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Cloning of tomato *SUMO1* and development of a CaMV *35S* based gene construct for plant transformation

Saad Imran Malik*, Abuzar Abdullah, SamraAzam

Department of Plant Breeding and Genetics, PMAS Arid Agriculture University, Rawalpindi, Pakistan

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Abstract

Tomato (*Solanum lycopersicum* L.) is one of most important vegetable which is affected by several biotic and abiotic stresses reducing its yield and quality. Stress related proteins may be modified by small ubiquitin-like modifiers (SUMOs) - the process known as SUMOylation, which involves many SUMO proteins and an enzymatic cascade for post-translational modification. SUMOylation is a well-studied process in *Arabidopsis* but little is known about its roles in crop plants including tomato. This research was aimed to develop a*SUMO1* gene overexpression construct under the influence of a CaMV35*S* promoter. Total RNA was extracted from tomato leaves through Trizol method followed by cDNA synthesis. The *SUMO1*gene specific primers having *Bgl*II and *BstE*II restriction enzymes sites at 5' ends were used to amplify full-length *SUMO1* coding sequence from cDNA via PCR. The fragment was purified and ligated into a TA cloning vector (pGEM-T) followed by sub-cloning in pCAMBIA1301 (a plant transformation vector) from which the native *GUS* gene was removed. All step-wise confirmations were done by restriction enzyme digestion and colony PCR followed by agarose gel electrophoresis analysis. The resulted plasmid based construct harboring *SUMO1* full-length coding sequence was named pCAMBIA: *SUMO1*. The construct was transformed in *Agrobacterium* strain LBA4404 through electroporation for subsequent *SUMO1* gene transfer in tomato through *Agrobacterium*-mediated transformation. Thetransgenic plants obtained after transformation will be used for *SUMO1* functional studies in tomato regarding biotic/abiotic stress tolerance and disease resistance.

* **Corresponding Author:** Saad Imran Malik ✉ saad.malik@uaar.edu.pk

Introduction

Modern genetic transformation techniques which involve gene isolation, cloning and direct gene transfer into plant genomes are now emerging as popular tools in the development of commercial crop cultivars with improved performance and quality (Que *et al*., 2010; Jungbauer 2010; Yang and Wan, 2011; Pulla 2016). *Bt* and glyphosate resistance crops are well-known examples in this regard (Duke, 2015; Yu, *et al*., 2011; Jungbauer, 2010). These approaches are marked as non-conventional breeding approaches which are independent of crossing and scarcity of germplasm resources. Tomato being the 2nd most consumed vegetable after potato is an excellent model for plant genetic and physiological research (Gupta *et al*., 2009) with its genome sequence published in 2012 (Sato *et al*., 2012). The ease of employing *Agrobacterium*-mediated transformation in tomato has made this crop a top choice for genetic and molecular studies (Park *et al*., 2003; She and Song, 2006; Sharma *et al*., 2009).

Improving genetic architecture of tomato has always remained the foremost target to increase its yield and quality (Monforte *et al*., 2014; Uluisik *et al*., 2016). Conventional breeding is limited only to controlled crossing and subsequent selection of plants with desirable traits (Gupta *et al*., 2009). Nevertheless, there are several examples of direct gene delivery in tomato to enhance stress tolerance, disease resistance and improve quality and shelf-life (Albacete *et al*., 2015; Folta *et al*., 2016; Li *et al*., 2016). Different transgenic tomato varieties have been produced by genetic transformation having longer shelf life as *Flavr Savr* tomato (Alexander *et al*., 2009), delayed ripening by incorporating *ACC SYNTHESES* gene (Klee *et al*., 1991), frost tolerance by engineering an anti-freeze gene (Lemaux, 2008), incorporation of a rice gene *OsYMB4* for drought tolerance (Vannini *et al*., 2007) and *AtNHX1* for salt tolerance (Zhang and Blumwald, 2001). Moreover, *DEL* and *ROS1* genes overexpression increased antioxidant amounts in tomato which have anti-cancerous properties (Butelli *et al*., 2008). Other examples include *Bt* gene for insecticidal tolerance and *Ceropin B* gene for tolerance to wilt diseases (Jan *et al*., 2010).

