

PLANT GROWTH AND GLUTAMINE SYNTHASE GENE *SCGS1* EXPRESSION IN SUGARCANE (*SACCHARUM* L. SPP. HYBRIDS) UNDER DIFFERENT NITROGEN LEVELS

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Abstract. The main aim of this study was to investigate the responses of agronomic traits, photosynthesis and glutamine synthase (GS) expression in different sugarcane (*Saccharum* L. spp. hybrids) varieties under different nitrogen levels. Sugarcane varieties GT11 (nitrogen inefficient) and GXB9 (nitrogen efficient) were planted at three levels of nitrogen supply, e.g. 0 kg/ha (low nitrogen level, L-N), 150 kg/ha (medium nitrogen level, M-N) and 300 kg/ha (high nitrogen level, H-N) of urea application. The agronomic traits, photosynthesis, and GS activity and expression were determined at different growth stages of sugarcane. The results showed that the plant height, stalk diameter, leaf area, chlorophyll content, net photosynthetic rate, GS activity and GS1 protein expression in GT11 increased with the increase of nitrogen application, while the plant height, stalk diameter, chlorophyll content, net photosynthetic rate, GS activity and GS1 protein expression in GXB9 reached the maximum on medium nitrogen level. The leaf area of GXB9 was always higher than that of GT11 on the same nitrogen level. Under the low and medium nitrogen levels, the activities of GS and expression of GS1 protein were higher in GXB9 than GT11. The transcription expression levels of *scGS1.a* and *scGS1.b* were down-regulated while that of *scGS1.c* up-regulated by the increase of nitrogen application level. In conclusion, the nitrogen inefficient sugarcane variety needs more nitrogen application, but excessive nitrogen application is not good for the nitrogen efficient sugarcane variety.

Keywords: *physiological characteristic, photosynthesis, molecular response, chlorophyll, nitrogen utilization*

Abbreviations: GS: Glutamine synthase; H-N: High nitrogen; L-N: Low nitrogen; M-N: Medium nitrogen

Introduction

Sugarcane is the most important sugar crop and a regenerative energy crop grown worldwide in tropical and subtropical areas, which contributes more than 90% of the total sugar in China (Li and Yang, 2015). Nitrogen is the main nutrition element of crops, it is required for protein synthesis, nucleic acid, phospholipid and many organic substances, and also plays an important role in photosynthesis. Previous studies have indicated that application of nitrogenous fertilizer is considered a significant measure to guarantee the productivity in sugarcane. However, excessive application of nitrogen fertilizer is common in sugarcane commercial production in China, and the application rate of urea is usually 500-700 kg/ha or higher, much higher than the world average. In our previous study, the effects of different nitrogen application levels (150, 300, 600 kg/ha urea) on

key enzymes of nitrogen metabolism and contents of related active substances in different sugarcane varieties were investigated, which indicated that the nitrogen metabolism of sugarcane was significantly enhanced with the increase of nitrogen application amount within a certain range of nitrogen application level (150-300 kg/ha urea) (Zhang et al., 2015). Over application of nitrogen fertilizer not only declines nitrogen use efficiency and increases the cost of sugarcane production, but also results in a series of negative effects such as cane quality degradation and agricultural environmental pollution (Bijay et al., 1995; Li et al., 2016).

Nitrogen assimilation is a vital process controlling plant growth and development. Plants absorb nitrogen in nitrate, ammonium and amino acid forms, and ultimately transform it into ammonium form. Majority of the organic nitrogen in plants is derived from the assimilation of ammonia into the amide position of glutamine by the enzyme GS (Lea and Mifflin, 2011). GS is a key enzyme involved in the nitrogen assimilation and metabolism of nitrogen compounds, and is also the first enzyme to transformed inorganic nitrogen into organic nitrogen in plants. GS pathway of ammonia assimilation is of crucial importance for crop growth and productivity. In most plant leaves, GS mainly exists in the form of GS1 and GS2; the expression of GS1 was evidently affected by the concentration and the form of nitrogen, while the expression of GS2 was mainly affected by light (Edwards et al., 1990). For higher plant, GS1 are mainly distributed in the companion cells of leaf vascular tissue phloem. The main functions of GS1 are assimilating ammonia produced by endogenous protein degradation, amino acid catabolism and stored nitrogen source, involving in the transfer of nitrogen source, and reusing finally (Swarbreck et al., 2011). GS1 was generally encoded by 3-5 genes, for Arabidopsis, tobacco and maize, GS1 was encoded by at least five homologous genes (Keegstra and Cline, 1999; Keiki et al., 2004; Li et al., 2006; Lightfoot et al., 1988; Lothier et al., 2011; Tingey et al., 1988), and it was encoded by three genes in rice (May and Soll, 1999). Previous researches indicate that GS1.a mainly participates in nitrogen remobilization during leaf senescence and nitrogen migration under low nitrogen condition, and affects grains' size, number and grouting (Antoine et al., 2006; Bernard et al., 2008; Kusano et al., 2011; Mayumi et al., 2005). GS1.b is mainly responsible for the primary NH_4^+ assimilation in roots, nitrogen remobilization during leaf ageing, assisting in the primary nitrogen assimilation of GS2 (Bernard et al., 2008; Kazuhiro et al., 2013). GS1.c can ease the ammonia poisoning, and also remission nitrogen assimilation in grain growth period (Goodall et al., 2013). Nogueira et al. (2005) have identified and characterized the *GS1* (*scGS1.a*, *scGS1.b* and *scGS1.c*) in sugarcane.

To our knowledge, there is no report about the impact of different nitrogen levels on the *GS1* gene expression in sugarcane. The purpose of this study was to investigate the effects of different nitrogen application rates on plant growth and photosynthesis in different sugarcane varieties, the response of *GS1* in sugarcane to nitrogen levels, and further reveal the role of GS1 in nitrogen metabolism and the related regulation mechanism, to provide a reference for efficient nitrogen utilization and regulation in sugarcane.

Materials and methods

Experimental materials

The experiment was conducted at College of Agriculture, Guangxi University in Nanning, China. The sugarcane variety GT11 (Nitrogen inefficient variety) and GXB9

(nitrogen efficient variety) were used for the experiment (Zhang et al., 2015). The soil used for experiment was red soil with pH 7.32, organic matter 4.23 g/kg, total nitrogen 0.27 g/kg, total phosphorus 0.33 g/kg, total potassium 7.85 g/kg, alkali-hydrolyzable nitrogen 21.55 g/kg, effective phosphorus 8.4 mg/kg, and available potassium 96 mg/kg. Urea (N 46%) was used as the nitrogenous fertilizer.

