BIOTECHNOLOGICAL APPROACHES FOR GENETIC IMPROVEMENT OF SORGHUM

A Dissertation

by

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ABSTRACT

Sorghum (*Sorghum bicolor* L., Moench) is the fifth most important cereal crop in the world and represents an important source of food, feed and energy in several countries. Recently, there has been an increasing interest in sorghum cultivation worldwide, since it is relatively more drought- and heat-tolerant than other cereal crops, and it is better suited for the predicted consequences of global warming. In Africa and Asia, sorghum is primarily used as food for more than 500 million people, while in the Americas and Australia, it is used mainly as a maize-substitute in livestock feed. In the United States, sorghum is also being used in the production of ethanol. In view of its diverse utility, sorghum offers a large number of target traits that could be modified to meet the required applications. In this work, we have used different genetic engineering approaches to address two important issues in sorghum: seed quality and nitrogen use efficiency. First, we examined the temporal and spatial activity of a rice glutelin gene (*GluA-2)* promoter, in transgenic sorghum. Results from quantitative and histochemical GUS assays, as well as from transcript analyses, showed that this promoter is highly active during the middle stages of sorghum seed development and that it controls transgene expression specifically in the seed endosperm. This means that the *GluA-2* promoter can serve as a useful tool in introducing novel traits into sorghum seed in order to improve the quality of this important cereal. Furthermore, we investigated the effects of cytosolic glutamine synthetase (GS1) and alanine aminotransferase (AlaAT) gene overexpression on nitrogen metabolism and plant growth in sorghum. T_2 generation

plants transformed with a sorghum GS1 gene (*Gln1*) driven by the maize ubiquitin promoter exhibited enhanced grain yield and biomass accumulation under optimal nitrogen levels.

DEDICATION

This thesis is dedicated to my parents, Enixia Simons and Rubén Urriola, my main examples of hard work and self-improvement.

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1. INTRODUCTION

Sorghum (*Sorghum bicolor* L.) constitutes an important source of food, feed and fodder in many parts of the world, and additionally, its role as a bioenergy crop is growing in some countries. Recently, there has been an increasing interest in sorghum cultivation worldwide, since it is relatively more drought- and heat-tolerant than other cereal crops, and it is better suited for the predicted consequences of global warming. In Africa and Asia, sorghum is primarily used as food for more than 500 million people, while in the Americas and Australia, it is used mainly as a maize-substitute in livestock feed. In the United States, sorghum is also being used in the production of ethanol. In view of its diverse utility, sorghum offers a large number of target traits that could be altered to meet the required applications. Genetic engineering offers an attractive means to improve sorghum seed-quality and confer novel traits to this crop in order to satisfy the nutritional needs of humans and animals, or to meet specific demands of the industry.

1.1 Seed-specific promoters for genetic improvement of sorghum seed quality

As is the case with other cereal grains, sorghum suffers from several nutritional deficiencies, such as low levels of vitamins and essential amino acids, and also low bioavailability of iron and zinc. Overcoming these deficiencies in the sorghum seed via genetic engineering involves either overexpression or silencing of gene(s), under the control of a promoter that can drive the transcription in a seed-specific manner. Usually,

this type of promoter will be expected to be active in the grain only. Prior to its use, it is important to determine the timing and strength of a promoter. Some traits may require the use of multiple promoters, each driving an individual gene, since the use of the same promoter to drive more than one gene is believed to result in homology-dependent gene silencing (Brusslan and Tobin, 1995; Park et al., 1996; Butaye et al., 2005). Therefore, in order to achieve regulated and stable transgene expression in the sorghum seed, the availability of promoters that provide endosperm-specific activity is of critical importance.

The promoters of the glutelin genes, encoding the main storage protein in rice (*Oryza sativa* L.), have been well characterized in transgenic rice and tobacco, showing activities exclusively in the endosperm (Okita et al., 1989). However, since it has been shown that the activity of a promoter can sometimes differ from that of the source organism when tested in a heterologous species, it is important to know the pattern and the level of rice *GluA-2* promoter activity in the sorghum plant. The **objective** of the first study in this dissertation was to examine the temporal and spatial activity of the rice glutelin 2 (*GluA*-2) gene promoter in transgenic sorghum. Studies conducted in transgenic rice showed that the *GluA-2* promoter directs strong beta-glucuronidase (GUS) expression in the outer portion of the endosperm (Qu et al., 2008). In addition, no GUS activity was detected in the embryo portion of the seed, roots, leaves, sheaths or stems. Based on these observations, my **hypothesis** is that in sorghum, the rice *GluA-2* promoter will also control transgene expression in an endosperm-specific manner. From our results, we expect to achieve a better understanding of the activity pattern and

strength of this promoter in the sorghum plant. Of course, a well-characterized promoter will be valuable in introducing novel traits into the sorghum seed.

1.2 Nitrogen use efficiency (NUE) in sorghum

Nitrogen (N) is a primary macronutrient in plants, and crop productivity highly depends on the use of nitrogen fertilizers, mainly in the form of nitrate and ammonium. N is an essential component of nucleic acids, proteins, cofactors and signaling molecules; hence, deficiency of this element results in poor plant growth and development. Due to the critical role that N fertilization plays in elevating yields in agriculture, the amount of N fertilizers applied to crops in the last 40 years has increased significantly, from 12 to 104 Tg/year (Mulvaney et al., 2009). However, it is estimated that only 30-50% of this N is taken up by plants, with the remainder contaminating soil, water and air.

The N that is lost from the plant-soil system causes soil erosion by killing the organisms that bind soil together, contaminates bodies of water, and also volatilizes in the form of nitrous oxide. Excessive N in aquatic ecosystems favors the growth of phytoplankton and algae, which deplete oxygen available for other species, resulting in the loss of animal life. Nitrous oxide is the third most abundant greenhouse gas, after carbon dioxide and methane. In fact, nitrous oxide is 300 times more potent than carbon dioxide in terms of its greenhouse effects (Johnson et al., 2007). For these reasons, production of N-dependent, high-yielding crops is often associated with environmental damage.

Sorghum is currently the fifth most important cereal worldwide. In semi-arid regions, sorghum is often cultivated on infertile soils by poor people who can hardly afford the high costs of N fertilizers. Both, from economic and environmental standpoints, there is a need for developing sorghum varieties that are more efficient in the use of this nutrient and therefore, require less input of N fertilizers.

There is limited understanding of the variables controlling N use in sorghum; nonetheless, knowledge derived from previous studies conducted in model species could allow researchers to develop novel strategies to understand and enhance NUE efficiency in this economically important crop. The **objective** of the second study in this dissertation is to investigate the effects, on the NUE in sorghum, of the overexpression of two different genes involved in nitrogen metabolism: cytosolic glutamine synthetase and alanine aminotransferase. I **propose** to generate and characterize at the molecular, biochemical and physiological levels transgenic sorghum plants overexpressing a cytosolic glutamine synthetase gene (*Gln1)* driven by the maize ubiquitin or CaMV 35S promoter, as well as transgenic lines transformed with a barley alanine aminotransferase (AlaAT) gene under the control of a tissue-specific aldolase gene promoter (Ald1) from brachypodium (*Brachypodium distachyon* L.*)*. I **hypothesize** that the overexpression of *Gln1* and AlaAT genes results in altered metabolite content and an increase in NUE in transgenic sorghum. By evaluating the effects of overexpressing these genes in sorghum, we will demonstrate their potential as candidate genes for controlling and enhancing NUE in this important crop.

2. LITERATURE REVIEW

2.1 Cereal seed storage protein gene promoters

Cereal seed storage protein genes are specifically and strongly expressed in seed tissues, since they are controlled by promoters with seed-specific activity. Cereal seed proteins are classified into albumins, globulins, glutelins and prolamins based on their solubility in various solvents. Albumins are soluble in water, globulins are soluble in saline solutions, glutelins are soluble in alkali, and prolamins are soluble in aqueous alcohol solutions. Promoters from cereal seed storage protein genes contain several conserved elements that regulate and confer seed-specificity, including RY repeats, the endosperm box, and ACGT and AACA motifs (Kawakatsu and Takaiwa, 2010).

RY repeats are recognized and activated by B3 transcription factors (Moreno-Risueno et al., 2008). These elements are present in monocot promoters, but are also known to be critical for the expression of seed storage proteins in dicots. When bound to RY repeats, B3 factors also interact with another type of factors, named Opaque2 (O2)-like basic leucine-zipper (bZIP) transcription factors. O2-like bZIP factors regulate the expression of seed storage protein genes by binding to an element called endosperm box, specifically to its GCN4 motif (TGA(G⁄C)TCA). O2 transcription factors were first identified in maize, and orthologues have been characterized in wheat (*Triticum aestivum* L.) (Albani et al., 1997), rice (Onodera et al., 2001) and barley (*Hordeum vulgare* L.) (Oñate et al., 1999).

The endosperm box (TGTAAAGTNAATNNGA/GTGAGTCAT), located 300 bp upstream of the transcription initiation site harbors an additional element called the prolamin box [P box; TG(T/C/A)AAAG] (Kawakatsu and Takaiwa, 2010). The P box is recognized by DNA binding with one finger (DOF) transcription factors, which have been identified in wheat, rice, maize *(Zea mays L.)* and barley (Vicente-Carbajosa et al., 1997; Mena et al., 1998; Conlan et al., 1999; Washio, 2001; Yamamoto et al., 2006). P box DOF transcription factors interact with O2-like bZIP factors and also with GAMYB transcription factors that bind to the AACA motif within seed storage protein gene promoters (Yamamoto et al., 2006). The AACA motif is a giberellic acid-responsive element (GARE) (Gubler et al., 1995). Furthermore, ACGT motifs have been identified; these elements are bound by O2-like bZIP factors.