SUMOs (Small ubiquitin-like modifiers) are small eukaryotic proteins of about 10-12 kDa which modify other cellular proteins by reversible covalent attachment at lysine residues altering their functions (Kurepa *et al*., 2003; Johnson, 2004; Novatchkova *et al*., 2004). The process as a whole is called SUMOylation. SUMOylation is now a mechanistically well understood phenomenon known to regulate a number of plant physiological processes like temperature stress tolerance (Yoo *et al*., 2006; Flick and Kaiser, 2009), plant reproduction (Jin and Hasegawa 2008; Augustine *et al*., 2016), plant defense (Miura *et al*., 2007; Jin *et al*. 2008, van den Burg *et al*., 2010) and root development (Lois *et al*., 2003; Zhang *et al*., 2010). SUMOylation is also involved in the regulation of various transcriptional factor and repressors (Miura *et al*.,2005; Miura *et al*., 2007; Chaikam and Karlson, 2010). Various eukaryotic cellular processes such as sub-nuclear localization, enzymatic stability and activity, regulation of cell cycle, innate immunity and DNA repair are linked to SUMOylation (Verger *et al*., 2003; Hay, 2005; Chosed *et al*., 2006). Hence SUMO genes are regarded as the key regulators of many environmental responses, growth and development.

In this study we aimed to develop a CaMV 35*S* based *SUMO1* gene construct for overexpression in tomato via *Agrobacterium*-mediated transformation. CaMV 35*S* promoter is the most commonly used promoter in plant transformation experiments for the constitutive overexpression of transgenes (Odell *et al*., 1985; Benfey and Chua, 1990; Yoo *et al*., 2005; Squires *et al*., 2007; Szwacka *et al*., 2009; Azizi *et al*., 2016). Following our targets, we isolated and cloned *SUMO1* full-length coding sequence (CDS) from tomato. This gene was sub-cloned in a plant transformation vector having a CaMV 35*S* promoter and a *NOS* terminator for subsequent transformation in tomato for overexpression. The construct developed will be used further for tomato transformation and *SUMO1* overexpression using standard tissue culture and *Agrobacterium*-mediated transformation procedures. This will lead to study the functional attributes and roles of *SUMO1* gene in tomato plants regarding stress tolerance and disease resistance.

Materials and methods

Plant material and growth conditions

The research was conducted in the Biotechnology Lab of the Department of Plant Breeding and Genetics, PMAS Arid Agriculture University Rawalpindi. The seeds of tomato (*Solanum lycopersicum* L.) cv. Rio Grande were obtained from a commercial seed store. They were germinated in a small three inch diameter pots using soil and peat moss mixture. The leaf tissues were used for the isolation of RNA.

Total RNA extraction

Then total RNA was isolated by Trizol method. Freshly collected leaves were ground in liquid nitrogen and transferred to pre-chilled microtubes. One ml TRIzol® (Invitrogen) was added to the tubes. The samples were thoroughly mixed by vortexing, centrifuged at 4 °C for 10 min at 12,000*g* and the clear top aqueous phase was collected in fresh tubes. Chloroform (200 µl) was added in the supernatant and the tubes were centrifuged 12,000*g*at 4 °C for 15 min. The top aqueous phase of about 600 µl was decanted into a freshmicrotube and precipitated with 300 µl isopropanol and NaCl/Na-citrate salt solution. The samples were centrifuged at 10,000*g* for 5 min at 4 °C and the pellet formed was washed with 1 ml of 75% ethanol diluted with DEPC-treated water. The pellet was suspended in 50 µl DEPC treated water and RNA samples were stored at –70 °C. RNA concentration was measured by spectrophotometric analysis and quality was verified by agarose gel electrophoresis followed by analysis under a Gel Doc XR (Bio-Rad, USA).

