Increased Seed Size Mediated by Cyamopsis Tetragonoloba Big SEEDS LIKE Silencing Is Positively Co-related to Galactomannan Content, Phytochemical Biosynthesis and Anti-diabetic Potential of Guar

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Abstract

In higher plants, seed size is an important agronomic trait that determines the crop yield and evolutionary fitness. Guar is an economically important legume crop because of the presence of galactomannan in its seed endosperm which is used as a stabilizer and thickener in pharmaceutical, paper, cosmetic and textile industries. The current study demonstrate the role of *CtBSL*, in regulation of seed size, weight and galactomannan content in two independent BSL12-25-15-31-3 and BSL70-21-25-4-16 T_4 transgenic lines of guar, via. pCMKCtBSL mediated post transcriptional *CtBSL* silencing approach. The upregulation of *CtCYCA2;4, CtCYCD3;2, CtCDKE-1*, *CtCYCT1;*5, *CtH2A, CtH2B, CtH3, CtH4, CtERF, CtWRKY43, CtGRF5* and *CtGIF1* genes in transgenic lines of guar contributed to enhanced cell proliferation and final seed size which was further strongly and positively correlated to phytochemical biosynthesis and their anti-diabetic, anti-AGEs and lipase inhibitory potential against *α-glucosidase*, *α-amylase*, *AGEs* and *lipase* enzymes, respectively. Therefore, here we conclude the potential role of *CtBSL* silencing in improving the crop yield and therapeutic treatment of diabetes and obesity by positive regulation of seed size and phytochemical biosynthesis, respectively, in guar.

1. Introduction

Clusterbean (*Cyamopsis tetragonoloba* (L) Taub) commonly known as guar, is an economical legume crop because of the presence of galactomannan or guar gum in its seed endosperm (Thakur and Prasad, 2020). The galactomannan, composed of linear chain of β (1->4) mannose residues cross-linked to galactose residues by α (1->6) linkages, is used as a cost-effective natural thickener, stabilizer and emusilfier in paper, pharmaceutical, textile, food, and cosmetic industries and additionally for the treatment of irritable bowel syndrome, high cholesterol and diarrhea (Thakur and Randhawa, 2018). Therefore, due to global demand of galactomannan there is a necessity to produce the high yielding guar varieties which can tolerate adverse climatic conditions.

Seed size is an important agronomic trait, selected during the process of domestication, that determines seed yield, evolutionary fitness and stress responses in crop plants. Large sized seeds are preferred over smaller seed size due to higher availability of reserves and better seedling survivorship (Li et al., 2008; Gresta et al., 2013). Seed size is a complex agronomic trait that is controlled by quantitative trait loci (QTLs) and affected by several environmental factors. However, signalling pathways, transcription factors and to some extent accumulation of seed reserves like storage proteins, sugars and fatty acids also plays a major role in determination of final seed size in angiosperms (Sreenivasulu and Wobus, 2013; Na and Yunhai, 2015).

Plants also produce secondary metabolities or phytochemicals in adverse environmental conditions, as a defense mechanism, for their normal growth and development. These phytochemicals, mainly phenolics, flavanoids, carbohydrates, terpenoids and tannins, holds a great pharmacological value in modern medicine as immune-modulators, antibiotics and therapeutic drugs for treatment of various diseases (Thirumurugan et al., 2018). Diabetes mellitus and obesity are among the most prevalent health disorders

worldwide that leads to several micro and macro-vascular complications in various individuals (Umar et al., 2018). In a study, about 366 million are expected to be diabetic by 2030, out of which 79.4 million are projected to be in India only (Firdous, 2014). Several compounds extracted from plant sources have been previously reported to show antidiabetic activity and are used for discovery and development of new type of antidiabetic molecule.

In current study we have demonstrated the role of *Cyamopsis tetragonoloba BIG SEEDS LIKE* (*CtBSL*) gene, in regulation of seed size, weight, sugars and other reserves, through RNAi mediated gene silencing approach in guar. Further the seed size is positively correlated to phytochemical biosynthesis which has anti-diabetic, anti-AGEs and anti-lipase bioactivity. Therefore, we expect this study to be useful for improving the seed yield, an important agronomic and economical trait, in guar and development of novel drug candidates for treatment of diabetes and obesity.

2. Material And Methods

2.1. Plant material and vector construction

The seeds of HG 2–20 variety of guar used in this study were procured from Central Arid Zone Research Institute (CAZRI), Jodhpur, Rajasthan, India. Ten days old *in vitro* grown guar plantlets at 25 to 28 ^{0}C temperature and 16h(light)/8h(dark) photoperiod were used for RNA extraction and subsequent cDNA preparation. A 354 bp fragment of *CtBSL* was amplified from cDNA in sense and sense orientation using following primers containing specific restriction sites: 5'-GAATTCATCATACCCATGTCGATT-3' (*Eco*RI/forward sense); 5'-GGTACCGCTGCAATGACACTTTCC-3' (*Kpn*I/reverse sense); 5'-GGATCCTAATCATACCCATGTCGA-3' (*Bam*HI/ forward antisense); 5'-AAGCTTGCTGCAATGACACTTTCC-3' (*Hin*dIII/ reverse antisense). Followed by directional cloning of amplified fragments in pKannibal intermediate vector (Fig S1a) and sub-cloning in pCAMBIA1302 plant transformation binary vector, the final construct, pCMKCtBSL (Fig S1b), was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by freeze and thaw method (Wesley et al., 2001).

2.2. Transformation, screening and phenotypic characterization

Fifteen days old cotyledon explants of HG 2–20 variety of guar transformed by *Agrobacterium*-mediated transformation were grown in selective medium containing 50 mg/L kanamycin. After the screening of putative transgenics the *in vitro* grown plantlets were transferred after 30 days to agro-peat, vermiculite and sand mixture (Fig S2). The acclimatized T_0 generation guar lines, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5, grown upto T_4 generation were phenotypically characterized for seed size and weight. The non-transformed *in vitro* grown acclimatized guar plantlets were used as control. All the images were analyzed using ImageJ (https://imagej.nih.gov/ij/) software.

2.3. SEM and FE-SEM

For SEM and FE-SEM analysis, thin seed sections of non transformed control (NTC), BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T_4 generation guar lines were fixed in formaldehyde (3.7 %) at 37 ^{0}C for 14 h followed by OsO_4 (1 %) at 4^0 C for 1 h. The thin seed sections dehydrated with series of ethanol washing up to critical dried point were imaged under SEM and FE-SEM QUANTA 200 FEG for measurement cell size and area (Huang et al., 2008). All the images were analyzed using NIH ImageJ software (https://imagej.nih.gov/).

2.4. Quantitative RT-PCR

For RT-qPCR analysis, 1 µg of total RNA isolated using Qiagen RNeasy Plant Mini Kit from seeds of NTC and BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T_4 generation guar lines at different developmental stages was subsequently used for first strand cDNA synthesis using Superscript II Invitrogen kit as per the manufacturer's instructions. The triplicate reaction was performed in Applied biosystems real-time PCR (Thermo Fisher) and the data was normalized using $^{\Delta\Delta}$Ct method. *GAPDH* was used as internal control.

The RT-qPCR analysis was performed using Applied biosystems real-time PCR (Thermo Fisher) and the data was normalized using $^{\Delta\Delta}$Ct method. *GAPDH* was used as an internal control. Followinf primer pairs were used for the reaction, *CtBSL* and *GAPDH* (FP- 5'ATGTTCATCGAGAATTTTAAGGTT3'/ RP-5'ATCACTTGTACTCGAGAATCAT3' and 5'AAGCCAGCATCCTATGACAGATTC3'/ RP-5'AAGCCAGCATCCTATGACAGATTG3'),performed in triplicates with the following cycling conditions: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec and 94°C for 30 sec.