Plant culture and nitrogenous fertilizer application

Single-budded seedcane sets of the two sugarcane varieties were planted in sands for germination under warm and wet condition on March 3, 2016. After 45 days, the healthy and consistent plants were selected and transplanted into plastic pots of 36.3 × 30 cm (height × diameter) filled with 20 kg soil, and two plants of the same variety were grown in each pot.

Three levels of nitrogen supply were set and applied after transplanting, that is, 0, 150 and 300 kg/ha, or 0, 1.55 and 3.10 g urea per pot were applied at seedling stage (May 15), referred to as the low nitrogen, medium nitrogen and high nitrogen levels, respectively. There was a low nitrogen content (total nitrogen 0.27 g/kg, alkali-hydrolyzable nitrogen 21.55 g/kg) in the soil used in the experiment, which could not be ignored. Therefore, we used the low nitrogen level to represent the actual nitrogen level in the soil even if we gave no nitrogen in the soil. Therefore, the experiment consisted of six treatments (two varieties and three nitrogen levels), and 9 replicates were arranged in a completely randomized block design, so total had 54 pots (*Fig. 1*).

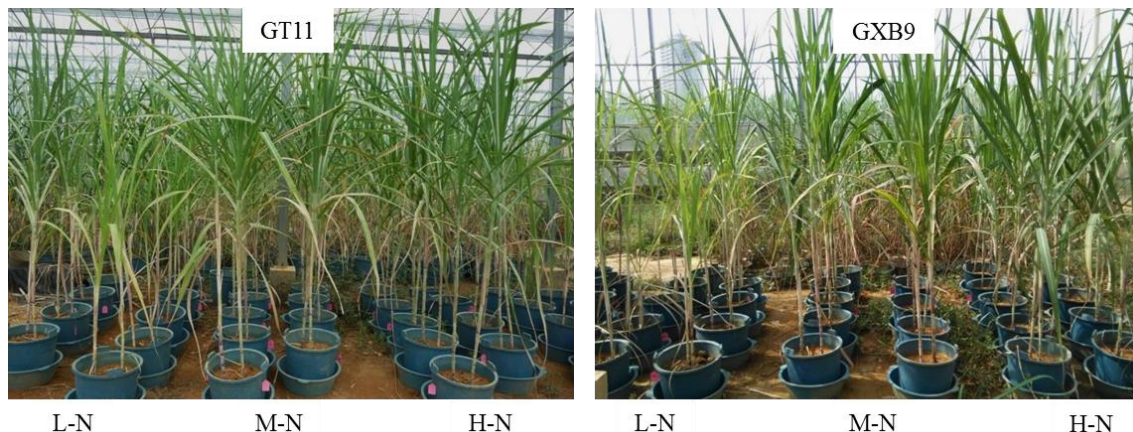


Figure 1. Growth photos of two sugarcane varieties (GT11 and GXB9) under three nitrogen application levels at sampling day (2016-07-12)

Sampling and assay

The leaf + 1 (the top visible dewlap leaf) was sampled on June 10, July 12, August 12, and September 15, respectively. Part of the fresh leaf sample was used for determination of GS activity with the method as described by Wei (2005), and the rest was frozen in liquid N₂ and kept at -80°C before use.

Investigation of agronomic traits

Nine plants were chosen randomly for agronomic trait investigation at sampling days after treatment. The plant height and stem diameter were estimated with conventional

methods. Leaf area of leaf + 1 was estimated using CI-203 Handheld Leaf Area Meter (CID Bio-Science, Inc., Camas, USA) and the leaf chlorophyll was estimated by SPAD readings measured with SPAD-502 chlorophyll meter (Konica Minolta, Inc., Tokyo, Japan).

Assay of photosynthetic rate

Net photosynthetic rate was measured using a LC Pro-SD portable photo system unit (Opti-Sciences Inc., Hudson, USA) at a photon irradiance of 1504 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The measurement was conducted at a gas flow rate 200 $\mu\text{mol s}^{-1}$. Nine plants in each treatment were randomly selected for measurement from 9:00 to 11:00 am in the morning of September 3 when it was sunny.

Quantification of *scGS1* gene expression on transcription level

The relative expression of *scGS1.a* (AY835453), *scGS1.b* (AY835454) and *scGS1.c* (AY835455) was quantified at the sampling days (June 10, July 12, August 12, and September 15, respectively) after treatment. The expression of *scGS1* in sugarcane leaf + 1 was performed in randomly selected 9 plants with a Light Cycler® 480II (Roche Applied Science, Basel, Switzerland) using the SYBR Premix Ex Tap™ II (TaKaRa, Dalian, China) in accordance with the product manual. The GAPDH gene of sugarcane (accession number EF189713) was used as an internal control to quantify the relative transcript level. Specific primers for those genes were designed using the Primer Premier 5 software based on their cDNA sequences (*Table A1* in the *Appendix*). The relative level of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ formula (Livak and Schmittgen, 2001).

Western blot analysis of *scGS1* protein in sugarcane

Total soluble protein was extracted from sugarcane leaf with 100 mM Tris (pH 7.8, containing 10 mM MgCl_2 , 100 mM 2-mercaptoethanol, 5% Polyvinylpyrrolidone) (Nogueira et al., 2005), and quantified using the method of Bradford (1976). For Western blot, denatured proteins were fractionated by SDS-PAGE [12.5% (w/v) polyacrylamide] according to Laemmli (1970) and transferred to nitrocellulose membrane as described by Towbin et al. (1979). Anti-GS1 primary polyclonal antibody was used to detect GS1 protein. Protein-antibody complexes were located using horse reddish peroxidase, and conjugated goat anti-rabbit immunoglobulin G. Pierce® ECL Western Blotting Substrate (Thermo, U.S.A.) was used for development and then observed under FluorChem HD2 (Alpha, Protein Simple, U.S.A.). The GS1 polyclonal antibody (AS08295, Agrisera, Sweden) and secondary antibody (Goat anti-rabbit IgG, HRP conjugated, CoWin Biosciences, Beijing, China) were purchased in a biological reagent company.

