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CLONING OF *OS*RGLP9 GENE OBTAINED FROM *Oryza sativa* IN *Nicotiana tabaccum*

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ABSTRACT

This investigation was carried out to clone rice germin-like protein gene (OsRGLP9) under the transcriptional control of a constitutive promoter in tobacco. For the purpose OsRGLP9 gene, 995 bp was first successfully cloned in pENTRD/Topo® cloning vector and the target gene was sub-cloned in pH7WG2 vector in sense direction downstream of CaMV35S promoter. Presence of insert in the destination vector was confirmed through sequencing. After confirmation recombinant plasmid containing target gene was prepared on large scale by alkaline lysis method and purified using Eppendorf Perfectprep® Gel Cleanup kit. Transgenic plants were obtained through Agro-bacterium mediated transformation. The transgenic plants were confirmed by polymerase chain reaction (PCR).

Keywords: Cloning, germin like protein, *Os*RGLP9, tobacco.

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INTRODUCTION

Plants grow in open environment and hence are exposed to both biotic and abiotic stresses constantly. Plants perceive these stresses and respond accordingly in a variety of ways. The molecular mechanisms by which plants offer responses to perceived stress related signals are not well characterized and understood yet. Cloning and characterization of stress responsive genes that are involved in these processes is very important, if the target is to understand the molecular mechanisms involved in stress related problems. The work in the area of genetic engineering is advancing rapidly and a considerable progress has been made in cloning of stress responsive

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genes including Germin and germin like protein genes. Different cereals like barley, maize, rice and wheat contain diverse group of proteins named as "Germins" (Lane, 2002). Initially these proteins were known as distinct marker of germination in wheat embryos and named as "germin" (Thompson and Lane, 1980). However, Jaikaran *et al*. (1990) characterized germin as a cell wall associated homopentameric glycoprotein with oxlate xoidase (OxO) activity (Lane *et al*., 1993; Dunwell *et al*., 2000). Oxalate oxidase activity is responsible for the production of hydrogen peroxide (H_2O_2) which gives protection against bacterial and fungal pathogens attack (Lane, 2002; Quan *et al*., 2008). Reactive oxygen species (ROS) production is increased by several environmental stresses, such as exposition to high levels of light, drought, heavy metals, salt concentrations, temperature extremes, air pollution, UV radiation, herbicides and pathogen attacks (Gratão *et al.*, 2005). The enzymatic ROS scavenging mechanisms in plants include: superoxide dismutase (SOD), the water-water cycle (WWC), the ascorbate-glutathione cycle (AGC), the glutathione peroxidase cycle (GPXC), and catalase (CAT) (Apel and Hirt, 2004). Till now three different enzymatic activities have been associated with these proteins. Oxlate oxidase activity is found in true germins, none of GLPs is known to possess oxalate oxidase (OXO) activity (Kim *et al*., 2004); SOD activity is associated with some germins and GLPs (Berna and Bernier, 1997; Kim *et al*., 2004). Germin like protein isolated from tobacco nectary and a moss have previously been reported to possess superoxide dismutase activity. Similarly, superoxide dismutase activity was found to be linked with many other GLPs studied later (Zimmermann *et al*., 2006). Barley germin is reported to possess both OxO and SOD activities i e. this protein is reported to be bi-functional (Woo *et al*., 2000; Mullineaux *et al*., 2006). The *Arabidopsis thaliana* genome contains multiple genes that encode germin-like proteins (GLPs). It has been reported that inhibition of the expression of an *A. thaliana* GLP is detrimental to plant growth. Therefore, GLPs are novel herbicide targets. The plant hormone auxin regulates cell elongation, division, differentiation and morphogenesis. Synthetic auxin analogues are also important selective herbicides. The best candidate for an auxin receptor is auxin-binding protein 1 (ABP1), first studied in membrane fractions 30 years ago and subsequently isolated and purified (Hertel *et al*., 1972; Venis, 1977; Jones, 1994). The ABP1 gene belongs to the ancient and functionally diverse germin/seed storage 7S protin superfamily (Woo *et al*., 2002) and therefore, GLPs family can be used as a novel class of herbicide targets. Many molecular biology approaches like overexpression of genes, upstream promoter analysis and gene silencing can be used to determine gene functions and