For real time expression analysis of *CtH2A, CtH2B, CtH3, CtH4, CtCYCA2;4, CtCYCD3;2, CtCDKE-1*, *CtCYCT1;*5, *CtERF, CtWRKY43, CtGRF5* and *CtGIF1* genes the list of primers used in given in Table S1.

2.5 Quantification of proteins, sugars and galactomannan

Bicinchoninic acid assay was used to determine the protein content in seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T_4 generation guar lines. Briefly, 100 µg of powdered seeds mixed with sodium bicinchoninic and $CuSO_4.5H_2O$ mixture (10:1) were incubated at 60^0 C for 35 mins. After cooling the solution's absorbance was measured at 562 nm. One mg/ml bovine serum albumin (BSA) was used as standard. For quantification of reducing and total soluble sugars, powdered seeds incubated in 80 % ethanol (70 ^{0}C for 90 min) were centrifuged at 14000 rpm for 10 min followed by supernatant extraction in rotary evaporator. The residuals obtained were dissolved in 0.5 mg/ml de-ionized water and the absorbance was noted at 620 nm and 490 nm, respectively (Nelson 1944; Somogyi 1952; Focks and Benning, 1998). For determination of galactomannan content, the dried residuals of powdered seeds extracted in phosphorus pentoxide (P_2O_5) and ethanol were hydrolyzed in 2 M trifluoroacetic acid (TFA) followed by membrane filteration and de-ionization in C_{18} column. The hydrolyzed galactose and mannose residues were separated using reverse phase high performance liquid chromatography (RP-HPLC) in water (H_2O) and acetonitrile (ACN) gradient elution mixture, where the ACN concentration increases from 5 % to 50 % (Tapie et al., 2008).

2.6. CtBSL *sub-cellular localization*

The CtBSL protein encoding gene was cloned in pENTRY-D (Invitrogen) vector containing green fluorescent protein (GFP) and subsequently sub-cloned in a gateway destination vector pMDC83, using LR reaction. The GFP signals were imaged using the Leica SP2 laser confocal microscope in tobacco epidermal cells as described by (Tian et al., 2014).

2.7. Yeast two hybrid assay

The full length *CtBSL* (1006 bp), *CtJAZ3* (1241 bp) and *CtNINJA* (1361 bp), *CtMYC2* (1062 bp) gene sequences were cloned in pGBKT7 (bait) and pGADT7 (prey) plasmids, respectively, and subsequently transformed into yeast two hybrid GOLD strain (Clontech), using a Frozen-EZ Yeast Transformation II Kit (Zymo Research). The protein interactions were analyzed on SD medium containing X-α-Gal (40 ng/mL) and Aureobasidin A (125 ng/mL), using quadruple dropout method (Lai and Lau, 2017). The list of primers used in this study is given in Table S1.

2.8. Phenolics and flavonoid estimation

The total phenolic content (TPC) was determined from methanol extract of dried seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T_4 generation guar lines using Folin-Ciocalteu (FC) reagent as described by Singleton and Rossi (Singleton and Rossi, 1965), at 630 nm. The gallic acid was used as standard and the TPC expressed as gallic acid equivalent (GAE/g). The total phenolic production (TPP) was calculated as follows: TPP (mg/L) = dry weight (g/L) ×TPC (mg/g). The total flavonoid content (TFC) was estimated at 415 nm using aluminum chloride ($AlCl_3$) colorimetric method (Ahmad et al., 2010). Quercetin was used as standard and the TFC expressed as quercetin equivalent (QE/g). The total flavonoid production (TFP) was calculated by the formula: TFP (mg/L) = dry weight (g/L) × TFC (mg/g).

2.9. Anti-diabetic, anti-advanced glycation end products and lipase inhibition assay

The *α-glucosidase* and *α-amylase* enzymatic activity of methanol extract of dried seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T_4 generation guar lines was measured at 405 nm using chromogenic method as described by Hano et al. (2013). The percentage *α-glucosidase* and *α-amylase* inhibition was calculated as: absorbance values in the presence of callus extracts - absorbance values in the absence of callus extracts. The inhibitory activity of *anti-advanced glycation end products* (*AGEs*) was determined as described by Kaewseejan and Siriamornpun (2015) at 330 nm and 410 nm excitation and emission wavelength, respectively, and the results were expressed as percent inhibition relative to equal volume of DMSO control. The *lipase* inhibitory activity of methanol extracts was determined as per Chen et al. (2018). The change in absorbance value (K) was determined at 400 nm and the percentage inhibition was calculated as: K (normal value) – K (experimental value) / K (normal value) × 100.

2.10. Statistical analysis

All the experiments were performed in triplicates. The means calculated from replicated data were analyzed by analysis of variance one-way analysis of variance (ANOVA) and compared by Student's t tests using IBM Statistical Package for the Social Sciences [SPSS] software, New York, USA at $P \leq 0.05$. Statistically significant differences were determined at a 5% level of probability for all comparisons. The results were expressed as mean ± SD of total experiments.

3. Results

3.1. Generation of transgenic guar lines

In total three transformation events of 100 cotyledons each, a total of 22 transgenic guar plants were generated out of which seven (BSL43-74, BSL9-21, BSL28-14, BSL14-11, BSL12-25, BSL56-28, BSL13-24, BSL41-23, BSL45-33, BSL70-21, BSL162-27, BSL124-31, BSL151-32, BSL152-23, BSL125-24, BSL153-28, BSL127-15, BSL142-17, BSL115-26 and BSL138-29) having the lowest *CtBSL* expression were grown up-to T_4 generation under controlled temperature in a green house (Fig. 1a, b). Out of seven two T_4 progenies BSL12-25-15-31-3 and BSL70-21-25-4-6 of BSL12-25 and BSL70-21, respectively, were selected on the basis of normalized *CtBSL* expression pattern and effective *CtBSL* silencing for phenotypic and genotypic analysis (Fig. 2).

3.2. CtBSL silencing mediated enhanced seed size and weight

The plants of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic guar lines were phenotypically identical and produced similar number of seeds per plant (Table 1), however, the seed mass (200 dry seed weight) of transgenic lines of guar was found to be significantly higher by 9.04 ± 3.1 % as compared to NTC. Therefore, next we observed the seed size during different stages of seed development and found that after 9 day post anthesis (DPA) the seed size of transgenic guar lines was significantly enhanced as compared to seed size in NTC, which remained visible even in mature seeds (Table 1, Fig S3). Further we observed a 9.01 ± 2.5 % and 5.57 ± 0.8 % increase in protein and sugar content, respectively, of transgenic guar lines as compared to NTC (Table 1).

Table 1
The characteristic summary of T_4 generation NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of clusterbean grown in field conditions.

Traits	Year	NTC	BSL12-25-15-31-3-3	BSL70-21-25-4-16-5
Height (cm)	2017	63.1 ± 1.6	66.5 ± 3.2	68.0 ± 3.6
	2018	70.9 ± 2.5	70.5 ± 2.6	67.2 ± 2.5
	2019	65.3 ± 1.4	60.2 ± 1.3	63.9 ± 1.4
	2020	68.5 ± 2.8	70.9 ± 1.5	70.5 ± 1.3
Pods/plant	2018	21.9 ± 1.4	23.8 ± 1.9	22.3 ± 1.3
	2019	25.7 ± 2.1	27.9 ± 1.6	26.4 ± 1.4
	2020	26.3 ± 1.1	24.4 ± 1.2	25.3 ± 1.7
Seeds/plant	2018	197.1 ± 12.6	214.2 ± 17.1	200.7 ± 11.7
	2019	231.3 ± 18.6	251.1 ± 14.4	237.6 ± 12.6
	2020	210.4 ± 8.8	219.6 ± 10.8	202.4 ± 13.6
Seeds/pod	2018	8.9 ± 1.5	8.8 ± 1.4	8.6 ± 1.2
	2019	9.1 ± 1.2	8.9 ± 1.3	8.7 ± 1.1
	2020	7.8 ± 1.2	8.1 ± 1.3	7.7 ± 1.2
Seed mass 200 dry seed/weight (mg)	2018	201.1 ± 3.9	276.4 ± 3.1	279.1 ± 2.5
	2019	225.6 ± 4.8	297.2 ± 3.6	298.8 ± 2.9
	2020	219.8 ± 2.6	286.4 ± 1.8	281.6 ± 3.1
Size (mm^2)	2018	1.4 ± 0.2	2.1 ± 0.1b	2.0 ± 0.2b
	2019	1.2 ± 0.2b	2.3 ± 0.2b	2.1 ± 0.3b
	2020	1.5 ± 0.2b	2.6 ± 0.3b	2.5 ± 0.2b
Protein (%w/w)	2018	21.8 ± 2.5	34.8 ± 1.9	31.2 ± 1.5
	2019	26.7 ± 3.6	33.7 ± 2.8	32.5 ± 2.7
	2020	25.8 ± 2.4	31.8 ± 2.6	33.2 ± 2.1

