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EXPRESSION OF *Oryza sativa* GALACTINOL SYNTHASE GENE IN MAIZE (*Zea may* L.)

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ABSTRACT

Galactinol synthase (GolS) is a key biological catalyst for the synthesis of the raffinose oligosaccharides (RFOs) which play important roles in abiotic stress adaptation of plants, especially drought tolerance. *GolS* gene has been isolated on a variety of plants in order to create material resources for generating transgenic plants resistant to adverse environmental factors. In our previous research, we have isolated a *GolS* gene from drought stress cDNA library of *Oryza sativa* L. Moctuyen (named *OsGolS)*. In this study, the expression vector pCAM-Rd/OsGolS carrying the isolated *OsGolS* gene under the control of stress-inducible *Rd29A* promoter was constructed and introduced into *Agrobacterium tumefaciens* LBA4404, which was used for maize transformation. PCR and Real-time PCR assay indicated that transgene was integrated in the genome of the regenerated *Zea mays* plants. Reverse transcription-PCR showed that the *OsGolS* was transcribed into mRNA in *Zea mays* and was highly expressed. These results provide a basis for the study of the function of *OsGolS* in drought responses and for the development of drought stress tolerant crops.

Keywords: Drought tolerance, maize transformation, Rd29A, OsGolS.

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BIỂU HIỆN GEN MÃ HÓA GALACTINOL SYNTHASE CỦA LÚA TRONG CÂY NGÔ *Zea may* L.

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TÓM TẮT

Galactinol synthase (GolS) là một enzyme chính, tham gia vào quá trình sinh tổng hợp raffinose oligosaccharide (RFO) và đóng vai trò quan trọng trong đáp ứng chống chịu điều kiện môi trường bất lợi của thực vật và đặc biệt đáp ứng chống chịu hạn. Gen *GolS* đã được phân lập từ nhiều loài thực vật khác nhau nhằm tạo ra nguồn vật liệu phục vụ các nghiên cứu tạo giống cây trồng biến đổi gen chống chịu điều kiện môi trường bất lợi. Trong nghiên cứu trước đây, chúng tôi đã phân lập gen *GolS* từ thư viện cDNA xử lý điều kiện hạn của giống lúa Mộc tuyền (đặt tên là *OsGolS*). Trong nghiên cứu này, để phục vụ mục tiêu tạo giống cây trồng biến đổi gen chống chịu hạn, chúng tôi đã thiết kế cấu trúc vector biểu hiện *OsGolS* và chuyển vào cây ngô (*Zea mays*) mô hình thông qua vi khuẩn *Agrobacterium tumefaciens*. Sự có mặt và số bản sao của gen chọn lọc *HPT* và gen đích *OsGolS* trong hệ gen của một số dòng ngô tái sinh đã được xác định bằng PCR and qPCR. Phân tích bằng RT-PCR đã chứng minh gen chuyển *OsGolS* đã được biểu hiện (ở mức độ mRNA) trong cây chuyển gen. Kết quả của nghiên cứu này là tiền đề cho các nghiên cứu tiếp theo về hoạt động chức năng của *OsGolS*, từ đó hướng tới việc tạo ra các giống ngô chuyển gen có khả năng chống chịu tốt với các điều kiện hạn.

Từ khóa: Biểu hiện gen mã hóa, cây ngô, khả năng chịu hạn, Rd29A, *OsGolS*.

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INTRODUCTION

Under abiotic stresses, active compound accumulation is an important response of plants to these conditions. These are compounds with small molecules, high solubility which do not interfere with normal cell metabolism when accumulated at high concentrations in the cytoplasm (Jewell et al., 2010). Organic soluble compounds protect cells from environmental stresses by varying the distribution of osmotic pressure, reducing the toxicity of oxidants, stabilizing membrane and structure of proteins and enzymes. When stored at high concentrations under drought conditions, these organic substances act as molecules that attract water and retain water from the cell, thereby maintaining cellular strength. Due to water-solubility properties, some organic soluble molecules such as betaine, trehalose, proline, etc. can adhere to the surface of proteins, protein complexes and membrane structures within the cell to maintain structural stability (Farooq et al., 2009).

