

Downregulation of galactomannan galactosyltransferase in *Cyamopsis tetragonoloba*

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Abstract

Guar is a legume of industrial importance due to the presence of gum (galactomannan) in its seed endosperm. The mannose (M)/galactose (G) ratio in galactomannan molecule is critical in deciding the physico-chemical properties of guar gum. Gum with reduced galactose content has better commercial value due to better viscosity. We report down regulation of *galactomannan galactosyltransferase (GMGT)* which links G residues to the M backbone in galactomannan molecule. The target *GMGT* sequence for RNAi was directionally cloned into pENTRTM/D-TOPO® vector, followed by a LR recombination reaction with pANDA vector. The constructed *gmgt* RNAi cassette was delivered into guar tissue using *Agrobacterium tumefaciens*-mediated gene transfer. The cotyledonary node explants were pre-cultured for 72 h and co-cultivated with *A. tumefaciens* strain harbouring the *gmgt* RNAi cassette for 24 h. Maximum number of putative transformed shoots regenerated from the cotyledonary node explants cultured *in vitro* on Murashige and Skoog (MS) medium containing 1.5 mg/L indole-3-acetic acid (IAA), 4.0 mg/L butyric acid (BA) and 1.0 mg/L gibberellic acid (GA₃). *In vitro* regenerated shoots were rooted on half-strength MS medium containing 4% sucrose and 2 mg/L indole-3-butyric acid (IBA). The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis showed reduced levels of *GMGT* expression in the seed endosperm of 35 days after flowering (DAF) stage pods. The present findings are expected to be beneficial in taking up of gene characterization and improvement of agronomic traits in guar.

INTRODUCTION

Guar [*Cyamopsis tetragonoloba* (L.) Taub.], also known as cluster bean, is a drought hardy subtropical legume. In earlier times, guar was cultivated for forage and green manure and guar beans were consumed as vegetable (Dwivedi et al. 1995; Jaiswal et al. 2018). Guar seeds have large endosperm with high content of galactomannan, which is commonly known as guar gum. Guar gum forms highly viscous aqueous solution at low concentrations (Dhugga et al. 2004). This property of guar gum makes it a cost-effective natural thickener. Due to its valuable gelling properties guar gum has found several applications in food, pharmaceutical, oil exploration, cosmetic, detergent and textile industry (Kuravadi et al. 2013). Additionally, guar gum is being further studied for its valuable properties in treating irritable bowel syndrome, diarrhea, diabetes and high cholesterol (Slavin and Greenberg 2003; Giannini et al. 2006; Butt et al. 2007; Tanwar et al. 2017).

Galactomannan is synthesized in Golgi lumen by two membrane-bound glycosyltransferases, mannansynthase (MS) and galactomannan galactosyltransferase (GMGT). MS catalyzes the synthesis of β -1, 4-linked mannan backbone and GMGT links galactose residues through α -1,6 linkage on the mannan backbone (Edwards et al. 1992, 1999, 2002; Reid et al. 2003; Dhugga et al. 2004). Mannose/galactose (M/G) ratio is the important parameter in deciding the physico-chemical properties of a galactomannan gum. The M/G ratio in guar varies from 1.5-2 (Whistler, 1948). As compared to locust bean gum obtained from carob trees which has higher M/G ratio in its galactomannan molecule, guar gum exhibits a lower tendency to form a gel. However, it takes 10 years of vegetative growth for a carob tree to flower and ultimately yield gum. Also, the labour-intensive harvesting of carob fruits makes

it a less favourable plant for commercial gum production. Therefore, guar gum is most commonly used galactomannan in various industries (Dionisio and Grenha 2012).

It is of immense importance to develop improved varieties of guar having better gum quality due to the industrial importance of guar gum. The development of new varieties of guar is difficult using conventional methods like hybridization, due to the constraints offered by the cleistogamy and small size of guar flowers. The variability in gum quality is also limited in the available germplasm to carry out such studies (Sandhu, 1988). An overexpression of α -galactosidase gene in guar endosperm is expected to result in the synthesis of galactomannan having an increased M/G ratio. The gene for α -galactosidase enzyme is highly expressed in *Senna occidentalis*. Joersbo et al. (2001) made an attempt to produce galactomannan with less substituted galactose residues by transforming guar plants with the α -galactosidase gene from *S. occidentalis*. Transformed plants showed considerably less reduction in galactose content than *S. occidentalis*. Also, the varieties which are regenerable are not highly transformable, and the varieties which are transformable are often not capable of rooting in culture (Dixon and Vaghchhipawala 2007). Except for few attempts (Joersbo et al. 1999; Joersbo et al. 2001; Dixon and Vaghchhipawala; 2007; Thakur et al. 2020), the genetic manipulation related research for improvement of guar agronomic traits is still in its infancy.