Traits	Year	NTC	BSL12-25-15-31-3-3	BSL70-21-25-4-16-5
Sugars (%w/w) (Total soluble sugars + Reducing sugars)	2018	41.3 ± 0.8a	51.3 ± 0.8	52.2 ± 1.2
	2019	45.6 ± 1.4a	53.4 ± 0.6	51.6 ± 0.1
	2020	44.4 ± 1.8b	50.4 ± 0.4	52.9 ± 0.3

Table 2
Galactomannan content analysis in seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of clusterbean

Sample	NTC	BSL12-25-15-31-3-3	BSL70-21-25-4-16-5
Galactose (% dry weight)	27.3 ± 1.3	35.5 ± 2.1	34.8 ± 1.5
Mannose (% dry weight)	54.7 ± 1.9	61.2 ± 1.7	59.9 ± 1.1
Ratio M:G	1.71	1.72	1.79
% Galactomannan (Galactose + Mannose)	82	96.7	94.7

The percent dry weight of galactose and mannose was enhanced by 8–9 % and 5–6 %, respectively, in seeds of BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic guar lines as compared to NTC (Fig. 3). The total percentage of galactomannan was found to be 82 %, 96.7 % and 94.7 % in seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of guar, respectively. Next, we measured the phytochemicals (phenolics and flavonoids) and observed twice higher phenolic and flavonoid content in seeds of BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of guar as compared to NTC (Fig. 4).

The change in seed size could be due to cell proliferation or cell expansion or both. So to understand the underlying mechanism we observed the thin section of cotyledon and seed coats under scanning electron microscope (SEM) and found a significant increase in cell number rather than cell size, which was nearly indistinguishable, in seeds of BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of guar as compared to NTC (Fig. 5, 6).

Upon analyzing the expression of genes related to cell division (*CtH2A, CtH2B, CtH3, CtH4, CtCYCA2;4, CtCYCD3;2, CtCDKE-1* and *CtCYCT1;5*) in response to cell proliferation and growth regulation (*CtERF, CtWRKY43, CtGRF5* and *CtGIF1*) we observed an significant increase in their expression levels in seeds of BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of guar as compared to NTC (Fig. 7), which signifies a positive correlation between *CtBSL* silencing and upregulation of cell division and growth

regulation genes. The *GAPDH* used as reference showed similar expression pattern in NTC as well as transgenic lines of lines (Fig. 7).

3.3. CtBSL forms a complex with repressor proteins

The yeast two-hybrid assay confirmed strong interaction of CtBSL protein with CtNINJA (repressor) protein, whereas, a very weak or no interaction was seen with another CtMYC2 (activator) protein. To confirm these results, we analyzed the interaction of CtJAZ3, a positive control, with both CtNINJA and CtMYC2 proteins. As shown in Fig. 8, CtJAZ3 showed a strong interaction with both activator and repressor proteins suggesting the formation of a repressor complex between CtBSL and other repressor proteins (Fig. 8).

3.4. Phytochemical activity analysis

We analyzed the anti-diabetic, anti-glycation end products (AGEs) and anti-lipase activity of phytochemicals in seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 transgenic lines of guar by percentage inhibition of *α-glucosidase*, *α-amylase*, *AGEs* and *lipase* enzymes and observed that the phytochemicals exhibited maximum inhibitory activity against *α-amylase* followed by *lipase* and *α-glucosidase* (Fig. 9).

4. Discussion

Guar or clusterbean is an economically important crop because of the presence of galactomannan in its seed endosperm which is used in paper, textile, pharmaceutical and cosmetic industries as a natural thickener and stabilizer. India is the largest producer of guar with 80 % of total world's production followed by the United States of America and Pakistan. However, recently a decline in its yield has been observed due to various biotic and abiotic stresses.

Seed size and weight are two major agronomic traits which are positively correlated to fitness of crop and its yield. The seedlings obtained from large sized seeds have better seedling survivorship as compared to smaller sized seeds (Orozco-Arroyo et al., 2015; Punjabi et al., 2018).

For crop improvement, conventional breeding programes are challenging in guar due to cleistagamous nature of flowers. Hence in this study we have used a reverse genetics approach to understand the role of *CtBSL* gene in guar plants. *CtBSL* is a member of BIG SEEDS gene family, having a conserved TIFY domain (Fig. S4). We observed that artificial RNAi mediated *CtBSL* silencing had a profound impact on cotyledon size, seed size, seed weight and other reserves in transgenic guar plants, which is consistent with the notion that seed size in legumes is correlated with cotyledon size (Lemontey et al., 2000). Further, the increase in cotyledon size of guar was mainly due to cell proliferation which is again in accordance with the previous studies in other plants (White 2006; Gonzalez et al., 2010, 2015; Hepworth and Lenhard, 2014).

Through *in silico* and *in vivo* studies in yeast, we observed that CtBSL a member of TIFY protein family interacts with repressor and co-repressor proteins to form a repressor complex which negatively regulates the expression of growth factors and other cell cycle genes in guar seeds. This confirms that in transgenic guar plants due to CtBSL silencing there is no formation of repressor complex which lead to increased expression or up-regulation of *CtCYCA2;4, CtCYCD3;2, CtCDKE-1*, *CtCYCT1;*5 cell cycle genes, *CtH2A, CtH2B, CtH3, CtH4* histone genes and *CtERF, CtWRKY43, CtGRF5* and *CtGIF1* transcription factors might have lead to increased primary cell proliferation and enhanced seed size in transgenic guar plants. Further the sub-cellular localization of CtBSL-GFP fusion protein (Fig. S5) confirms the role of CtBSL as putative transcription regulator. Similar results were also reported in previous studies where certain transcription factors negatively regulate the organ growth in certain plants including model plant *Arabidopsis* (Horiguchi et al. 2005; Pauwels et al., 2010; Cuéllar Pérez et al., 2014; Ge et al., 2016).

Plant produces various phytochemicals (phenolics and flavonoids), in response to external biotic and abiotic stress as a defense mechanism for their normal growth and development (Ali et al., 2006), which play therapeutic role in development of relevant medicines (Mohale et al., 2014; Tungmunnithum et al., 2018). Diabetes is one the major health concern and formation of AGEs is one of the major complications faced by diabetic patients in hyperglycemic conditions. Another big concern is obesity, as per world health organization (WHO) it leads to 3.4 million deaths every year, as per WHO (Ezzati et al., 2002; Chen et al., 2017). One way to control obesity is to inhibit pancreatic lipase activity which results in 50–70 % fat decomposition (Chen et al., 2017). In this study, we have reported the anti-diabetic, anti-advanced glycation end products (AGEs) and anti-lipase activity of phytochemicals (phenolics and flavonoids) extracted from *CtBSL* silenced transgenic guar plants. We observed that phytochemicals showed higher *α-amylase* inhibitory activity, which contributed more to its anti-diabetic potential, as compared to *α-glucosidase.*

Further, the phytochemicals extracted from seeds of BSL70-21-25-4-16-5 showed maximum inhibition (31.2 ± 1.68 % and 37.6 ± 1.36 %) followed by BSL12-25-15-31-3-3 (28.3 ± 1.28 % and 35.8 ± 1.12 %) as compared to wild (19.2 ± 1.61 % and 20.6 ± 1.42 %) guar plants, which is again consistent with the observations made by Grillo et al. (2008) and Hano et al. (2013).