The raffinose family oligosaccharides (RFOs) are extensively distributed in higher plants and have important functions in carbon storage, photosynthate translocation and seed physiology, and are synthesized from sucrose by addition of activated galactose moieties donated by galactinol (Ayre et al., 2003). Galactinol synthase (GolS) is a

member of glycosyltransferases family involved in the first step of RFO biosynthesis. GolS catalyzes the transfer of UDP-D-galactose to *myo*inositol and is considered the main regulator of this biosynthetic pathway (Peterbauer & Richter, 2001). Recently, it was reported that the expression of enzymes is related to the biosynthesis of galactinol and RFOs, of which intracellular accumulation in plant cells are closely associated with the responses to environmental stresses (Peters et al., 2007). Nishizawa et al. (2008) identified ten *AtGolS* genes in the *Arabidopsis* genome, and *AtGolS1*, *AtGolS2* and *AtGolS3* were up-regulated under abiotic stresses. Transgenic tobacco plants overexpressing *Cucumis sativus CsGolS1* showed an increased accumulation of galactinol resulting in the increased tolerance to biotic stress, drought and high salinity (Kim et al., 2008). In *Brassica napus*, accumulation of *BnGolS1* mRNA in developing seeds was associated with the acquisition of tolerance to desiccation and coincided with the formation of raffinose and stachyose (Li et al., 2011). Another article reported the differential regulation of three GolS isoforms in *Coffea arabica* (*CaGolS1*, *CaGolS2*, *CaGolS3*) under water deficit, high salt and heat shock conditions that enhanced raffinose and stachyose formation during these stresses (dos Santos et al., 2011). These results demonstrate that the *GolS* gene plays an essential role in galactinol accumulation that helps plants to enhance resistance to environmental hazards.

In the previous study, we have isolated *OsGolS* from the cDNA library of Moctuyen rice variety (Nguyen & Pham, 2012). To study further the function of *OsGolS* in abiotic stress response, in this study, we designed the *OsGolS* expression vector and transformed it into the model maize plants through *A. tumefaciens*. This research is the beginning of the development of drought tolerant transgenic maize varieties from the local gene resources.

MATERIALS AND METHODS

The maize variety used for genetic transformation was K7 from the Vietnam Maize Research Institute.

Bacterium *E. coli* DH5α and *Agrobacterium tumefaciens* LBA4404 were obtained from Thermo Fisher Scientific (US) and Clontech Laboratories (US), respectively. pCAMBIA1300 vector was purchased from Gene Technologies (US); pGEM/OsGolS (Nguyen & Pham, 2016) and pCAMBIA1301/Rd29A (Pham et al., 2014) vectors were provided by the Molecular Pathology Department, Agriculture Genetics Institute, Hanoi.

The primers (table 1) were synthesized by Invitrogen (US) and Sigma (US).

Table 1. Oligonucleotides used in the research

Name	Sequence	Size of PCR product (bp)	Gene/vector
Actin-F	5'-CCTGGGATTGCCGATCGT -3'	146	*Actin1*
Actin-R	5'-CTGCTGAAAAGTGCTGAGAG-3'		
Hyg-F	5'-AAACTGTGATGGACGACACCGT-3'	294	*Hygromycin* (pCAM-Rd29A)
Hyg-R	5'-GTGGCGATCCTGCAAGCTCC -3'		
Rd-F	5'-AAGCTTCGACTCAAAACAAACTTA-3'	2063	[*Rd29A:Nos*] (pCAM-Rd29A)
Nos-R	5'-AGACCGGCAACAGGATTCAA-3'		
GolS-RT-F	5'-TGCCAACTACGGTGCAAGTA-3'	235	*OsGolS*
GolS-RT-R	5'-ACGAATGACATCTCGTAGGG-3'		
GolS-F	5-CGATGGCTCCTCCCCAGC-3'	1026	*OsGolS*
GolS-R	5'-TCACGCGGCCGAGGGCG-3'		

Construction of binary vector

To generate the pCAM-Rd overexpression vector, the DNA fragment containing *Rd29A* promoter and *NOS* terminator was released from the pCAMBIA1301-Rd29A vector (Pham et al., 2014) by digestion with *Eco*RI/*Hin*dIII and fused into pCAMBIA1300 vector. Subsequently, the ORF of *OsGolS* gene from the cloning vector pGEM/OsGolS (Nguyen & Pham, 2016) was cloned into a binary vector at *Bam*HI-restriction site downstream of *Rd29A* promoter. The resulting construct (pCAM-Rd/OsGolS) was pre-checked using PCR with specific primers (GolS-F/GolS-R, GolS-F/Nos-R and Rd-F/Nos-R) and digestions by *Hin*dIII/*Eco*RI and *Bam*HI, and finally confirmed by sequencing.