cDNA synthesis and confirmation

Revert Aid first-strand cDNA synthesis kit (Thermo Scientific™) was used for cDNA synthesis using RNA according to manufacturer's instructions. RNA sample and the kit reagents were allowed to thaw on ice. The reaction was constituted bymixing 7 µl nuclease free water, 5 µl total RNA and 1 µl oligo $(dT)_{18}$ primers in a 1.5 ml microtube. The reaction mixture was 4 µl 5X reaction buffer, 1 µl RNAse inhibitor, 1 µl M-MuLV RT and 1 µl of dNTPs (10 mM each).

The cocktail was incubated at 42 °C for 60 min for cDNA synthesis and the reaction was terminated by heating at 70 °C for 15 min. Nuclease free water (40 µl) was added to the mixture and cDNA preps were stored at -70 °C.

A polymerase chain reaction (PCR) was setup to confirm quality and integrity of cDNA using *ACTIN* gene primers *SlACTIN* (F) GATGCATATGTTGGTGATGAAGC and *SlACTIN* (R) GTGGTGAACATGTAACCTCTCTC. The reaction mixture was prepared using 16.6 µl water, 0.5 µl dNTPs (10 mM), 2.5 µl 10X *Taq* buffer, 1 µl *SlACTIN* forward and reverse primers, 1 µl $MgCl_2$ (50 mM), 0.4 µl *Taq* DNA polymerase and 2 or 4 µl cDNA. PCR conditions were used in three steps viz. denaturation, annealing and extension at 94 °C, 55 °C and 72 °C for 20 s, 20 s, and 30 s, respectively with initial denaturation of 94 °Cfor 1 min and final extension at 72 °Cfor 5 min. Resultant PCR products were confirmed by agarose gel electrophoresis using 1.5% agarose prepared in TAE buffer.

PCR amplification of full-length SUMO1 gene

The coding sequence (CDS) of *SUMO1* was amplified using *SUM1-Bgl*II forward primer ATAGATCTATGTCAGGCGTCACTCAACA having *Bgl*II restriction site (underlined) and *SUM1-BstE*II reverse primer TGGTGACCCTAAGACAAAGATCCACCAGT having *BstE*II restriction site (underlined). The primers were flanked by specific restriction sites which were present in pCAMBIA1301 plant expression vector from which *GUS* gene was removed by restriction digestion for the ligation of *SUMO1* full-length CDS. The primers were used for PCR base amplification of full-length CDS using Phusion® high fidelity DNA polymerase (Thermo Scientific).

Gel purification and cloning of SUMO1 in pGEM-T vector

The PCR amplified *SUMO1* fragments were resolved by agarose gel electrophoresis and visualized under UV with a 1 kb DNA ladder (Thermo Scientific) as size standard.

The amplified fragments were sliced from agarose gel and purified by using Silica Bead DNA gel extraction kit (Thermo Scientific) according to manufacturer's instructions. The final elute was 40 µl in DEPC-treated water which was quantified on 2% agarose gel using 100 bp DNA ladder as standard.

The resulting DNA fragments were A-tailed using *Taq* DNA polymerase at 72 °C for 15 min and cloned in pGEM-T vector (Promega, USA) according to manufacturer's instructions. The ligation mixture having pGEM-T vector and *SUMO1* CDS was transformed into *E. coli* strain DH5α by heat shock method (Sambrook and Russel, 2001). The transformed cells were plated on LB + 50 mg/L ampicillin plates having IPTG and X-Gal for blue-white colony selection.

Confirmation of transformants by PCR and restriction enzyme digestion

The single white colonies were picked and mixed in 50 µl water and used 1 µl in the PCR. A standard PCR using T7 and SP6 primers was setup and the products were run on 2% agarose gel for analysis.

