ISOLATION AND CLONING OF *OsGSL8* GENE AND ITS OVEREXPRESSION IN *Arabidopsis thaliana*

to be submitted as Major Project II in partial fulfilment of the requirement for the degree of

MASTER OF TECHNOLOGY
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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled "**Isolation and cloning of *OsGSL8* gene and its overexpression in *Arabidopsis thaliana"*** submitted by **Sanjay S (DTU/14/MTECH/095)** in the partial fulfilment of the requirements for the reward of the degree of Bachelor of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by him under the supervision of Dr Ananda K Sarkar in the National Plant Genome Research Institute and the guidance of Dr. Navneeta Bharadvaja, Department of Biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honoring of any other degree.

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DECLARATION

This is to certify that the thesis of Major Project II entitled ***"*Isolation and cloning of *OsGSL8* gene and its overexpression in *Arabidopsis thaliana*"** in the partial fulfilment of the requirements for the reward of the degree of Mater of Technology, Delhi Technological University (Formerly Delhi college of Engineering, University of Delhi), is an authentic record of the my own work carried out under the guidance of project supervisor ***Dr. Ananda K Sarkar***, Staff Scientist, National Institute of Plant Genome Research, Delhi - 67. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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ABSTRACT

Rice is the most widely consumed cereal grain in the world. It is responsible for one-fifth of the caloric intake of humans worldwide. Hormones play an important role in plant growth and development. Gibberellic acid (GA) is an important plant hormone which regulates various aspects of plant biology, such as stem elongation, seed germination, plant stature, leaf expansion, and control of apical dominance. The *GAST* (Gibberellic Acid Stimulated Transcript) gene family is a family of genes whose expression is induced by various cellular processes regulated by GA. Genes of *GAST* family regulate plant developmental processes such as cell elongation, cell division, seed germination, root and flower development and also involved in biotic and abiotic stress response. In rice, initially, three genes *OsGSR1*, *OsGASR1,* and *OsGASR2* were identified. However, currently 9 genes have been defined as part of the GAST gene family in rice. All genes of this family encode proteins of 80-152 amino acids, each characterized by a conserved C-terminal domain of 59-64 amino acids. Within this domain, a residue of 12 cysteine residues is perfectly conserved. Some members of the rice-*GAST*-like family have not yet been characterized. In this study, characterization of a member of the *GAST*-like gene family, *OsGSL8* has been attempted. An overexpression construct of this gene was prepared and cloned into pCAMBIA1301 binary vector, which was transformed into *Arabidopsis thaliana* plant via *Agrobacterium*, to create an overexpression line of *OsGSL8* in *Arabidopsis*. In future, overexpression line of *OsGSL8* could be used for the functional characterization of this gene. A reporter line of *proOsGSL8* was also prepared, for observing tissue-specific expression. The T_1 plants of reporter line showed strong GUS expression in leaves. Further phenotypic analysis of these transgenic line and analysis of its homolog mutant in *Arabidopsis* is necessary to uncover its conserved or distinct role in plant development and stress response.

INTRODUCTION

Rice (*Oryza sativa*) is one of the major cereal food crops of the world population. It belongs to the Poaceae family and is an important carbohydrate source. Besides this, it provides fibre and contains minerals such as potassium, magnesium and iron. The germ layer of rice contain aleuronic layer which is rich source of contains starch and vitamins. After wheat and maize, rice serves as a staple food to a large population. Rice provides around 1528 KJ of energy for per 100g. In 1985, International Scientific community declared rice as model plant for cereals. In Asia, rice serve as a staple food for more than half of the population and provide more than 70% of caloric value (Ammar et al., 2007). In addition, among monocotyledonous plants, rice serves as a model plant for genome and proteome analysis. The compact genome size is of around 430Mbp. From germination to maturity the life cycle of rice plant takes around 110-150 days, which depends on the environment condition. The development of rice plant can be divided into three stages of development:

- Vegetative (Germination to panicle initiation)
- Reproductive (panicle initiation to heading)
- Ripening or Maturation (Heading to maturity)

Rice can grow under different environmental conditions but is most commonly cultivated in irrigated lands and rainfall conditions, 57% rice is grown in irrigated land, 25% in rainfall region, 10% in upland, 6% in deep water and 2% in wet land conditions. The crop yield is affected by various biotic and abiotic stresses which affect its productivity rate (Ren et al. 2010). For better crop yield and to increase the nutritive value, it is necessary to overcome these different stresses.

ROLE OF HORMONES IN PLANT DEVELOPMENT:

Plant hormones play a crucial role in plant development. Auxin, a phytohormone promotes the plant growth by inducing shoot and root branching, changes in growth direction and vascular differentiation (Laskowski et al., 1995, Leyser et al., 2001). Auxin is transported to plant parts either by auxin influx or efflux carrier proteins (Muday and De long 2001). It is synthesized in the growing tip and transported to other parts such as stem and root (Crozier et al., 2000). AUX, LAX1 and PIN-FORMED are examples of auxin influx and efflux carrier protein. In rice silencing of this PIN gene line showed reduced adventitious root growth (Min Xu et al., 2005).

Cytokinin is a signalling hormone molecule which promotes the plant development. In rice endosperm the cell division and cell elongation activity was found to be regulated by the cytokinin hormone (Yang et al, 2002). Cytokinins synthesized in plant root tips which interact with auxin and this interaction leads to the regulation of lateral root development in rice (Wightman et al, 1980; Hinchee and Rost, 1986).

Abscisic acid (ABA) is another phytohormone also known as stress hormone, as its level gets highly accumulated during stress condition (Christmann et al., 2006; Yamaguchi Shinozaki and Shinozaki, 2006; and Qin et al., 2006). For example, ABA stimulation was high in guard cells during osmotic stress condition (Yamaguchi- Shinozaki and Shinozaki, 2006). Root growth is highly affected during stress condition such as water deficient and high salinity condition and these stresses leads to high level of ABA in root (Zhang and Davies., 1987; Jia et al., 2002). Under water deficient conditions, ABA induce the root development (Sharp and Lenoble, 2002;Sharp et al., 2004).

Gibberellic acid (GA) is a plant hormone which plays an important role in both cellular and developmental process (Li Wang et al 2009). It promote the plant growth by inducing the stem elongation, seed germination, plant stature, leaf expansion, flower, fruit and reproductive organ development (Inada et al., 2000;Steber and McCourt, 2001; Yang et al., 2003; Mussig, 2005). Bioactive GA promotes stem elongation, leaf expansion, and root growth after germination (Davies, 1995; Yaxley et al., 2001). For example, in *Arabidopsis* GA is essential for development of stamens and petals during flower development (Koornneef and van der veen, 1980). The synthesis of GA proceeds through three stages and regulated by three dioxygenase enzymes including GA 20, GA 3-, and GA 2-oxidases. Among these three enzymes GA 2-oxidase enzymes is the major GA deactivation enzyme and the rest two enzymes catalyse the final step in GA biosynthesis (Yamaguchi,2008).

Apart from hormones root development is also regulated by coordinated expression of various genes both spatially and temporarily. Recent findings show that in *A. thaliana*, GA induced the endodermal cell expansion in the elongation zone of primary root (Ubeda- Tomas et al., 2008). In rice, GA deficiency increased the adventitious root formation (Lo et al., 2008). Gibberellic acid induces the expression of GAST family gene and regulate the plant development.

GAST family genes and their role in plant development:

Many GAST family genes were identified in numerous plant species and it was found that, these genes expression was up regulated by GA at different stages of plant development.

Gibberellic Acid Stimulated Transcript (*GAST*) genes have been identified in both monocot and dicot plants as gibberellins responsive gene. *GAST* genes regulate the plant developmental process in different stages of development including cell elongation, cell division, seed germination, root and flower development. Certain *GAST* genes were responsible for plant resistances to abiotic and biotic stresses. Till now, many *GAST* family genes have been identified in different plants, for example *GEG* in *Gerbera hybrida* (Kotilainen *et al.*, 2002), *GASA* genes in *Arabidopsis thaliana* (Herzog *et al.*, 1995, Roxrud *et al.*, 2007), *GIP* genes in Petunia hybrid (Ben-Nissan and Weiss, 1996), *Snakin* genes in Potato (Sergura *et al.*,1999; Berrocal-Lobo *et al.*, 2002); *GAST1* gene in tomato (Shi *et al.*, 1992).