Down-regulation of *GMGT* expression in guar seed is expected to result in the biosynthesis of galactomannan having improved gum properties. We report here the RNAi of *GMGT* in guar by *Agrobacterium*-mediated delivery of RNAi cassette. The transformed plants showed downregulation of *GMGT* in guar seed endosperm.

MATERIALS AND METHODS

Plant material

The seeds of guar variety RGC-936 were obtained from Central Arid Zone Research Institute (CAZRI), Jodhpur, India.

Bacterial strains and plasmids

The bacterial strains and plasmids used in the present study are given in Table S1.

Media and growth conditions

Luria Bertani (LB) medium (HiMedia, Mumbai, MH, India) was used for growing *Agrobacterium tumefaciens* and *Escherichia coli* strains. Kanamycin sulphate (HiMedia), hygromycin B (HiMedia) and cefotaxime (HiMedia) were added to the LB medium for selection. Murashige and Skoog (MS) medium (HiMedia) supplemented with various growth hormones (HiMedia) was used for *in vitro* shoot and root regeneration of guar. The guar tissue cultures were kept under 16 h of light/8 h of dark regime at $25 \pm 2^\circ\text{C}$ for growth.

Germination of guar seeds

In vitro germination of guar seeds was done as reported by us earlier (Verma et al. 2013).

Pre-culturing of cotyledonary node explants

Cotyledonary nodes of guar were excised from 10 days old *in vitro* germinated guar seedlings. The cotyledonary node explants were transferred to a 250 mL Borosil Glass Erlenmeyer Flask (Cole Parmer, Mumbai, MH, India) containing MS medium supplemented with 1.5 mg/L indole-3-acetic acid (IAA), 4.0 mg/L butyric acid (BA) and 1.0 mg/L gibberellic acid (GA₃) for pre-culturing. The pre-culturing of explants was carried out for three (24, 48 and 72 h) different time periods.

Co-cultivation of cotyledonary node explants with *Agrobacterium* strain

A loopful of *A. tumefaciens* DPSV1 culture was inoculated in 5 mL of LB liquid medium containing 50 mg/L kanamycin sulfate and incubated overnight at 28°C. Bacterial culture of OD₅₄₀ 0.521 was centrifuged at 10,000 rpm for 10 minutes and the pellet was resuspended in 5 mL MS liquid medium. Pre-cultured cotyledonary node explants were immersed in 5 mL of bacterial suspension. After 15 min, the explants were taken out and blotted dry on a sterilized filter paper. The explants were then transferred to shoot regeneration medium containing 1.5 mg/L IAA, 4.0 mg/LBA and 1.0 mg/LGA₃ for co-cultivation with *A. tumefaciens* DPSV1 strain. All pre-cultured explants were co-cultivated for 24, 48 and 72 h.

In vitro regeneration of putative transformed shoots from cotyledonary nodes

After co-cultivation, the cotyledonary node explants were transferred to a 100 mL Borosil Glass Erlenmeyer Flask (Cole Parmer) containing MS medium supplemented with 1.5 mg/L IAA, 4.0 mg/LBA and 1.0 mg/LGA₃, 50 mg/L kanamycin sulfate and 200 mg/L cefotaxime. The flasks containing cotyledonary node explants on the selective MS medium were kept in a plant tissue culture room under the conditions described earlier. After 15–20 days, the cotyledonary node explants were observed for *in vitro* regeneration of shoots, and percentage of shoot regeneration was calculated.

Preparation of RNA interference cassette for guar galactomannan galactosyltransferase

Polymerase chain reaction (PCR) was carried out in a thermal cycler (Veriti™, Applied Biosystems, Waltham, MA, USA) to amplify a 543 bp fragment (Fig. S1) of guar *GMGT* from plasmid pKPSD1. The primers were designed according to the guidelines of pENTR™ Directional TOPO® cloning kit (Life Technologies, Carlsbad, CA, USA). The sequences of forward and reverse primers were 5'-CACCACCAGTTATACCATGGACAA-3' and 5'-TCTCGTATTCCGGAGTCTGT-3', respectively. Initial denaturation was carried out at 94°C for 5 min. This was followed by 30 cycles of amplification; each cycle consisted of denaturation at 94°C, annealing at 60°C and extension at 72°C, for 1 min each. Final extension was done at 72°C for 10 min. The PCR product was run on 0.8% agarose gel. The amplified DNA fragment was eluted from the gel using PureLink™ Quick gel extraction kit (Life Technologies) and directionally ligated to pENTR™ D-TOPO® vector (Life Technologies) following manufacturer's instructions. The resulting recombinant plasmid pSW1 was used to transform TOP 10 *E. coli* cells and the kanamycin-resistant transformants were selected on LB solidified medium containing 50 µg/mL