Maize transformation

The binary vector pCAM-Rd/OsGolS was electroporated into *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium*-mediated transformation of the model maize variety K7 was performed according to the protocol of the Vietnam Maize Research Institute (unpublished). Briefly, from 10 to 12-day-old immature embryos were co-cultured with *A. tumefaciens* for 3 days. The medium containing cefotaxime (200 mg/L), vancomycin (100 mg/L) and hygromycin (15 mg/L) was used to select calli derived from these embryos. Then, the antibiotic-resistant calli were transferred onto regeneration medium (containing 100 mg/L myoinositol, 400 mg/L casein, 1 mg/L kinetin and coconut water) for the regeneration of transgenic plants.

Genotyping transgenic plants

DNAs were isolated from transgenic plants using CTAB methods as described by Doyle and Doyle (1990).

PCR method (Sambrook & Russel, 2001) with specific primers (Actin-F/Actin-R, Hyg-F/Hyg-R, GolS-RT-F/Nos-R) was used to screen regeneration plants. The components of PCR were 20 ng of genomic DNA, 10 X *Taq* DNA polymerase buffer, 0.1 mM dNTP, 2.5 µM each primer and 1.0 unit of Taq DNA polymerase in overall volume of 25 µL. The thermal cycling conditions of PCR were as follows: 35 cycles of 94^{o}C for 30 sec, 56^{o}C for 20 sec and 72^{o}C for 45 sec.

The transgene copy number in the genome of the transgenic plants was determined by a real-time quantitative PCR method as described by Zhang et al. (2003), using both *Hygromycin phosphotransferase* (*HPT*) and *OsGolS* genes specific primers Hyg-F/Hyg-R and GolS-RT-F/GolS-RT-R, respectively. *Actin* gene was used as an internal reference gene.

Analysis of transgene expression

Total RNAs were isolated from PCR-positive T1 transgenic plants using the *RNAspinTM Total RNA Extraction* Kit (Intron, Korea). cDNA was synthesized from 5.0 µL RNAs in a 20 µL volume using the First Strand cDNA Synthesis Kit (Thermo Scientific). PCR reaction was performed in a 25 µL volume with 150 nM of each primer (GolS-RT-F/GolS-RT-R) using the parameters as follows: 30 cycles of 94^{o}C for 30 sec, 60^{o}C for 20 sec and 72^{o}C for 30 sec. Each PCR reaction was performed in triplicate and a no-template control was included; *Actin* gene was used as an internal control; cDNA sample from wild-type plant was used as a negative control. Agarose gel electrophoresis image of PCR products were analyzed using ImageJ software.

RESULTS

Designing the *OsGolS* expression contruct drived by stress-inducible *Rd29A* promoter

To generate the expression vector pCAM-Rd/OsGolS under the control of stress-inducible *Rd29A* promoter for maize transformation, the DNA fragment containing ORF of *OsGolS* was inserted into multi-cloning site between *Rd29A* promoter and *NOS* terminator (fig. 1). The generated expression vector was verified using PCR with specific primers. The amplification of 1026 bp (fig. 2A - lane 1), 1176 bp (fig. 2A - lane 3) and 2063 bp (fig. 2A - lane 3) sequences on the recombinant vector resulted

in the DNA products of the predicted size corresponding to the primer pairs GolS-F/GolS-R, GolS-F/Nos-R and Rd-F/Nos-R, respectively. Both double digestion with *Hin*dIII/*Eco*RI (fig. 2B - lane 2) and single digestion with *Bam*HI (fig. 2B - lane 3) confirmed further that *OsGolS* has been successfully cloned into pCAM-Rd vector.