Role of GAST genes in root development:

A study was performed by (Zimmermann et al., 2010) to identified the role of *GAST* genes in lateral root development in maize (*Zea mays)*. They identified 10 *GAST* genes from maize and six *GAST* genes form rice. They found that among ten genes *ZmGSL2, ZmGSL4, ZmGSL6, ZmGSL9* expressed during lateral root development. In maize, *ZmGSL1* gene exert alternate splicing into *ZmGSL1a* and *ZmGSL1b*. *Zm*GSL1a expressed strongly in lateral root primordial and *ZmGSL1b* was expressed in cortical cells surrounding the lateral root, which suggested their involvement in lateral root development.

GAST genes exhibit pathogen resistant activity

Some plants have the ability to resist the pathogen infection. Antimicrobial peptides, pathogen related proteins, certain organic compounds such as phytoalexins and phytoanticipins are important components in plant defense mechanism. Several families of antimicrobial peptides were reported in plants (Garcia olmedo et al., 1992, 1995). Over expression of these compounds or a specific gene make the plant resistant to the pathogen infection (Osbourn 1996, 1999). Potato Snakin (*St*SN2) gene was identified as pathogen resistant gene and found that it was expressed in tubers, stem, flowers, shoot apex and in

leaves (Berrocal-Lobo *et al*., 2002). *St*SN2 share 38% sequence similarity with previously identified *St*SN1 gene. *St*SN1 was located on chromosome 4 and *St*SN2 is on chromosome 1. Their amino acid sequences show similarity with the previously identified GAST 1 gene in tomato and with GASA gene in *Arabidopsis (*Vanesa Nahirñak *et al., 2012).* Both proteins share several characteristic features of antimicrobial peptides. *StSN2* accumulate in tubers along with *StSN1* and other plant defensin such as *St*PTH1, the complementary and synergistic activity between these two peptides is responsible for the broad spectrum activity against pathogen (Berrocal Lobo *et al*., 2002).

GAST genes in fruit development:

Strawberry is a non-climacteric fruit, during ripening cell wall relaxation occurs whereas in other plants ripening process is mediated by both enzymatic and non-enzymatic reactions and other Reactive Oxygen Species (ROS) are also involved (Fry 1998; Rodriguez *et al*., 2002; Foreman *et al*., 2003,Liszkay et al., 2004). A study on strawberry *Fa*GAST1 gene demonstrated that it is highly expressed in roots and in some vegetative parts including leaves and flowers (de la Fuente et al., 2006). Commonly all *GAST* family genes were up regulated by GA. A recent study on strawberry *FaGAST2* gene revealed that it is not regulated by GA instead the expression was regulated by ethephon, an intracellular generator of ethylene (MoyanoCanete et al., 2012). They reported that *Fa*GAST2 gene highly expressed throughout fruit receptacle development and ripening, and the expression was also increased during oxidative stress condition. *FaGAST2* gene share 31% amino acid sequence similarity and 15.7% nucleotide sequence similarity with *FaGAST1* gene. Overexpression of *FaGAST2* leads to delayed plant growth with reduction in cell size due to smaller parenchyma cells. Silencing of *FaGAST2* gene leads to increase the expression of *FaGAST1* and it was also found that there was no alteration in fruit cell size in *FaGAST2* RNAi lines. The results of study also demonstrated that, both the genes synergistically act to determine the cell size for fruit development.

GAST genes responses to stress conditions:

Many study demonstrated that GAST gene have role in plant developmental processes. Recent researches on GAST gene found that it has the ability to confer tolerance to stress, which usually affect the plant growth. The expression of GAST gene also induced under stress condition (Segura et al., 1999). In *Arabidopsis thaliana* which is a model dicotyledonous plant, 14 GAST genes were identified *GASA1-GASA14 (*Roxrud et al., 2007).

Recently the functional characterization of *GASA14* was studied and it was observed that that it has role in leaf expansion and in abiotic stress. It has the ability to modulate the ROS and provide the abiotic stress. *GASA14* genes have these properties because it contains both GASA domain and PRP (proline rich protein) domain coding sequence (Sun *et al*., 2013). It was found that it show expression in elongation zone of root. It shows 73.3% amino acid identity with PRGL in *Gerbera hybrida* (Peng et al, 2010). *GASA4* gene expression in *Arabidopsis* has been shown to cause increased heat tolerance (Chang-Beom Ko, 2007).

Moreover recently, GAST genes were also identified in cotton (*Gossypium hirsutum)* and found to have role in fiber development. A summary of identified GAST-like genes form various plant species is given in **table-1**.

Table 1: List of GAST-like genes and their roles in plant developmental processes.

S:No	SPECIES NAME	GAST GENE	FUNCTION	REFERENCE
1	*Lycopersicum esculentum*	*GAST 1*	Shoot development	Li Fang Shi *et al.,* (1992)
2	*Arabidopsis thaliana*	*GASA 4*	Flowering and seed development	Roxrud *et al*.,(2007)
3	*A. thaliana*	*GASA 5*	Control flowering time & stem growth	Auberts *et al*., (1998)
4	*A. thaliana*	*GASA 14*	Leaf expansion and abiotic stress tolerant	Sun *et al.,* (2013)
5	*Petunia hybrid*	*GIP*	Cell elongation and cell division	Ben-Nissan *et al*., (2004).
6	*Fragaria spp*	*FaGAST 1 and 2*	Control of fruit size	MoyanoCanete *et al.*,(2012)
7	*Gossypium hirsutum*	*GhGASL*	Fiber development	Zhang et al.,(2012)
8	*Zea mays*	*ZmGASL*	Early root development	Zimmermann *et al.*, (2010)
9	*Oryza sativa*	*OsGASR1 &OsGASR2*	Panicle differentiation	(Furukawa *et al.*, (2006)
10	*Oryza sativa*	*OsGSR1*	Hormone crosstalk	Wang *et al*.,(2009)
11	*Gerbera hybrid*	*GEG*	Corolla and Carpel development	Mika Kotilainen *et al.*, (1999)
12	*Glycine soja*	*GsGASA1*	Cold-induced root growth	Kun-Lun Li *et al*., (2011)

			inhibition	
13	*Fragaria* x *ananassa*	*FaGAST2*	Fruit development and ripening	Enriqueta Moyano-Cañete *et al*., (2013)

GAST gene family in Rice:

Recently in rice, three GAST genes *i.e.OsGASR1*, *OsGASR*2 and *OsGSR1* were identified and characterized. Expression of *OsGASR1* and *OsGASR2* was identified in panicle region (Furukawa et al, 2006). It was reported that *Os*GASR1 protein show 38.7% sequence identity with *Os*GASR2 protein and phylogenetic analysis showed that the *Os*GASR1 and OsGASR2 were related to *St*SN1 and *At*GASA2 and *At*GASA3, respectively. *OsGASR1* and *OsGASR2* were found to be expressed in shoot apical meristems (SAM) and root apical meristems (RAM). Though *OsGASR1* was moderately expressed in root cap and in vascular tissues in roots but *OsGASR2*was expressed only in root cap and its expression was not found in vascular tissues. Both *OsGASR1* and *OsGASR2* genes showed high level of expression in meristems and in panicle development which was related to cell proliferation. Another rice GAST gene *Os*GSR1 was found to be involved in hormone crosstalk with brassinosteroids hormone and this interaction mediated the plant development (Li wang et al, 2009). Brassinosteroid (BR) is a plant steroidal hormone, which down regulates the expression of *Os*GSR1 gene whereas expression of *Os*GSR1 was up regulated by GA. *Os*GSR1 is required for normal gibberellic acid response, including for α-amylase activity and for internode elongation. The transgenic RNAi lines of *OsGSR1* shows altered phenotypes such as short seminal roots and dwarfism which is similar to brassinosteroids deficient plant. The dwarf phenotype of the plant was recovered by exogenous apply of 24-eBL steroid hormone. The study also revealed that *OsGSR1* directly regulate BR synthesis by interacting with BR biosynthetic enzyme and to achieve optimum plant growth development the two hormone pathways (GA and BR) may interact with each other (Li wang et al., 2009). From the previous studies it is evident that *GAST* family genes exhibit many functions which are essential for plant development. The *GAST*-like gene family has been identified in monocot plants like maize and rice. The genes of this family characteristically encode small polypeptides that contain a C-terminal domain having 12 perfectly conserved *Cys* residues. The *GAST* genes *OsGASR1*, *OsGASR2* and *OsGSR1* genes have been characterized. However, many genes such as *OsGSL1*, *OsGSL5*, *OsGSL6*, *OsGSL7*, *OsGSL8*, *OsGSL9*, *OsGSL12* are yet to be characterised. Among these *OsGSL8* gene has not yet been characterized and may be responsible for important developmental or stress-related functions.