Several promoters from genes encoding seed storage proteins have been isolated from rice (Okita et al., 1989; Leisy et al., 1990; Wu et al., 1998; Qu and Takaiwa, 2004; Kawakatsu et al., 2008; Qu et al., 2008), maize (Russell and Fromm, 1997; Hood et al., 2003), wheat (Lamacchia et al., 2001; Wiley et al., 2007), barley (Cho et al., 1999; Choi et al., 2003; Furtado et al., 2008), and sorghum (Mishra et al., 2008; Ahmad et al., 2012), and used in homologous or heterologous systems. A list of cereal seed promoters evaluated by homologous expression is shown in Table 1.

2.2 Rice glutelin promoters

Amongst the above-mentioned promoters, rice glutelin promoters have been studied most extensively. The glutelin multigene family consists of fifteen genes, and

has been classified into *GluA, GluB, GluC and GluD* subfamilies (Kawakatsu et al., 2008). To date, the promoters of 10 of these genes have been evaluated in stably transformed transgenic rice plants. It has been reported that promoters of *GluA* and *GluB s*ubfamilies direct high-level gene expression in aleurone and subaleurone layers, but weak expression in the inner part of the endosperm. In contrast, promoters of *GluC* and *GluD* subfamilies drive expression throughout the endosperm. These differences in the expression pattern, controlled by the glutelin gene promoters, result from mutations in *cis*-elements involved in endosperm specificity.

Comparison of glutelin promoter sequences revealed that changes in the sequences of three conserved *cis*-elements are critical in defining their spatial expression pattern or strength. Mutations in GCN4 change gene expression from the outer region of the endosperm to the inner portion of the endosperm (Wu et al., 1998), and also cause a reduction in the expression level. The P box and the ACGT motif play a role in determining the level of expression directed by glutelin promoters. Mutations in the P box and ACGT have been shown to decrease expression ten-fold and four-fold, respectively, without affecting spatial activity (Wu et al., 2000).

The temporal and spatial patterns of the rice *GluA*-2 gene promoter have been studied in both homologous and heterologous systems. The protein encoded by the *GluA*-2 gene is synthesized and accumulates throughout rice seed development, starting at five days post-anthesis (dpa), and reaching maximum level around 15 dpa (Okita et al., 1989). Studies conducted in transgenic rice showed that the *GluA-2* promoter directs strong beta-glucuronidase (GUS) expression in the outer portion of the endosperm

Table 1 Seed storage protein promoters analyzed in homologous systems

Species	Gene	Protein	Expression	Remarks	References
Rice	*GluA-1/Gt-2*	Glutelin	I		(Qu et al., 2008)
	GluA-2/Gt-1	Glutelin	I		(Qu et al., 2008)
	GluA-3	Glutelin	I		(Wu et al., 1998)
	GluB-1	Glutelin	I		(Wu et al., 1998)
	GluB-2	Glutelin	I		(Qu and Takaiwa, 2004)
	GluB-3	Glutelin	II	Pseudogene	(Qu et al., 2008)
	GluB-4	Glutelin	I		(Qu and Takaiwa, 2004)
	GluB-5	Glutelin	I		(Qu et al., 2008)
	GluC-1	Glutelin	III		(Qu et al., 2008)
	GluD-1	Glutelin	III		(Kawakatsu et al., 2008)
	Glb-1	α-Globulin	IV		(Wu et al., 1998)
	REG2	7S Globulin	V		(Qu and Takaiwa, 2004)
	NRP33	13 kD prolamin	III		(Wu et al., 1998)
	PG5a	13 kD prolamin	II		(Qu and Takaiwa, 2004)
	RP10	10 kD prolamin	I	Leaky w/o 3'UTR	(Qu and Takaiwa, 2004)
	RP16	16 kD prolamin	I		(Qu and Takaiwa, 2004)
	RAG1	Albumin	IV		(Wu et al., 1998)
Maize	*PGNpr2*	7S globulin (Glb-1)	V		(Hood et al., 2003)
	Z27	27 kD γ -zein	III		(Russell and Fromm, 1997)
Wheat	*LMW Glu*	LMW glutenin	VI		(Stöger et al., 2001)
	HMW Glu-1D1	HMW glutenin	III		(Lamacchia et al., 2001)
Barley	*Hor2-4*	B1-hordein	III	Leaky in rice	(Cho et al., 1999; Furtado et al., 2008)
	Hor3-1	D-hordein	III	Leaky in rice	(Cho et al., 1999; Furtado et al., 2008)

I, high activity in aleurone and subaleurone layers, weak activity in inner starchy endosperm; II, aleurone and subaleurone layers; III, endosperm; IV, inner starchy endosperm; V, embryo and aleurone layers; VI, subaleurone layers (Kawakatsu and Takaiwa, 2010).

(Qu et al., 2008). GUS activity was first detected in the aleurone and subaleurone layers at 7 dpa, and as the seed matured, it gradually spread to the inner part of the endosperm, remaining much weaker in the central area. In addition, no GUS activity was detected in the embryo portion of the seed, roots, leaves, sheaths or stems. In maize, the rice *GluA-2* promoter has also been shown to control endosperm-specific transgene expression (Russell and Fromm, 1997). Since it has been demonstrated that the activity of a promoter can differ from that of the source organism when tested in a heterologous species, it would be interesting to know if there is any change in the pattern or the level of rice *GluA-2* promoter activity in other cereals.

2.3 Primary routes of nitrogen assimilation and nitrogen use efficiency in plants

Plant nitrogen metabolism consists of two major phases: uptake and utilization. Utilization can be further divided into two steps: assimilation and remobilization (Zhang, 2011). Plants take up nitrogen from the soil mostly in the form of nitrate and ammonium through nitrate- and ammonium transporters, respectively. Nitrate is taken up by two main families of transporters, NRT1 and NRT2. Once nitrate enters the cytoplasm, it is reduced to nitrite by the enzyme nitrate reductase (NR), and then transported to the plastids or chloroplasts, where it is reduced to ammonium by nitrite reductase (NiR).

Ammonium, whether taken directly from the soil or derived from the reduction of nitrate, is assimilated into glutamine and glutamate through the glutamine synthetase/glutamate synthetase (GS/GOGAT) cycle (Zhang, 2011; Xu et al., 2012). Glutamine is then converted to asparagine, and glutamate to aspartate, by asparagine

synthetase and aspartate aminotransferase, respectively. These aminoacids are considered long-distance nitrogen transport compounds, and are translocated via the xylem to mesophyll cells for utilization in carbon assimilation or storage in the chloroplasts. During senescence and grain filling, various enzymes, including GS and glutamate dehydrogenase (GDH) (McAllister et al., 2012), participate in the remobilization of stored N into the maturing fruits or grains.

N use efficient plants are more capable of uptake, assimilation, translocation or remobilization of N available to them for the development of fruits or grains. Different equations can be used to measure NUE and other related physiological traits (Table 2). These equations take into account either shoot or grain weight, and their use depends on the harvested product of the plant species being evaluated. In grasses and cereals, NUE refers to the efficiency with which applied nitrogen is converted into biomass and grain yield. NUE can also be defined as the product of N uptake efficiency (NUpE) and N utilization efficiency (NUtE). The ability of a plant to uptake and utilize N in an efficient manner is determined by many variables, including genotype, soil N availability, and plant N and carbon (C) status. Several strategies have been used to study genes and proteins involved in plant primary N metabolism and identify those that could have the greatest potential to control and increase NUE.

2.4 Transgenic approaches for the development of nitrogen use efficient plants

Genetic engineering represents a valuable tool to study NUE-candidate genes (Good et al., 2004; Zhang, 2011; McAllister et al., 2012; Xu et al., 2012). The function

and regulation of nitrate and ammonium transporters have been well-characterized in plants; however, there have been few studies showing the effect of their overexpression on NUE (Katayama et al., 2009). The only successful attempt that has been reported to date is the overexpression of a rice NRT2.1 gene controlled by the CaMV 35S promoter in arabidopsis, which resulted in increased shoot weight (Liu et al., 1999; Fraisier et al., 2000; Hoque et al., 2006; Kumar et al., 2006; Katayama et al., 2009). There is also a small number of studies reporting the overexpression of NR and NiR genes. In general, overexpression of NR and NiR has led to a reduction in nitrate levels and an increase in N uptake, without affecting NUE. This can be due to the complex transcriptional, translational and post-translational regulation of NR and NiR genes/enzymes.

Numerous studies have been performed on transgenic plants overexpressing genes that encode enzymes involved in amino acid biosynthesis (Good et al., 2004; McAllister et al., 2012; Xu et al., 2012). Constitutive overexpression of an *Escherichia coli* asparagine synthetase (AsnA) gene in lettuce *(Lactuca sativa* L.*)* and canola *(Brassica napus* L.*)* improved vegetative growth and N status, as well as seed yield and N harvest index, respectively (Seiffert et al., 2004; Giannino et al., 2008). Tobacco *(Nicotiana tabacum)* plants overexpressing an alfalfa *(Medicago sativa* L.*)* NADH-dependent GOGAT (NADH-GOGAT) gene driven by the CaMV 35S promoter showed higher shoot weight (Chichkova et al., 2001). Tobacco and maize (*Zea mays* L.) plants with ectopic expression of an *E. coli* glutamate dehydrogenase (GDH) gene under the control of the CaMV 35S promoter showed increased biomass (Ameziane et al., 2000; Mungur et al., 2006; Lightfoot et al., 2007).

Genes encoding signaling and regulatory factors, or enzymes implicated in processes such as translocation, remobilization and senescence, have also been investigated in an attempt to improve NUE (Good et al., 2004; McAllister et al., 2012; Xu et al., 2012). Overexpression of the rice cytokinin oxidase (CKX2) gene resulted in an increase in panicle and grain numbers (Ashikari et al., 2005). Bi et al. (2009) found that overexpression of the ENOD-93-1 mitochondrial membrane protein gene driven by the ubiquitin promoter led to an increment in the dry biomass in rice. Yanagisawa et al. (2004) observed that the constitutive overexpression of the maize *Dof1* gene in arabidopsis enhanced growth rate under low N conditions.