So collectively, on the basis of results obtained we can conclude that *CtBSL* gene expression is strongly correlated to seed size in guar, which further linked to enhanced phytochemical biosynthesis and its anti-diabetic, anti-AGEs and anti-lipase potential.

5. Conclusion

The post-transcriptional silencing of *CtBSL* results in favourable agronomic traits like increased seed size and weight along with the enhanced accumulation of commercially important galactomannan in seeds of transgenic clusterbean plants. Further we observed a strong positive co-relation between seed size and phytochemical biosynthesis which is directly related to enhanced anti-diabetic, anti-AGEs and lipase inhibitory activities. Thus our finding paved a way of improving the yield of agronomically and

economically important clusterbean and galactomannan, respectively. Simultaneously our results indicate the potential of the phytochemicals obtained from clusterbean for the treatment of two important health issues, diabetes and obesity, which would be useful in development of effective strategies to significantly use clusterbean for therapeutic purposes.

Declarations

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Author contributions

Omika Thakur and Ramasare Prasad conceived and designed research. Omika Thakur conducted experiments, plant transformation and bioinformatics analyses. Ramasare Prasad supervised the project and contributed new reagents or analytical tools. Omika Thakur and Ramasare Prasad analyzed the data and wrote manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

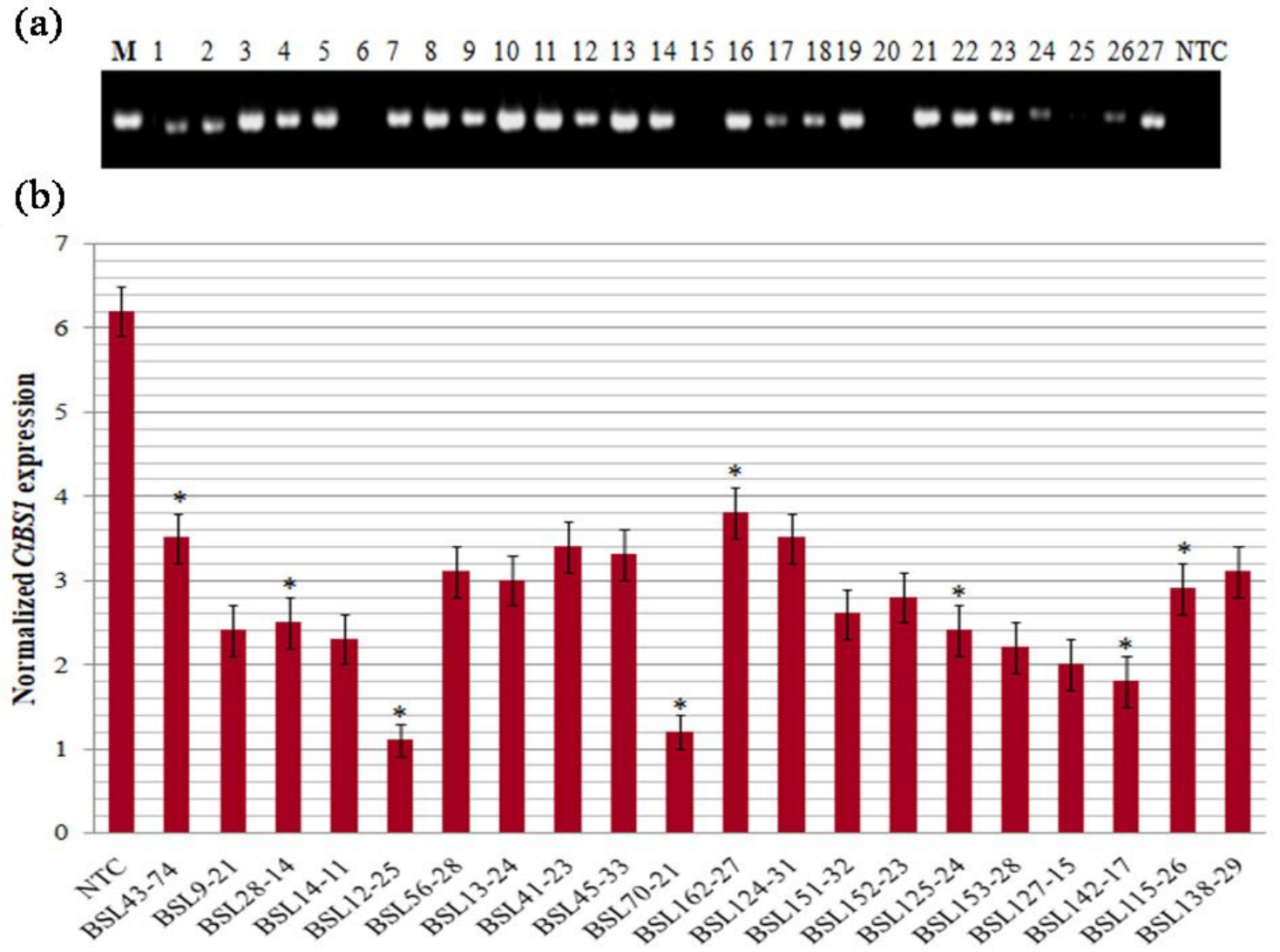

Figure 1

Screening of transgenic clusterbean plants. (a) RT-qPCR analysis of CtBSL gene expression in NTC and T0 transgenic plants of clusterbean using gene specific primers containing restriction sites; (b) qRT-PCR expression analysis of CtBSL in NTC and T1 transgenic seeds of clusterbean. *Indicates significant differences at p = 0.05 (n = 3).