The positive clones were grown in liquid LB media overnight with 50 mg/L ampicillin. The plasmids were isolated using GenJet plasmid miniprep kit (Thermo Scientific). Isolated plasmid preps were analyzed on agarose gel and used in a PCR for confirmation for the presence of *SUMO1* gene fragment using gene specific primers listed above. The plasmid preps were digested with *Bgl*II and *BstE*II restriction enzymes and the mixture was analysed on 2% agarose gel.

Ligation of digested fragments in pCAMBIA1301

The *SUMO1* gene fragment was gel-eluted and purified for sub-cloning in pCAMBIA 1301 (*GUS* removed) having *Bgl*II and *BstE*II restriction sites using a ligation mixture having 0.8 µl T_4 DNA ligase and 1 µl 10X ligation buffer. The ligation mixture was transformed in *E. coli* competent cells. Single transformed colonies were picked and grown overnight in liquid LB with kanamycin (50mg/L). After confirmation by PCR and double digestion with restriction enzymes, the selected plasmid preps were transformed in *Agrobacterium* strain LBA4404 by electroporation using 2 mm cuvette and a Gene Pulser (Bio-Rad, USA) employing 25 µF capacitance, 2400 V and 200 Ω resistance and a pulse length of 5 ms. This mixture was plated on LB + kanamycin (50mg/L) + rifampicin (25mg/L) plates and placed at 28 °C for 2-3 days in dark. Single colonies obtained after 3 days were confirmed by colony PCR using *SUMO1* gene specific primers.

Results

Obtaining full-length coding sequence of tomato SUMO1 (SlSUMO1)

SlSUMO1 gene was searched using Sol Genomics database; an online gene search tool for solanaceae family using the Arabidopsis *SUMO1*(*At*5G55160) nucleotide sequence as query sequence.

The best hit was identified on long arm of chromosome 7 of tomato genome annotated as*Solyc*07g064880. This gene was found to have a genomic DNA sequence length of 2469 bp including intron, exons, 3' UTR (un-translated regions) and 5' UTRs with acDNA length of 573 bp including UTRs. However, the CDS of *SUMO1*was found to be300 bpin size with 100 amino acid residues long protein chain for precursor SUMO1 protein. Agarose gel electrophoresis of RNA isolated from leaf tissues revealed intact 28*S* and 18*S* ribosomal RNA sub-units and a 5*S* transfer RNA unit (Fig. 1A).

This verifies the good quality and purity of RNA suitable for subsequent cDNA synthesis using an oligo $(dT)_{18}$ primer. cDNA synthesis using this RNA prep was validated by using *SlACTIN* primers in two independent PCRs. Since *ACTIN* genes are present and expressed ubiquitously in all plant cells, the validation using *SlACTIN* primers demonstrated clear bands of 425 bp (Fig. 1B) confirming the suitability of cDNA prep for full-length CDS amplification of *SUMO1* gene by PCR.