2.5 Glutamine synthetase as a candidate gene for improving nitrogen use efficiency

Since all the N, whether derived from nitrate or ammonium, is assimilated into organic compounds through the reactions catalyzed by GS, this enzyme is likely to be an important checkpoint controlling NUE (Miflin and Lea, 1976). GS catalyzes the ATP dependent reaction between ammonia and glutamate to produce glutamine. In higher plants, GS has been classified into cytosolic (GS1) and plastidic (GS2) isoforms (Hirel and Gadal, 1982; Zhang, 2011). In most species, GS1 is encoded by a family of up to five genes, while GS2 is encoded by a single gene (Swarbreck et al., 2010). In arabidopsis and maize, five genes encode GS1, and in rice, only three. GS1 genes are differentially expressed in various plant tissues, suggesting that each one has specific functions in N assimilation. GS2 is predominant in leaf mesophyll cells and other photosynthetic tissues, indicating that it might be involved in photorespiration.

Table 2 Definitions and formulas used to describe nutrient use efficiency in plants

Equation	Term	Formula	Definition	Comments
1	Nitrogen use efficiency	NUE=Sw/N	Sw, shoot weight (DW); N, nitrogen content of shoots (DW)	Does not account for biomass increase
2	Usage index	UI=Sw*(Sw/N)	Sw, shoot weight; N, nitrogen in shoots	Takes into account absolute biomass increase
3	Nitrogen use efficiency (grain)	NUE=Gw/Ns	Gw, grain weight; Ns, nitrogen supply (g per plant)	Reflects increased yield per unit applied nitrogen
4	Uptake efficiency	UpE=Nt/Ns	Nt, total nitrogen in plant; Ns, nitrogen supply (g per plant)	Fraction of nitrogen converted to grain
5	Utilization efficiency	UtE=Gw/Nt	Gw, grain weight; Nt, total nitrogen in plant	Fraction of nitrogen converted to grain
6	Agronomic efficiency	$AE=(Gw_F-Gw_C)/N_F$	N_F, nitrogen fertilizer applied; Gw_F, grain weight with fertilizer; Gw_C, grain weight of unfertilized control	Measures the efficiency of converting applied nitrogen to grain yield
7	Apparent nitrogen recovery	$AR=(N_F$ uptake-N_C uptake)/ N_F*100	N_F uptake=plant nitrogen (fertilizer); N_C uptake=plant nitrogen (no fertilizer); N_F =Nitrogen fertilizer applied	Measures the efficiency of capture of nitrogen from soil
8	Physiological efficiency	$PE=(Gw_F-Gw_C)/(N_F$ uptake-N_C uptake)	Nitrogen fertilizer applied Gw_F, grain weight (Fertilizer); Gw_C, grain weight (no fertilizer)	Measures the efficiency of capture of plant nitrogen in grain yield

Equation 1 in essence measures the carbon:nitrogen ratio of the plant. The "Utilization Index" factors for the absolute amount of biomass produced as well as for the ratio of biomass per unit nitrogen. "NUEg" is calculated as a grain production per unit of N available. There are two primary components of NUEg, which are referred to as "uptake efficiency" (the efficiency with which the total plant N is used to produce grain) (Eqns 4, 5, respectively). For simplicity, fertilizer applied is often substituted for nitrogen supply (designated N_s) and nitrogen in the above ground tissues substituted for total nitrogen (designated Nt). Three fertilizer efficiency parameters have been defined, including agronomic efficiency (AE), apparent nitrogen recover (AR) and physiological efficiency (PE) (Eqns 4-6). AR reflects the efficiency of the crop in obtaining nitrogen-based fertilizer from the soil, whereas PE can be viewed as the efficiency with which crops use nitrogen in the plant for the synthesis of grain. These equations (Eqns 3-8) can also be expanded to include additional factors, including physiological ones (Good et al., 2004).

Multiple studies have been conducted on the overexpression of GS as candidate genes for improving NUE (Table 3). Overexpression of GS1 gene driven by the CaMV 35S promoter in tobacco and poplar resulted in growth improvement and leaf dry weight (DW) increase, respectively (Gallardo et al., 1999; Oliveira et al., 2002; Fu et al., 2003). Wheat plants overexpressing a GS1 gene driven by the light-induced rubisco small subunit *(rbcS)* promoter had higher root and grain DW, and enhanced capacity to accumulate N, mainly in the grain (Habash et al., 2001). In rice, overexpression of a GS1 gene under the control of a ubiquitin promoter led to increased spikelet yield under high N conditions (Brauer et al., 2011); however, no positive effect on NUE was observed when the CaMV 35S promoter was used (Cai et al., 2009). Martin et al. (2006) demonstrated that overexpression of a maize *gln1-3* gene driven constitutively by the cassava vein mosaic virus (CsVMV) promoter increased grain number.

2.6 Alanine aminotransferase as candidate gene for improving nitrogen use efficiency

Alanine aminotransferase (AlaAT) seems to play an important role in N metabolism and NUE. This enzyme catalyzes the reversible transfer of an amino acid group from glutamate and pyruvate to form alanine and 2-oxoglutarate (Zhang, 2011). Plants express multiple AlaAT isoforms encoded by a multigene family (Kendziorek et al., 2012). Between two to six forms of AlaAT have been found in plant tissues, including roots, leaves, flowers and seeds. Different isoenzymes are involved in diverse metabolic pathways, depending on their subcellular localization. Some isoforms appear

to be expressed constitutively and have functions in general amino acid metabolism. Other AlaAT isozymes seem to have light-dependent expression, suggesting a potential role in photorespiration. A cytosolic AlaAT isoform is found in both mesophyll and bundle sheath cells, and has been implicated in the transfer of C units between these cell types (Son and Sugiyama, 1992). In addition, an AlaAT isozyme localized in the peroxisomes is induced by hypoxia and may have a function in anaerobic stress (Muench and Good, 1994; Muench et al., 1998; Liepman and Olsen, 2003).

In an effort to improve NUE, some groups have studied the effects of AlaAT gene overexpression (Table 3) (Good et al., 2004; McAllister et al., 2012). Good et al. (2007) found that transgenic canola (*Brassica napus* L.) overexpressing a barley (*Hordeum vulgare* L.) AlaAT gene under control of a tissue-specific promoter resulted in increased biomass and seed yield under low N conditions. Shrawat et al. (2008) showed that rice plants with ectopic overexpression of the barley AlaAT gene driven by a tissue-specific promoter led to increments in biomass and seed yield under high N conditions.

Table 3 Transgenic approaches to improve plant nitrogen use efficiency by overexpression of glutamine synthetase and alanine aminotransferase genes

Gene source (accession number)	Transgenic approach	Host plant (s)	Characteristic of NUE	
			Growth condition	Grain yield/biomass N uptake/metabolism
Glutamine synthetase				
PsGS1 (EC 6.3.1.2)	CaMV 35S	Tobacco	MS	Growth improved, leaf TAA decreased
PsGS1 (EC 6.3.1.2)	CaMV 35S	Poplar	HS	Leaf DW increased (112% under LN AND 6% under HN)
PvGS1	Rubisco small subunit	Wheat	Peat-based compost	Root and grain DW increased, enhanced capacity to accumulate N, mainly in grain
MsGS1 (EC 6.3.1.2)	CaMV 35S	Tobacco	MS	Shoot DW increased 70% and root DW increased 100% under LN
GmGS1	CaMV 35S	Lotus	MS	DW did not change
OsGS1;1 (AB037595)	CaMV 35S	Rice	Field	Yield decreased 25%-33%
			HS	TN increased under both LN and HN
OsGS1;2 (AB180688)	CaMV 35S	Rice	Field	Yield decreased 7%-25%
			HS	TN increased under both LN and HN
OsGS1;2 (AB180688)	Ubiquitin	Rice	Soil (growth chambers)	Spikelet yield increased 29%-35% under HN NUE increased 30%-33%
			Soil	No change No change
OsGS2 (X14246)	CaMV 35S	Rice	MS	No change in soluble protein and free ammonium
ZmGS1	CvMV	Maize	Soil	Grain yield increased 45% under LN
Alanine aminotransferase				
HvAlaAT (Z26322)	*btg26*	Canola	Soil-less mixture	Seed yield increased 32.7%, DW increased 55%-64% under LN
			HS	DW increased 30%-75 under LN
HvAlaAT (Z26322)	*OsAnt1*	Rice	Soil-less mixture	Spikelet yield increased 31%-54%, DW increased 30%-34%
			HS	TN increased 36%-61%

Abbreviations: *btg26,* canola root-specific promoter; *CaMV 35S,* cauliflower mosaic virus 35S promoter; DW, dry weight; HN, high nitrogen concentration; HS, hydroponic solution; LN, low nitrogen concentration; MS, Murashige and Skoog medium; Os*Ant1*, aldehyde dehydrogenase promoter; TAA, total ami no acids; ubiquitin, maize ubiquitin promoter, TN, total nitrogen content (Xu et al., 2012).

3. ACTIVITY OF A RICE GLUTELIN PROMOTER IN TRANSGENIC SORGHUM

3.1 Synopsis

Grain sorghum is an important source of food and feed worldwide, and more recently, it is also being utilized as a biofuel feedstock. In view of its diverse utility, sorghum grain offers a large number of target traits that could be improved to meet the required usage. As in the case of other cereal crops, genetic engineering offers an attractive means to enhance sorghum seed-quality to satisfy the nutritional needs of humans and animals or to fulfill specific demands of the biofuel industry. Thus, promoters that provide regulated transgene expression in a seed-specific manner are of critical importance to obtain the desired quality grain. Whether a promoter is homologous or heterologous, it must first be evaluated in the target plant species. In this study, we examined the temporal and spatial activity pattern of a rice glutelinA-2 gene (*GluA-2)* promoter in transgenic sorghum. A 1,832 bp long sequence of the *GluA-2* promoter was ligated at the 5'-end of the *gus*A reporter gene, and this construct was introduced into sorghum by *Agrobacterium*–mediated transformation of immature embryos. Quantitative GUS analysis of homozygous T_2 seeds from three independent transgenic lines showed detectable expression of the transgene at 14 days post anthesis (dpa), which increased as the seed matured. Furthermore, histochemical GUS assay confirmed this pattern of activity and also revealed that the *GluA-2* promoter directs GUS expression in the inner starchy endosperm. In addition, the absence of detectable

GUS expression in other tissues such as embryo, leaf, stem, root, pollen and inflorescence showed that the activity of this promoter is specific to the endosperm portion of the seed. Furthermore, transcript analyses showed high *gus*A mRNA abundance at 14 and 17 dpa. Our results show the efficacy of the rice *GluA-2* promoter as an endosperm-specific promoter in sorghum and suggest that it can serve as a valuable tool in improving the seed quality of this important cereal.

