to recover in ۳۰۰ µl LB broth medium free antibiotic by incubated at ۳۷ °C for ۱-۲ hr with shaking. These recovered cells were plated onto LB agar plates containing ampicillin, IPTG (Fermentas) and X-Gal (Fermentas) to screening of blue and white colonies and incubated at ۳۷ °C for ۱۸ hr. Several white and blue colonies were randomly selected from each agar plate and inoculated in a LB medium containing ampicillin and incubated at ۳۷ °C for ۱۸ hr.

Confirmation of Cloning of SAG۱ gene into pTZ۵۷R/T vector. The plasmid was purified from white and blue colonies of bacteria by Accuprep plasmid Extraction Kit (BioNEER), following the manufacturer's protocol. After plasmid extraction, following experiments were performed for improving cloning of SAG۱ gene into pTZ۵۷R/T vector:

۱) Comparison of extracted plasmids on ۰,۸٪ agarose gel. ۳µl of each plasmid extracted from white (pT-SAG۱) and blue (pTZ۵۷R/T) colonies bacteria were loaded on a ۰,۸٪ agarose gel and were electrophoresis and photographed. Then, plasmid bands on agarose gel were compared.

۲) PCR amplification of SAG۱ gene using by pT-SAG۱ plasmids. Plasmid DNA extracted form white colonies bacteria (pT-SAG۱) was used as a template to amplify the SAG۱ gene by PCR performed in ۲۵ µl of solution under condition previously description in part ۳. The PCR product were analyzed by electrophoresis on a ۱٪ agarose gel and photographed. The size marker used to estimate PCR products were the ۱۰۰bp and ۱kbp DNA ladders (Fermentas).

۳) Enzyme digestion of pT-SAG۱ plasmids. With regard to designed HindIII and EcoRI restriction enzymes sites respectively on forward and reverse primers and present them in recombinant plasmids extracted from white colonies bacteria (pT-SAG۱), these plasmids were digested by HindIII and EcoRI enzymes. For this propose each enzyme digestion reaction was performed in ۲۰ µl of solution containing ۱۰ µl plasmid , ۱ µl restriction enzyme, ۲ µl ۱۰X buffer and ۷ µl D.W, after spin and vortex, this mixture was incubated in ۳۷ °C for overnight. Because of being different of restriction enzyme buffers, each enzyme digestion was performed

separately. Total of enzyme digestion products by EcoRI was loaded on a ۱٪ agarose gel and the band resulting from digestion by EcoRI enzyme containing SAG۱ fragment was extracted from agarose gel by DNA extraction Kit from agarose gel (Fermentas) and second enzyme digestion by HindIII was performed on it. Products from digestion by HindIII were loaded on a ۱٪ agarose gel and the band resulting from digestions by two enzymes was analyzed by electrophoresis.

۴) Nucleotide sequencing of the SAG۱ cloned in pTZ۵۷R/T vector. The plasmids extracted from white colonies bacteria (pT-SAG۱) were sequenced by Faza Biotech Company.

RESULTS

DNA extraction. Fig.۲ shows that genomic DNA has been extracted by using lysis buffer and proteinase K followed by phenol /chloroform method.

PCR amplification. Fig.۳ shows that DNA fragment PCR amplified was about ۹۶۰bp and similar to the *Toxoplasma gondii* SAG۱ gene size and no any genes was amplified exception with SAG۱ gene. Thus, these designed primers are specific for amplifying of SAG۱ gene. Results from electrophoresis of PCR products with extracted plasmids pT-SAG۱ using specific primers and remembered conditions showed that a ۹۶۰bp fragment of SAG۱ gene was amplified and this gene has been cloned into PTZ۵۷R/T plasmid (Figure ۶).

Ligation of SAG۱ gene into pTZ۵۷R/T Cloning vector. According to the figure ۱, there were two patterns for ligation of SAG۱ gene into pTZ۵۷R/T cloning vector. In pattern ۱, introduced HindIII recognition site of SAG۱gene is near to EcoRI restriction site of pTZ۵۷R/T cloning vector but in pattern ۲, introduced HindIII recognition site of SAG۱gene is far from EcoRI restriction site of pTZ۵۷R/T cloning vector.