tobacco is being used as a model plant, worldwide, for the genetic transformation of many plant genes to explore their specific functions. For the present study, rice GLP has been selected as it is a very important, not only because it is a food crop, but also because its genome has been sequenced completely. The sequencing of rice genome has enabled plant scientists to analyze and elucidate the functions of rice genes (Chrispeels and Sadava, 2003). Furthermore rice is also reported to express, different GLP genes (Membre and Bernier, 1998), however, their function/biochemical role is not elucidated yet.

MATERIALS AND METHODS

Plant Material

Rice cultivar Nipponbare and tobacco cultivar xanthi were used as experimental materials. Seeds of rice were obtained from National Agricultural Research Centre Islamabad Pakistan and that of toacco from Professor Dr. R.W. Thornburg, Prof of Biochemistry Iowa State University USA. Nipponbare was tested for DNA isolation for further genetic manipulations, while *Nicotiana tabacum* cultuvar Xanthi was used as model plant for transformation and overexpression of *Os*RGLP9 gene.

Surface Sterilization and Germination of Rice Seeds

Rice seeds (*Oryza sativa japonica* cultivar Nipponbare) were surface sterilized at room temperature. About 24-30 seeds were carefully dehusked taking care not to damage embryo. Dehusked seeds were washed with commercial liquid detergent in a 50 ml falcon tube thoroughly till the seeds were visible clean and free from dust. Then seeds were washed with tap water three times to remove detergent. The seeds were again washed 3 times with distilled water. Seeds were treated with 70 % sodium hypochlorite (Bleach) under shaking conditions for 30 minutes. After bleach treatment,seeds were washed 3 times with sterile distilled water, each time for ten minutes, dried and inoculated in test tubes containing half strength N6 medium. The tubes were kept in illuminated growth chamber under 25±1 ^{o}C and 16 hour photoperiod conditions.

Surface Sterilization and Germination of Tobacco Seeds

Tobacco (*Nicotiana tabacum* cultivar xanthi) seeds were surface sterilized at room temperature, with the following procedures. Required numbers of seeds were incubated in 70 % ethanol for 2 minutes in a falcon tube. After ethanol treatment seeds were incubated in 30% commercial bleach for 5 minutes while shaking at intervals. Then seeds were incubated in 20% commercial bleach for 5 minutes while shaking as above. Seeds were given five times washes with sterile distilled water. Sterilized seeds were dried on sterile filter

paper and inoculated on ½ strength Murashige and Skoog (MS) medium. Plates were placed in illuminated incubator at 25 ± 1 °C and 16 hour photoperiod conditions. The plants obtained from germinated seeds were maintained by nodal fragmentation for further use.

Isolation of Genomic DNA from Rice (*Oryza sativa japonica* cultivar Nipponbare) Plants

DNA was extracted from *Oryza sativa* cultivar Nipponbare, by CTAB (Cetyl Dimethyl Ethyl Ammonium Bromide) method with minor modifications in basic protocol of (Richards, 1997).

Amplification of *Os*RGLP9 Gene

Primer Design

Following Topo adapted primer pair was used for amplification of *Os*RGLP9 gene

Gateway Topo Primers:

Upper primer 5/-CACC-ATGGCCTCCTCTTCCTTATTTC-3/

Lower primer 5/-TCAGTAGTTGTTCTCCCAGAAC -3/

Topo adapted primers were designed on Chromosome 8 of *Oryza sativa* CV Nipponbarae to amplify 8p.OsRGLP9 gene using Invitrogen pENTR/D-TOPO cloning kit cat # K2400-20. Feasibility of primer annealing was checked using primer designing software such as NCBI Primer blast. Kozak sequence CACC was added as overhang to 5/ end of forward primer according to instructions given by manufacturer to allow directional cloning of target gene.