3.2 Introduction

Grain sorghum (*Sorghum bicolor* L.) constitutes an important source of food and feed in many parts of the world, and additionally, its role as bioenergy feedstock is growing in various countries. Recently, there has been an increasing interest in sorghum cultivation worldwide, since it is relatively more drought- and heat-tolerant than other cereal crops, and it is better suited for the predicted consequences of global warming. In Africa and Asia, sorghum grain is primarily used as food for more than 500 million people, while in the Americas and Australia, it is used mainly as a maize-substitute in livestock feed. In the United States, sorghum seed is being sought for use in the production of ethanol. In view of its diverse utility, sorghum seed offers a large number of target traits that could be enhanced to meet the required applications. Now that sorghum genome has been sequenced (Paterson et al., 2009) and that there is an efficient transformation system available (Kumar et al., 2011), genetic engineering offers an attractive means to incorporate novel traits into sorghum grain in order to satisfy

different nutritional needs of humans and animals or to meet specific demands from the energy industry.

Introduction of new traits into sorghum seed via genetic engineering involves either overexpression or silencing of gene(s) under the control of a promoter that can drive transcription in a seed-specific manner. Usually, this type of promoter will be expected to be active in the grain only and will also determine the timing and level of transgene expression during the development of the seed. Some traits may require the use of multiple promoters in order to control various genes, since the use of the same promoter to drive more than one gene is not advisable (Brusslan and Tobin, 1995; Park et al., 1996; Butaye et al., 2005). Therefore, the availability of a seed-specific promoter that has been tailored to a desired strength and temporal activity for a particular application is of critical importance.

Regulatory sequences from genes encoding cereal seed storage proteins represent important sources of seed-specific promoters, since they are specifically and highly expressed in the endosperm tissue, resulting in high levels of accumulation of these proteins. The promoters of several albumin, globulin, glutelin and prolamin genes from barley, maize, rice and wheat have been well-studied to date (Okita et al., 1989; Leisy et al., 1990; Russell and Fromm, 1997; Wu et al., 1998; Cho et al., 1999; Lamacchia et al., 2001; Choi et al., 2003; Hood et al., 2003; Qu and Takaiwa, 2004; Wiley et al., 2007; Furtado et al., 2008; Kawakatsu et al., 2008; Qu et al., 2008). Amongst these, promoters of glutelin genes, which encode the main storage protein in rice (*Oryza sativa* L.), have been characterized most extensively. The glutelin multigene family consists of fifteen

genes that have been classified into four sub-families: *GluA, GluB, GluC and GluD* (Kawakatsu and Takaiwa, 2010).

In this study, we have examined the temporal and spatial activity profile of a 1.8 kbp promoter sequence from the rice *GluA*-2 gene (Okita et al., 1989) in transgenic sorghum . The activity of the *GluA*-2 gene promoter has been previously analyzed in transgenic rice, showing activity exclusively in the endosperm (Qu et al., 2008). However, since it has been shown that the activity of a promoter can differ from that of the source organism when tested in a heterologous species, it is necessary to investigate if there is any variation in the pattern or the level of activity of the rice *GluA-2* promoter in the sorghum plant. The promoter region of *GluA*-2 gene was ligated to the *gus*A reporter gene to create a construct, which was introduced into sorghum by means of *Agrobacterium*-mediated transformation. Results presented in this report suggest that, in sorghum, the rice *GluA-2* promoter controls transgene expression in an endosperm-specific manner, thus demonstrating its potential as a useful tool in improving sorghum seed quality via genetic engineering.

3.3 Experimental procedures

3.3.1 Assembly of binary vector

A binary vector was assembled by excising a *Pst*I/*BamH*I fragment containing a 1,832 bp long sequence of rice *GluA-2* promoter from the plasmid, pYW102 and ligating it at the 5'-end of the *gus*A reporter gene sequence in a pCAMBIA-based vector. Plasmid was mobilized by electroporation into *Agrobacterium tumefaciens* strain NTL4

harboring the disarmed Chry5 Ti plasmid designated pTiKPSF2 (Palanichelvam et al., 2000). The laboratory designation for this vector was LCT87.

3.3.2 Plant transformation

The cassette was introduced into *S. bicolor* L. genotype P898012 by *Agrobacterium*-mediated transformation of immature embryos (14 days post-anthesis, dpa) as described by Kumar et al. (2011).

3.3.3 Southern blot analysis

Southern blot analysis was performed to confirm the number of transgene copies integrated into the genome of transformed sorghum plants. Genomic DNA was isolated from sorghum leaves of T_0 plants (Dellaporta et al., 1983), and 15 µg were digested with *BamH*I and fractionated on a 1% agarose gel. The membrane was hybridized with a ^{32}P-labeled *gus*A gene fragment.

3.3.4 Fluorometric and histochemical GUS assays

Quantitative, fluorometric GUS analyses were performed on T_2 seeds from three independent, homozygous, single-copy lines according to the method described by Jefferson et al. (1987). Assays were conducted on endosperm and embryos from seeds harvested at 10, 11, 12, 13, 14, 17, 21 and 28 dpa. In addition, these assays were carried out on shoots and roots of one week old T_2 seedlings, leaves and stalks of two month old T_2 plants, pollen, and inflorescence tissue. In order to determine the spatial pattern of

activity in different-aged seeds, longitudinal sections of T_2 seeds at 14, 17, 21 and 28 dpa were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc).

3.3.5 RNA expression analysis

RT-PCR analysis was conducted to compare the transcription activity of the rice *GluA-2* promoter-driven *gus*A gene and the endogenous sorghum α-kafirin gene (*a-Kaf*), encoding the major seed storage protein in sorghum (GenBank accession number EU424175) (De Mesa-Stonestreet et al., 2010; Kumar et al., 2012). Total RNA was isolated from seeds at 10, 14, 17, 21 and 28 dpa as described previously (Wang et al., 2012). A sample of 800 ng of total RNA was reverse-transcribed using MultiScribe Reverse Transcriptase from Applied Biosystems. The primer sets used were 5'-GGTCGTCATGAAGATGCGGAC-3'and 5'- ACGGTTCAGGCACAGCACATC-3' for *gus*A, 5'-ATGGCTACCAAGATATTTGTCCTCCTTGCG-3' and 5'-AATCTAGAAGATGGCACTTCCAACGATGGG-3' for *a-kaf*, and 5'-GGGTTTAGACCGTCGTGAGA-3' and 5'-TTCAGTCATAATCCGGCACA-3' for 18S rRNA.

3.4 Results

3.4.1 Generation of transgenic sorghum lines

In order to examine the temporal and spatial activity pattern of the rice *GluA-2* promoter in transgenic sorghum, we assembled a binary vector designated LCT87 (Figure 1), harboring a 1,832 bp long sequence of the promoter driving the *gus*A reporter gene. The

construct also contained the hygromycin phosphotransferase *(hptII)* gene under the control of the 35S cauliflower mosaic virus (CaMV 35S) promoter for selection of transgenic events. Figure 2 shows the sequence of the rice *GluA-2* promoter utilized in this study, indicating putative *cis*-elements for cereal seed storage protein gene regulation [prolamin (P) box, AGCT, GCN4 and AACA] identified using the Plant *Cis*-acting Regulatory DNA Elements (PLACE) database (Higo et al., 1999). The LCT87 vector was introduced into sorghum by *Agrobacterium*-mediated transformation of immature embryos. Hygromycin-resistant calli were selected and shoots were obtained upon transfer to regeneration media. Plantlets with well-developed roots were transferred to soil. Southern blot analysis was performed on putative transformants to confirm transgene integration and copy number. A total of 26 independent transgenic lines were generated from transformation experiments. Among these 26 transgenic events, lines 87-3B, 87-8B and 87-21B showed single transgene copy integration within their genomes (Figure 3) and were used for detailed analyses.

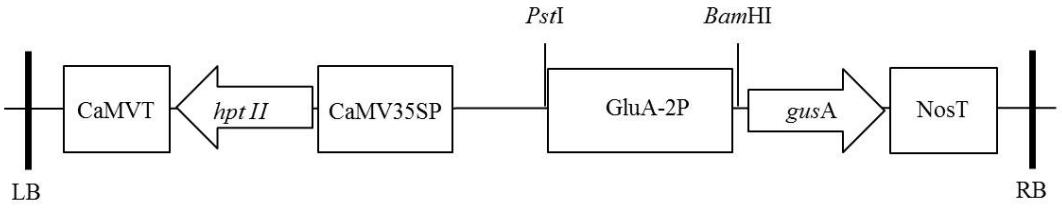

Figure 1 T-DNA portion of the LCT87 vector used to generate transgenic sorghum lines.