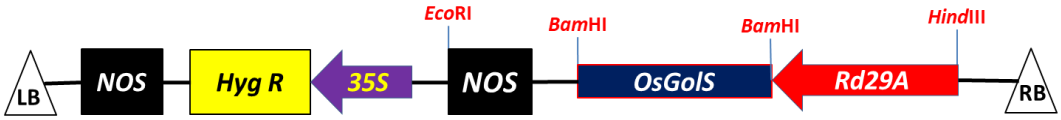

Figure 1. Schematic drawing of pCAM-GolS vector

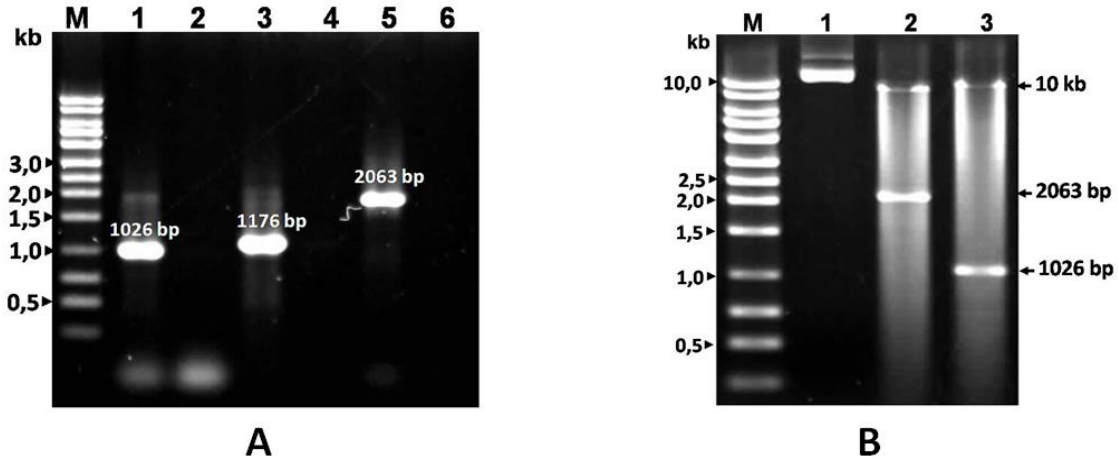

Figure 2. Confirmation of pCAM-Rd/OsGolS vector construction. (A) PCR; lane 1 & 2: PCR with GolS-F/GolS-R primers; lane 3 & 4: PCR with GolS-F/NOS-R primers; lane 5 & 6: PCR with RD-F/NOS-R primers; lane 2, 4 & 6: Negative controls (without template DNA). (B) Digestion; lane 1: Original vector; lane 2: Digested with *Hin*dIII/*Eco*RI; lane 3: Digested with *Bam*HI. Lane M: DNA Marker 1 kb

To identify that *OsGolS* encoding sequence was inserted into the vector pCAM-Rd without any mutation, the recombinant vector was sequenced. Analysis of sequencing results showed that *OsGolS* has been correctly cloned at *Bam*HI site on vector pCAM-Rd (fig. 3).

In the past, constitutive promoters, including *35S*, *Ubiquitin* and *Actin* were effectively used in plant transformtion. However, in some cases, the continuous expression of abiotic stress-responsive transgenes resulted in the negative effects on plant growth under normal conditions (Nakashima et al., 2014). One solution to this problem is to use stress-inducible promoters, such as *Lip9*, *OsNAC6* and *OsLEA3-1* (rice), *HVA22* (barley). The promoter *Rd29A* is one of the stress-inducible promoters isolated from *Arabidopsis thaliana* and proven to be induced by drought, salinity and cold conditions (Yamaguchi-Shinozaki & Shinozaki, 1993). Some model transgenic plants such as *Nicotiana tabacum*, *Solanum tuberosum*, *Glycine max*, *Arachis hypogaea*, *Triticum aestivum* and rice which expressed *DREB1* gene drived by *Rd29A* promoter showed the increased tolerence to stress without any abnormal phenotype (Nakashima et al., 2014). In this study, the expression vector containing rice galactinol sythase-encoding sequence under the control of the *Rd29A* promoter was designed to generate drought-resistant transgenic maize plants.

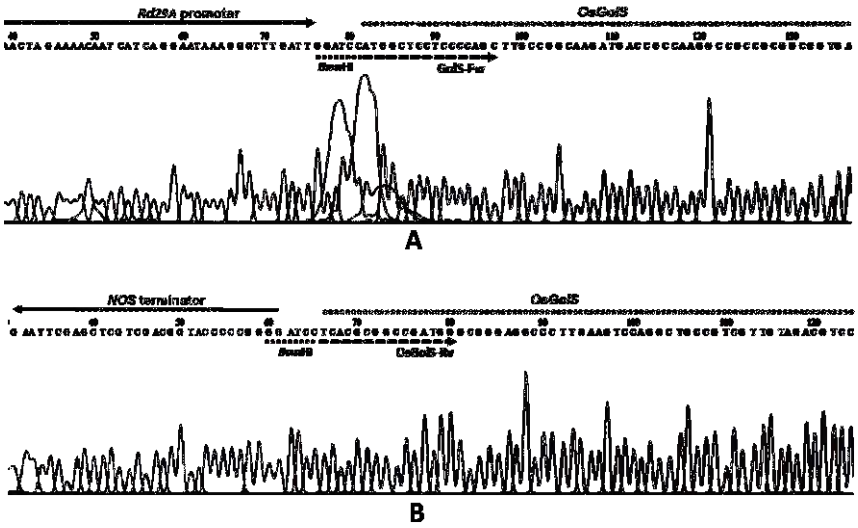

Figure 3. Sequencing analysis of pCAM-Rd/OsGolS vector. (A) Sequencing result using Rd-F primer. (B) Sequencing result using Nos-R primer