PCR with *Pfu* for Topo Cloning

As per instructions given in manual of Invitrogen pENTR/D-TOPO cloning kit cat # K2400-20, a 50 µl PCR reaction was performed using *Pfu* polymerase (Fermentas Lithuania, UAB) at annealing temperature of 58 °C to prepare PCR product for use in Topo cloning. PCR reaction contained 1X *Pfu* Buffer with $MgSO_4$, 0.04 mM dNTP mix, 0.5 *p*M each forward and reverse primers, 1 µg template DNA and 2.5 U of *Pfu* DNA polymerase. PCR was performed as initial denaturation at 95 °C for 3 min, then thirty five cycles comprising denaturation at 95 °C for 30 S, annealing at 58 °C for 30 S, and extension at 72 °C, with a final extension of 15 min.

Topo Cloning Reaction to Create Entry Clone

Whole 50 µl of *Pfu* amplified *Os*RGLP9 amplicon was gel purified using Eppendorf Perfectprep® Gel Cleanup kit Cat # 7955152051 according to manufacturer's instructions. Topo cloning reaction was performed using PENTR/D topo cloning kit (Cat # k 2400 20) according to manufacturer's instructions. Three ng of purified amplicon was subject to topo cloning reaction. The topo reaction was transformed into One Shot® Competent *E. coli* cells by heat shock method according to written protocol

Positive Clone Selection and Confirmation by PCR Analysis

Two colonies of putative clones from kanamycin selective plates were picked and subjected to overnight culture at 37 °C in Luria Brothe (LB) containing Kanamycin @ 50 µg/ ml. A 3 ml aliquot from overnight cultures were subject to plasmid isolation (miniprep) by alkaline lysis method as described by (Birnboim and Doly, 1979) to screen out positive clones. The isolated plasmids were analyzed as templates in PCR using gene specific primers to confirm presence of insert in the putative recombinant vectors. After PCR the reactions were resolved and analyzed on 1.0 % Agarose gel, along with super-coiled 1 Kb ladder (Fermentas Lithuania, UAB) as DNA marker, using gel documentation system.

LR Recombinatorial Cloning Reaction with Expression Vector pH7WG2 to Create Over Expression Construct

A 3 ml aliquot from overnight grown cultures of confirmed positive clone was subject to plasmid isolation using Genomed Jetquick plasmid miniprep spin kit Cat # 400050, according to manufacturer's instructions, to have purified plasmid for use in LR recombinatorial reaction for preparing overexpression construct. To prepare over expression construct of *Os*RGLP9 gene, LR cloning reaction was performed (Gateway® LR Clonase™ II Enzyme Mix from Invitrogen) according to manufacturer's instructions. pH7WG2 vector (Karimi *et al*., 2002) was used in this reaction to over express *Os*RGLP9 gene, under the control of 35S promoter, in tobacco plants.

Transformation of *E coli* DH5α with pH7WG2/*Os*RGLP9 Plasmid

Transformation of E coli strain DH5α with pH7WG2/*Os*RGLP9 Plasmid was performed by electroporation method using E. coli Porator (Life Technologies GBCO BRL). Two µl of LR reaction was used for transformation of electrocompetent *E. coli* cells. LR reaction and electrocompetent cells transformation mix was subjected to 2.5 kV/0.15 cm voltage. Then 1 ml of sterile LB was added to electroporated cells and cells were incubated at 37 °C under constant shaking conditions at 250 rpm for one hour. Hundred µl of transformation mix was placed on LB antibiotic containing plates (Hygromycin @ 50 µg /ml and Streptomycin @ 20 µg /ml). The plates were incubated at 37 °C overnight to allow growth and selection of positive clones.

Selection and analysis of putative Clones Containing pH7WG2/*Os*RGLP9 Over-expression Construct.