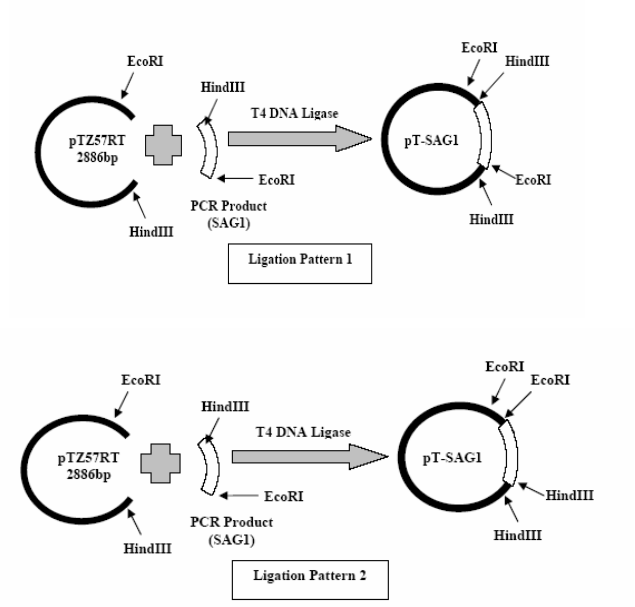


Figure ۱. Ligation patterns for construction of recombinant pT-SAG۱ plasmid with pTZ۵۷R/T (cloning vector) and SAG۱ gene.

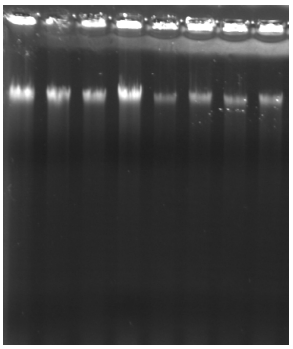


Figure ۲. Genomic DNA extraction from *Toxoplasma gondii* tachyzoites was performed by lysis buffer and phenol : chloroform method and electrophoresed in ۰٫۸٪ agarose gel.

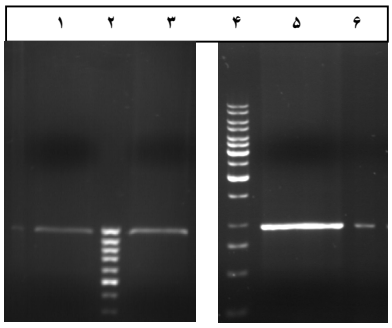


Figure ۳. PCR amplification and gel electrophoresis of PCR product. *Lane ۱, ۲, ۵ and ۶*: PCR product (approximately ۹۶۰bp); *Lane ۳*: ۱۰۰bp DNA ladder; *Lane ۴*: ۱Kbp DNA ladder.

Comparison of extracted plasmids on ۰,۸٪ agarose gel. Electrophoresis of extracted plasmids showed that both of plasmids (pTZ۵۷R/T and pT-SAG۱) had three bands (linear, open circular and super-coil plasmids respectively from up to down) in which pT-SAG۱ bands placed above of pTZ۵۷R/T bands on agarose gel (Figure ۴).

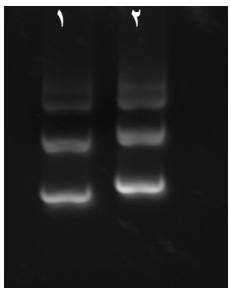
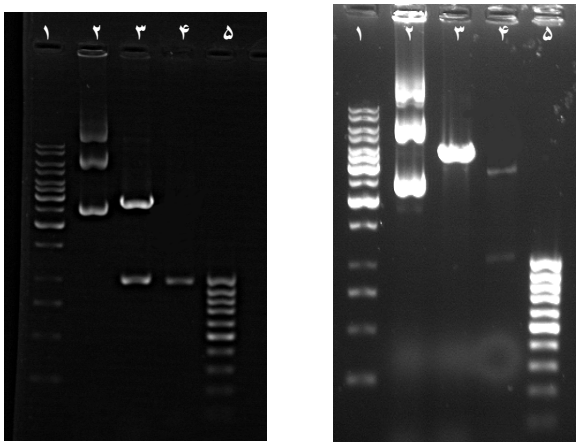


Figure ۴. Comparison of extracted plasmids on ۰,۸٪ agarose gel showed that both of plasmids (pTZ۵۷R/T and pT-SAG۱) had three bands (open circular, linear and super-coil plasmids respectively from up to down) in which pT-SAG۱ bands placed above of pTZ۵۷R/T bands on agarose gel : pTZ۵۷R/T (*Lane ۱*) and pT-SAG۱ (*Lane ۲*).