kanamycin sulfate. The transformants containing plasmid pSW1 were confirmed by colony PCR. M13 sequencing primers (pENTR™ Directional TOPO® cloning kit; Life Technologies) with forward, 5'-GTAAAACGACGGCCAG-3' and reverse, 5'-CAGGAAACAGCTATGAC-3', sequence, were used to amplify an 868 bp fragment (Fig. S2) which contained 543 bp fragment of the *GMGT* and flanking sequences of plasmid pSW1. The colonies showing amplification of 868 bp fragment were grown on LB medium containing 50 µg/mL kanamycin sulfate and the strain was named as *E. coli* SV2. The plasmid pSW1 was isolated from *E. coli* SV2 strain using PureLink™ HiPure Plasmid DNA Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) and got sequenced (SciGenom, Cochin, KL, India) to confirm positive clones (Fig. S3).

The LR recombination reaction between the plasmid pSW1 and pANDA was carried out using Gateway® LR Clonase™ II enzyme mix (Life Technologies). The resulting recombinant plasmid pSW2 was used to transform TOP 10 *E. coli* cells. The kanamycin and hygromycin resistant transformants were selected on LB medium containing 50 µg/mL kanamycin sulfate and 50 µg/mL hygromycin B. A colony was further cultured and the strain was named as SV3. The plasmid pSW2 was isolated from *E. coli* SV3 cells using PureLink™ HiPure Plasmid DNA Purification Kit (ThermoFisher Scientific) and used to transform competent *A. tumefaciens* LBA4404 strain. *A. tumefaciens* transformants were selected on LB medium containing 50 µg/mL kanamycin sulfate and 50 µg/mL hygromycin B. The transformants containing plasmid pSW2 were confirmed by colony PCR. Gene specific primers were used to amplify *GMGT* and β -glucuronidase (*GUS*) fragments. The primer sequence and PCR programme for *GMGT* sequence amplification has been described earlier. The sequences of forward and reverse primers for *GUS* were 5'-CATGAAGATGCGGACTTACG-3' and 5'-ATCCACGCCGTATTCGG-3', respectively. The annealing temperature used for the amplification of 636 bp fragment of *GUS* was 55°C. The colonies showing amplification of *GUS* and *GMGT* fragments were grown on LB medium containing 50 µg/mL kanamycin sulfate and 50 µg/mL hygromycin B and named as *A. tumefaciens* SV1 strain.

In vitro regeneration of putative transformed plantlets of guar

In present study, the experiments carried out for standardization of transformation parameters for guar variety RGC-936, using *A. tumefaciens* DPSV1 strain, maximum number of transformed shoots showing *GUS* activity regenerated from the cotyledonary node explants pre-cultured and co-cultivated for 72 h and 24 h, respectively. The same methodology was followed for obtaining maximum number of transformed plantlets of guar variety RGC-936 producing the hairpin loop for *GMGT*. An OD₅₄₀ of 0.521 liquid culture of *A. tumefaciens* SV1 strain was prepared in MS liquid medium containing 50 mg/L kanamycin sulfate and 50 mg/L hygromycin B. After 24 h of co-cultivation with *A. tumefaciens* SV1 strain, the cotyledonary node explants were transferred to MS medium containing 1.5 mg/L IAA, 4.0 mg/L BA and 1.0 mg/L GA₃, 50 mg/L kanamycin sulfate, 50 mg/L hygromycin B and 200 mg/L cefotaxime. The *in vitro* regenerated shoots were rooted on half-strength MS medium containing 4% sucrose and 2 mg/L indole-3-butyric acid (IBA). The hardening of *in vitro* regenerated putative transformed guar plantlets was done as reported by us earlier (Verma et al. 2013). The plantlets obtained were grown till maturity in a green house.

Analysis of galactomannan galactosyltransferase expression in the endosperm of transformed guar plants

Seeds were harvested from the control (non-transformed) plants and *in vitro* regenerated putative transformed plantlets at 35 days after flowering (DAF) stage. Endosperm was excised from guar seeds and total RNA was isolated from the endosperm using TRI-reagent® (Sigma-Aldrich, St. Louis, MO, USA) as per manufacturer's instructions. The isolated RNA was quantified using Varian Cary 50 Bio UV-VIS Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) and treated with DNaseI, RNase-free (Life Technologies) as per the manufacturer's protocol. cDNA was synthesized from 1 µg of total RNA using a Verso cDNA synthesis kit (Life Technologies) as per manufacturer's instructions. The primers for *GMGT* and *actin-11* (*ACT11*) for carrying out quantitative Real Time-PCR (qRT-PCR) were designed as per the primer designing guidelines by Power SYBR® Green PCR Master Mix (Life Technologies). The sequences of forward and reverse primers for *GMGT* were 5'-ATGGATGTGTGGGTGAGCAT-3' and 5'-ATGGATGTGTGGGTGAGCAT-3', respectively. The sequences of forward and reverse primers for *ACT11* were 5'-GTGCGATGTTGATATCAGGAAGGACC – 3' and 5'-CTCTCAGGTGGTGCTACAACCTTG – 3', respectively. The qRT-PCR mixture was prepared using Power SYBR® Green PCR Master Mix (Life Technologies) and the qRT-PCR reaction was carried out in StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The activation was carried out at 95°C for 10 min. This was followed by 40 cycles of amplification; each cycle consisted of denaturation at 95°C for 15 sec, annealing at 55°C for 1 min and extension at 55°C for 1 min. The C_T values obtained were used to calculate the relative time-fold decrease in *GMGT* expression using 2^{-ΔΔCT} method (Livak and Schmittgen2001).