Two colonies of putative clones from hygromycin plus streptomycin selective plates were picked and subjected to overnight culture at 37 °C in LB broth with hygromycin @ 50 µg/ ml and streptomycin @ 20 µg/ ml. A 3 ml aliquot from overnight cultures were subjected to plasmid isolation by alkaline lysis method as described by (Birnboim and Doly 1979) to screen out positive clones. The isolated

plasmids were analyzed as templates in PCR using gene specific primers to confirm presence of pH7WG2 insert in the putative recombinant vectors. After PCR the reactions were resolved and analyzed on 1.0 % Agarose gel along with super-coiled 1 Kb ladder (Fermentas Lithuania, UAB) as DNA marker, using gel documentation system.

Large Scale preparation of pH7WG2/OsRGLP9 Plasmid

Cultures containing confirmed pH7WG2/OsRGLP9 plasmid were sub cultured further on larger scale and then subjected to plasmid isolation by alkaline lysis method as described by (Birnboim and Doly 1979). The plasmid preparation was purified using Eppendorf Perfectprep® Gel Cleanup kit Cat # 7955152051 with slight modification in basic protocol that plasmid preparation was directly mixed with membrane binding solution instead of prior running on agarose gel.

Agrobacterium Mediated Transformation of Tobacco by Leaf Disc Transformation Method.

Transformation of Agrobacterium with pH7WG2/*Os*RGLP9 Plasmid

LBA4404 strain of Agrobacterium was electroporated with pH7WG2/OsRGLP9 plasmid. Electrocompetent cells of LBA4404 were prepared by the same method as described previously *E coli* DH5 α with the only exception that agrobacterium culture was incubated at temperature of 28 °C for a period of 2 days to reach to the required growth OD600 of 0.4.

Electroporation

Two µl of pH7WG2/OsRGLP9 midi-prep were mixed with 50 µl electrocompetent cells of LBA4404 in a microfuge tube. The mix was then added to electroporation cuvette, whose electrodes were 0.15 cm apart. The electrocompetent cells and recombinant plasmid mix, contained between two electrodes, was subjected to an electric pulse of 2500 KV/cm for a period of 5 mints. After shock the elctroporated cells were brought to recovery phase by adding 1 ml of sterile LB broth. Subsequently transformation mix suspended in sterile LB broth was incubated at 28 °C for a period of 2 hours under shaking conditions at 250 RPM. After recovery phase 100 µl of transformation mix was spread on antibiotic selective plates containing streptomycin and hygromycin. The plates were incubated at 28 °C for 2 days. Colonies that appeared on antibiotic selection plates were considered as positive and were proceeded further for plant transformation.

Preparation of Agrobacterium Culture

A single colony of recombinant Agrobacterium growing on streptomycin / hygromycin selective plate was picked up with sterile tooth pick and transferred to 25 ml of sterile LB both containing

streptomycin and hygromycin @ 20 mg and 50 mg/ ml respectively. This was the subject to incubation at 28 ° under constant shaking conditions in dark, till its OD600 reached to 0.4.

Conditioning of Leaf Discs

For preconditioning about 35 sterile leaf discs per plate, as described in materials and methods, were placed upside down on MS0 medium. The plates were sealed with parafilm and incubated for a period of two days at 25±1 °C under 16/8 hours photoperiod conditions.

Co-Cultivation

Agrobacterium culture equipped with pH7WG2/OsRGLP9 plasmid was poured into a sterile beaker, while maintaining sterile conditions. Preconditioned tobacco leaf disc as prepared earlier were then infected with Agrobacterium, by dipping them into culture for 15 minutes. After infection the discs were removed from culture, dried on sterile filter paper, rinsed with sterile distilled water, were ultimately transferred to fresh hygromycin MS selection medium

Selection of Transgenic Plants

Transformants were chosen on the basis of Hgromycine resistance and these transgenic plants were screened for the presence of *Os*RGLP9 gene.

Confirmation of Transgenic Nature of Plants

Genomic DNA from both control and transgenic tobacco was isolated by CTAB method. PCR using gene specific primers was performed by using DNA as template from control and transgenic plants.

Regeneration of Transformed Plants

Plasmid pH7WG2/OsRGLP9 bears streptomycin/Hygromycin resistance gene as a selectable marker for selection of transformed plants. After two weeks transformed leaf tissues were transferred to fresh hygromycin MS selection media for two weeks and this process was repeated after every two weeks.