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-1832  AATTCCTCTG TTTTGTTTTT CACCCTCAAT ATTTGGAAAC ATTTATCTAG GTTGTTTGTG TCCAGGCCTA

-1762  TAAATCATAC ATGATGTTGT CGTATTGGAT GTGAATGTGG TGGCGTGTTC AGTGCCTTGG ATTTGAGTTT

-1692  GATGAGAGTT GCTTCTGGGT CACCACTCAC CATTATCGAT GCTCCTCTTC AGCATAAGGT AAAAGTCTTC

-1622  CCTGTTTACG TTATTTTACC CACTATGGTT GCTTGGGTTG GTTTTTTCCT GATTGCTTAT GCCATGGAAA

-1552  GTCATTTGAT ATGTTGAACT TGAATTAACT GTAGAATTGT ATACATGTTC CATTTGTGTT GTACTTCCTT

-1482  CTTTTCTATT AGTAGCCTCA GATGAGTGTG AAAAAAACAG ATTATATAAC TTGCCCTATA AATCATTTGA

-1412  AAAAAATATT GTACAGTGAG AAATTGATAT ATAGTGAATT TTTAAGAGCA TGTTTTCCTA AAGAAGTATA

-1342  TATTTTCTAT GTACAAAGGC CATTGAAGTA ATTGTAGATA CAGGATAATG TAGACTTTTT GGACTTACAC

-1272  TGCTACCTTT AAGTAACAAT CATGAGCAAT AGTGTTGCAA TGATATTTAG GCTGCATTCG TTTACTCTCT

-1202  TGATTTCCAT GAGCACGCTT CCCAAACTGT TAAACTCTGT GTTTTTTGCC AAAAAAAAAT GTATAGGAAA

-1132  GTTGCTTTTA AAAAATCATA TCAATCCATT TTTTAAGTTA TAGCTAATAC TTAATTAATC ATGCGCTAAT

-1062  AAGTCACTCT GTTTTTCGTA CTAGAGAGAT TGTTTTGAAC CAGCACTCAA GAACACAGCC TTAACCCAGC

 -992  CAAATAATGC TACAACCTAC CAGTCCACAC CTCTTGTAAA GCATTTGTTG CATGGAAAAG CTAAGATGAC

 -922  AGCAACCTGT TCAGGAAAAC AACTGACAAG GTCATAGGGA GAGGGAGCTT TTGGAAAGGT GCCGTGCAGT

 -852  TCAAACAATT AGTTAGCAGT AGGGTGTTGG TTTTTGCTCA CAGCAATAAG AAGTTAATCA TGGTGTAGGC

 -782  AACCCAAATA AAACACCAAA ATATGCACAA GGCAGTTTGT TGTATTCTGT AGTACAGACA AAACTAAAAG

 -712  TAATGAAAGA AGATGTGGTG TTAGAAAAGG AAACAATATC ATGAGTAATG TGTGAGCATT ATGGGACCAC

 -642  GAAATAAAAA GAACATTTTG ATGAGTCGTG TATCCTCGAT GAGCCTCAAA AGTTCTCTCA CCCCGGATAA

                        P box
 -572  GAAACCCTTA AGCAATGTGC AAAGTTTGCA TTCTCCACTG ACATAATGCA AAATAAGATA TCATCGATGA

 -502  CATAGCAACT CATGCATCAT ATCATGCCTC TCTCAACCTA TTCATTCCTA CTCATCTACA TAAGTATCTT

                                       ACGT
 -432  CAGCTAAATG TTAGAACATA AACCCATAAG TCACGTTTGA TGAGTATTAG GCGTGACACA TGACAAATCA

 -362  CAGACTCAAG CAAGATAAAG CAAAATGATG TGTACATAAA ACTCCAGAGC TATATGTCAT ATTGCAAAAA

 -292  GAGGAGAGCT TATAAGACAA GGCATGACTC ACAAAAATTC ATTTGCCTTT CGTGTCAAAA AGAGGAGGGC

 -222  TTTACATTAT CCATGTCATA TTGCAAAAGA AAGAGAGAAA GAACAACACA ATGCTGCGTC AATTATACAT

                                                                      GCN4
 -152  ATCTGTATGT CCATCATTAT TCATCCACCT TTCGTGTACC ACACTTCATA TATCATGAGT CACTTCATGT

             AACA                                                     TATA box
  -82  CTGGACATTA ACAAACTCTA TCTTAACATT TAGATGCAAG AGCCTTTATC TCACTATAAA TGCACGATGA

  -12  TTTCTCATTG TT
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Figure 2 Sequence of 1,832 bp *GluA-2* promoter. Putative *cis*-elements for cereal seed storage protein gene regulation are underlined.

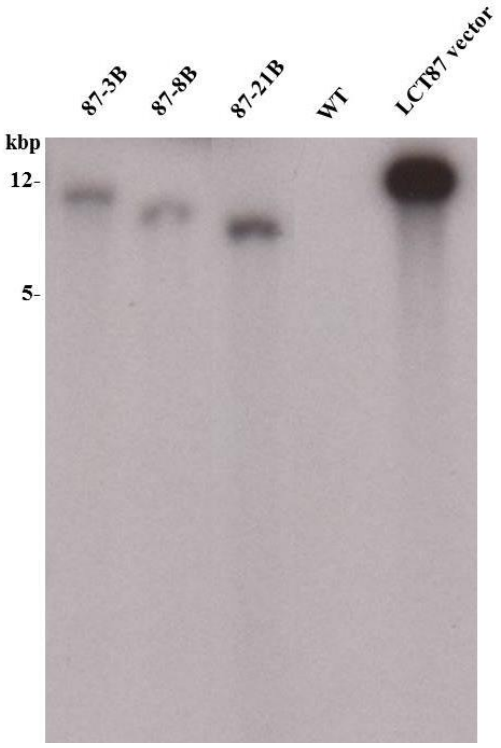

Figure 3 Southern blot analysis of genomic DNA from transgenic lines 87-3B, 87-8B and 87-21B, and a wild-type control plant. Genomic DNA was digested with *BamH*I and hybridized with a *gus*A gene fragment.

3.4.2 Temporal activity pattern of the rice *GluA-2* promoter in transgenic sorghum

The temporal activity pattern of the rice *GluA-2* promoter in transgenic sorghum was analyzed by quantitative, fluorometric GUS assays (Figures 4A and 4B). GUS activity driven by the *GluA-2* promoter was evaluated in the endosperm tissue of seeds harvested at 10, 11, 12, 13, 14, 17, 21 and 28 dpa from T_2 homozygous lines. Using the fluorometric GUS analysis, the first detectable GUS activity was observed at 14 dpa in the seed endosperm tissue. As the seed matured, there was an increase in the level of GUS activity. However, a significant decrease in GUS activity was observed at 28 dpa,

with values comparable to those obtained at 14 dpa. Average GUS activity in the seeds from three independent transgenic lines ranged from 26 to 98 nmol 4-MU/mg protein/min at 14 dpa, 1,108 to 1,989 nmol 4-MU/mg protein/min at 17 dpa, 393 to 510 nmol 4-MU/mg protein/min at 21 dpa, and 3 to 41 nmol 4-MU/mg protein/min at 28 dpa (Figure 4A). Endosperm being a storage organ, its protein levels are expected to increase as the seed develops. Results shown for protein levels per seed during its development confirm this (Figure 5). Thus, GUS activity, presented as a function of protein level in the endosperm, is not likely to parallel the increase in the transgene activity, especially if the rate of increase in seed protein content is significantly higher. With this in mind, we also calculated GUS activity per seed and the results are presented in terms of nmol 4-MU/seed/min (Figure 4B).

These results were confirmed by histochemical GUS analyses. T_2 seeds harvested at 14, 17, 21 and 28 dpa from homozygous transgenic plants were sectioned longitudinally and stained with X-Gluc. A representative seed at each time point is shown in Figure 6. X-Gluc staining was visible in seeds at 14 dpa (Figure 6A). As the seeds developed, the intensity of GUS staining increased (Figures 6B and 6C), reaching a maximum level at 21 dpa. As it also occurred in fluorometric GUS assays, a decrease in GUS staining was observed at 28 dpa (Figure 6D).

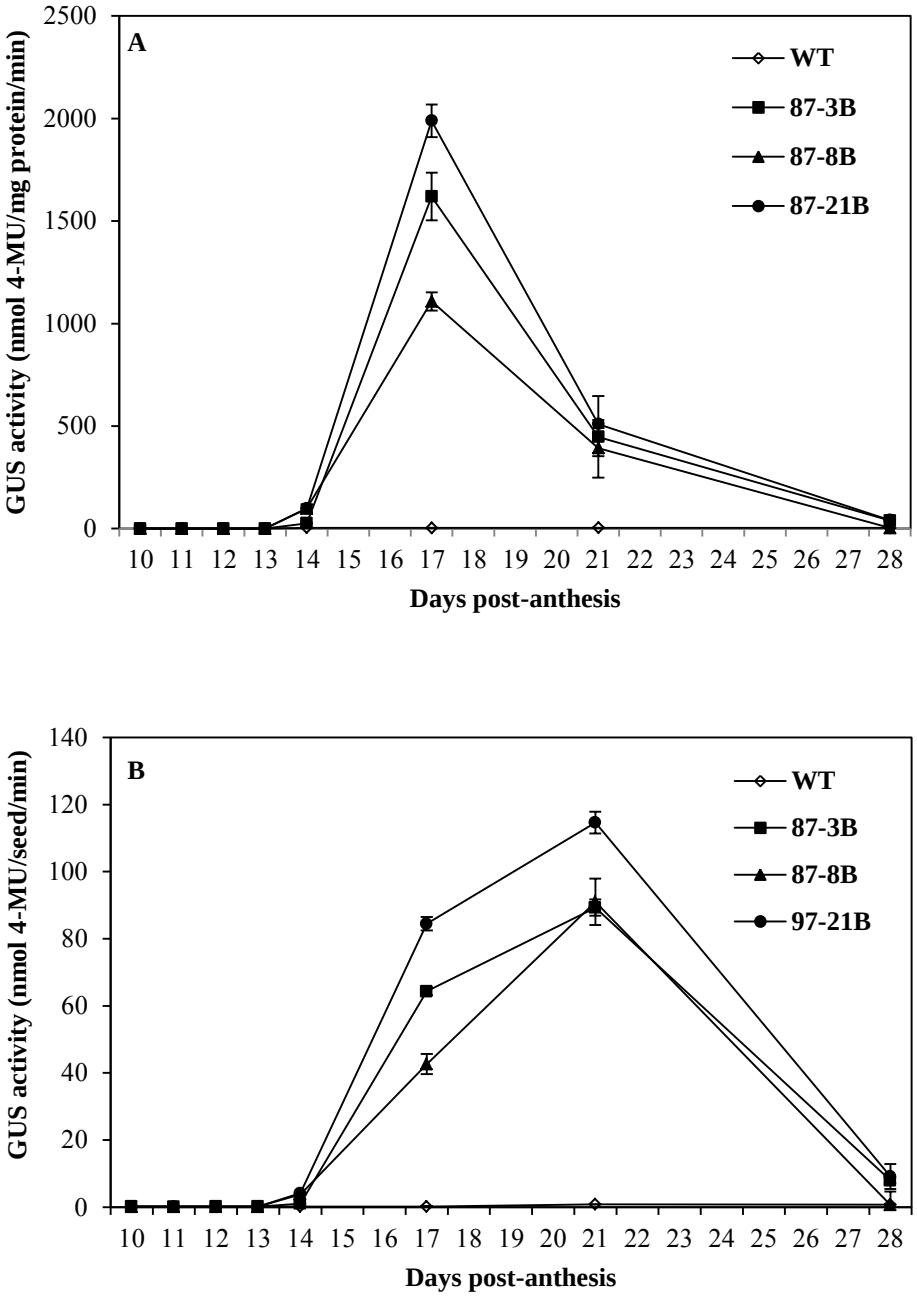

Figure 4 GUS activity driven by the *GluA-2* promoter in the endosperm tissue of transgenic sorghum seeds. GUS activity is presented as **(A)** nmol 4-MU/mg protein/min, and **(B)** nmol 4-MU/seed/min. Results shown are mean (±SEM) values from three individual plants.