Transformation of *OsGolS* into maize

After transforming the *Agrobacteria* carrying the pCAM-Rd/OsGolS vector into maize, the transgenic plants have been selected through antibiotic selection. As a result, 48 plants were regenerated from 3925 immature embryos (table 2). Shoot regeneration ratios of selected embryos varied from 15.0% to 34.3%. The transgenic plants were detected by PCR amplification of genomic DNA using wild-type plants as negative control and the pCAM-Rd/OsGolS plasmid DNA as a positive control. Expected fragments of *OsGols* gene appeared in the amplified product of 12 transgenic individuals (fig. 4, table 2); the average transformation efficiency is about 0.3%. This result indicated the presence of *OsGolS* gene in the genome of transgenic maize.

Table 2. Transformation of *OsGolS* into model maize variety

Batch No.	No. infected embryos	No. regenerated plants	No. PCR positive plants/No. analysed plants[1]			Transformation efficiency[2] (%)	No. plants/No. analysed plants[3]	
			Actin1	*HPT*	*OsGolS*		1 copy	>1 copy
I	1000	12	12/12	3/12	3/12	0.3	1/3	2/3
II	2050	27	27/27	7/27	7/27	0.342	2/7	5/7
III	875	9	9/9	2/9	2/9	0.23	1/2	1/2
Total	3925	48	48/48	12/48	12/48	0.29	4/12	8/12

[1]Regeneration plants were tested by PCR with *Actin1*, *HPT* and *OsGolS* primers.
[2] Transformation efficiency was calculated according to the equation: E = (No. PCR-positive plants/No. total infected embryos) * 100%.
[3]Number copy of transgene was estimated by qPCR via $2^{\Delta Ct}$ value; *Actin1* was used as an internal control. Values are mean of data taken from three replicated experiments.

Then, all plants revealed to be positive by PCR were analyzed further using qPCR to determine the copy number of the transgene. The results indicated that 4 plants had one copy of transgene and the remaining plants had two or more copies of the transgene (table 2). Additionally, the similar results between two either PCR (fig. 4) or qPCR (data not shown) experiments using *HPT* and *OsGolS* specific primers indicated that there

was no T-DNA rearrangements during the process of chromosomal integration. All plants (T0) with the single copy of transgene (named as G1.10, G2.8, G2.21 and G3.1) were grown in green-house for seed colection and gene expression experiments (T1).

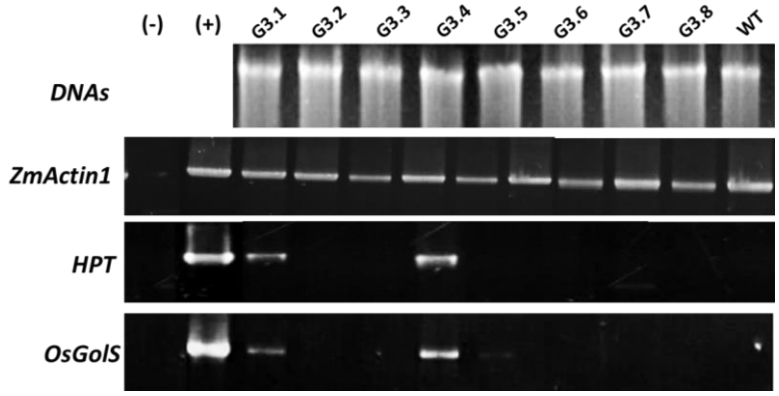

Figure 4. PCR detection results of *HPT* and *OsGolS* gene in regenerated plants (Batch III). DNA was extraced from T? transgenic (G3.1–G3.8) and non-transgenic (WT) plants and amplified by PCR with internal control (*ZmActin1*), and transgene (*HPT* and *OsGolS*) primers. (-) Blank control; (+) Positive control