Root Formation inTransgenic Plants

The transgenic shoots were allowed to grow in jars till they attained 1-2 node stage. The transgenic shoots (1-2 nodes high) were transferred to fresh jars containing rooting medium with reduced antibiotic concentration. After rooting transgenic plants were transferred to pots containing soil with poly ethylene bags. After one week, poly ethylene bags completely removed from plants when they got acclimatized to open atmosphere.

Control Plants

To get control plants, young leaves of wild type tobacco plants cv Xanthi were regenerated on MS regeneration medium without selective antibiotics. After two weeks the plants turned brown and died

after a month.

RESULTS AND DISCUSSION

Isolation and Cloning of *Os*RGLP9 Gene

Full length *Os*RGLP9 gene (995 bp long) was isolated by PCR amplification, on chromosome 8 sequence (Accession no NC_008401 REGION: 7993408.7994721) using gene specific Topo adapted primers, from genomic DNA template. Fragment of about 1 Kb size was produced when separated by agarose gel (Fig-1) suggesting the specificity of primers to *Os*RLP9 only. The resulting fragment cloned PENTRD/Topo vector as host.

Transformation of *E. coli* with pENTRD/Topo *Os*RGLP9 Vector

One shot top10 chemically competent cells from Invitrogen were transformed with pENTRD/Topo/*Os*RGLP9 recombinant vector by heat shock method. Two of the transformed, kanamycin resistant, colonies were randomly selected and plasmid was isolated. The size of the product increased from 2.58 Kb (actual size of ENTRD/Topo) to 3.575 Kb after insertion of *Os*RGLP9 gene (Fig-2.).

Confirmation of *Os*RGLP9 In pENTRD/Topo by PCR

The insertion of *Os*RGLP9 gene in pENTRD/topo vector was confirmed by PCR using gene specific primers. Plasmid preparations as described in previous section (Fig-2.) were used as templates for PCR to analyze putative clones. PCR amplified *Os*RGLP9 (Fig-3.) from plasmid preparations and confirmed the success of topo cloning reaction.

Preparation of *Os*RGLP9 Over-expression Construct

Expression vector pH7WG2 and pENTRD/Topo/ *Os*RGLP9 entry clone were combined in a LR recombinatorial reaction to prepare pH7WG2 /*Os*RGLP9 overexpression plasmid. *E. coli* cells were transformed with pH7WG2/*Os*RGLP9 plasmid by electroporation. Two of the streptomycin and hygromycin resistant colonies were subjected to overnight culture at 37^{o}C. Plasmids were isolated from overnight cultures by alkaline lysis method and finally analyzed by PCR to confirm presence of *Os*RGLP9 gene in the destination expression vector. PCR amplified *Os*RGLP9 gene only from colony 1 of the two randomly selected colonies as analyzed by 1% agarose gel electrophoresis (Figure-4.).

Genetic Transformation of Tobacco By Agrobacterium Method

Once confirmed, culture from colony 1 was further processed for large scale isolation of pH7WG2/*Os*RGLP9 plasmid to be used for plant transformation. Tobacco cv. Xanthi was used as model plant for transformation of *Os*RGLP9 gene by agrobacterium mediated transformation method as described in material and methods section. After transformation the tissues were placed back onto regeneration

medium for two days and then MS selection media for two weeks for regeneration of transformants

Root Formation in Transgenic Plants

At two nodes stage, regenerated shoots were transferred to rooting media. Root formation started after 5-6 days of the shoot on rooting media and plants were then transferred to soil when large number of roots was established.

Wild Type Control Plants

Regeneration of control plants started 8-10 days after growth on regenerating media resulting in small shoot formation. These small regenerated plants were shifted to fresh media and finally transferred to rooting media.

Confirmation of Transgenic Nature of Plants

Transformed plants were screened both through hygromycin media and PCR. Out of total 12 transformed leaves, 8 regenerated on selection media revealing 67% transformation efficiency.