Data analysis

All the data were analyzed using Microsoft Excel 2010 and SPSS 21.0. ANOVA test was used to compare the mean values of each treatment. Significant differences between the means of parameters were determined by using the LSD test ($P < 0.05$).

Results

Effects of different nitrogen levels on agronomic traits in different sugarcane varieties

The data in *Table 1* show the growth rate of two sugarcane varieties under medium and high nitrogen levels increased with the course of growth, and the highest growth rate occurred between August and September. In general, the plant height and stem diameter in the variety GT11 increased with the level of nitrogen application. For the variety GXB9, the highest plant height and stem diameter were recorded on medium nitrogen level (*Table 1*). The leaf area of two sugarcane varieties significantly increased with the level of nitrogen application (*Table 1*). Under the same nitrogen level, the leaf area is basically characterized by GXB9 > GT11 (*Fig. A1* in the *Appendix*).

Table 1. *Effects of different nitrogen levels on agronomic traits in different sugarcane varieties*

Variety		GT11			GXB9		
Date nitrogen levels		L-N	M-N	H-N	L-N	M-N	H-N
2016-06-10	Plant height (cm)	27.1 ± 3.4 ^a	32.3 ± 3.7 ^a	32.7 ± 4.5 ^a	26.9 ± 3.1 ^a	27.2 ± 3.5 ^a	27.5 ± 3.5 ^a
	Stalk diameter (mm)	10.5 ± 0.7 ^c	11.9 ± 0.8 ^b	12.7 ± 0.7 ^a	7.8 ± 0.8 ^c	9.5 ± 0.7 ^a	8.6 ± 0.7 ^b
	Leaf area (cm ²)	80.1 ± 8.4 ^a	89.21 ± 8.52 ^a	89.2 ± 7 ^a	88 ± 9.6 ^a	87.6 ± 2.5 ^a	87.8 ± 7.7 ^a
	SPAD	36.7 ± 0.9 ^a	38.5 ± 0.8 ^a	40.4 ± 1.4 ^a	39.9 ± 1.8 ^b	45.2 ± 2 ^a	42 ± 1.5 ^{ab}
2016-07-12	Plant height (cm)	43.8 ± 1.8 ^c	49.2 ± 2.3 ^b	55.5 ± 2.5 ^a	43.6 ± 3 ^b	49.8 ± 2.6 ^a	48.7 ± 3.2 ^a
	Stalk diameter (mm)	15.6 ± 0.6 ^c	16.3 ± 0.9 ^b	17.7 ± 0.6 ^a	13.4 ± 0.6 ^c	15.5 ± 0.6 ^a	14.9 ± 0.7 ^b
	Leaf area (cm ²)	317 ± 17.4 ^a	330 ± 18.5 ^a	343 ± 11.4 ^a	353 ± 15.7 ^a	366 ± 31.4 ^a	386 ± 36.1 ^a
	SPAD	34 ± 1.7 ^b	37.1 ± 1.4 ^{ab}	39.8 ± 1.5 ^a	35.2 ± 1.8 ^b	47.8 ± 1.9 ^a	48 ± 1.7 ^a
2016-08-12	Plant height (cm)	73.2 ± 4.3 ^c	81.3 ± 5.2 ^b	93.8 ± 5.5 ^a	82.7 ± 4.6 ^b	97.5 ± 4.5 ^a	95.5 ± 3.9 ^a
	Stalk diameter (mm)	13.9 ± 0.7 ^b	15.3 ± 0.8 ^a	15.1 ± 0.7 ^a	14.5 ± 0.8 ^b	16.9 ± 0.7 ^a	17 ± 0.7 ^a
	Leaf area (cm ²)	313 ± 15.2 ^c	393 ± 24.9 ^b	537 ± 22 ^a	356 ± 10 ^c	467 ± 20 ^b	582 ± 27.5 ^a
	SPAD	28 ± 1.8 ^b	39.1 ± 2. ^a	39 ± 2.3 ^a	29.9 ± 1.2 ^b	51.8 ± 1.8 ^a	49.6 ± 1.7 ^a
2016-09-15	Plant height (cm)	102 ± 5.6 ^c	146 ± 2.4 ^b	154 ± 4.7 ^a	113 ± 4.1 ^c	175 ± 5 ^a	165 ± 4.1 ^b
	Stalk diameter (mm)	14.5 ± 0.6 ^c	15.5 ± 0.9 ^b	16.2 ± 0.6 ^a	14.7 ± 0.6 ^c	17.9 ± 0.6 ^a	17.1 ± 0.7 ^b
	Leaf area (cm ²)	263 ± 16 ^c	388 ± 20 ^b	553 ± 25 ^a	343 ± 30 ^c	476 ± 30 ^b	609 ± 23 ^a
	SPAD	23.2 ± 0.8 ^b	35 ± 0.8 ^a	37 ± 1.4 ^a	30.9 ± 1.1 ^b	40.6 ± 0.8 ^a	42.2 ± 1 ^a

Data are presented as mean ± SE, and data points with different letters are significantly different ($P < 0.05$) between different nitrogen levels from the same sugarcane variety at the same time. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

Effect of different nitrogen levels on chlorophyll content in different sugarcane varieties

Overall, the chlorophyll relative content in the variety GT11 is characterized by high nitrogen > medium nitrogen > low nitrogen levels, increased with the levels of nitrogen application. The chlorophyll relative content in the variety GXB9 showed the order of medium nitrogen > high nitrogen > low nitrogen levels (*Table 1*).

Effect of different nitrogen levels on net photosynthetic rate in different sugarcane varieties

At booming stage of sugarcane (September 3), the net photosynthetic rate in the variety GT11 was significantly higher in the high nitrogen treatment than the low

nitrogen treatment. For the variety GXB9, the net photosynthetic rate was significantly higher under medium nitrogen level than low and high nitrogen levels. Under low and medium nitrogen levels, the net photosynthetic rate was higher in the variety GXB9 than the variety GT11, but had no significant difference between them under high nitrogen level (*Fig. 2*).