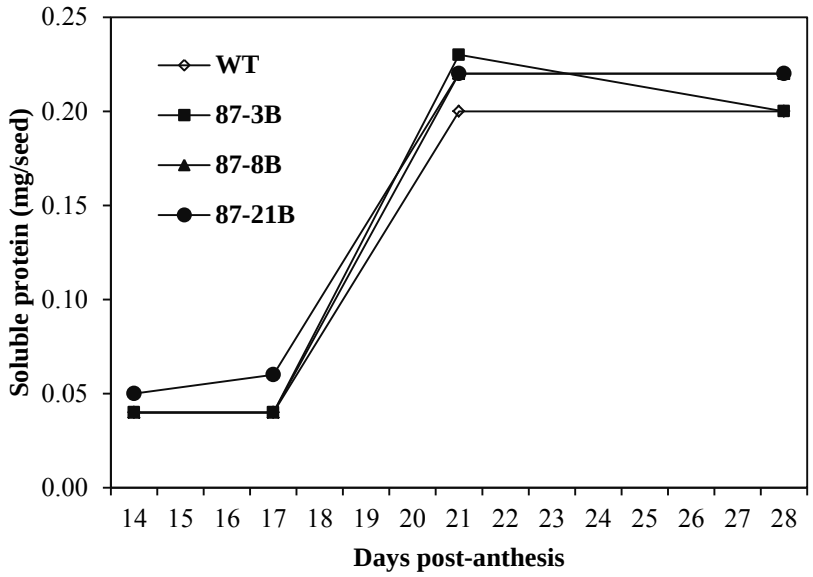

Figure 5 Soluble protein content in the seeds from transgenic lines 87-3B, 87-8B and 87-21B, and a wild-type control plant.

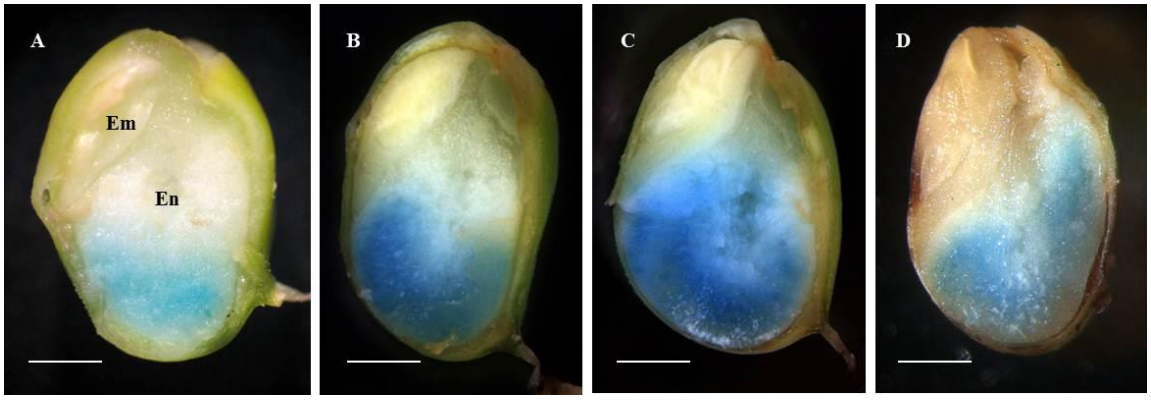

Figure 6 Histochemical localization of GUS activity in the developing seeds from transgenic line 87-21B. Seed at **(A)** 14 dpa, **(B)** 17 dpa, **(C)** 21 dpa, **(D)** 28 dpa. Bar = 1 mm.

3.4.3 Spatial activity pattern of the rice *GluA-2* promoter in transgenic sorghum

Histochemical GUS assay also revealed the spatial activity pattern of the rice *GluA-2* promoter in transgenic sorghum. X-Gluc staining was observed in a portion of

the inner part of the endosperm of seeds harvested at 14 dpa (Figure 6A). As the seed matured, GUS expression spread throughout the starchy endosperm (Figures 6B and 6C); nonetheless, no GUS staining was observed in the seed embryo.

At the whole plant level, fluorometric GUS analyses confirmed the tissue-specificity of the *GluA-2* promoter in transgenic sorghum. Fluorometric GUS assays were conducted in stem, leaf, pollen, inflorescence and seed-embryo from T_2-homozygous independent transgenic lines. Results presented in Table 4 revealed that the promoter had no activity in these tissues of the transgenic plants.

Table 4 GUS specific activity in various tissues of a T_2-homozygous transgenic sorghum plant and in a control plant

	GUS activity (nmol 4MU/mg protein/min)					
	Stem	Leaf	Root	Pollen	Inflorescence	Embryo
WT	0.32 ± 0.03	0.25 ± 0.01	0.59 ± 0.02	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
87-3B	0.36 ± 0.04	0.22 ± 0.02	0.63 ± 0.07	0.03 ± 0.02	0.03 ± 0.01	0.09 ± 0.01
87-8B	0.31 ± 0.01	0.37 ± 0.04	0.29 ±0.16	0.02 ± 0.01	0.03 ± 0.01	0.14 ± 0.06
87-21B	0.33 ± 0.04	0.32 ± 0.04	0.66 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01

Results shown are mean (±SEM) values from three individual plants.

3.4.4 Transcript expression of the *GluA-2* promoter-driven *gus*A gene and the sorghum endogenous *α-Kaf* gene

For future applications of this promoter for sorghum seed quality improvement, it is important to know the timing of the promoter activity that is derived from a heterologous species. Therefore, mRNA expression profiles of the *gus*A gene controlled by the rice *GluA-2* promoter were ascertained and compared with the endogenous,

sorghum *a-Kaf* gene promoter by RT-PCR analysis (Figure 7). Transcript expression was evaluated in seeds harvested at 10, 14, 17, 21 and 28 dpa from T_2 homozygous plants. RT-PCR results showed high abundance of *gus*A mRNA in the seeds at 14 and 17 dpa, but not at other time points. In contrast, *a-Kaf* mRNA was strongly expressed at 10 and 14 dpa, with a decrease as the seed matured.

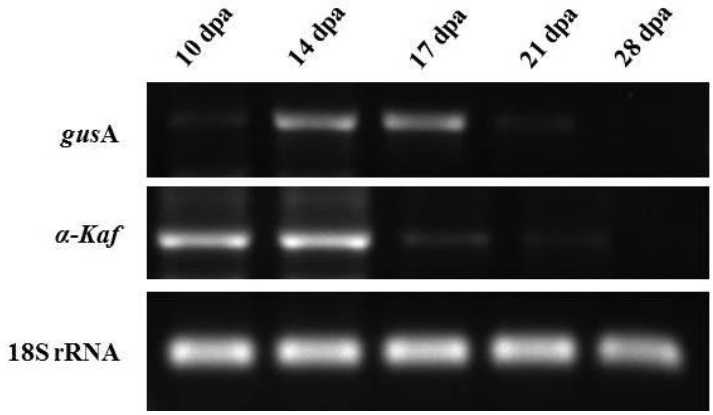

Figure 7 RT-PCR analysis of the expression pattern of the *GluA-2* promoter–driven *gus*A gene in comparison with the sorghum endogenous *a-Kaf* gene in transgenic line 87-21B.

3.5 Discussion

Rice glutelin promoters have been studied extensively in homologous systems (Okita et al., 1989; Wu et al., 1998; Qu and Takaiwa, 2004; Kawakatsu et al., 2008; Qu et al., 2008). Moreover, they have been utilized to drive endosperm-specific expression of transgenes for different biotechnological purposes in this crop. In the current study, we characterized the temporal and spatial activity pattern of a 1,832 bp long rice *GluA-2* gene promoter in transgenic sorghum. The findings support our hypothesis that, in

sorghum, the rice *GluA-2* promoter can confer strong transgene expression in an endosperm-specific manner.

Fluorometric analysis of GUS activity was useful in studying the temporal pattern of expression controlled by the rice *GluA-2* promoter in sorghum. Cereal seed storage proteins are known to start synthesis early in seed development, reaching a maximum level of expression at middle stages of the maturation process. Our results showed that transgene expression directed by the rice *GluA-2* promoter in sorghum started at approximately 14 dpa and increased thereafter until around 21 dpa (Figure 4B). Subsequent decrease in GUS activity suggests that the activity of the rice *GluA-2* promoter in transgenic sorghum either began to decline significantly or ceased (Figures 4A and 4B).