Screening of Transformed Plants

To confirm transgenic nature of hygromycin resistant plants amplification of *Os*RGLP9 was carried out through PCR with gene specific primers showing one kb fragment of *Os*RGLP9 gene obtained from transformed plants while DNA from control plant did not amplified.

The present research investigates cloning of full length *Os*RGLP9 gene of about 995 bp long located on chromosome 8 sequence (Accession no NC_008401 REGION: 7993408.7994721) using gene specific Topo adapted primers, from genomic DNA template. Isolation of DNA fragment of about 1000 base pair suggested the specificity of the tested primers primers to *Os*RLP9 only. The isolated DNA trasnfered to competent cells by heat shock method were selected carring kanamycin resistant genes as selectable markers. The isolated plasmid was cloned into ENTRD/Topo which increased the size of the plasmid inot 3.575 Kb. Analysis of the plasmid by gene specific primers confirmed cloning of the subject gene into ENTRD/Topo vector. These results suggest good preparation of the plasmid and confirmed that the topo cloning reaction was working properly. After confirmation **e**xpression vector pH7WG2 and pENTRD/Topo/ *Os*RGLP9 entry clone were mixed in a LR recombinatorial reaction for the construction of pH7WG2 /*Os*RGLP9 over-expression plasmid which was then transformed to *E. coli* cells by electroporation. The streptomycin and hygromycin resistant colonies confirmed the presence of the expression vector. After confirmation of the colony for the presence of expression vector containing *Os*RGLP gene, tobacco cv. Xanthi was transformed by agrobacterium. The transformed tissues were placed back onto regeneration medium for

two days and then MS selection media for two weeks for regeneration of transformants. After shoot regeneration, tissues were transferred to rooting media. Transformed plants were screened both through hygromycin media and PCR. The data indicated agrobacterium transformation of the tested vector revealed 67% efficiency. Transgenic plants were confirmed by hygromycin resistance and amplification of *Os*RGLP9 from genomic DNA by PCR. From these studies it can be concluded that tobacco can be rountinely cloned with different kind of genes with more efficiency. Moreover, the tested vectors can be used for accurate insertion of gene of interest.

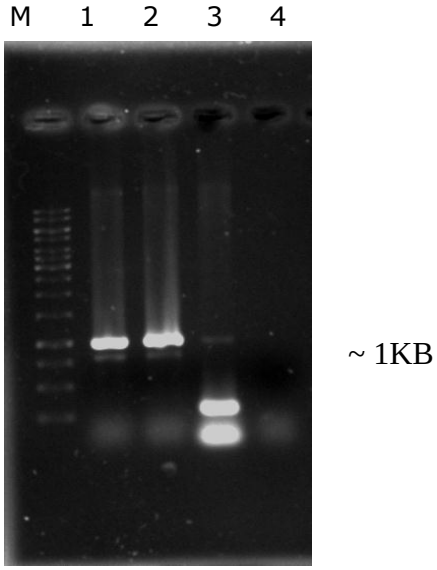

Figure-1. PCR amplification of *Os*RGLP9
Lanes 1 and 2 contain *Os*RGLP9 amplified products. Lane 3 is positive control with Ubiquitin Primers and lane 4 is control.
M is 1 Kb marker (Fermentas Lithuania UAB).