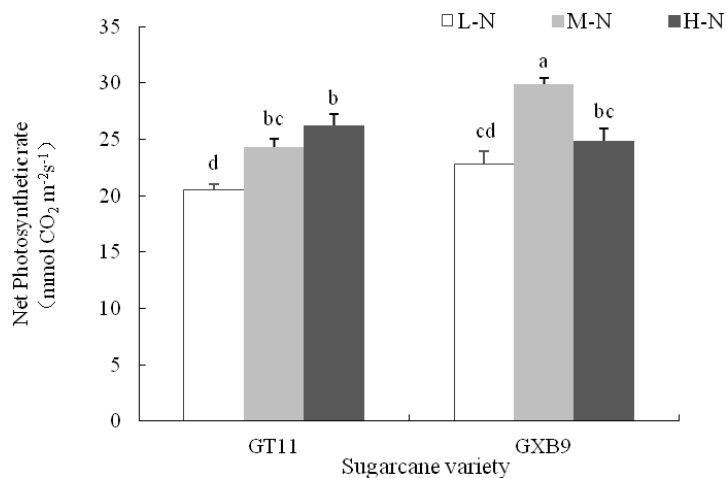
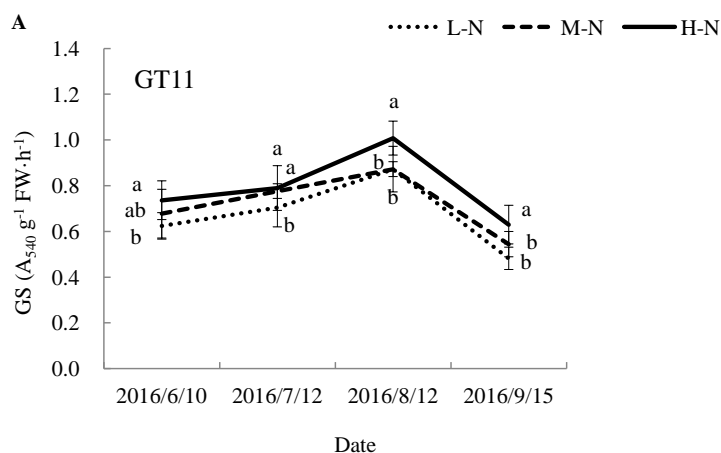


Figure 2. Photosynthetic rate in leaves of two sugarcane varieties under different nitrogen levels measured on September 15, 2016. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

Effect of different nitrogen levels on GS activity in different sugarcane varieties

The data in *Figure 3* showed that the GS activity in leaves of the variety GT11 increased with the level of nitrogen application (*Fig. 3A*), and peaked on August 12 in all the treatments except for the high nitrogen treatment in GXB9. The GS activity was significantly higher on high nitrogen level than that on low and medium nitrogen levels. The highest GS activity was recorded in the variety GXB9 under medium nitrogen level on August 12 (*Fig. 3B*). Under low and medium nitrogen levels, the GS activity was always higher in the variety GXB9 than the variety GT11 (*Fig. A2*), and this may be related to the higher biological nitrogen fixation ability and nitrogen efficiency of the variety GXB9.



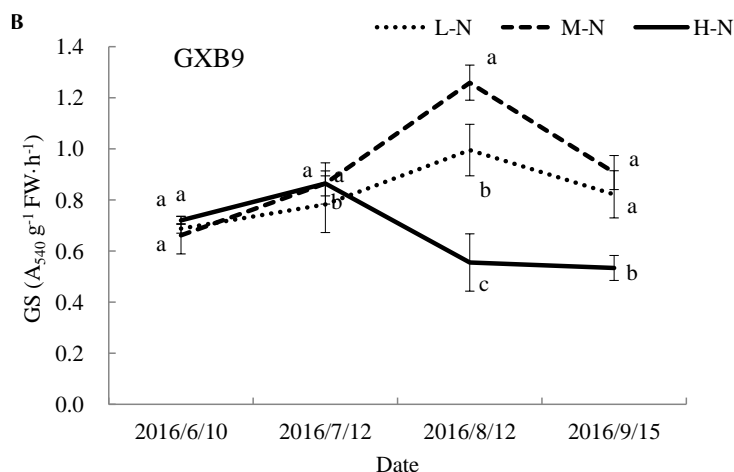


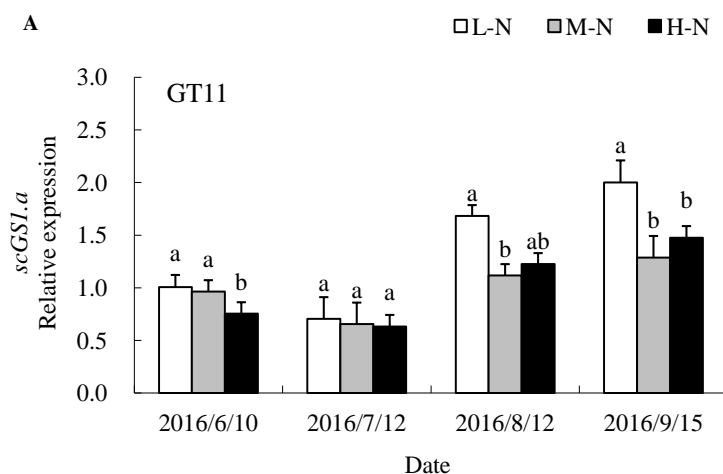
Figure 3. GS activity in leaves of two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

Effect of different nitrogen levels on *scGS1* expression in different sugarcane varieties

From Figure 4, we can see that for most treatments, *scGS1.a* expression was higher in August and September than June and July. *scGS1.a* expression tended to decrease with the level of nitrogen application in both sugarcane varieties. Under low nitrogen level, *scGS1.a* expression was higher in GXB9 than GT11 (Fig. A3A), while under high nitrogen level, *scGS1.a* expression in GXB9 is lower (Fig. A3B).

From Figure 5, we can find that in June and July, *scGS1.b* expression tended to decrease with the level of nitrogen application in both two sugarcane varieties except for that in GT11 in August and September when it showed the highest under medium nitrogen level.

The data in Figure 6 showed that the *scGS1.c* expression tended to increase with the level of nitrogen application from June to September and the growth course from June to August in both sugarcane varieties. For different sugarcane varieties, the *scGS1.c* expression showed the order of GXB9 > GT11 under the same nitrogen level (Fig. A4).