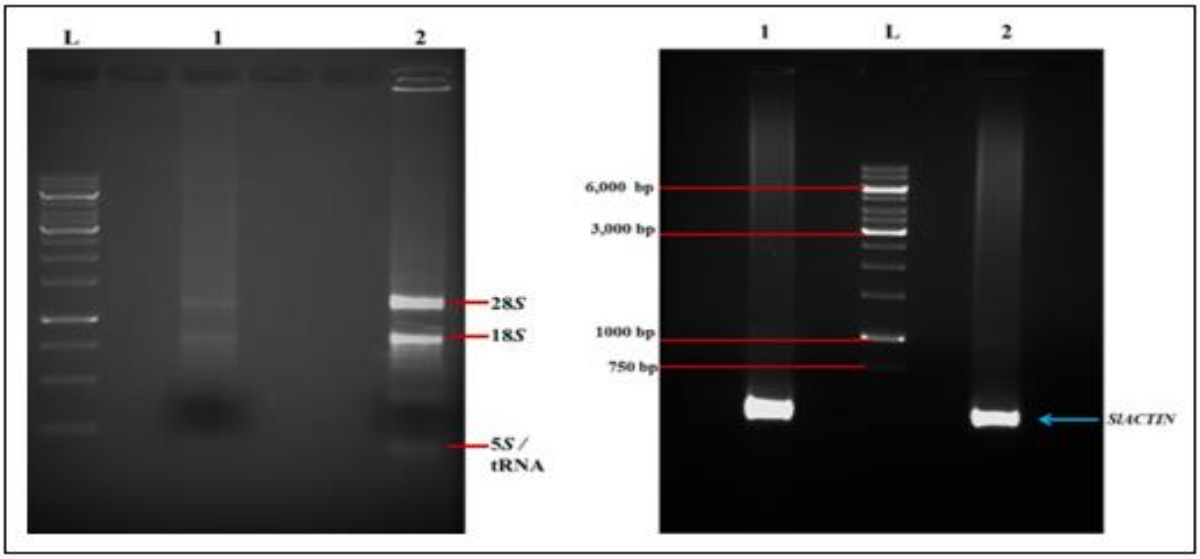

Fig. 1. Agarose gel electrophoresis pictures where nucleic acids are stained with ethidium bromide and photographed under UV using gel documentation system. L represents 1 kb DNA ladder(**A)** Total RNA(1 µl in lane 1 and 3 µl in lane 2) of tomato cv. Rio Grande extracted from leaves and revealed by 28*S* and 18*S* intact bands on 1.5% agarose gel, (**B)**Both lane 1 and 2, PCR products of *Actin* gene using *SlActin* primers confirm the synthesis of cDNA after gel electrophoresis.