A **B**

Figure ۵A. Agarose gel electrophoresis of Digestion of extracted pT-SAG۱ (according to ligation pattern ۱) after transformation; *Lane ۱:* ۱Kbp DNA ladder; *Lane ۲:* pT-SAG۱ has three bands (linear, open circular and super-coil plasmids respectively from up to down); *Lane ۳:* pT-SAG۱ digested by EcoRI has two bands in that, one is less than ۱۰۰۰bp (down) and the other is less than ۳۰۰۰bp (up); *Lane ۴:* Second digestion by HindIII on less than ۱۰۰۰bp band (containing SAG۱); *Lane ۵:* ۱۰۰bp DNA ladder.

Figure ۵B. Agarose gel electrophoresis of Digestion of extracted pT-SAG۱ (according to Ligation pattern ۲) after transformation; *Lane ۱:* ۱Kbp DNA ladder; *Lane ۲:* pT-SAG۱ has three bands (linear, open circular and super-coil plasmids respectively from up to down); *Lane ۳:* pT-SAG۱ digested by EcoRI has one band (approximately less than ۴۰۰۰bp); *Lane ۴:* Second digestion by HindIII on pT-SAG۱ digested by EcoRI, has two bands in that, one is ۹۶bp (down) and the other is less than ۳۰۰۰bp (up); *Lane ۵:* ۱۰۰bp DNA ladder.

Comparison of bands of extracted plasmids from white and blue colonies bacteria shows that bands of plasmids extracted from white colonies are heavier than plasmids extracted from blue colonies and thus, SAG۱ gene has been cloned into pTZ۵۷R/T (Figure ۴).

Enzyme digestion. According to the ligation patterns (figure ۱), when plasmids extracted from white colonies are digested by EcoRI restriction enzyme, digestion products may have two different electrophoresis patterns. Figure ۵,۱ showed that electrophoresis of digestion products obtained from the first digestion by EcoRI had two bands in that, one was less than ۱۰۰۰ bp and the other was less than ۳۰۰۰ bp. Figure ۵,۲ showed that when plasmids extracted from white colonies were digested by EcoRI restriction enzyme, one band (approximately less than ۴۰۰۰ bp) was observed. In both above sets of enzyme digestion, electrophoresis of digestion products obtained from the second digestion by HindIII (that performed on digestion products obtained from the first digestion by EcoRI) showed a ۹۶۰ bp band (Figure ۵,۱ and ۵,۲). Figure ۵,۲ showed that second digestion by HindIII on pT-SAG۱ digested by EcoRI, had two bands in that, one was ۹۶ bp (down) and the other was less than ۳۰۰۰ bp (up). Results from enzyme digestion revealed that if the plasmid extracted from white colonies bacteria (pT-SAG۱) were digested with EcoRI and HindIII, a ۹۶۰ bp band was cut and separated that this is SAG ۱ gene, and thus the SAG۱ gene has been cloned into pTZ۵۷R/T.

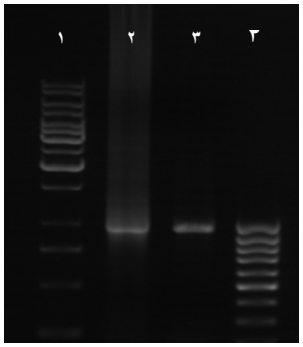


Figure ۶. Agarose gel electrophoresis of PCR amplification of SAG۱ gene products with pT-SAG۱ and Genomic DNA; *Lane ۱:* ۱Kbp DNA ladder; *Lane ۲:* PCR amplification of SAG۱ gene with pT-SAG۱; *Lane ۳:* PCR amplification of SAG۱ gene with Genomic DNA; *Lane ۴:* ۱۰۰bp DNA ladder.