As a part of the Rice *GAST*-like gene family, its functions need to be understood for fully understanding the mechanism of plant development. Hence, the present study aims at the functional characterization of the *OsGSL8* gene and exploring its role in plant development.

AIMS OF THE STUDY

- To obtain full-length genomic, CDS and protein sequence of *OsGSL8* from Rice genome annotation database.
- To prepare overexpression and reporter constructs of *OsGSL8* in binary vector pCAMBIA1301.
- Generation of transgenic *OsGSL8* overexpression line of *Arabidopsis* in *Agrobacterium* mediated plant transformation.
- To perform phylogenetic analysis of *OsGSL8* gene to identify related orthologous genes from other plant species.

MATERIALS AND METHODS

Media preparation:

Half Strength Murashige and Skoog (MS):

S.No	Chemical Name	Company	gm/l
1	Murashige and Skoog Media (MS)	Hi Media	2.2
2.	Sucrose	Fischer Scientific	10
3.	Agar Extra Pure	Hi Media	0.8

Luria Bertani Broth:

S.No	Chemical Name	Company	gm/l
1	Yeast Extract	Hi Media	5
2	Tryptone	Hi Media	10
3	SodiumChloride	Hi Media	10

Luria Bertani Agar Media:

S.No	Chemical Name	Company	gm/l
1	Yeast Extract	Hi Media	5
2	Tryptone	Hi Media	10
3	SodiumChloride	Fischer Scientific	10
4	Bacteriological Agar	Hi Media	15

Isolation of Genomic DNA

Plant grown hydroponically for 2 weeks was used to isolate total genomic DNA. Around one gram of whole seedling was ground into fine powder using mortar and pestle in liquid nitrogen. The ground material was taken in 1.5 ml microcentrifuge tubes containing 200µl of miniprep buffer (1.4 M NaCl, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 2% β-Mercaptoethanol, 2% CTAB). The contents of the tube were mixed vigorously. The sample was then centrifuged at 13000 rpm for 15 min. The supernatant was taken in a fresh tube with equal volume of isopropanol. After incubation in ice for 20-30 min, the sample was again centrifuged at 13000 rpm for 15 min. The supernatant was discarded and the pellet was

washed with 70% ethanol, by centrifuging at 13000 rpm for 5 min. The supernatant was again removed and the sample was air-dried at RT. The sample was finally dissolved in 30µl of TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA, pH 8.0) at 4^{o}C.

Preparation of cDNA

Isolation of total RNA

For designing the primers, cDNA sequence of *OsGSL8* was used, which was obtained from Rice Genome Annotation Database. Primer was designed according to the sequence by the use of primer3 software (http://primer3.ut.ee/). Total RNA was isolated from different tissues and from roots of hormone treated plants by Trizol method. For total RNA isolation tissues were homogenized properly by using liquid nitrogen with the help of autoclaved RNase free mortar and pestle. After homogenization 1ml of Trizol reagent was added to grounded powder. The homogenized samples were incubated at room temperature for 5 minutes which allow the nucleoprotein complex to dissociate. To this 200 µl of chloroform was added and contents were transferred to RNAs free micro centrifuge tube. The contents were mixed properly by shaking the tube for 15 seconds and incubated for 2-3 minutes at room temperature, followed by centrifugation at 12,000xg for 15minutes at 4^{0}C. The aqueous phase transferred to a new micro centrifuge tube and to this 500µl of 100% isopropanol was added and incubated for 10minutes at room temperature. After incubation the samples were centrifuge at 12,000xg for 10 minutes at 4^{0}C. After centrifugation, supernatant was removed and pellet was washed with 70% ethanol by centrifuge at 7500xg for 5 minutes at 4^{o}C. After the spin, ethanol was removed, pellet was air dried and dissolved in RNase free water.

cDNA synthesis

After checking the quality and spectrophotometric quantification, total RNA (2-3 µg) was converted into cDNA using M-MLV Reverse Transcriptase (Invitrogen,USA). The RT reaction consisted of total RNA, 0.1µl of 10 mM dNTP mix, 2 µl of random hexamer primer, 4µl of 5X reaction buffer, 1µl Ribonuclease Inhibitor and 1µl of M-MLV Reverse Transcriptase enzyme in a final volume of 20 µl. Reaction was carried out at 25^{o}C (10 min) / 37^{o}C (1 h) followed by termination at 85^{o}C for 5 min.

Cloning for preparation of overexpression and reporter construct in binary vector

Overexpression construct

Full length nucleotide sequence of *OsGSL8* is found to be present on chromosome 6 (LOC_Os06g51320.1) in rice genome (Rice genome annotation database). For preparation of overexpression construct of *OsGSL8*, primers were designed for cloning full-length CDS in binary vector. For amplification of *OSGSL8* gene cDNA of IR 64 Indica rice was used as a template and 50µl of reaction contains, 1µl of cDNA, 5 µl of 10X reaction buffer, 1.0 µl of 10mM dNTP mix, 1µl of each primer and 1µl of Taq polymerase (Sigma, USA). Following Primer sequences were used:

***OsGSL8OE* Forward primer**- 5'- AGATCTAATGGCCTCCTCCGGCTCCA -3';

***OsGSL8OE* Reverse primer**- 5'- GGTGACCTCATGGGCACTTGAGCTT -3';

Reaction was carried out at 95^{o}C (5 min) / {95^{o}C (30 sec)/ 60^{o}C (60 sec)/ 72^{o}C (60 sec) x 35}/72^{o}C (10min) followed by storing at 4^{o}C for 5 min. The amplified product obtained after the PCR was ligated into pJET1.2 vector and cloning was confirmed by restriction digestion analysis and sequencing.

For cloning of *OsGSL8* in pCAMBIA1301 binary vector, the confirmed clone *OsGSL8*:pJET and binary vector pCAMBIA1301 were digested with *BglII* and *BstE*II restriction enzymes, to release the insert and linearization of vector, respectively. Restriction digested fragments were gel-eluted and ligated. Ligation mix was then transformed in *E.coli* competent cells. Cloning was confirmed by isolation of plasmid, followed by restriction and sequencing.

Reporter construct

For preparation of reporter construct of *OSGSL8*, primers were designed to amplify 1087 kb sequence from the upstream region of *OsGSL8* gene. The forward primer contained *Hind*III and reverse primer contained *Nco*I restriction sites.

Forward Primer: 5' AAGCTTACCAGACAAGGCATGTGAT3'

Reverse Primer: 5' CCATGGGGATGGATCTGATTCTGA3'

. The promoter region of *OsGSL8* was PCR amplified using genomic DNA of IR64 cultivar rice cultivar as template. The reaction was carried out at 95^{o}C (5 min) / {95^{o}C (30 sec)/ 60^{o}C (60 sec)/ 72^{o}C (60 sec) x 35}/72^{o}C (10min) followed by storing at 4^{0}C for 5 min. PCR product was ligated to pJET cloning vector. Ligation product was transformed to *E.coli* DH5α competence cells. Cloning was confirmed by restriction digestion and sequencing.

For the cloning of pro*OsGSL8* into the binary vector pCAMBIA1301 between *Hind*III and *Nco*I restriction sites, the confirmed clone pro*OsGSL8:*pJET and pCAMBIA1301 vector were both digested with above mentioned restriction enzymes, to release 1087 bp insert and linearization of vector, respectively. R. digested fragments were gel-eluted and ligated. Ligation mix was then transformed in *E. coli* competent cells. Cloning was confirmed by isolation of plasmid, followed by restriction digestion.