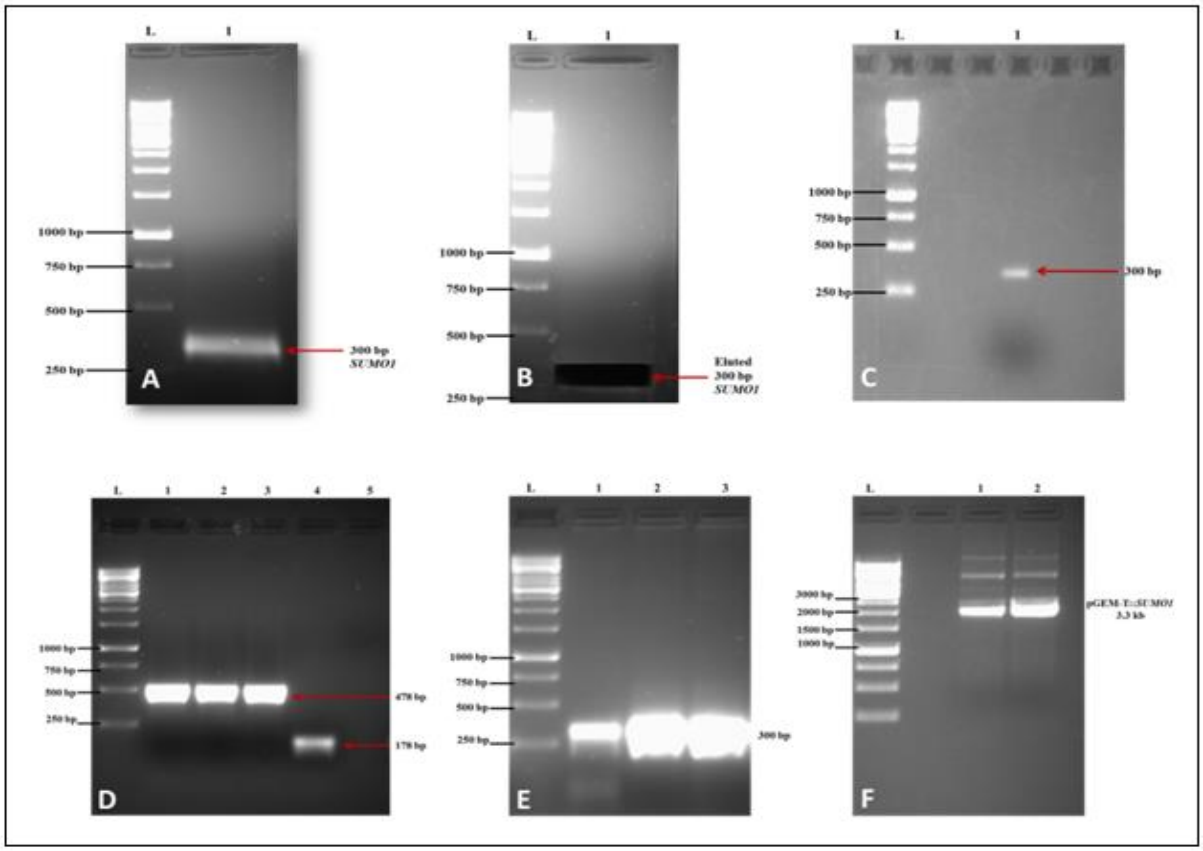

Fig. 2. L represents 1 kb DNA ladder and lanes are represented as numbers (A)Confirmation of amplified PCR product using Phusion high-fidelity DNA polymerase, (B) excision of *SUMO1* gene fragment from agarose gel for purification, (C) quantification and confirmation of gel eluted *SUMO1*,(D) Colony PCR-based confirmation of transformed *E. coli* cells using T7 and SP6 border primers, (E) Gene specific colony PCR of overnight grown cultures, and (F) confirmation of plasmid isolated from overnight grown selected cultures.

PCR based amplification and cloning of SlSUMO1 CDS

High-fidelity DNA polymerase was used to reduce nucleotide incorporation errors and a correct full-length open reading frame (ORF) amplification by PCR. The primers having restriction sites at 5' end amplified a correct size fragment of ~300 bp corresponding to *SUMO1* gene after PCR (Fig. 2A). In order to clone this gene, the PCR product was excised from the agarose gel (Fig. 2B) and purified for subsequent cloning in a TA cloning vector and confirmed later by running the extracted fragment on agarose gel which showed a correct size band of 300 bp length (Fig. 2C). The quantification of the eluted fragments was found to be ~3ng/µl. This purified fragment was later cloned in a TA cloning vector by performing a ligation reaction and the mixture was transformed in *E. coli* competent cells.