RESULTS

Selection of 72h pre-culture and 24h co-cultivation time periods for in vitro regeneration of transformed shoots

Parameters for transformation of guar variety RGC-936 were standardized using *A. tumefaciens* DPSV1 strain. The results showed that the variation in pre-culture and co-cultivation time periods had a profound effect on explant survival, shoot regeneration and transformation efficiencies (Table S2). It was observed that the *in vitro* shoot induction frequency increased with an increase in pre-culturing time and decrease in co-cultivation time. The GUS assay results revealed that none out of the putative transformed shoots were positive for transformation, which were obtained after pre-culturing and co-cultivation of the cotyledonary node explants for 24 h-48 h, 24h-72h and 48 h-72 h time periods, respectively. The highest percentage of regenerated transformed shoots was obtained from cotyledonary node explants pre-cultured for 72 h and co-cultivated for 24 h.

Cassette for RNA interference of galactomannan galactosyltransferase

The RNAi construct for *GMGT* was made as described in materials and methods section. The resulting recombinant plasmid harbouring the RNAi cassette was named as pSW2 and introduced into *A.*

tumefaciens LBA 4404 strain. The *A. tumefaciens* strain harbouring the RNAi cassette was named SV1 and used for transformation of guar. A schematic representation of RNAi cassette construction is being made (Fig. 1).

In vitro regeneration and growth of putative transformed plants

Ten days old *in vitro* germinated guar seedlings (Fig. 2a) were used as source of cotyledonary node explants. The explants were precultured for 72h on MS medium. The cotyledonary node explants were co-cultivated with *A. tumefaciens* SV1 culture for 24 h (Fig. 2b). Direct *in vitro* shoot regeneration occurred after 20–25 days of culture of the cotyledonary node explants on selective shoot regeneration MS medium. *In vitro* regenerated putative transformed shoots of guar have been shown in Fig. 2c and Fig. 2d. *In vitro* root regeneration occurred after 10–15 days of transfer of shoots to half-strength MS rooting medium. Figure 2e and Fig. 2f show *in vitro* regenerated complete plantlet of guar with well-developed root and shoot system. The plantlets were transferred to cocopeat mixture (Fig. 3a). The plantlets showed proper growth on transfer to pots containing soil, sand and farm yard manure (Fig. 3b) and soil in green house (Fig. 3c). The setting of flowers and pods on transformed guar plants has been shown in Fig. 3d.

Downregulation of galactomannan galactosyltransferase expression in the endosperm of transformed guar plants

The real-time qRT-PCR studies showed comparatively lesser levels of *GMGT* in the seed endosperm of transformed guar plantlets. The time-fold decrease in gene expression calculated by the formula $2^{-\Delta\Delta C_t}$. Out of the 30 *in vitro* regenerated plantlets, 4 showed down-regulation of *GMGT* in the endosperm tissue. Relative time-fold decrease in gene expression in the transformed plantlets as compared to the control non-transformed guar plant was found to be 1.80, 1.91, 1.95 and 1.98. Bar-graph showing the relative time-fold decrease in level of *GMGT* in endosperm tissue of transformed guar plantlets has been presented (Fig. 4).

DISCUSSION

Guar is a legume of industrial and medicinal importance but many efforts have not yet been made to work towards the genetic improvement of this crop. Recently, some studies have identified different sets of molecular markers from different tissue of guar which could assist molecular breeding programmes (Kuravadi et al. 2014; Tanwar et al. 2017; Thakur et al. 2018). Since past many years, no attempts have been made to genetically manipulate this crop for various quality and quantity characteristics. RNAi is being deployed for elucidation of gene functions and production of genetically manipulated plants with improved characteristics. Till today, no reports are available for standardization of a RNAi protocol which could assist gene characterization and manipulation of gum quality-related characteristics in this legume. Such studies have also lagged due to the unavailability of suitable transformation protocols. The transformation protocols are often variety-dependent and affected by recalcitrancy towards regeneration (Dixon and Vaghchhipawala 2007). Therefore, we report here a protocol for exploiting the potential of

RNAi technology in this industrially and medicinally important legume. Several parameters which could affect *Agrobacterium*-mediated transformation efficiency are also being highlighted.