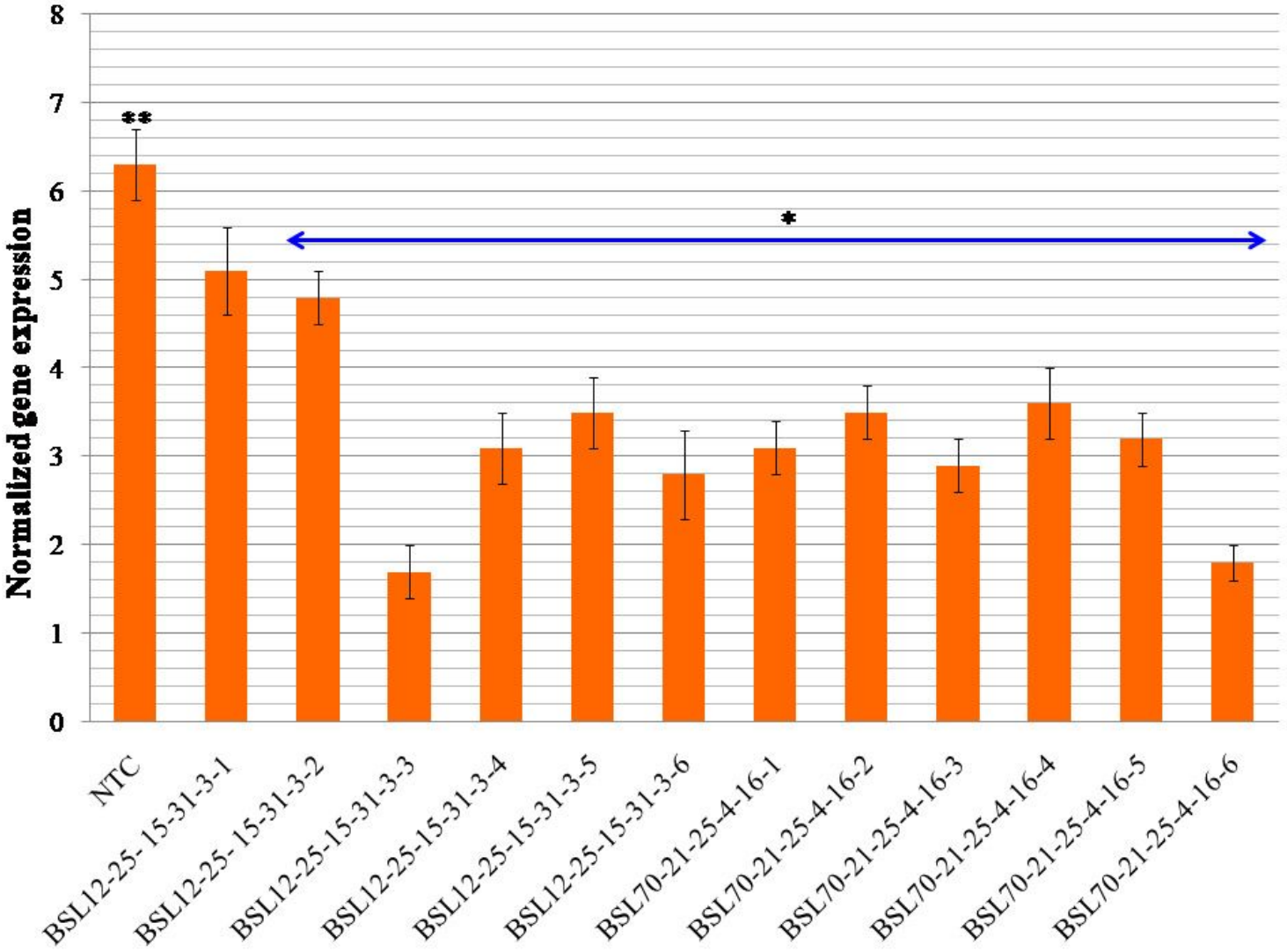

Figure 2

The RT-qPCR analysis of CtBSL expression in T4 transgenic progenies of BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 and NTC in clusterbean. Shown are the mean ± S.D. for n=3. **, Student's t-test, $p<0.001$.

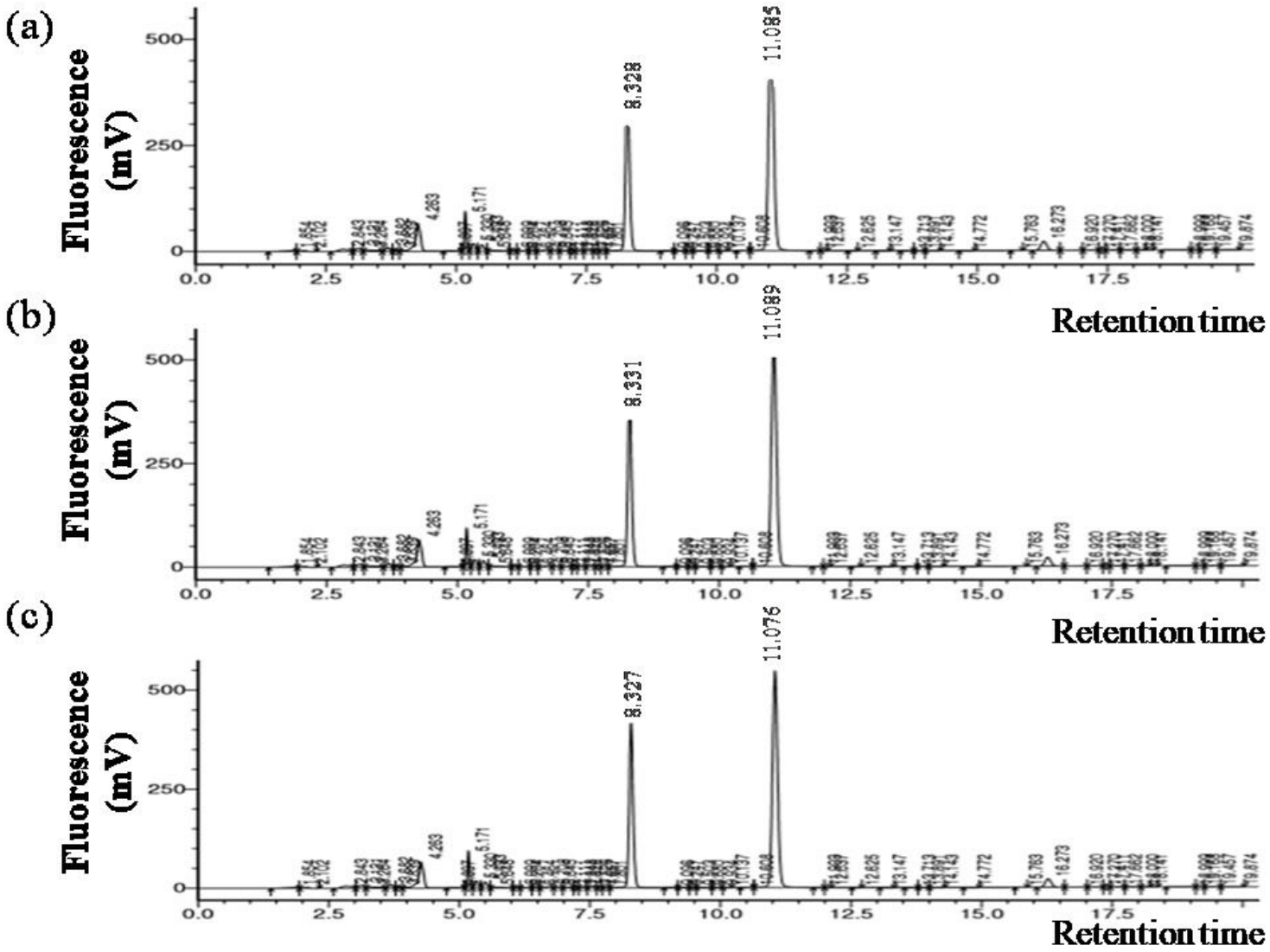

Figure 3

Reverse phase high performance liquid chromatography (RP-HPLC). (a) Separation of galactose, mannose and internal standard myo-inositol in NTC; (b) BSL12-25-15-31-3; (c) BSL70-21-25-4-16 transgenic lines of clusterbean.