Nucleotide Sequencing. Nucleotide sequence analysis of the SAG۱ cloned in pTZ۵۷R/T vector revealed ۱۰۰٪ sequence identity with P-Br strain (GenBank Accession No. AY۱۸۷۲۷۸,۱), P strain (GenBank Accession No. S۸۵۱۷۴,۱), and C-strain (GenBank Accession No.S۶۳۹۰۰,۱). Sequence analysis of the SAG۱ region revealed high homology of ۹۸٪ with RH strain (GenBank Accession No. AY۲۱۷۷۸۴,۱) and ZS۱ Strain (GenBank Accession No. S۷۳۶۳۴,۱).

DISCUSSION

Genetic typing methods of *T. gondii* strains have been extensively perfected in recent years. From a technical point of view, many tools usable for genetic studied on single-copy loci have been used: RFLP, PCR-RFLP, sequencing, random amplified polymorphic DNA PCR (RAPD-PCR) and isoenzyme analysis. Most of these studies were performed on a small sampling of stocks and described the use of only one locus, mainly SAG۲ locus, for genetic typing (Ajzenberg *et al* ۲۰۰۲b).

We describe the cloning and sequence analysis of the gene which encodes the major surface antigen (SAG۱ or P۳۰) of *T. gondii*. Results showed that the P۳۰ gene is a single copy, contains no introns and can extract it from genomic DNA of tachyzoite stage. Results also showed that SAG۱ gene is cloned in PTZ۵۷R/T plasmid, forming pT-SAG۱ recombinant plasmid and *E. coli* TG۱ strain is the best host for pT-SAG۱ transformation.

Burg *et al* (۱۹۸۸), Hunter *et al* (۱۹۹۹) and Kimbita *et al* (۲۰۰۱) also showed that SAG۱ is stage specific, being detected only in the tachyzoite stage, but absent in the sporozoite and bradyzoite stages and this antigen is abundant and homogeneously distributed on the surface of both extracellular and intracellular tachyzoites. Kimbita *et al* (۲۰۰۱) and Biemans *et al* (۱۹۹۸) also resulted that the gene encoding SAG۱ occurs as a single copy, without introns.

Results also showed that according to the ligation patterns (figure ۱); digestion products may have two different electrophoresis patterns (figure ۵,۱ and ۵,۲) and different ligation patterns have no effect on cloning and sequencing.

Sequence analysis of SAG۱ gene cloned into pTZ۵۷R/T shows that the sequence has ۱۰۰٪ identity with P-Br strain, P strain and C Strain and high homology of ۹۸٪ with RH strain and ZS۱ strain. This result shows that SAG dimorphism and chromosomal localization are windows through which are population biology of *T. gondii* can be observed and is similar to reports of other researchers (Letscher-Bru *et al* ۲۰۰۳, Burg *et al* ۱۹۸۸) about highly conserved of SAG۱ sequence in *T. gondii* strains and Lekutis *et al* (۲۰۰۱) that believed that in addition to developmentally regulated differences in SAG expression, there is measurable allelic variation between the three prototypic strains of *T. gondii*. Interestingly, just to alleles were identify at the SAG۱ and SAG۲A luci when Type I, II and III strains were compared. In fact, most SAGs are dimorphic.

Burg *et al* (۱۹۸۸) showed that comparison of near-full length cDNA to genomic DNA by sequence and restriction mapping (as well as full length protection of the ۵′ end of P۳۰ mRNA with genomic DNA) demonstrate that the P۳۰ gene contains no introns and northern blot analysis shows that the P۳۰ mRNA is about ۱۵۰۰ nucleotides in length and accumulates to very high levels and predicted size for P۳۰ primary translation product deduced from the cDNA sequence is ۳۴,۷ kDa also showed that there are two potential methionines for SAG۱; although translational machinery most often utilizes the methionine codon it encounters, some data suggest that the second methionine codon of P۳۰ is used to initiate translation. Since a signal sequence of ۴۷ amino acids is unprecedented, this potential signal sequence cleavage site would also suggest the second methionine codon as the initiator of the

primary translation product (with a signal peptide of ۳۰ amino acids). Therefore, the amplified ۹۶۰ bp DNA segment in this work utilizes the second methionine codon of P۳۰ and it is used to initiate translation.

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