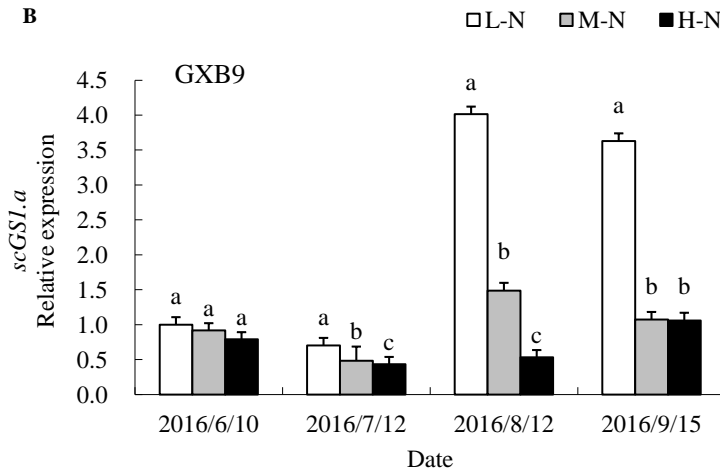


Figure 4. Expression of *scGS1.a* mRNA in leaves of two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

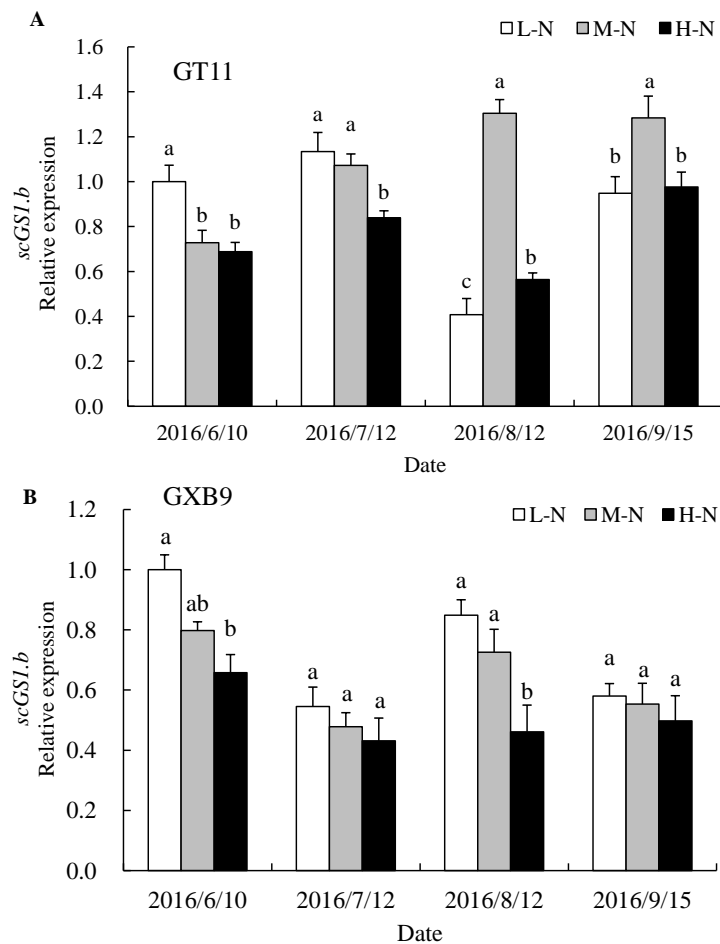


Figure 5. Expression of *scGS1.b* mRNA in two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

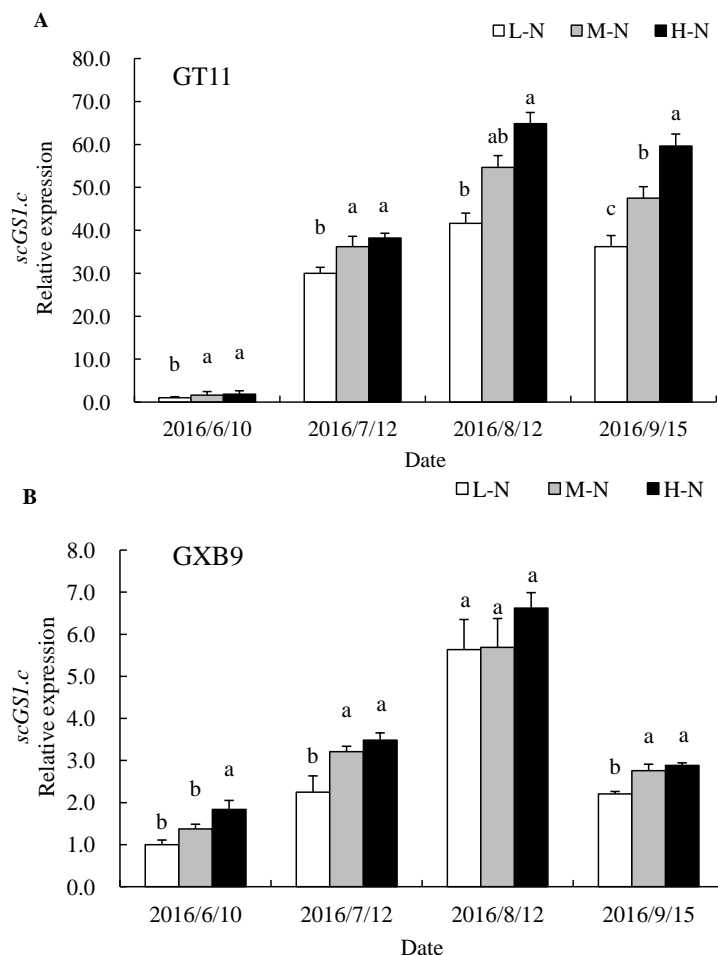


Figure 6. Expression of *scGS1.c* mRNA in two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date. L-N, low nitrogen level; M-N, medium nitrogen level; H-N, high nitrogen level

Influence of different nitrogen levels on GS1 protein expression in different sugarcane varieties

The GS1 protein expression in the variety GT11 increased with the level of nitrogen application, and that in GXB9 reached the maximum under medium nitrogen level (Figs. 7 and 8). The GS1 protein expression is higher in GXB9 than GT11 under low and medium nitrogen levels (Fig. A5).