In other studies, the efficiency of maize transformation varied from 0 to 50%, depending in both co-cultivation and regeneration steps (Bohorova et al., 1995; Pranjal et al., 2016). Although cells from different tissues including immature embryo, mature embryo, stamen, bud, buds regenerated from calli were used for gene transformation, the transformation studies using immature embryo showed the highest efficiency (Torney et al., 2007). In Vietnam, Tran et al. (2017) transformed sweet potato *IbOr* gene into immature embryos of two inbred maize lines H145 and H95 with 8.6% and 6.2% of transformation efficiency. In our work, the transformation efficiency was very low (0.3%) while the regeneration efficiency was quite high (up to 34.3%), which indicated that it was mainly due to the low efficiency in T-DNA integration into the K7 maize genome.

Analysis of *OsGolS* gene expression in transgenic plants

The expression of *OsGolS* transgene was detected through semi-quantitative RT-PCR using the specific primers. Total RNA was extracted from drought-treated PCR-positive T1 transgenic and non-transgenic plants and was subsequently analyzed using RT-PCR. Among 5 plants analyzed, G1.10 and G3.1 showed a specific amplified band to *OsGolS* gene; while G2.8, G2.21 and wide-type plant did not show this band (fig. 5). This result demonstrated that *OsGolS* was successfully transcribed into mRNA and expressed in the transgenic maize.

In many previous studies, the analysis of transgene expression was often performed with homozygous T2 transgenic plants (Hu et al., 2006). However, several studies analyzed transgenic plants in the generation T1 or T2 in order to investigate the relationship between genotype and phenotype. For example, Hur & Kim (2014) analyzed the expression of transgene *OsMAPK2* in T1 transgenic *A. thaliana* by northern blot assay. The expression of the *OsNAC6* under the control of *35S* promoter in T1 transgenic rice lines was also demonstrated by Realtime RT-PCR (Rachmat et al., 2014). Here, we analyzed the expression of *OsGolS* in 4 T1 transgenic maize plants using a RT-PCR method. Our results indicated that transgenic plants represent one genetically independent event. All *OsGolS* transgenic plants which showed

the expression of transgene will be further analyzed for drought tolerance in subsequent generations to demonstrate the function of *OsGolS* in abiotic stress responses.

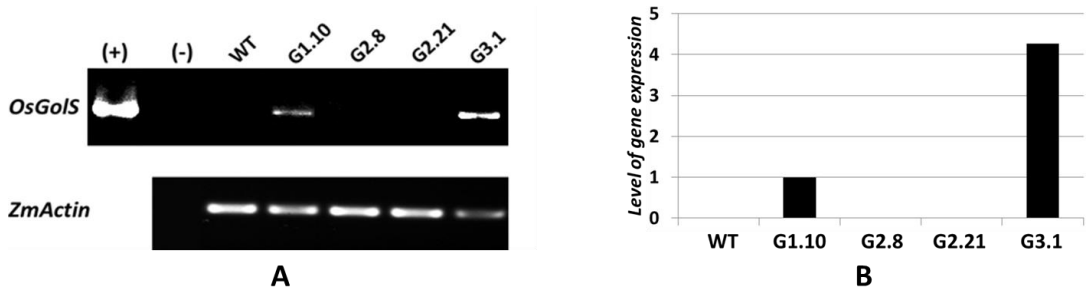

Figure 5. Expression of *OsGolS* in T1 transgenic maize plants. (A) Amplification of transgene (*OsGolS*) and internal control gene (*ZmActin*) by RT-PCR. (B) Gene expression correlation of *OsGolS* in transgenic and non-transgenic plants. (+): positive control (pCAM-Rd/OsGolS); (-): blank control; (WT): non-transgenic plant; (G1.10, G2.8, G2.21, G3.1): transgenic plants; level of transgene expression in G1.10 plant was equal to 1. Values shown in the graphs are the mean of data taken from three replicated experiments

CONCLUSION

In this study, we contructed *OsGolS* expression vector under the control of stress-inducilble promoter *Rd29A*. T-DNA was successfully transformed into model maize variety K7 through *A. tumefaciens* with 0.29% efficiency. Transgenic plants were analyzed using PCR and qPCR for the presence and copy number of transgene in the genome of transformant, respectively. Using semi-quantitative RT-PCR, the expression of *OsGolS* gene was detected in drought-treated transgenic plants. These results are the basis for further functional studies of *OsGolS*, which in turn improve the drought tolerance of crops by genetic engineering technology.

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