We have earlier reported efficient *in vitro* regeneration of guar plants using cotyledonary node explants of variety RGC-936. Therefore, we started our work with cotyledonary node explants of this variety and standardized various transformation parameters. We found that maximum number of transformed shoots regenerated from cotyledonary node explants pre-cultured for 72 hours and co-cultivated with *A. tumefaciens* DPSV1 bacteria for 24 hours. In previous two reports (Joresbo et al. 1999, 2001), co-cultivation with *A. tumefaciens* was carried out for 72 h, but we got best results by carrying out co-cultivation for 24 h in this variety.

Using the above-mentioned preculture and cocultivation conditions, a RNAi cassette containing sense/antisense *GMGT* sequence was transformed into guar. The *in vitro* regenerated putative transformed shoots were rooted on a separate rooting medium without antibiotics. As per earlier studies, *in vitro* regeneration of roots from transformed guar shoots has been reported to be difficult. Joersbo et al. (1999, 2003) carried out rooting of transformed shoots by grafting them on a non-transformed decapitated stock tissue. However, *in vitro* rooting of transformed shoots has been achieved by Dixon and Vaghchhipawala (2007) and the overall transformation and regeneration success has been observed to be highly variety dependent.

We could successfully obtain transformed plantlets of a high yielding Indian guar variety RGC-936, with *in vitro* established shoot and root system, which on transfer to soil, matured to healthy pod-bearing plants. From the previously reported expressed sequence tag (EST) data, the highest level of *GMGT* expression was detected at 35 DAF stage in the seed endosperm (Naoumkina et al. 2007). Hence, the seeds of 35 DAF stage were used for real-time *GMGT* expression analysis in the present study. Reduced expression of *GMGT* was observed in the seed endosperm of the transformed plants. In this study, we have also discussed in detail the parameters for transformation of guar. With some empirical modifications, the protocol could be helpful for carrying out gene characterization and functional genomics studies in this economically important legume. Thus, present study may be helpful in paving a way for further research on the improvement of gum-related and other agronomical traits in this legume.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Author contributions

SV and GSR designed the experiments. SV did the experimental work and analyzed the data. KSD and KSG helped in providing research material and important insights regarding regeneration, cloning and guar transformation experiments and critically revised the manuscript. SV and GSR wrote the manuscript.