Discussion

Nitrogen is the biggest nutrient element for crop growth and development demand. However, excessive nitrogen fertilizer application will reduce plant nitrogen utilization efficiency and cause serious environmental pollution. Zhou et al. (2006) found that different sugarcane varieties responded differently to different nitrogen levels, and the sugarcane variety RB72-454 with high biological nitrogen fixation ability has better growth potential than others under low nitrogen condition. In this study, the plant height, stem diameter increased with the level of nitrogen application in the nitrogen

inefficient sugarcane variety GT11 while showed the highest at medium nitrogen level in the nitrogen efficient sugarcane variety GXB9 with high biological nitrogen fixation ability, which suggested that the responses of different sugarcane varieties to nitrogen application are varied, and nitrogen efficient sugarcane variety needs less nitrogen fertilizer application.

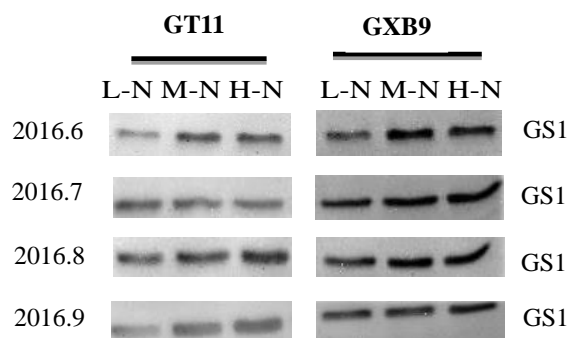


Figure 7. Western blotting analysis of sugarcane *GS1* protein under different nitrogen levels. *L-N*, low nitrogen level; *M-N*, medium nitrogen level; *H-N*, high nitrogen level

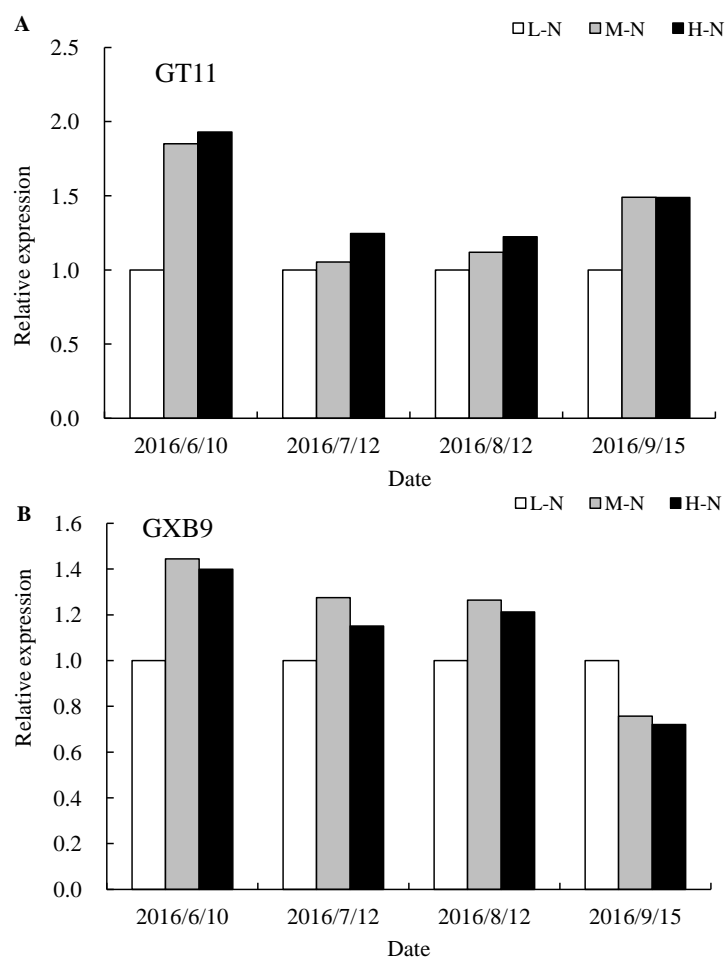


Figure 8. Effects of different nitrogen levels on the expression of *GS1* protein in different sugarcane varieties. *L-N*, low nitrogen level; *M-N*, medium nitrogen level; *H-N*, high nitrogen level

Accumulation distribution rate of nitrogen reached 60 to 80% in leaves, and the proportion of nitrogen in leaves increased with the growth course of sugarcane (Jiang, 2016). Sugarcane leaf segments had contrasting amount of chlorophyll, nitrogen and sugars (Mattiello et al., 2015). Nitrogen fertilizer application can obviously increase the chlorophyll content and leaf area in wheat (Li et al., 2007). In this study we found that sugarcane leaf area significantly increased with the increase of nitrogen application levels. We also found that appropriate nitrogen fertilizer application could increase the content of chlorophyll in leaves, which is consistent with the results reported by Shao et al. (2009).

Wu and Zhao (2010) reported that nitrogen has the best level range for promoting photosynthetic rate. Out of this range, photosynthetic rate decreases. Nitrogen efficient wheat genotypes have strong photosynthetic capacity under low nitrogen condition, but inefficient genotypes need sufficient fertilizer to get higher photosynthetic capacity and higher yield (He et al., 1999). N supply and the sugarcane ability to respond to N influenced photosynthesis (Bassi et al., 2018). In this study we proved that the photosynthetic rate increased with the increase of nitrogen level in the nitrogen inefficient variety GT11, while it showed the highest on the medium nitrogen level in the nitrogen efficient variety GXB9, and significantly higher in the latter than the former.