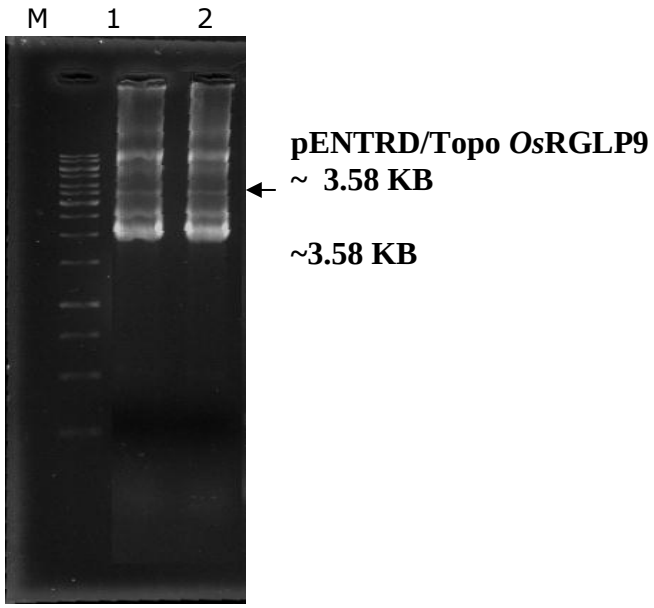

Figure- 2. Agarose gel electrophoresis of pENTRD/Topo /*Os*RGLP9 vector. Lane 1 and 2 contain pENTRD/Topo vector (2.58 Kb) with 8p. OsRGLP9 insert (~ 1 Kb) M is 1 Kb marker (Fermentas Lithuania UAB).

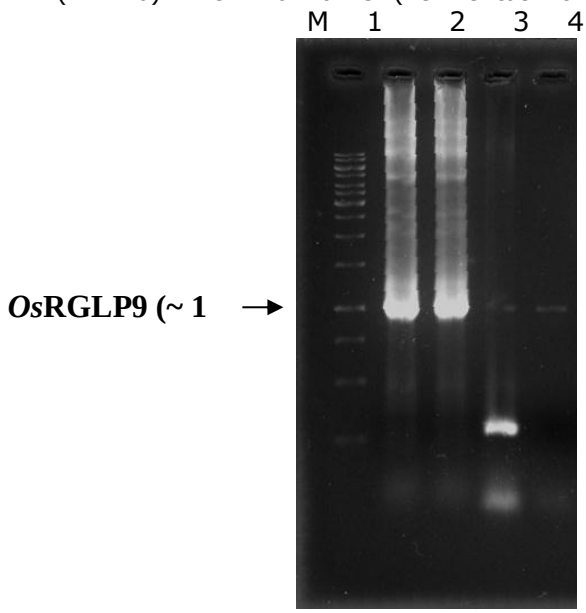

Figure- 3. PCR confirmation of *Os*RGLP9 in pENTRD/Topo vector Lane 1 and 2 contain amplified *Os*RGLP9 products. Lane 3 is positive control with Ubiquitin Primers and lane 4 is negative control and M is 1 Kb marker (Fermentas Lithuania UAB).

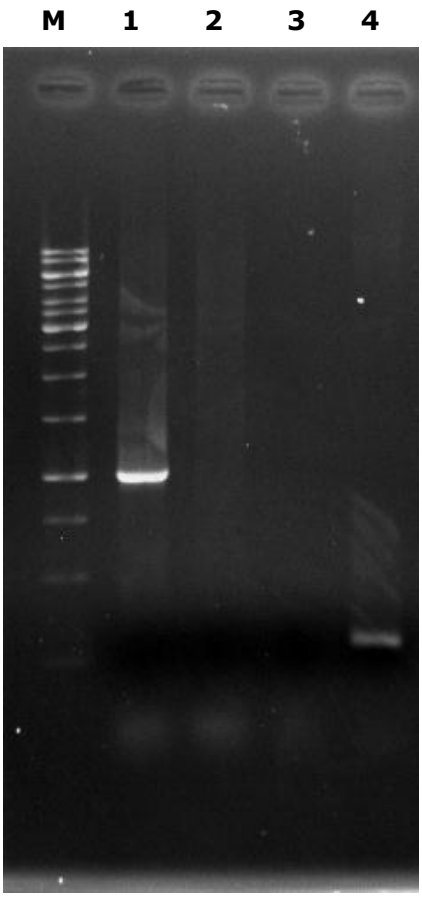

Figure-4. Clone analysis of the putative destination clone
Lane 1 contains amplified *Os*RGLP9 gene in destination vector.
Lane 2 shows no amplification from selected colony 2.
Lane 3 is a negative control.
Lane 4 is positive control with Ubiquitin primers.
M is 1 KB ladder (Fermentas Lithuania UAB).

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