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Supplementary Tables

Supplementary Table S2 is not available with this version

Figures

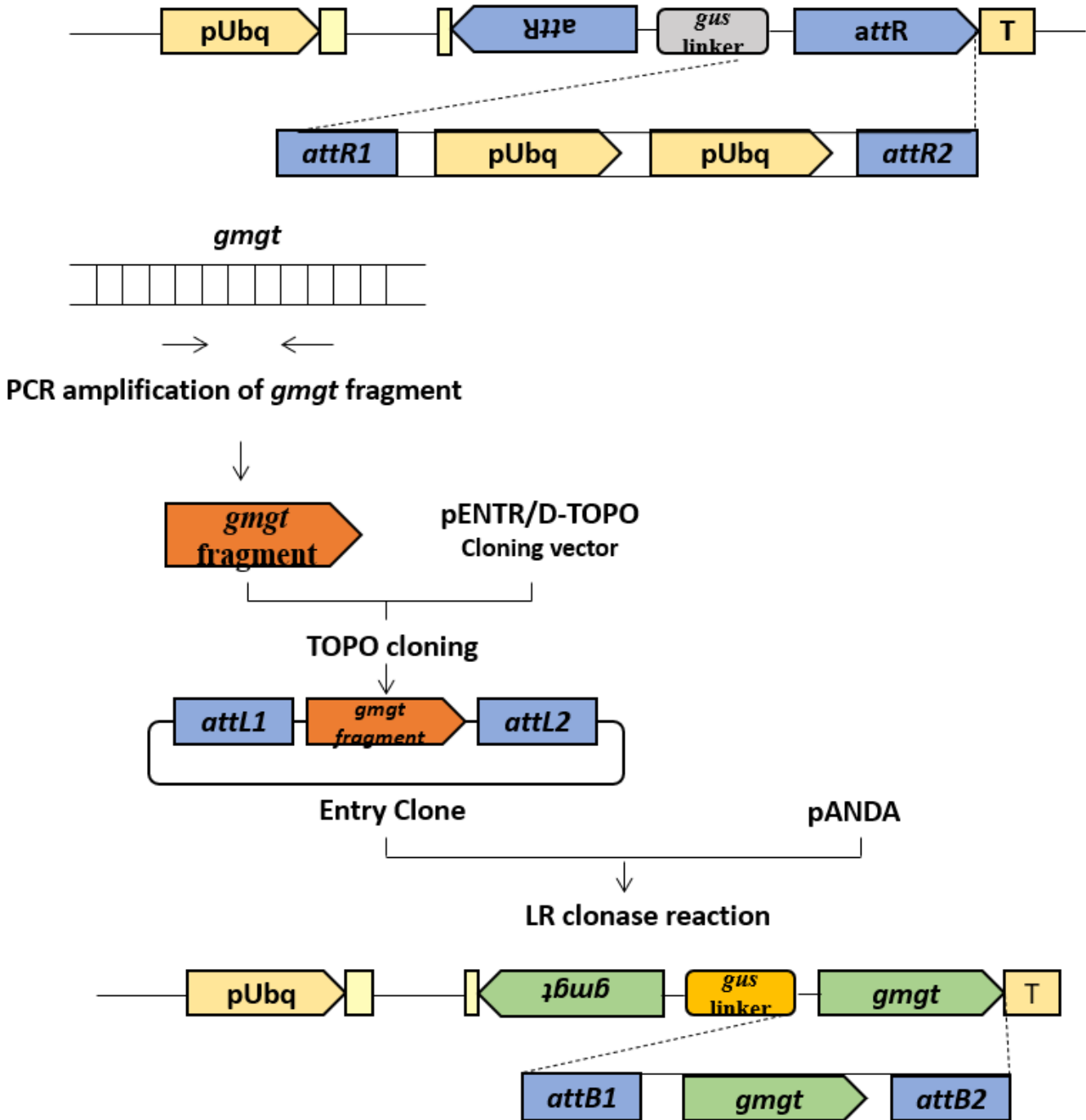


Fig. 1

Figure 1

Schematic representation of RNAi cassette construction: The *GMGT* fragment was amplified by PCR reaction using gene-specific primers and cloned into the pENTR/D-TOPO cloning vector. The final RNAi cassette was produced by an LR reaction between the pANDA destination vector and the entry clone containing *GMGT* sense-antisense sequence.