In this study, the GS activity in all the treatments presented a typical single peak during whole detection duration, and most reached peak on August 12. August belongs to the booming stage, and the plant growth needs large amount of nitrogen. Luo (2007) pointed out that nitrogen can significantly improve GS activity in rice leaf, and the GS activity presented a single peak during whole growth period and reached peak at the heading stage. In this study, the GS activity in the variety GT11 increased with the increase of nitrogen level, while that in the variety GXB9 presented medium nitrogen > low nitrogen > high nitrogen from August to September, which is similar to the result reported by Gui (2007). The variety GXB9 also showed high GS activity under low nitrogen condition, and it always showed significantly higher than the variety GT11, but it seems that high nitrogen has inhibitory effect on the GS activity in the variety GXB9. Nitrogen could induce GS gene expression (Higashi et al., 1998; Ortega et al., 2001). The expression of *GSI-1* in wheat was raised when the nitrogen supply decreased, and high NH_4^+ supply specifically induced *GSI-3* gene expression in barley and sorghum (Caputo et al., 2009; Elomari et al., 2010; Goodall et al., 2013). In the present study, the *scGSI.a* expression decreased with the increase of nitrogen application in the variety GXB9, while the highest under low nitrogen in the variety GT11, which is consistent with the results in wheat reported by Caputo et al. (2009). The relative expression of *scGSI.a* in plant showed the highest level under low nitrogen supply could reduce plant damage due to nitrogen deficiency. Robinson et al. (2007) used NH_4^+ labeled ^{15}N as nitrogen source in a sugarcane hydroponic cultural experiment, and found marked nitrogen transport from xylem to leaves accumulated in the form of glutamic acid and glutamine, forming a marked small nitrogen library. In the present study, the expression of *scGSI.a* was significantly higher in the variety GXB9 than the variety GT11 at the booming stage of sugarcane under the medium and low nitrogen condition, reflecting that the variety GXB9 could have stronger ability for transfer of nitrogen from library to source, which could be related with its low nitrogen resistance characteristics. At the early stage of the sugarcane growth, the expression of *scGSI.b* decreased with the increase of nitrogen application rate while the expression of

scGS1.c increased with the increase of nitrogen application rate for both sugarcane varieties. The enzyme *scGS1.c* can accelerate the assimilation of NH_4^+ and reduce ammonia poisoning. The expression of *scGS1.c* is 15 times higher in the variety GXB9 than the variety GT11 in June, indicating that the NH_4^+ assimilation ability of the variety GXB9 is significantly higher than the variety GT11.

The results in the present study showed that, application of 150 kg/ha urea can keep or significantly increase the GS1 protein expression in the leaves of sugarcane variety GXB9, but more nitrogen fertilizer application rate was needed for the variety GT11. The GS1 protein expression level was higher in the variety GXB9 than the variety GT11, which indicated that the former has stronger low nitrogen endurance than the latter. GS1 protein in sugarcane was commonly encoded by the genes *scGS1.a*, *scGS1.b* and *scGS1.c*. We found that the relative expression ratio of *scGS1.a* : *scGS1.b* : *scGS1.c* on transcription level is approximately 60 : 26 : 0.6, which indicated that *scGS1.a* is the most highly expressed sugarcane *GS1* gene. We also found that the GS1 protein relative expression increased with the increase of nitrogen application in sugarcane. However, there was big difference between transcription expression and enzyme activity due to the influences of various factors. Ortega et al. (2001) reported that there is no GS enzyme activity increase when the GS1 gene transcription increased in alfalfa. GS enzyme in plants is in a dynamic process of biosynthesis and decomposition, and enzyme synthesis is positively correlated with the substrate concentration (Guan et al., 2007). Under low nitrogen condition, lack of GS substrate will cause primary oxidative modification of GS activity sites His residues, followed by protein conformation change or enzyme split, and the process will only be suspended until the GS peptides is hydrolyzed. It is thus clear that lack of substrate prompts enzyme decomposition under insufficient nitrogen condition, and rich substrate helps GS enzyme synthesis and keeps its stability under rich nitrogen conditions. It is also clarified that GS1 transcription level increased but the GS protein expression quantity decreased under low nitrogen condition in the present study.

Conclusion

The nitrogen inefficient sugarcane variety GT11 had the best performance in plant height, stalk diameter, leaf area, SPAD, net photosynthetic rate, GS activity and GS1 protein under high nitrogen levels, while the nitrogen efficient sugarcane variety GXB9 performed the best under medium nitrogen level. Under the low and medium nitrogen levels, the activities of GS and expression of GS1 protein were higher in GXB9 than GT11. The genes *scGS1.a* and *scGS1.b* had higher expression levels in leaves under low nitrogen level while the gene *scGS1.c* increased the relative expression level with the increase of nitrogen application rate. It is suggested that *scGS1.a* and *scGS1.b* be more important for nitrogen metabolism, possibly taking part in the nitrogen assimilation in sugarcane under low nitrogen level.

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Conflict of interests. The authors declare that they have no conflict of interests.

Author contribution. L. T. Y. and Y. R. L. designed the study. K. Z. and D. Y. conducted the experiments. K. Z. and D. Y. analyzed data. K. Z., D. Y., L. T. Y. and Y. R. L. wrote the manuscript. L. T. Y. and Y. R. L. revised and finalized the manuscript.

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APPENDIX

Table A1. The primers of *scGS1.a*, *scGS1.b*, *scGS1.c* and *GAPDH* used to detect the relative expressions in sugarcane

Primer	Primer sequence	
<i>GS1.a</i>	-AGACAGAGCAGAACGGCAAG-3'	F1
	-CTTCCAGAGGATGGTGGTGT-3'	R1
<i>GS1.b</i>	-CAAAGCAAGGACGGTGAAA-3'	F1
	-TGAGGGTAGAGGATGACTTCG-3'	R1
<i>GS1.c</i>	-CTGGGATGGATGTGAGGAGC-3'	F1
	-GTCTCTGAAGATGGCCCGAG-3'	R1
<i>GAPDH</i>	-CTCTGCCCAAGCAAAGATG-3	F1
	-TGTTGTGCAGCTAGCATTG-3'	R1

Figure A1. Leaf area of two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date