Histochemical GUS assays provided further insight into the timing pattern of the rice *GluA-2* promoter in transgenic sorghum. GUS staining was detectable at 14 dpa and its intensity increased at 17 dpa (Figures 6A and 6B). Despite the decline in fluorometric GUS activity shown after 17 dpa in Figure 4A, GUS staining at 21 dpa was higher than at 17 dpa (Figure 6C). These mismatched observations are likely to be due to the fact that concentration of total soluble proteins in a seed harvested at 21 dpa is higher than at 17 dpa. When fluorometric GUS activity is expressed in terms of nanomoles of 4-Methylumbelliferone (4-MU) per seed per minute (Figure 4B), rather than as a function of protein level, the temporal pattern observed for the rice *GluA-2* promoter corresponds to that of histochemical GUS analyses.

The spatial expression pattern directed by the rice *GluA-2* promoter in sorghum seed differed from the pattern observed previously in the rice seed. According to Qu et al. (2008), the activity of a 2.3 kbp fragment of the *GluA-2* promoter was specific to the outer portion of the rice seed endosperm, while we found that the promoter was active in the inner part of the sorghum seed endosperm. One possible reason for this discrepancy may be that the promoter sequence we evaluated in sorghum was partial. More extensive analysis needs to be performed to confirm this. Nevertheless, the sequence tested in the current investigation can be used for altering sorghum seed quality. In addition, the absence of detectable GUS activity in the vegetative and floral tissues confirms that the rice *GluA-2* promoter is suitable for seed-specific regulation of transgene expression in sorghum.

Transcript analyses showed that the expression of mRNA for the *GluA-2* promoter driven-*gus*A gene and the sorghum *a-Kaf* gene differed from each other during sorghum seed development (Figure 7). This suggests that both promoters could be used in a stepwise fashion, each controlling a different gene, to obtain a desired trait. In conclusion, our results demonstrate that the rice *GluA-2* promoter characterized in this study can be used to control endosperm-specific transgene expression in sorghum and thus, it can serve as a useful tool in introducing novel traits into sorghum seed by means of genetic engineering.

4. EFFECT OF GLUTAMINE SYNTHETASE AND ALANINE AMINOTRANSFERASE GENE OVEREXPRESSION ON SORGHUM NITROGEN USE EFFICIENCY

4.1 Synopsis

Nitrogen is a primary macronutrient in plants, and nitrogen fertilizers play a critical role in crop production and productivity. However, it is estimated that only between 30% to 50% of applied nitrogen is taken up by plants, with the remainder contaminating soil, water and air. Therefore, from both economic and environmental standpoints, there is a considerable interest in developing plants that take-up and utilize nitrogen in a more efficient manner. In this study, we investigated the effect of glutamine synthetase (GS) and alanine aminotransferase (AlaAT) gene overexpression in nitrogen metabolism and plant growth in sorghum (*Sorghum bicolor* L.). GS catalyzes the ATP dependent reaction between ammonia and glutamate to produce glutamine, while AlaAT is involved in the transfer of an amino acyl group from glutamate and pyruvate to form alanine and 2-oxoglutarate. The 1,071 bp long coding sequence of a sorghum cytosolic GS gene (*Gln1*) was ligated at the 3'-end of the maize ubiquitin (Ubq) and cauliflower mosaic virus (CaMV 35S) promoters. Both vectors were introduced into sorghum immature embryos by *Agrobacterium*–mediated transformation. In parallel, a third construct harboring a 1,049 bp long sequence of a barley AlaAT gene driven by a brachypodium aldehyde dehydrogenase (*Ald1*) gene promoter was also transferred to sorghum. Neither plants transformed with the AlaAT

gene nor the *Gln1* gene under the control of the CaMV 35S promoter showed increase in transgene overexpression or enzyme activity in comparison to the wild-type. However, plants transformed with the G*ln1* gene driven by the ubiquitin promoter did exhibit higher accumulation of the transcripts and GS activity compared to the controls. When grown under optimal nitrogen conditions, these *Gln1* transgenic plants showed improved vegetative biomass production and grain yield.

4.2 Introduction

Nitrogen (N) is a primary macronutrient for plants, and plant growth and crop productivity highly depend on the application of N fertilizers. Due to the critical role that N fertilization plays in increasing yields in agriculture, world demand for N will grow by 1.3% per year from 2012 to 2016, according to the Food and Agriculture Organization of the United Nations. However, while N fertilizers can contribute to enhanced agricultural productivity, they can also lead to environmental degradation. In fact, it is estimated that only 30 to 50% of applied N is taken up by plants, with the remainder causing soil, water and air pollution. For this reason, there has been an increasing interest in improving N use efficiency (NUE) in plants, which is their efficiency to take-up N from the soil and utilize it in the production of biomass. Genetic engineering has played an important role in the study of candidate genes thought to improve NUE.

Among the multiple biotechnological strategies used to date to enhance NUE in plants, overexpression of genes encoding enzymes such as glutamine synthetase (GS)

and alanine aminotransferase (AlaAT) seems to have been most successful (Good et al., 2004; McAllister et al., 2012; Xu et al., 2012). GS is known to catalyze the ATP dependent reaction between ammonia and glutamate to produce glutamine. This enzyme is likely to be an important checkpoint controlling NUE, since it is involved in the assimilation of all the N, derived either from nitrate or ammonium, into organic forms (Miflin and Lea, 1976). GSs are present as cytosolic (GS1) or plastidic (GS2) isoforms (Hirel and Gadal, 1982; Zhang, 2011), are differentially expressed in various plant tissues, and have specific functions in N assimilation. As is the case with GS, AlaAT is also expressed differentially in different plant tissues and is involved in multiple metabolic pathways. AlaAT catalyzes the transfer of an amino group from glutamate and pyruvate to form alanine and 2-oxoglutarate. Various reports have shown that overexpression of GS genes resulted in improved plant biomass or grain yield in rice, poplar, wheat, maize, and tobacco growing under different N concentrations (Gallardo et al., 1999; Habash et al., 2001; Oliveira et al., 2002; Martin et al., 2006; Cai et al., 2010). Moreover, Brauer et al. (2011) recently reported that the overexpression of a GS gene in rice significantly increased spikelet yield under high N conditions. In contrast to GS, AlaAT was generally not considered to have any significant effect on plant NUE. However, Good et al. (2007) and Shrawat et al. (2008) demonstrated that transgenic canola and rice overexpressing a barley (*Hordeum vulgare* L.) AlaAT gene under low and optimal N conditions, respectively, exhibited increased biomass and seed yield. Furthermore, as part of an effort to commercialize this technology, field trials of

transgenic canola, wheat and barley overexpressing AlaAT have been recently conducted, obtaining promising results (McAllister et al., 2012).

In this study, we investigated the effect of overexpression of GS1 and AlaAT genes in sorghum. The 1,071 bp long coding sequence (CDS) of a cytosolic GS gene (*Gln1*) was placed downstream of the maize ubiquitin promoter or the 35S cauliflower mosaic virus (CaMV 35S) promoter in two separate binary vectors. In addition, a plasmid harboring the 1,049 bp long CDS of a barley AlaAT gene driven by the brachypodium (*Brachypodium distachyon* L.*)* aldehyde dehydrogenase gene (*Ald1*) promoter was used (Good et al., 2007; Shrawat et al., 2008). All constructs were introduced into sorghum by means of *Agrobacterium*-mediated transformation. Transformants showing higher transgene expression and enzyme activity than the wild-type plants were subjected to biomass and yield evaluations, as well as to nitrogen analyses.

4.3 Experimental procedures

4.3.1 Assembly of binary vectors

The CDS of a sorghum *Gln1* gene (Martin et al., 2006) was obtained by RT-PCR from mRNA isolated from the leaves of sorghum, genotype P898012. Primers Gln1-F1: 5'-GGATCCATGGCCTCCCTCACCGA-3' and Gln1-R1: 5'-GAGCTCTCAGGGCTTCCAGAGGATG-3' were used to amplify a 1,071 bp long sequence and incorporate *BamH*I and *Sst*I cloning sites at the 5' and 3' ends, respectively. A binary vector was assembled by ligating this fragment at the 3'-end of

the maize ubiquitin promoter in a pCAMBIA-based vector. The laboratory designation for this vector was LCT93. In parallel, primers Gln1-F2: 5'-CCATGGATGGCCTCCCTCACCGA-3' and Gln1-R2: 5'-GGATCCTCAGGGCTTCCAGAGGATG-3' were used to amplify the same sequence, but incorporating *Nco*I and *BamH*I cloning sites at the 5' and 3' ends, respectively. This fragment was ligated at the 3'-end of the tobaco etch virus (TEV) translation enhancer under the control of the CaMV 35S promoter in a pCAMBIA-based vector. The laboratory designation for this second vector was LCT94. A third plasmid, provided by Dr. Alan Good, harbouring the 1,469 bp long CDS of a barley AlaAT gene (Good et al., 2007; Shrawat et al., 2008) under the control of the brachypodium *Ald1* promoter was designated LCT91. Each plasmid was mobilized by electroporation into *Agrobacterium tumefaciens* strain NTL4 containing the disarmed Chry5 Ti plasmid designated pTiKPSF2 (Palanichelvam et al., 2000) that was then used to transform sorghum.

4.3.2 Plant transformation

The transgene expression cassettes were introduced into sorghum genotype P898012 by *Agrobacterium*-mediated transformation of immature embryos (14 dpa), and putative transformants were obtained following selection on hygromycin (Kumar et al., 2011).

4.3.3 Molecular characterization of transgenic events

Southern blot analysis was performed to confirm the number of transgene copies integrated into the genome of transformed sorghum plants. Genomic DNA was extracted from sorghum leaves of T_0 plants (Dellaporta et al., 1983) and 15 μg were digested with *BamH*I and fractionated on a 1% agarose gel. Membranes were hybridized with ^{32}P-labeled *hpttII* gene fragments.

Northern blot analyses were conducted on total RNA isolated from leaf or root tissues using TRIzol® Reagent (Ambion). Membranes were hybridized with ^{32}P-labeled *Gln1* or AlaAT gene fragments.