Transformation of *Arabidopsis thaliana*

Transformation in *Agrobacterium*

To carry out the genetic transformation in *Arabidopsis thaliana* plant, the over expression and promoter constructs were first mobilized into *Agrobacterium tumefaciens* competent strain GV350. *OsGSL8*OE:pCAMBIA1301 and pro*OsGSL8*:pCAMBIA1301 positive clones were transformed in *Agrobacterium* competent cells and selection was done on rifampicin and kanamycin containing LB agar plates. Transformation was confirmed by isolation of plasmid from *Agrobacterium,* followed by restriction.

Floral dip for transformation in *Arabidopsis* plants

For functional characterization of *OsGSL8* gene, the over expression and promoter construct were transformed into wild type *A .thaliana* plant using floral dip method (Clough and Bent. 1998).

Plant Handling

The seeds of *A. thaliana* were washed with 500 µl of wash buffer (70 % ethanol and 1 % TritonX-100) for 10 minutes followed by washing with sterile MQ-water. Then the seeds were placed on ½ MS plates and kept at 4^{0}C for two days. After two days the plates were transferred into growth chambers. After 6-8 days of germination, the seedlings were transferred to soil and placed under 16/8 h (day and night cycle) at 25^{0}C.

For plant transformation, an overnight primary culture was raised in 5 ml of LB media by inoculating a single colony of *OsGSL8*OE:pCAMBIA1301 and pro*OsGSL8*:pCAMBIA1301. Secondary culture was grown for the respective clones, by inoculating 1 ml of primary culture in 250 ml of LB medium containing rifampicin and kanamycin. Culture was incubated for 16 h at 28^{o}C with constant shaking at 200 rpm. After incubation, *Agrobacterium* cells were pelted at 4000 rpm for 20 min at 4^{o}C. Cells were re-suspended in 500 ml of a solution containing 5% sucrose and .05% Silwet (surfactant). *Arabidopsis* plants were transformed by dipping the flowers and unopened buds in *Agrobacterium* suspension for 1 minute with constant shaking. After performing floral-dip plants were kept in dark for 24 hours. Transformed plants were kept in growth room (18/6 hr day/night cycle and 22^{0}C temperature) for maturation of seeds. T1 seeds were harvested and inoculated on ½ MS plates containing hygromycin for selection of transformants.

GUS Assay

The T_1 transformants of the reporter line selected from hygromycin plates were grown and then used for GUS assay. Small leaves from each transformant were isolated gently from the plant and taken in a 1.5ml MCT, containing the GUS assay solution (1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid in 50 mM Na_2HPO_4, pH 7.0 and 0.1% Triton X-100). The samples were incubated at 37^{o}C overnight for about 12 hours. After incubation, they were taken and checked for the blue colored GUS stain. The positive samples were taken for further growth and harvesting.

Phylogenetic analysis

Comparisons and phylogenetic analysis of protein sequences of the *OsGSL8* with the sequences available in the database were performed using 'MEGA 7.0' software which involves the implementation of bootstrap Neighbor-Joining (NJ) algorithm (Saitou N *et al*., 1987). for multiple alignment and building of phylogenetic tree.

General cloning procedure

Elution of DNA from gel

DNA fragments (band) of desired size were eluted out from the respective gel slices using the FavorPrep GEL/PCR purification Mini Kit (Favorgen, Taiwan). Three volumes of buffer

FADF buffer (supplied with the kit) was added to 1 volume of the gel (100 mg ~100 µl) in an MCT. The tube was kept at 60^{o}C for 10 min (or until the gel slice was completely dissolved) and mixed at regular intervals by gently inverting during the incubation. The mixture was then applied to the column placed in a collection tube and centrifuged at 13000 rpm for 1 min in a microcentrifuge. The flowthrough was discarded and 0.75 ml of wash buffer was added to the column for washing followed by centrifugation for 1 min. After discarding the flow-through, the sample was centrifuged for an additional 2 minutes to remove any traces of the wash buffer. The column was then placed in a new MCT, and 30µl of elution buffer (10 mM Tris-HCl, pH 8.5) was added to the center of the column and centrifuged for 1 minute for the complete elution of DNA.

Ligation

Ligation was carried out using vector and insert in a ratio of 1:3, in the presence of 1X ligation buffer and 1 U of T4 DNA ligase (Thermo Scientific, USA). All ligations were set up on ice and finally incubated at 22^{0}C for 20-22 h in a cooling water bath. The ligation reaction mixture was directly used for bacterial transformation.

Preparation of competent bacterial cells

E.coli host strain DH5α was made chemically competent for transformation. A single colony of the host was inoculated in 5 ml LB containing appropriate antibiotics. The starter culture was incubated at 37^{o}C for 200 rpm, overnight. The following morning, 200-300µl of the starter culture was sub-cultured in 100 ml of LB containing appropriate antibiotic. The culture was incubated at 37^{o}C and 200 rpm, till the OD_{600} reached up to 0.3-0.4. The bacterial culture was then transferred into a 30 ml polypropylene tube and pelleted by centrifuging at 4^{o}C at 4000 rpm for 10 min. Pellet was resuspended in 20 ml of ice-cold 15% glycerol and incubated on ice for 5minutes. After incubation, the cells were again pelleted by centrifugation at 1500 g for 5 minutes at 4^{0}C. Supernatant was discarded and pellet was resuspended in 20 ml of ice-cold buffer and spun again at 1500 g for 5 minutes at 4^{0}C.Finally the pellet was dissolved in 4ml chilled buffer, mixed well and stored it in -80^{0}C after aliquoting into MCTs.

Bacterial transformation

For bacterial transformation, ligation reaction mixture (2 to 10 µl) was added to an aliquot (200 µl) of frozen competent *E.coli* DH5α cells and incubated on ice for 30 min. The cells

were given heat shock at 42^{o}C for 90 sec followed by immediate chilling in ice-bed for 5 min. To the transformation mix, 1 ml of plain LB broth was added and components were incubated at 37^{o}C for 1 hour with constant shaking at 250 rpm. Then the suspension was centrifuged at 5000 rpm for 5 min to pellet down the bacterial culture. The volume of the media was reduced to 200 µl by centrifugation, mixed with the pelleted cells and plated on LB-agar plate containing appropriate antibiotic(s). Antibiotic concentrations used were 50 µg/ml ampicillin or 50 µg/ml kanamycin, depending upon the type of plasmid used for bacterial transformation. The plates were incubated at 37^{o}C overnight (15-16 h) in an incubator. The colonies were then screened for recombinant plasmids carrying cloned fragments.

Plasmid isolation by alkaline lysis method

Single well isolated white colonies were picked and inoculated in 4-5 ml of LB-broth containing appropriate antibiotics with the help of sterile pipette tips. The culture was grown at 37^{o}C for 16 h with constant shaking at 250 rpm in an incubator shaker. Bacterial cells were pelleted in a 2 ml microcentrifuge tube by centrifugation at 4^{o}C, 12000 rpm for 1 min. The supernatant was discarded and pellet was resuspended in 300 µl of alkaline lysis solution I (50mM glucose; 25mM Tris-HCl, pH 8.0and 10mM EDTA, pH 8.0), and mixed by vortexing. Then, 300 µl of freshly prepared solution II (0.2 N NaOH and 0.1% SDS) was added to the microcentrifuge tubes, contents were mixed gently by inverting 4-6 times and incubated at room temperature for not more than 5 min. To this, 300 µl of chilled solution III (100 ml of solution III contains 60.0 ml of 5 M potassium acetate and 11.5 ml glacial acetic acid) was added, mixed thoroughly and incubated on ice for 5 min. After the incubation is over, to the tube 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was gently inverted 2-3 times and then centrifuged at 13000 rpm for 10 min. The aqueous phase was transferred to a fresh micro centrifuge tube. The solution containing plasmid DNA was precipitated using 0.6 volume of isopropanol, mixed thoroughly and followed by centrifugation at 4^{o}C, 13000 rpm for 15 min. The pellet was washed with 70% ethanol by centrifugation at 13000 rpm for 5 min, then air-dried and resuspended in 30 µl of Milli-Q water containing RNase A (20 µg/ml) and incubate at 60^{0}C for 10 minutes. Plasmid DNA was checked on agarose gel containing 0.5 µg/ml ethidium bromide by electrophoresis for quality and quantity. Plasmid DNA was stored at 4^{0}C. Quantification of DNA was done at 260 nm by using spectrophotometer.