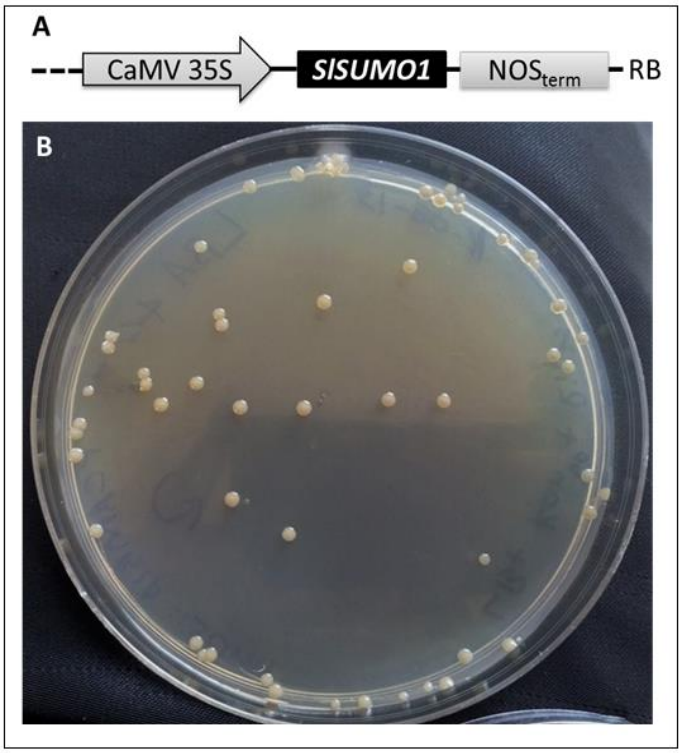

Fig. 4. (A) Resulting cassette (pCAMBIA: *SUMO1*) for *SUMO1* overexpression having a CaMV 35S promoter and NOS terminator, **(B)**Transformed *Agrobacterium* LBA4404 colonies on LB selection media having rifampicin and kanamycin as selection antibiotics.

The transformed cells developed colonies on LB selection media. Colony PCR for 4 randomly selected clones using T7 and SP6 primers yielded 3 positive clones showing a band of about 450 bp length (Fig. 2D), which include cloning vector multiple cloning sites on both ends of *SUMO1* fragment along with flanking regions within T7 and SP6 primer regions. However, clone 4 did not carry *SUMO1* fragment (Fig. 2D). On further validation of three positive selected clones, PCR was performed by using *SUMO1* gene specific primers and the products after agarose gel electrophoresis revealed 3 correct size bands of *SUMO1* fragment (Fig. 2E) confirming its presence.

The selected clones were grown in liquid cultures and isolated plasmids run on agarose gel approved the isolation of plasmids in substantial amount from positive clones having *SUMO1* fragment (Fig. 2F).

Sub-cloning SlSUMO1 full-length CDS in pCAMBIA1301

The isolated plasmid prep when subjected to restriction digestion using *Bgl*II and *BstE*II restriction enzymes released a correct size *SUMO1* fragment after gel electrophoresis (Fig. 3B). However, the plasmid miniprep chosen to be used for this restriction digestion was first confirmed by PCR for the presence of correct size SUMO1 fragment using gene specific primers (Fig. 3A).

The release SUMO1 fragment as in Fig 3A was extracted from the gel, purified and sub-cloned in a cut/*GUS* released pCAMBIA1301 vector which had a native *GUS* gene between *Bgl*II and *BstE*II restriction sites. Out of several colonies obtained after transformation of *GUS* removed pCAMBIA1301 and *SUMO1* fragment ligation mixture, two were selected for confirmation by PCR using *SUMO1* gene specific primer pair and showed a correct size 300 bp band on agarose gel (Fig. 3C). After this confirmation, the two previously confirmed transformed *E. coli* liquid cultures were subjected to plasmid isolation via miniprep and confirmed by agarose gel electrophoresis showing ample amount of plasmid obtained (Fig. 3D). The resulting construct within the pCAMBIA vector was named pCAMBIA::*SUMO1* having an upstream 35*S* promoter and a NOS terminator at the right border (Fig. 4A). The plasmid obtained after bacterial liquid culture miniprep was confirmed via PCR for the presence of correct size band of 300 bp for *SUMO1* (Fig. 5A) and later by restriction enzyme digestion using *Bgl*II and *BstE*II restriction enzymes releasing a correct size 300 bp fragment (Fig. 5B).