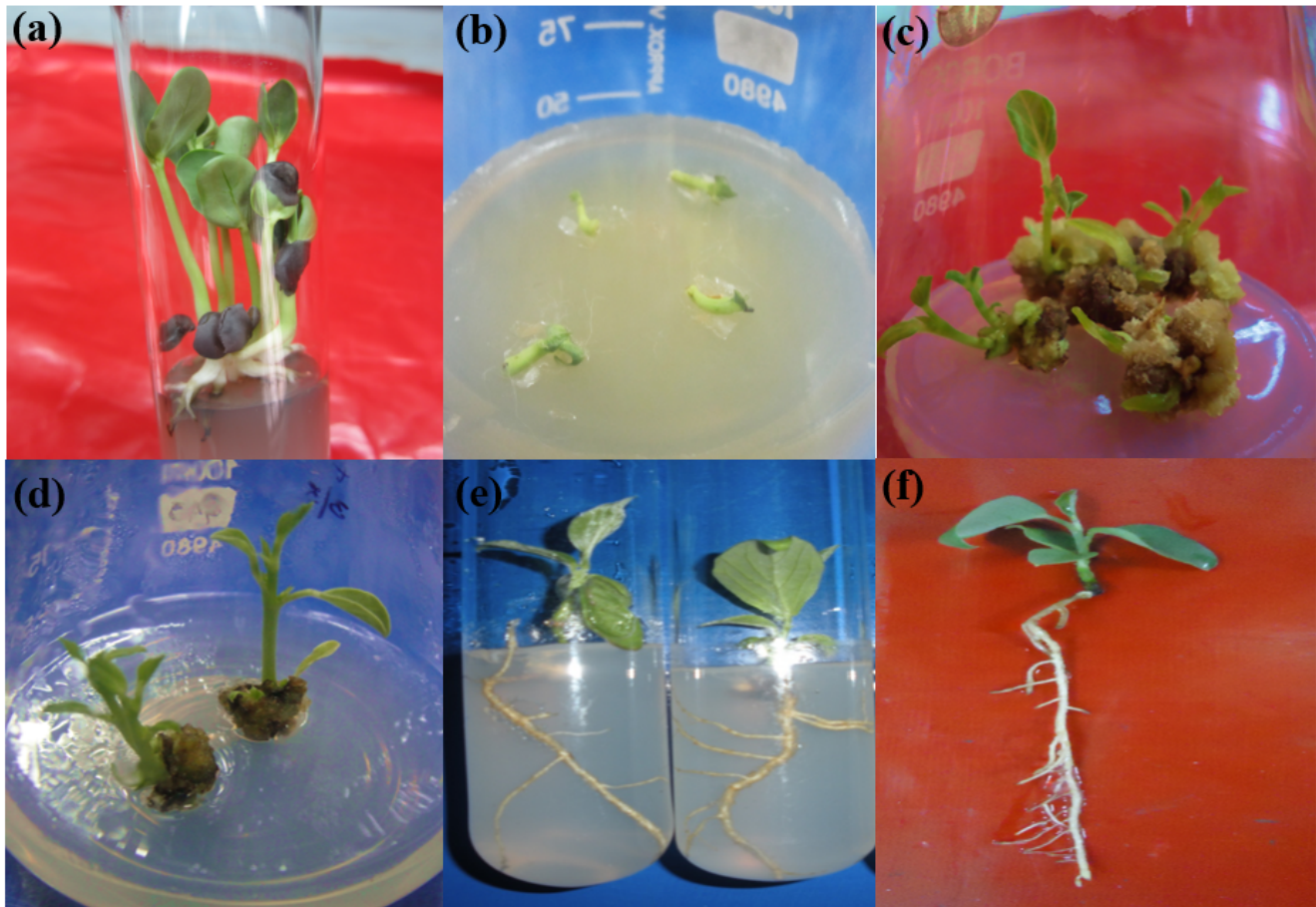


Fig. 2

Figure 2

- a** *In vitro* germinated, 10 days old seedlings of guar.
- b** Cotyledonary node explants co-cultivated with *A. tumefaciens* strain SV1 harbouring RNAi cassette for *GMGT*
- c** *In vitro* regenerated 25 days old putative transformed shoots of guar variety RGC-936.
- d** *In vitro* regenerated 40 days old putative transformed shoots of guar variety RGC-936.
- e** *In vitro* regenerated roots from putatively transformed *in vitro* regenerated guar shoots.

f *In vitro* regenerated guar plantlets taken out of rooting medium for transfer to pots.