RESULTS AND DISCUSSION

For characterization, of the *OsGSL8* gene, the full-length genomic, CDS and protein sequence was obtained from the Rice genome annotation Data Base (http://rice.plantbiology.msu.edu/).

Cloning of *OsGSL8* cDNA into pJET vector

The full-length CDS obtained from Rice genome annotation DataBase was used for designing primers for preparation of overexpression construct. *OsGSL8* gene was amplified using cDNA of IR64 *indica* rice cultivar as a template by using designed primers. A gradient PCR was performed to identify the annealing temperature which was optimized at 60^{o}C. **Figure1.1** shows the gradient PCR performed at temperatures 56, 58, 60, 62 and 64^{o}C.

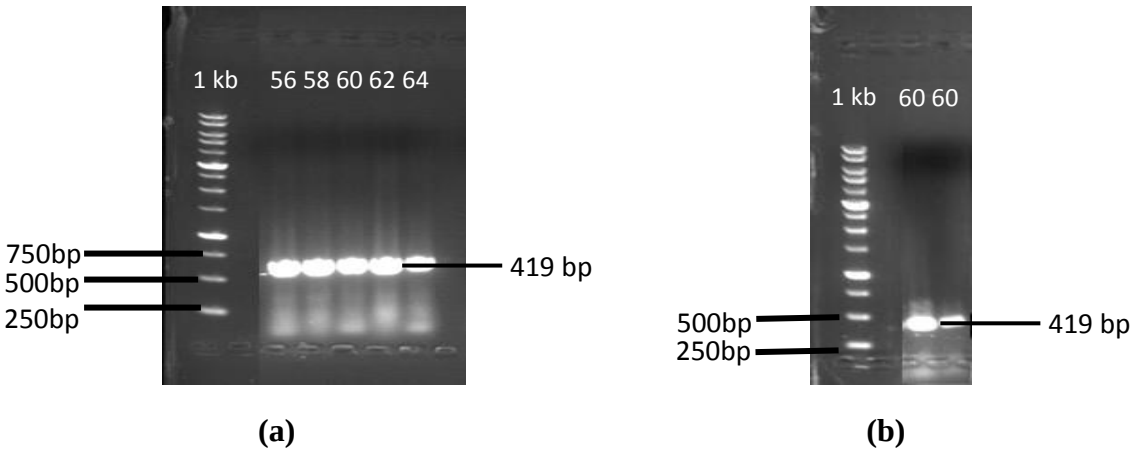

Figure 1.1) Gradient PCR Amplification of *OsGSL8*.

1.2) PCR amplification for gel elution.

The 419 bp PCR products were cloned into pJET vector. The *OsGSL8OE*:pJET construct was transformed into *E.coli* DH5α competent cells by heat shock method. The transformation was confirmed by performing colony PCR as shown in **Figure 2.1**.

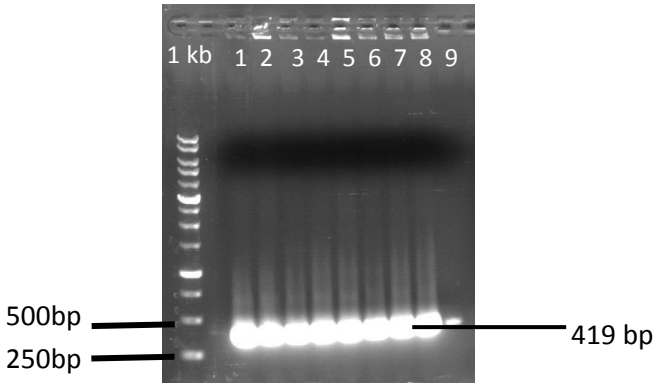

Figure 2.1: Colony PCR confirmation of *OsGSL8*OE:pJET construct in *E.coli* DH5α cells (lanes 1-8). The final lane contains PCR-amplified product

The p*OsGSL8*OE:pJET plasmid was isolated and the cloning was confirmed by restriction by *Xba*I and *Xho*I restriction enzymes which release the 419 bp product as shown in **Figure 2.2**.

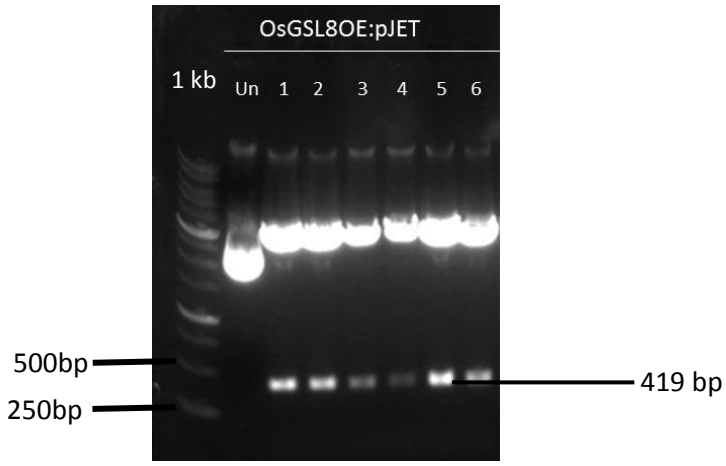

Figure 2.2: Restriction confirmation of *OsGSL8*OE:pJET by *Xba*I/*Xho*I giving expected 419 bp product in lanes 1-6 (Un-Uncut construct)

The positive clones are subjected to sequencing for confirmation of sequence. Sequencing with pJET reverse primer gives sequence which is similar to database sequence of *OsGSL8* as shown by **figure 3.1**. A snap shot of sequencing is depicted in **figure 3.2**.