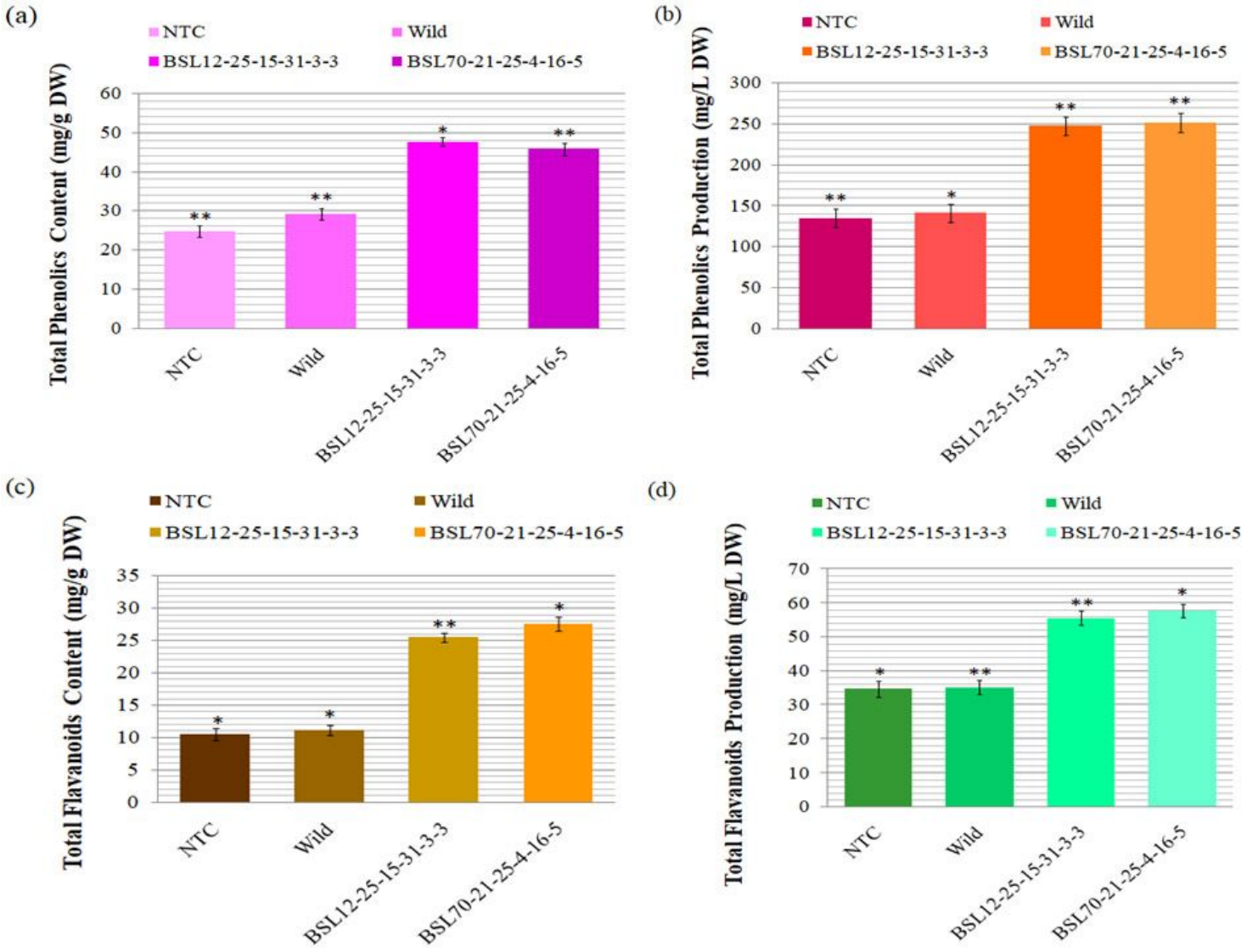

Figure 4

Estimation of phenolics and flavonoid accumulation. (a) Total phenolic content (mg/g DW); (b) Total phenolic production (mg/L DW); (c) Total flavonoid content (mg/g DW); (d) Total flavonoid production (mg/L DW) in NTC, wild, BSL12-25-15-31-3 and BSL70-21-25-4-16 transgenic lines of clusterbean. Values represent means ± standard errors from triplicates.

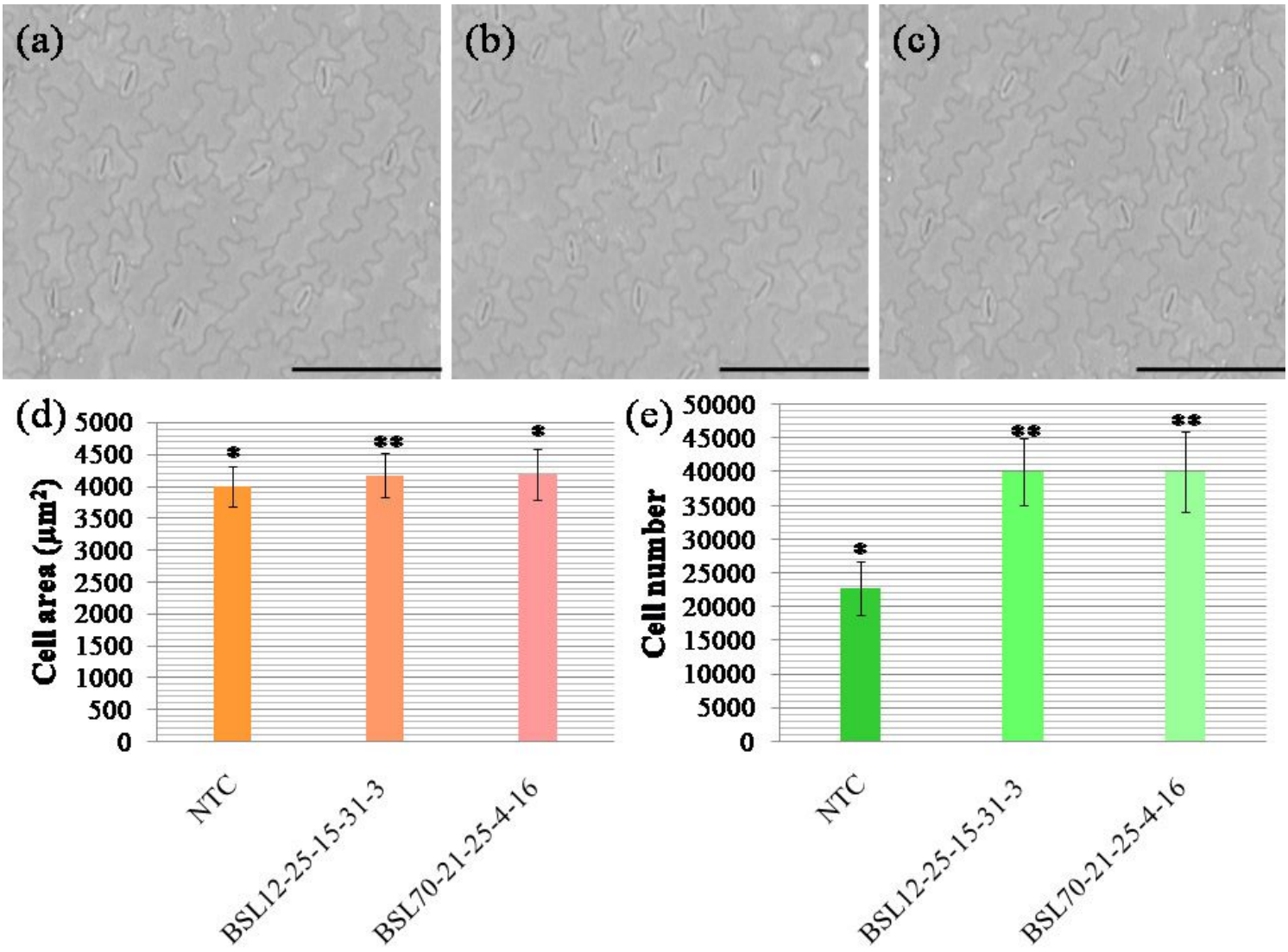

Figure 5

Cotyledon morphology in seeds of NTC, BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 T4 transgenic lines of clusterbean. (a-c) SEM images of cotyledons in seeds of NTC, BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 transgenic plants; (d-e) Quantification of epidermal cell area and cell number in seed coats of NTC, BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 T4 transgenic lines of clusterbean. Shown are the mean ± S.D. for n=5. **, Student's t-test, $p<0.001$. Scale bar, 200 µm.