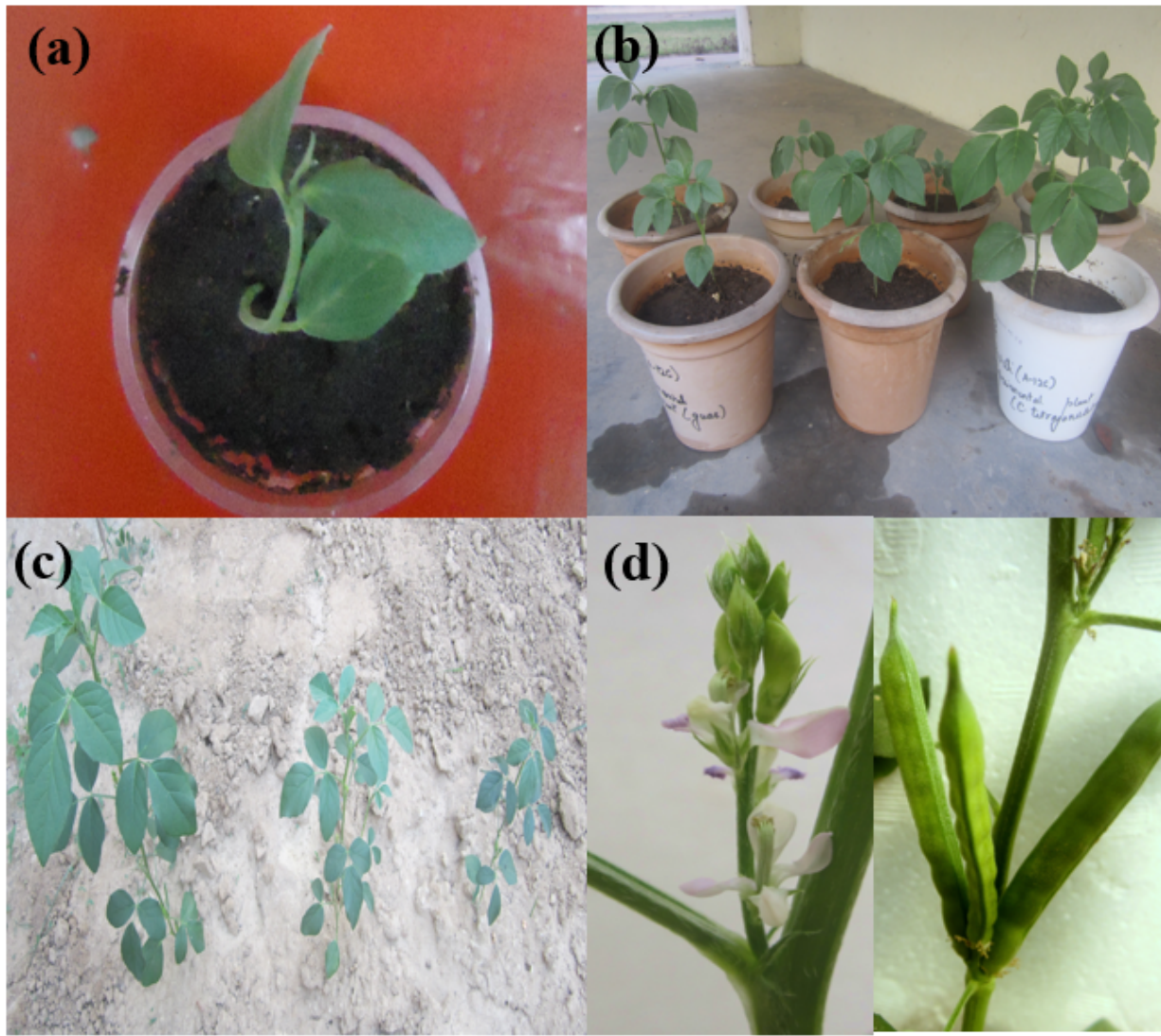


Fig. 3

Figure 3

a Putative transformed plantlets of guar variety RGC-936 transplanted to Cocopeat mixture.

b Putative transformed plantlets showing healthy growth on transplantation to pots containing soil, sand and farm yard manure.

c Transformed plantlets transplanted to soil in green house.

d Flower and pod setting on transformed plants.

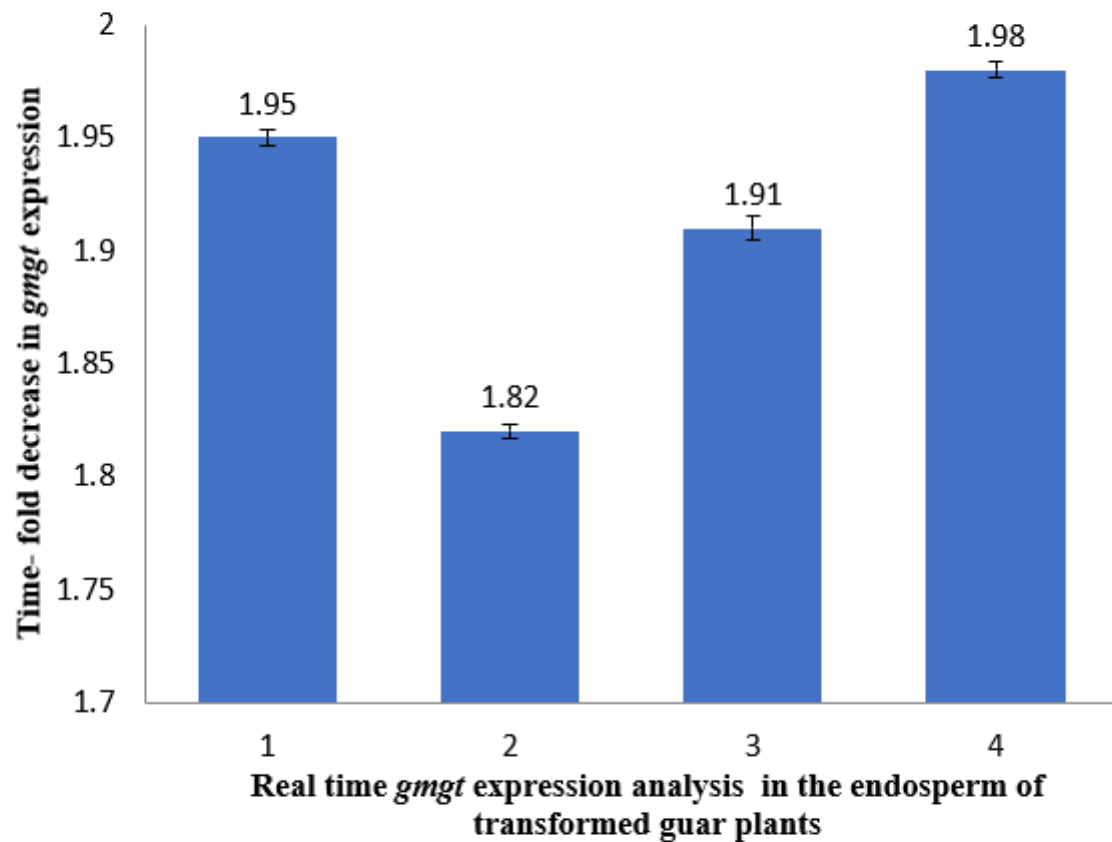


Fig. 4

Figure 4

Real-time RT-PCR analysis of *GMGT* expression in endosperm tissue of transformed guar plants. Bar graph represents the fold decrease *GMGT* expression quantified by normalization to the β -actin as an internal control. Mean values $\pm$ SE (n = 3). **: $P < 0.001$.

Supplementary Files

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- [SUPPLEMENTARYTABLES.docx](#)
- [FS1.png](#)
- [FS2.png](#)

- [FS3.png](#)