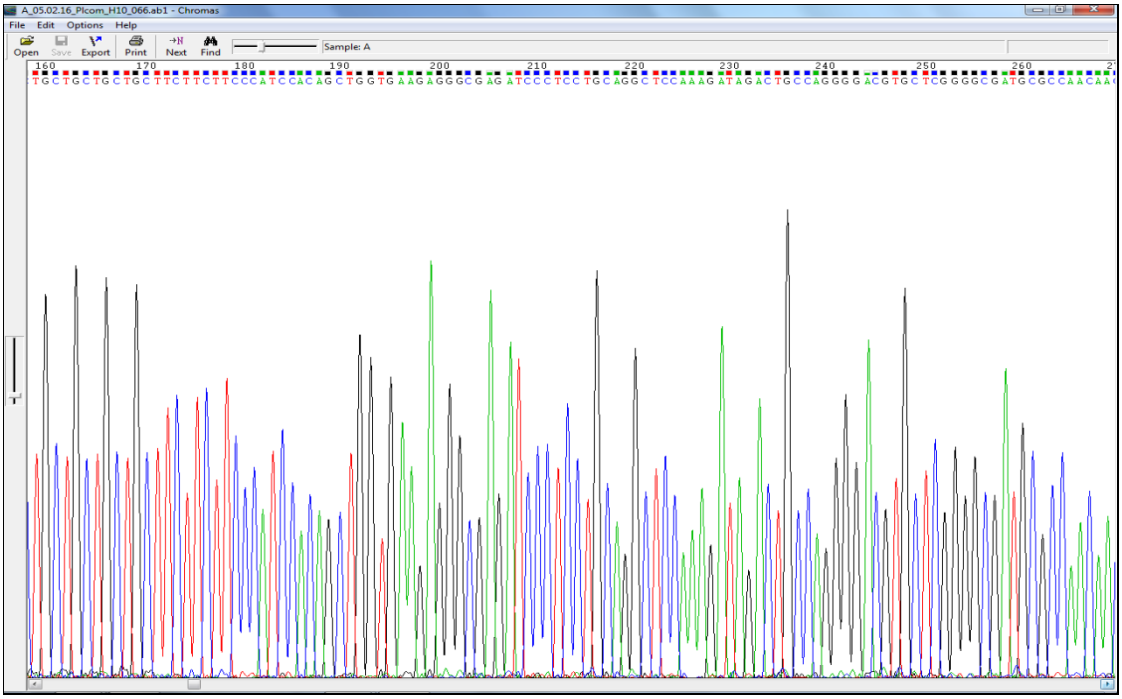

Figure 3.1: A snapshot of sequencing of *OsGSL8OE* sequence clone in pJET

- Adenine - Guanine - Cytosine - Thymine

```
LOC_Os06g51320.1    TACTACCCCTTCATGCTATTACTACTGCTGATG-TTTCTTGCGGTTTCATGTAATTGTCA  523
                    ||||||||||||||||||||||||||||||||| |||||||||||||||| ||||||||
      OsGSL8:pJET   TACTACCCCTTCATGCTATTACTACTGCTGATGTTTTCTTGCGGTTTCATATAATTGTCA  180

LOC_Os06g51320.1    CTAGATACTTTTTAATGTTGAGTGCTCGTCCTTTTTACTTATGAAGTTATATATTCTTCG  583
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      OsGSL8:pJET   CTAGATACTTTTTAATGTTGAGTGCTCGTCCTTTTTACTTATGAAGTTATATATTCTTCG  240

LOC_Os06g51320.1    TATAGGTATACTATATTAGCAATGCATATTGTGTCACAAATATAGTATATCATATAGCTT  643
                    |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
      OsGSL8:pJET   TATAGGTATACTATATTAGCAATGCATATTGTGTCACAAATATAGTATATC--ATAGCTT  298

LOC_Os06g51320.1  TAATTAAGAATAAATAAATTGTTTATAGTAAAAGTTGAAATACACATGACCAACGGTGAC  703
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      OsGSL8:pJET TAATTAAGAATAAATAAATTGTTTATAGTAAAAGTTGAAATACACATGACCAACGGTGAC  358

LOC_Os06g51320.1  AAGTAAATGAGCACATAGCTAGCTAGGGATTAATCTACTAGCTAGTACGTACAATTGGGC  763
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      OsGSL8:pJET AAGTAAATGAGCACATAGCTAGCTAGGGATTAATCTACTAGCTAGTACGTACAATTGGGC  418
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Figure 3.2: Alignment of *OsGSL8OE* sequence clone derived from IR 64 cDNA with the sequence present in database**.**

The positive clone *OsGSL8*OE:pJET, which was confirmed in sequence, were further selected for cloning in binary vector pCAMBIA1301. For this, *OsGSL8*OE:pJET was digested with *BstE*II and *Bgl*II restriction enzymes which releases a 400 bp product. The restriction digestion product was gel-eluted. The binary vector pCAMBIA1301 was also

digested with *BstE*II and *Bgl*II and vector back bone was gel eluted. The vector and insert was ligated and transformed in *E. coli* DH5α competent cells. The positive clones were screened by colony PCR. The selected positive colonies were inoculated for plasmid isolation and positive clones were confirmed by restriction digestion with *Pml*I and *Bgl*II restriction enzymes as shown in **Figure 4.1**. Here the restriction product of 400 bp (in lanes 4 and 5) confirms the positive cloning of *OsGSL8OE* in pCAMBIA1301 binary vector. The representation of the *OsGSL8*OE:pCAMBIA1301 is given in **Figure 4.2.**

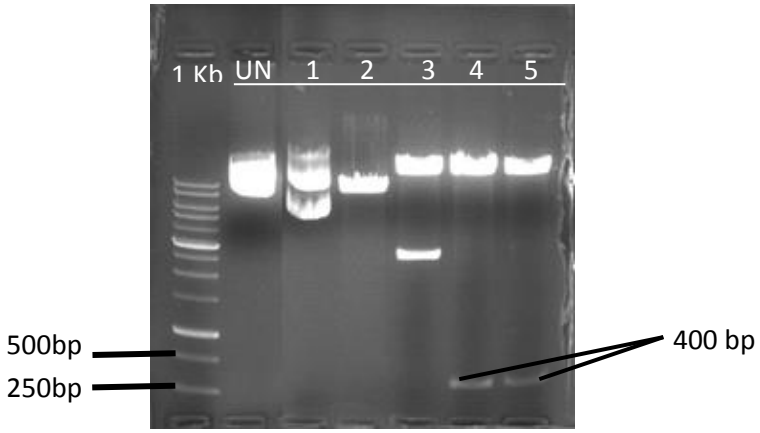


Figure 4.1: Confirmation of *OsGSL8*OE:pCAMBIA1301 by restriction using *Pml*I/*Bgl*II in lanes 4 and 5 (UN – Uncut construct)

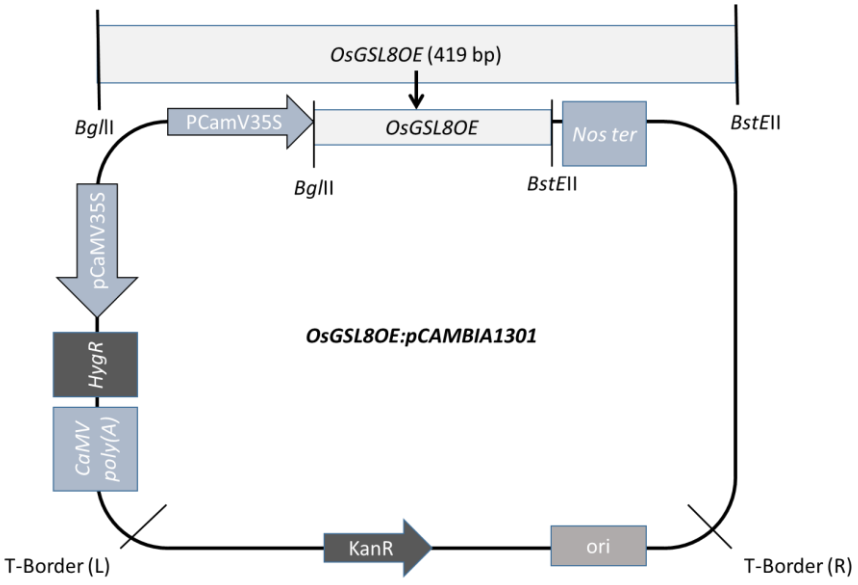


Figure 4.2: Representation of *OsGSL8*OE:pCAMBIA1301 construct

Cloning and Analysis of *OsGSL8* promoter

Cloning of *OsGSL8* in pJET vector

To understand the tissue-specific expression of *OsGSL8*, a reporter construct was prepared. The 1087 bp 5' upstream region of translation start site of *OsGSL8* was selected for construction of reporter construct. *proOsGSL8* was amplified using genomic DNA of IR64 as a template and designed primers. The amplified product obtained in the PCR was gel eluted and ligated to pJET vector. The cloning was confirmed by restriction by *Hind*III restriction enzymes as shown in **Figure 5.1**. The forward orientation clone gave a band of 1750 bp while the reverse orientation clone gave a band of 500 bp.

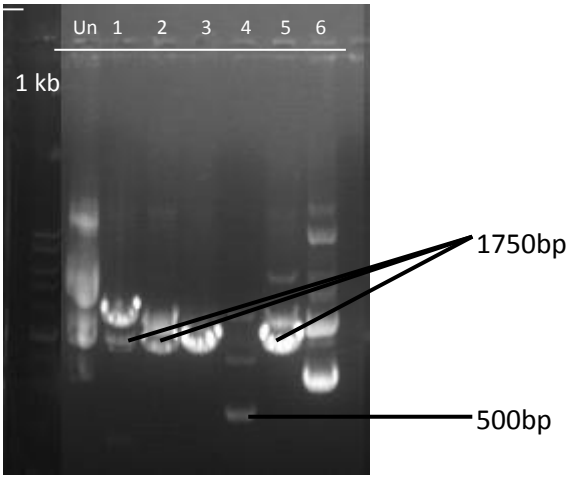


Figure 5.1: Confirmation of pro*OsGSL8*:pJET by restriction with *Hind*III. Here lanes 1,2 and 5 confirm forward orientation with 1750 bp band while lane 4 confirms reverse orientation with 500 bp band (Un – Uncut construct).

Sequencing was done with pJET forward and reverse primer separately and then assembled to obtain the full sequence which was found to be similar to database sequence of *OsGSL8,* as depicted in **Figure 5.2**. The 1087 bp product is aligned with the Database sequence of *OsGSL8* using ClustalW software (http://www.genome.jp/tools/clustalw/).

Figure 5.2: Alignment of cloned pro*OsGSL8*:pJET with the sequence of OsGSL8 promoter sequence obtained from the database**.**

Cloning of promoter *OsGSL8* in pCAMBIA1301 binary vector