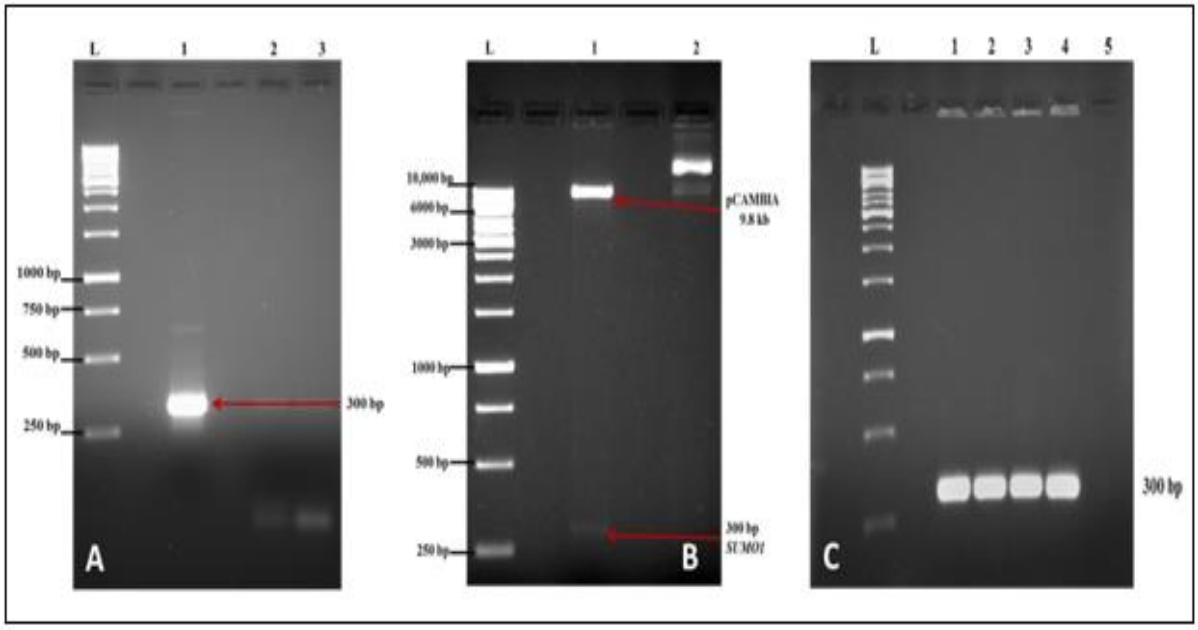

Fig. 5. L represents 1 kb DNA ladder(A)PCR based confirmation of plasmid miniprep for *SUMO1* full-length CDS in pCAMBIA using *SlSUMO1* gene specific primers, (B) double digestion of pCAMBIA: *SUMO1* plasmid with a 300 bp*SUMO1* fragment released, and (C) colony PCR confirmation of pCAMBIA: *SUMO1* in *Agrobacterium* strain LBA4404.

Transformation of pCAMBIA: SUMO1 in Agrobacterium competent cells

The transformed *Agrobacterium* LBA4404 colonies were obtained after electroporation on the selection media (Fig. 4B). Four *Agrobacterium* colonies were confirmed for the presence of *SUMO1* gene fragment within the restriction sites of digested pCAMBIA1301 vector by PCR using *SUMO1* gene specific primers and showed clear bright bands of 300 bp each confirming the presence of SUMO1 gene within the newly engineered gene cassette (Fig. 5C).

Discussion

Obtaining high quality and intact RNA and precise genetic engineering procedures were found as the major factors for successful cloning of *SUMO1* gene towards the development of a 35*S* based gene cassette. Gene promoters used to drive gene expression may either be constitutive or inducible. Different types of constitutive promoters have been used in transformation experiments for gene functional analysis by overexpression of a particular gene or, on the other hand, knocking down its expression by RNAi-based gene silencing.

However, 35*S*-based gene promoter driving the expression of a certain gene is the most widely used promoter both in monocots and dicots, and is equally effective in both types of plants (Odell *et al*., 1985; Skuires *et al*., 2007). Nevertheless, the level of gene expression varies in different tissues as well as in lines overexpressing a gene under its influence (Custers *et al*., 1999; Skuires, *et al*., 2007). Data presented in this report is a step forward in order to establish putative roles of *SUMO1* in tomato. Transgenic plants obtained from the present research are expected to be a valuable source of *SUMO1* gene functional studies in tomato regarding biotic/abiotic stress tolerance and developmental studies, especially, flowering and fruit set. The SUMO1 gene construct developed can also be used to transform other plants species for gene functional studies in order to uncover the novel functions of SUMOylation in the regulation of plant processes.

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