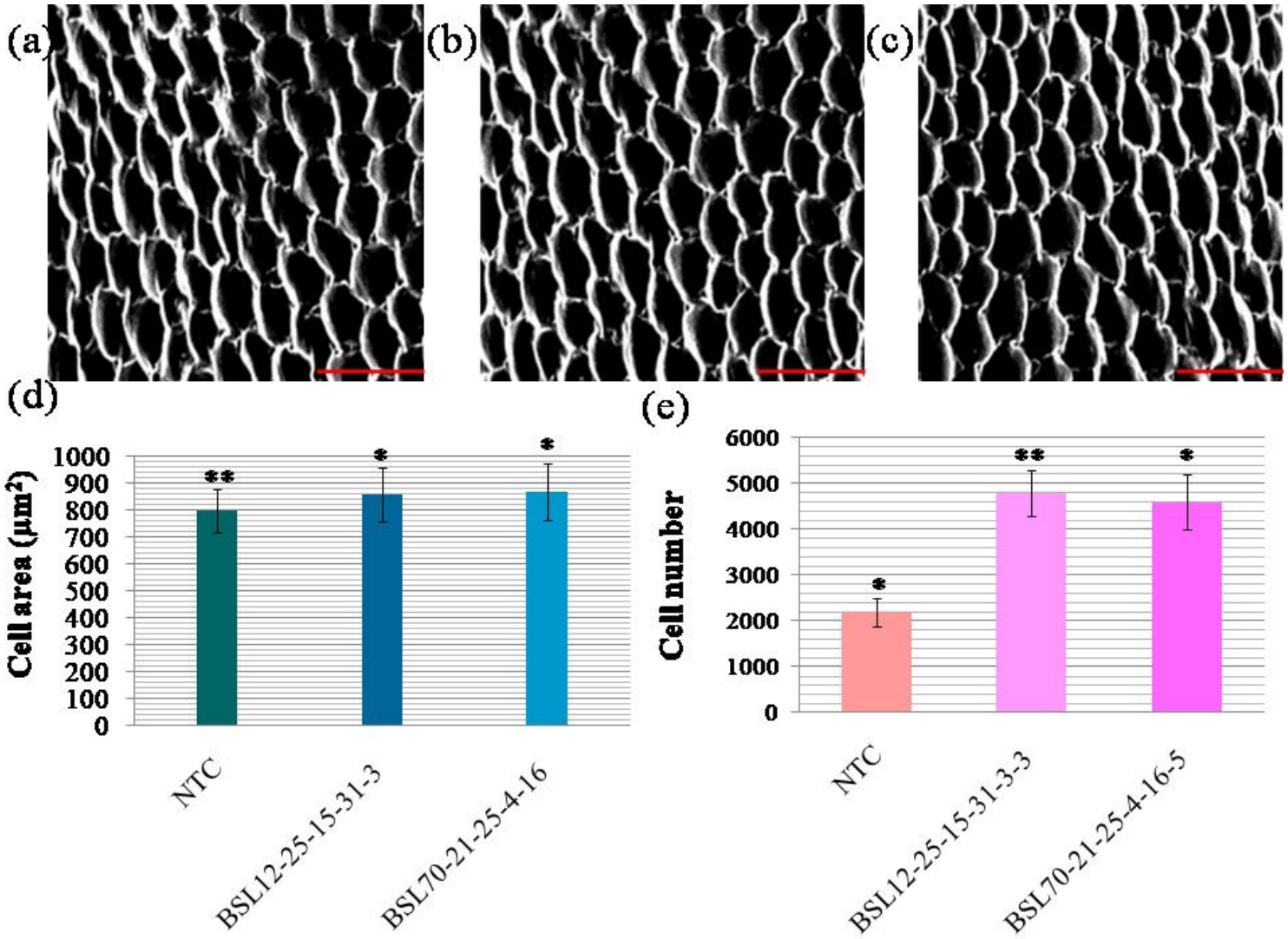

Figure 6

Cell size is not altered in CtBSL silenced plants of clusterbean. (a-c) FE-SEM images of seed coat (45 DPA) epidermal cells in NTC, BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 T4 transgenic lines of clusterbean; (d-e) Quantification of epidermal cell area and cell number in seed coats of NTC, BSL12-25-15-31-3-3, BSL70-21-25-4-16-5 T4 transgenic lines of clusterbean. Shown are the mean ± S.D. for n=5. **, Student's t-test, $p<0.001$. Scale bar, 100 µm.

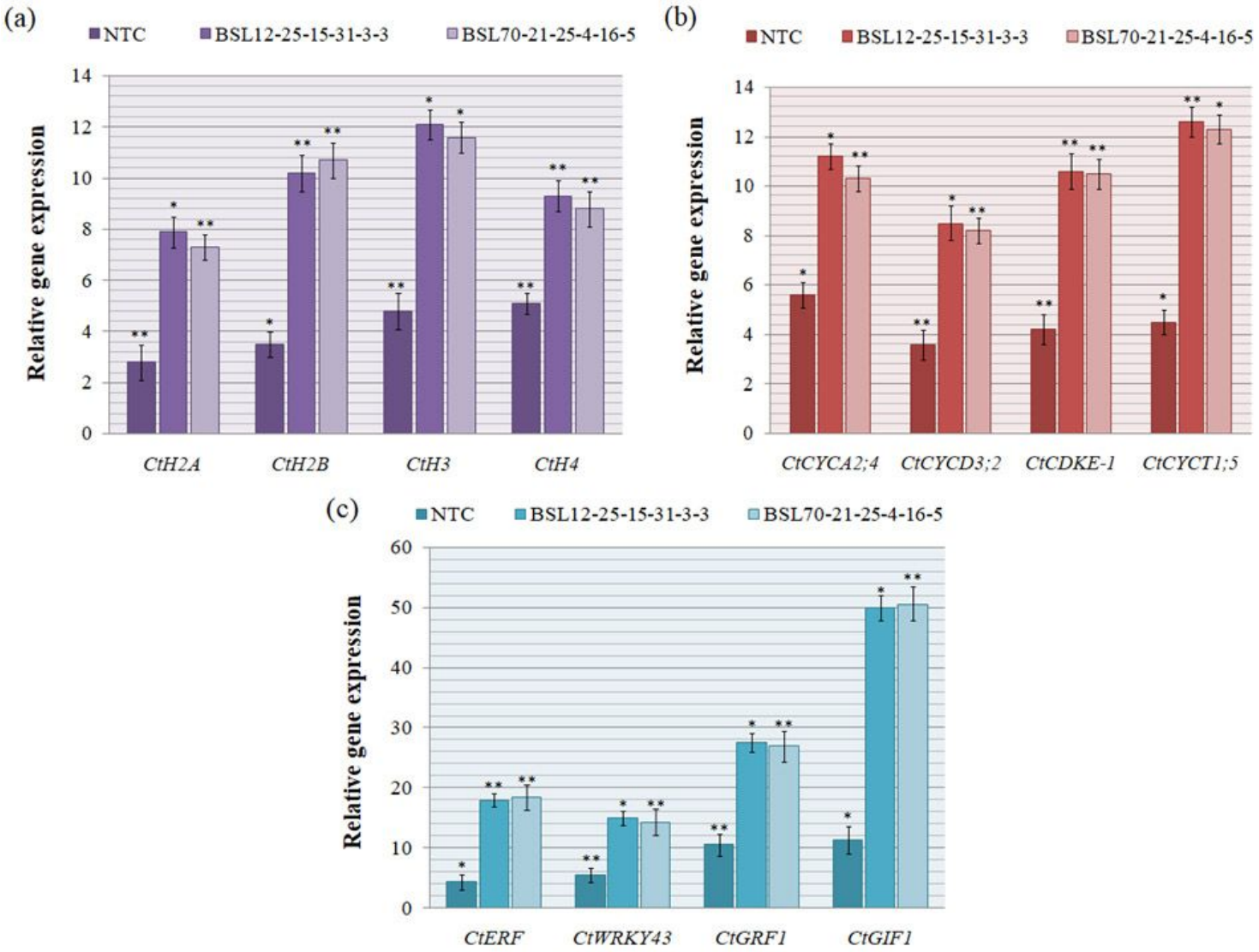

Figure 7

The RT-qPCR expression analysis. (a) CtH2A, CtH2B, CtH3, CtH4 histone gene expression. (B) CtCYCA2;4, CtCYCD3;2, CtCDKE-1, CtCYCT1;5 CCC gene expression. (C) CtERF, CtWRKY43, CtGRF5, CtGIF1 gene expression in seeds of NTC, BSL12-25-15-31-3-3 and BSL70-21-25-4-16-5 T4 transgenic lines of clusterbean. Shown are the mean ± S.D. for n=3. **, Student's t-test, $p<0.001$.