The positive clone pro*OsGSL8*:pJET were further selected for cloning in binary vector pCAMBIA1301. For this, pro*OsGSL8*:pJET was digested with *Hind*III and *Nco*I restriction enzymes which releases a 1087 bp product. The restriction digestion product was gel-eluted. Binary vector pCAMBIA1301 was also digested with *Hind*III and *Nco*I and vector back bone was gel eluted. The vector and insert was ligated and transformed in *E. coli* competence cells. The positive clones were screened by colony PCR as shown in **Figure 6.1**.

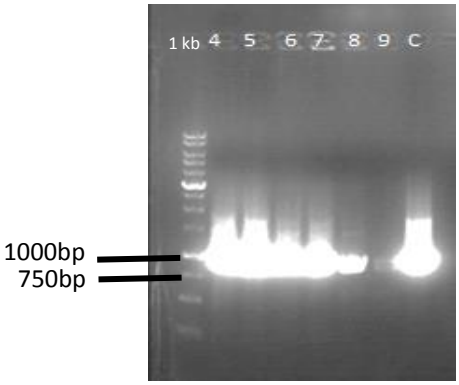


Figure 6.1: Colony PCR: (pro*OsGSL8*:pCAMBIA1301) positive clones in lanes : 4, 5, 6, 7, 8. Positive clone in pJET vector was was used as control

The selected positive colonies were inoculated for plasmid isolation and positive clones were confirmed by restriction digestion with *Pml*I and *Bgl*II restriction enzymes (**Figure 6.2**). A representation of the pro*OsGSL8*:pCAMBIA1301 construct is given in **Figure 6.3**.

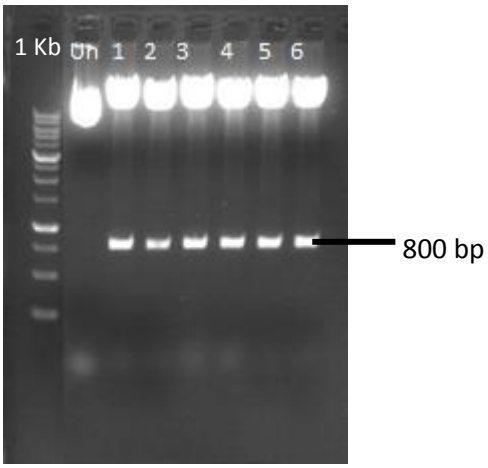


Figure 6.2: Restriction digestion of proOsGSL8:pCAMBIA1301 by *Pml*I and *Bgl*II

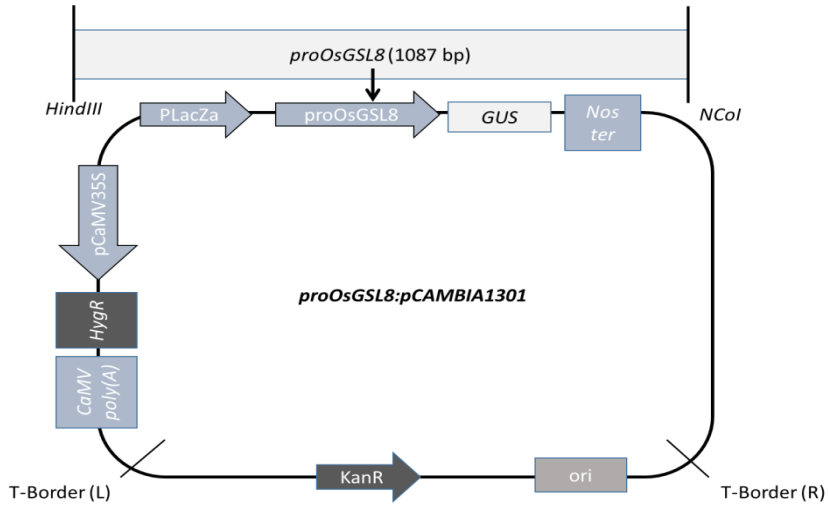


Figure 6.3: Depiction of pro*OsGSL8*:pCAMBIA1301

Plant transformation

For functional characterization of *OsGSL8* gene, the overexpression (*OsGSL8*OE:pCAMBIA1301) and promoter construct (pro*OsGSL8*:pCAMBIA1301) were transformed into wild type *A.thaliana* plant. After performing floral-dip plants were kept in dark for 24 hours. Transformed plants were kept in growth room (18/6 hr day/night cycle and 22^0C temperature) for maturation of siliques **(Figure 7)**. T_1 seeds were harvested and inoculated on ½ MS plates containing hygromycin for selection of transformants.

(a) *(b)*

Figure 7: *Arabidopsis* plants after floral-dip. Over expression (*OsGSL8*OE:pCAMBIA1301) and promoter construct pro*OsGSL8*:pCAMBIA1301) was transformed into wild type *A.thaliana* plant, (a) and (b) respectively.

GUS Assay

GUS staining was performed on leaf samples isolated from the selected T_1 transformants of reporter line pro*OsGSL8*:pCAMBIA1301. Leaf sample for the plant transformed with only pCAMBIA1301 vector was used as control, which has CaMV35S promoter driving the GUS expression **(Figure 8)**. Plants showing positive staining were taken for further growth and harvesting.

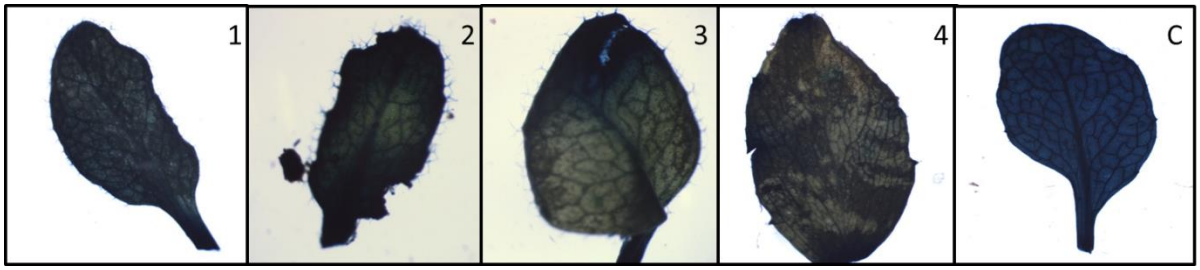


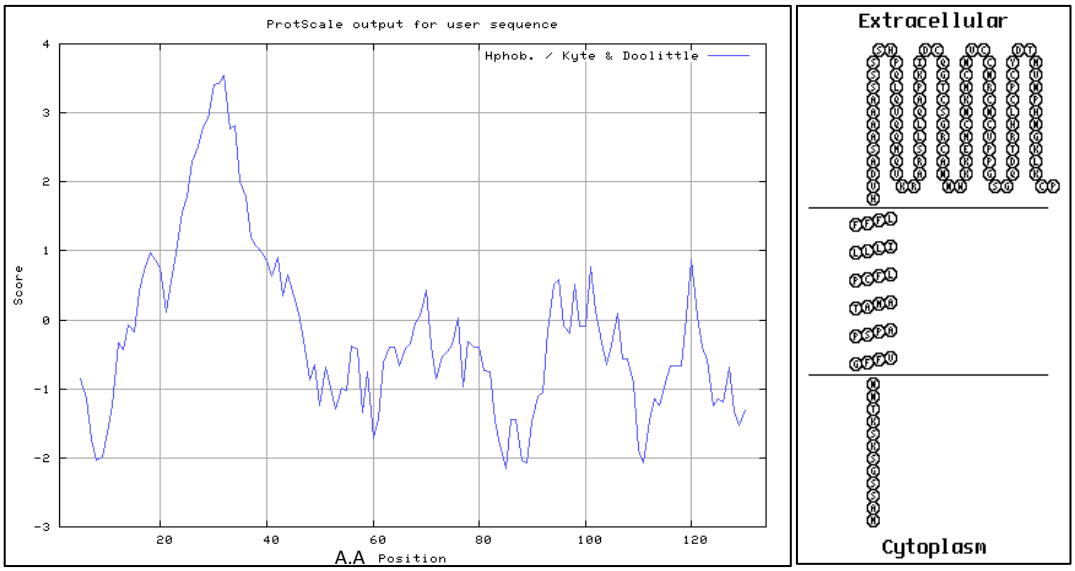
Figure 8: GUS assay of leaf samples from T_1 generation of reporter line pro*OSGSL8*:pCAMBIA1301. Samples 1, 2 and 3 show positive staining while sample 4 shows negative staining. Control sample is taken from plant transformed with binary vector pCAMBIA1301 only.

Analysis of *OsGSL8* protein and its phylogeny

The full length clone of *OsGSL8OE*:pJET was sequenced from both ends. The CDS was found to be 405 bp long with 57.03% GC content (A=85, T=89, G=98 and C=133 bases), starting with ATG and terminated with TGA. The predicted protein sequence contains 133 amino acids with a molecular weight of 14.40 KDa (http://web.expasy.org/cgi-bin/protparam/protparam). The grand average of hydropathicity (GRAVY) calculated for this

protein was -0.177. The predicted protein is rich in cysteine (9.8%), followed by serine (9.8%) and alanine (9.0%). Total number of negatively charged residues (Asp+Glu) is 5 while total number of positively charged residues (Arg+Lys) is 14. The theoretical isoelectric point (pI) of the predicted protein is 9.01, while the instability index (II) is computed to be 40.68, which classify this protein to be unstable in nature.

The protein sequence of *OsGSL8* was also in silico analysed for the presence of hydrophobic regions (http://web.expasy.org/cgi-bin/protscale/) (Hydropathy plot, Kyte and Doolittle, 1982) as shown in Figure. The regions between amino acids 20-40, 80-100 and 120 are hydrophobic in nature as they showed values more than zero in hydrophobicity scale **(Figure 9a)**. One transmembrane helices were predicted when *OsGSL8* amino acid sequence was subjected to analysis by HMMTOP used for the prediction of transmembrane helices and topology of proteins; http://www.enzim.hu/hmmtop; Tusnady and Simon, 1998; 2001) (**Figure 9b**).



(a) (b)