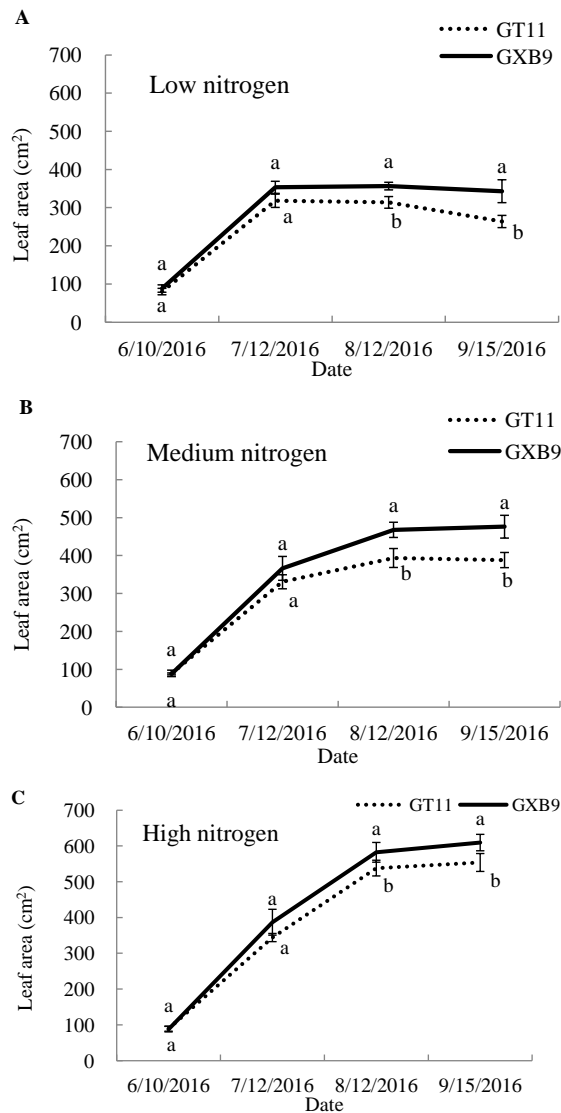


Figure A2. GS activity in two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date

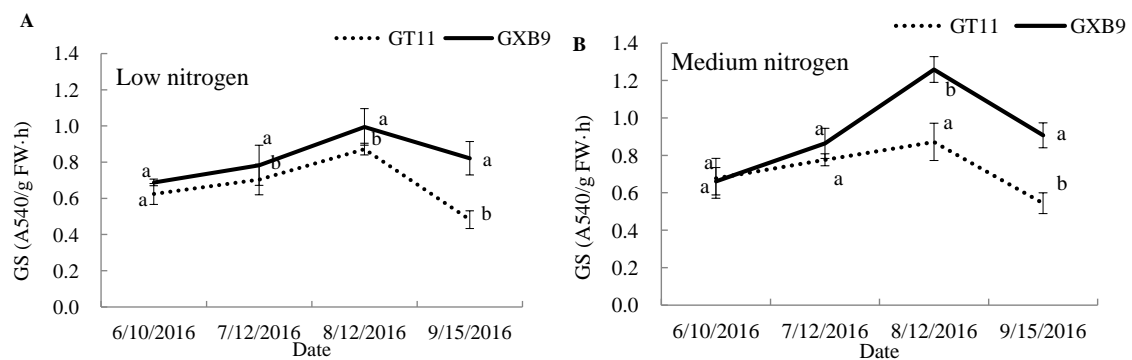


Figure A3. Expression of *scGS1.a* mRNA in two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date

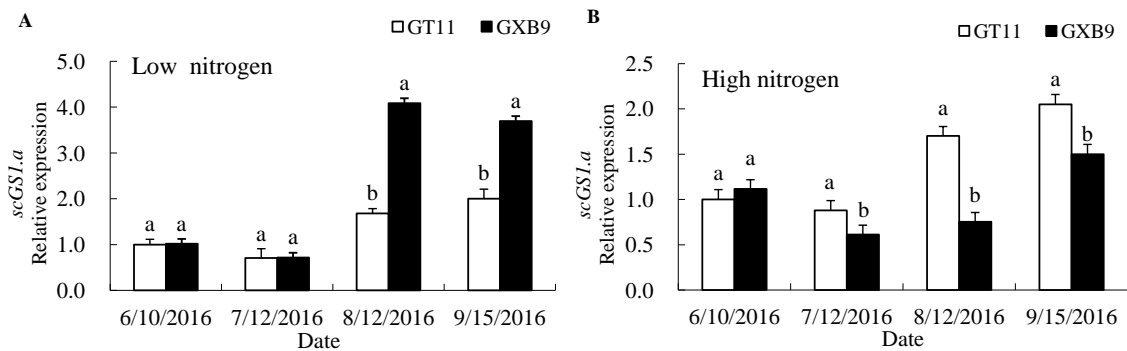


Figure A4. Expression of *scGS1.c* mRNA in two sugarcane varieties under different nitrogen levels. Treatments labeled with different small letters represent statistically significant difference at $P < 0.05$ at the same date

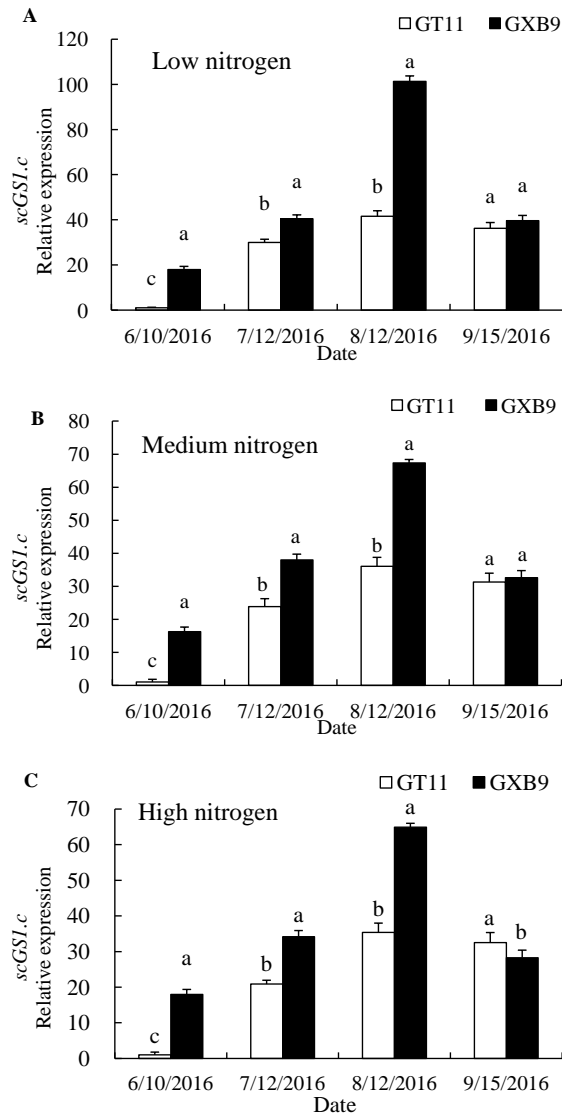


Figure A5. Expression of *GS1* protein in two sugarcane varieties under different nitrogen levels