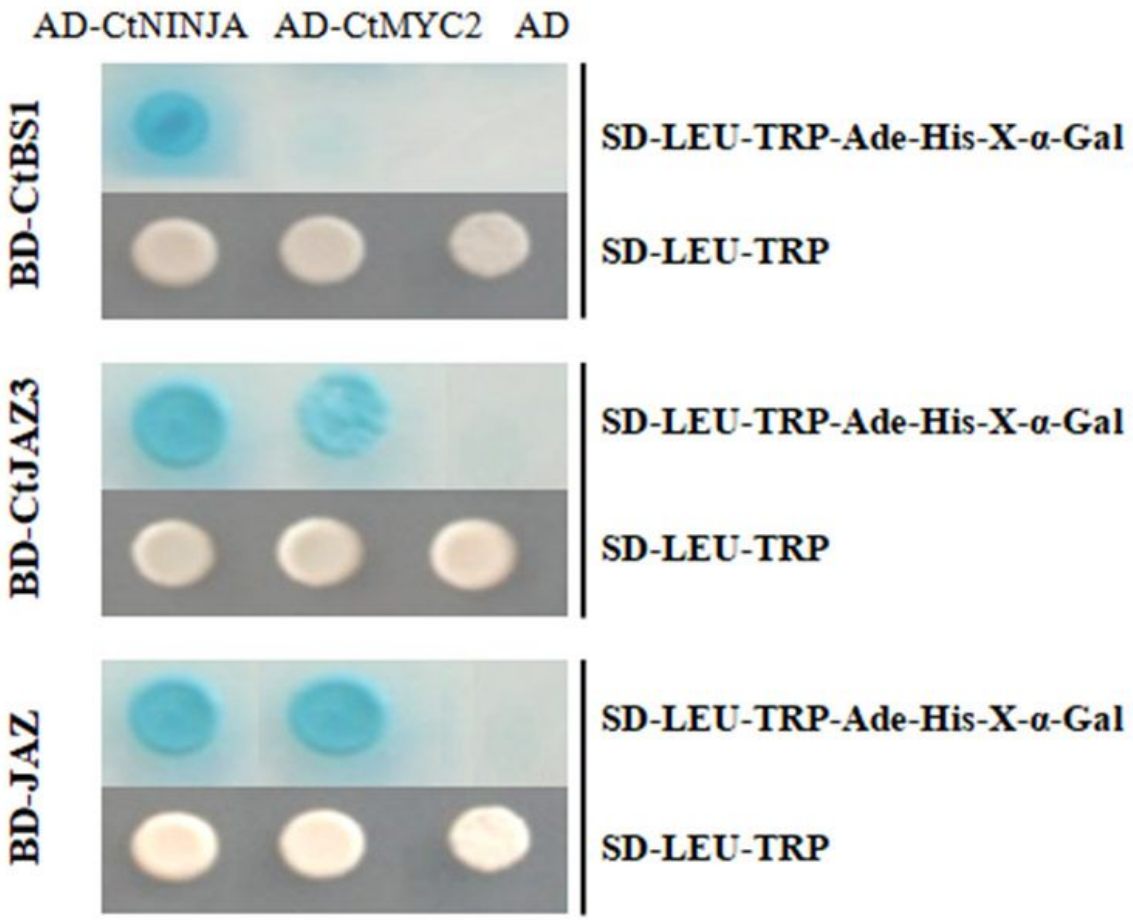

Figure 8

Screening of CtBSL interactions using yeast two hybrid assay. (a) CtBSL interacts with CtNINJA repressor protein but not with CtMYC2 activator protein; (b) The control CtJAZ3 interacts with both CtNINJA repressor protein and CtMYC2 activator protein; (c) Interaction of JAZ-Domain with CtNINJA and CtMYC2 protein.

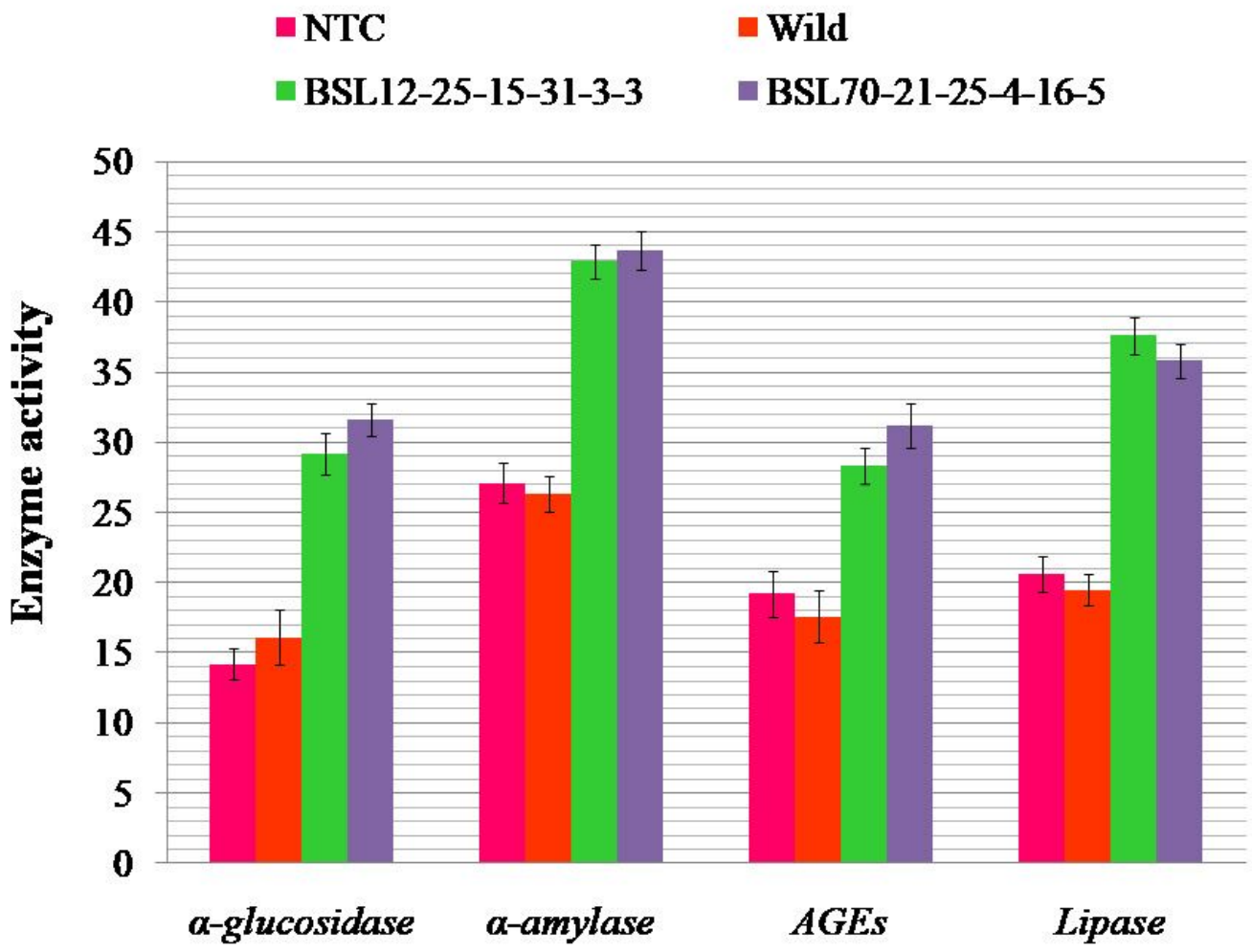

Figure 9

Anti-diabetic, anti-AGEs, lipase inhibitory potential of NTC, wild, BSL12-25-15-31-3 and BSL70-21-25-4-16 transgenic lines of clusterbean.

Supplementary Files

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