Figure 9: (a) Hydrophobicity Plot of *OsGSL8* protein **Kyle-Doolittle** scale is widely used for determining the hydrophobicity of proteins. The horizontal scale represents the position of amino acid in the sequence while the vertical scale shows the hydrophobicity score. In the plot (a), regions above 0 are hydrophobic in nature. A pictorial view of transmembrane helices generated by **SACS HMMTOP** transmembrane prediction software is shown (b).

Phylogenetic Study of obtained amino acid sequence of *OsGSL8*

Sequence analysis using ClustalW software indicated that the *OsGSL8* has a conserved functional GASA domain with 100% homology in the domain region with different members of GAST gene family. The GAST family genes share a common structural feature which contain cysteine residues in their c-terminal region and so called as GASA domain. GASA

domain is responsible for the biochemical activity, 3-dimensional structure and for their interaction.

Phylogenetic study of the obtained amino acid sequence of OsGSL8 with the members of GAST family members reported from other plant species was carried out. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N *et al*., 1987). The evolutionary distances were computed using the WAG model and are in the units of the number of amino acid substitutions per site. The analysis involved 30 amino acid sequences. All positions containing gaps and missing data were partially deleted. Evolutionary analyses were conducted using MEGA7 software. In the Phylogenetic analysis the OsGSL8 showed close relation to AtGASA14, as depicted in the phylogenetic tree in **Figure 10.** The accession numbers of protein sequence used for the study is given in **appendix-1**.

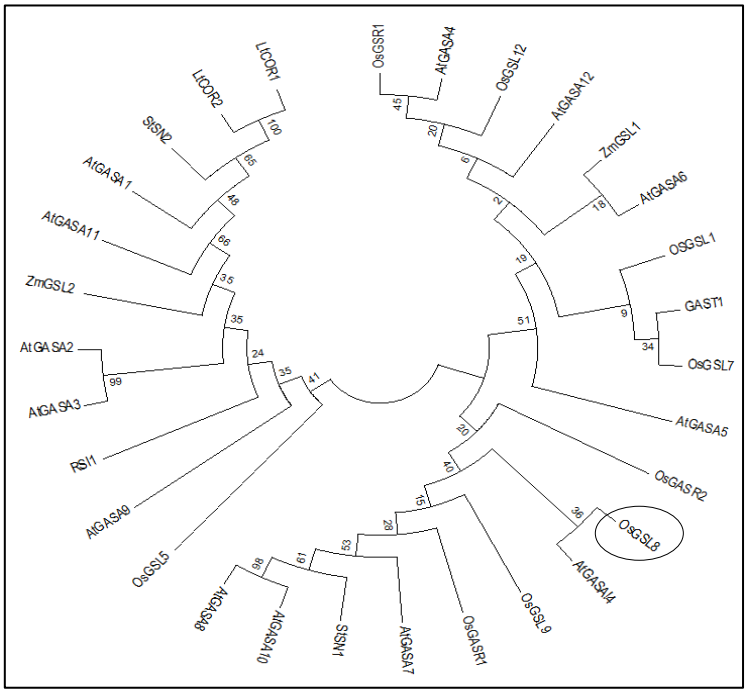


Figure 10: Unrooted neighbor-joining tree comparing the amino acid sequence of *OsGSL8* with homologs from other species

CONCLUSION

OsGSL8 belongs to the *GAST* gene family. ClustalW analysis reveals that it contains the 12 cysteine – rich region which is typical of members of the *GAST* gene family. Some of the gene family members of *Arabidopsis GAST* family have a role in plant development. The phylogenetic analysis showed that homolog of *OsGSL8* in *Arabidopsis* is *AtGASA14*, which is reported to have role in leaf expansion and stress responses. This indicates that *OsGSL8* could also have role in plant development or stress responses, which needs to be studied. To understand the function of *OsGSL8* gene, I have made an overexpression line in *Arabidopsis* and also made a GUS reporter line for understanding tissue-specific expression of this gene. Further phenotypic analysis of these transgenic lines or a mutant of its *Arabidopsis* homolog could uncover its conserved or distinct role in plant development and stress response.

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Appendix-1: List of protein sequences used in phylogenetic analysis

S.NO	GAST GENE NAME	ACCESSION ID	GAST GENE SPECIES
1	*ZmGSL1a/b*	AC206247	*Zea mays*
2	*ZmGSL2*	AC190635	*Zea mays*
3	*OsGSR1*	AY604180	*Oryza sativa*
4	*OsGASR1*	AB192574	*Oryza sativa*
5	*OsGASR2*	AB192575	*Oryza sativa*
6	*OsGSL1*	Os05g0376800	*Oryza sativa*
7	*OsGSL5*	Os05g0432200	*Oryza sativa*
8	*OsGSL7*	Os03g0607200	*Oryza sativa*
9	*OsGSL8*	Os06g0729400	*Oryza sativa*
10	*OsGSL9*	Os07g0592000	Oryza sativa
11	*OsGSL12*	Os09g0414900	*Oryza sativa*
12	*StSN1*	AAD015118	*Solanum tuberosum*
13	*St SN2*	CAC44012	*Solanum tuberosum*
14	*RSI1*	AAA20130	*Solanum tuberosum*
15	*GAST 1*	P27057	*Solanum tuberosum*
16	*LtCOR11*	AAB62947	*Lavatera thuringiaca*
17	*LtCOR12*	AAC15460	*Lavatera thuringiaca*
18	*AtGASA 1*	At1g75750	*Arabidopsis thaliana*
19	*AtGASA2*	At4g09610	*Arabidopsis thaliana*
20	*AtGASA3*	At4g09600	*Arabidopsis thaliana*
21	*AtGASA4*	At5g15230	*Arabidopsis thaliana*
22	*AtGASA5*	At3g02885	*Arabidopsis thaliana*
23	*AtGASA6*	At1g74670	*Arabidopsis thaliana*
24	*AtGASA7*	At2g14900	*Arabidopsis thaliana*
25	*AtGASA8*	At2g39540	*Arabidopsis thaliana*
26	*AtGASA9*	At1g22690	*Arabidopsis thaliana*
27	*AtGASA10*	At5g59845	*Arabidopsis thaliana*
28	*AtGASA11*	At2g18420	*Arabidopsis thaliana*
29	*AtGASA12*	At2g30810	*Arabidopsis thaliana*
30	*AtGASA14*	At5g14920	*Arabidopsis thaliana*