4.3.4 Plant material for molecular and physiological studies

T_2 seeds obtained from the transgenic lines and corresponding null segregant (NS) plants were germinated on filter paper. One week-old seedlings were planted in 500 ml size plastic cups (with holes in the bottom) containing sand, for the harvesting of leaf or root samples at the vegetative stage. The experiment was conducted in a greenhouse and plants were irrigated daily with a nutrient solution containing either 4 mM NO_3^- (low N concentration) or 12 mM NO_3^- and 2mM NH_4^+ (optimal N concentration) (Tables 5 and 6). The pH of each solution was adjusted to 5.8 before use. At each irrigation, solution was allowed to drain out from the bottom of the plastic cups. At the five-leaf stage, roots and the three youngest fully expanded leaves were harvested for subsequent use in transcription, enzyme and metabolite analyses. For the long-term studies, plants were grown in rockwool cubes (Grodan, Ontario, Canada) in a

greenhouse and irrigated every other day with the nutrient solutions as described above. At maturity, shoot tissue and seeds were harvested for metabolite analyses and yield determinations.

Table 5 Macronutrient composition of nutrient solutions

	Low N (mM)	Optimal N (mM)
KNO_3	4	4
$Ca(NO_3)_2$	---	3
NH_4NO_3	---	2
$CaCl_2$	3	---
$MgSO_4$	0.7	0.7
KH_2PO_4	0.7	0.7
K_2HPO_4	0.2	0.2

Table 6 Micronutrient composition of nutrient solutions

	mg/L
Na_2MoO_4 x $2H_2O$	0.06
H_3BO_3	1.5
$MnSO_4$	2
$CuSO_4$ x $5H_2O$	0.25
$ZnSO_4$ x $7H_2O$	1
EDTA-Fe	0.6

4.3.5 Enzymatic assays

AlaAT and GS enzyme activities were determined in freshly harvested leaf and root tissues using spectrophometric assays as described previously by Muench et al

(1998) and O'Neal and Joy (1973), respectively. Measurement of soluble protein was performed spectrophotometrically using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

4.3.6 Evaluation of growth and biomass production

Shoot and root dry weights (DW) were determined for transgenic and null-segregant plants at the five-leaf developmental stage. In addition, flowering time, height of the primary tiller, grain number, grain weight and vegetative shoot DW were determined in mature transgenic plants and null-segregant controls at 35 days post anthesis (dpa). The first long-term experiment was first started on October 3, 2012 and, in order to evaluate its reproducibility, a second long-term experiment was initiated on February 11, 2013. Both these experiments were conducted in exactly the same manner, however, because of the timing of experiments, the plants experienced different temperatures, light quantity, and photoperiods as these conditions could not be fully controlled in our greenhouses.

4.3.7 Ammonium and nitrogen analyses

Dry plant material was used for ammonium and nitrogen analyses. Ammonium was extracted with 60% methanol and determined by the salicylate-type colorimetric method described previously by Husted et al. (2000). Nitrogen content was estimated by the Dumas combustion method as described by Palle et al. (2013).

4.4 Results

4.4.1 Generation of transgenic sorghum lines

In order to investigate the effects of overexpressing *Gln1* and AlaAT genes in the improvement of NUE in sorghum, we used three different plasmid constructs (Figure 8). We assembled two binary vectors harboring the 1,071 bp long CDS of a sorghum *Gln1* gene under the control of the maize ubiquitin promoter or the CaMV 35S promoter, designated LCT93 and LCT94, respectively. Additionally, we used a binary vector carrying the 1,469 bp long CDS of a barley AlaAT gene driven by the brachypodium *Ald1* gene promoter, designated LCT91. All the constructs contained the hygromycin phosphotransferase *(hptII)* gene downstream of the CaMV 35S promoter for plant selection and were introduced into sorghum by *Agrobacterium*-mediated transformation of immature embryos. Calli that were resistant to hygromycin were selected and regeneration was induced by transfer to regeneration media. Plantlets with well-developed roots were transferred to soil. PCR-screening using *hptII* primers was conducted on T_0 plants. Distinct bands of expected size were obtained from 6 transgenic events generated from transformations performed with LCT91 vector, three for LCT93 and two for LCT94. Southern blot analysis was conducted to determine transgene copy number on LCT91 lines. Among LCT91 transgenic events, 91-1A, 91-3A, 91-5A, 91-5B and 91-6A showed integration of a single transgene copy within their genomes (Figure 9).

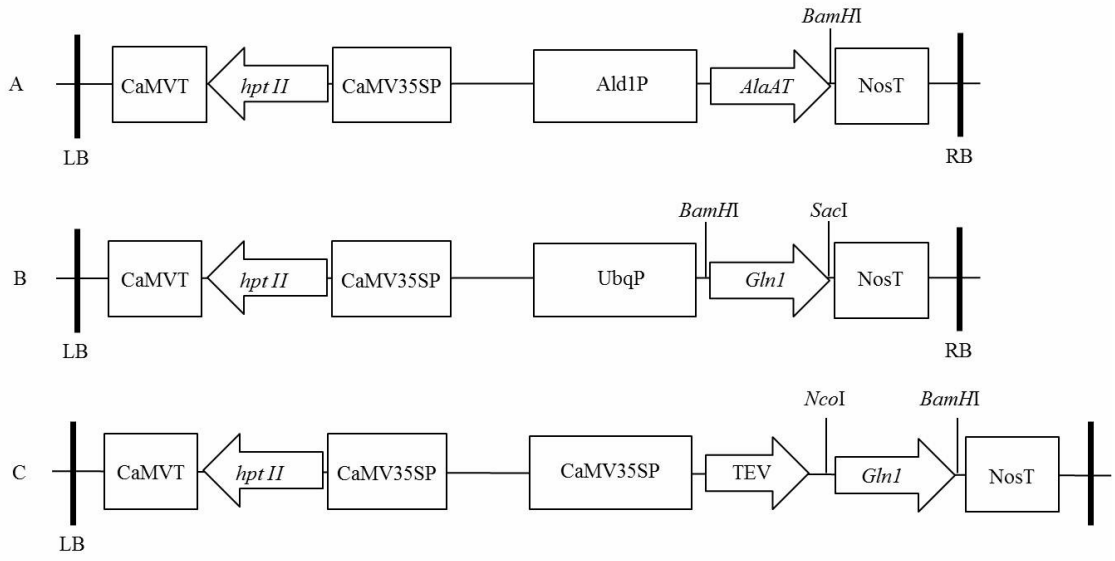

Figure 8 T-DNA portion of vectors used to generate transgenic sorghum lines. **(A).** LCT91, **(B)** LCT93, **(C)** LCT94

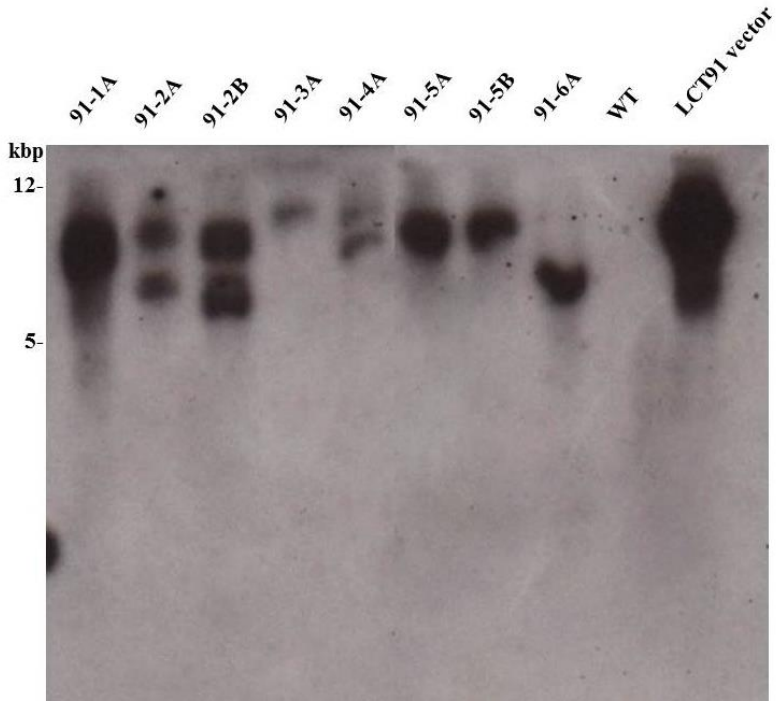

Figure 9 Southern blot analysis of genomic DNA obtained from the leaves of LCT91 transgenic lines and a wild-type control plant. Genomic DNA was digested with *BamH*I and hybridized with an *hptII* gene fragment.

4.4.2 Evaluation of gene transcription and enzyme activity

The levels of mRNA encoding the barley AlaAT in LCT91 transgenic lines or the sorghum GS1 in LCT93 and LCT94 plants were assessed by Northern blot hybridization. Transcript accumulation was evaluated in both leaf and root tissue from T_2 homozygous plants and the corresponding null segregants grown under low or optimal N conditions until the five-leaf developmental stage. Northern blot analyses of AlaAT transformants showed that only lines 91-2A and 91-4A grown in a low N concentration exhibited slightly higher amounts of AlaAT gene transcripts in their leaves in comparison to the null segregants; however, no difference was observed for lines 91-1A or 91-5B (Figure 10). Also, there was no major difference in the levels of AlaAT mRNA present in leaf samples from LCT91 transgenic lines grown under optimal N conditions. Furthermore, no difference in AlaAT gene transcription was observed in root samples from LCT91 plants grown under either low or optimal N regimen. By contrast, transgenic lines carrying the *Gln1* gene driven by the maize ubiquitin promoter, and grown under either low or optimal N conditions, showed higher abundance of *Gln1* transcripts in both leaves and roots, in comparison to the null segregants (Figure 11). However, in transgenic plants transformed with the *Gln1* gene under the control of the CaMV 35S promoter, no major difference in mRNA synthesis was found compared to the null segregant controls in leaf or root tissues, or between N treatments (Figure 12).

The enzyme activities in AlaAT and *Gln1* transformants were evaluated in the leaves and roots of T2 homozygous plants and null segregants grown under low or optimal N conditions. AlaAT activities in lines 91-1A and 91-5B were analyzed in a

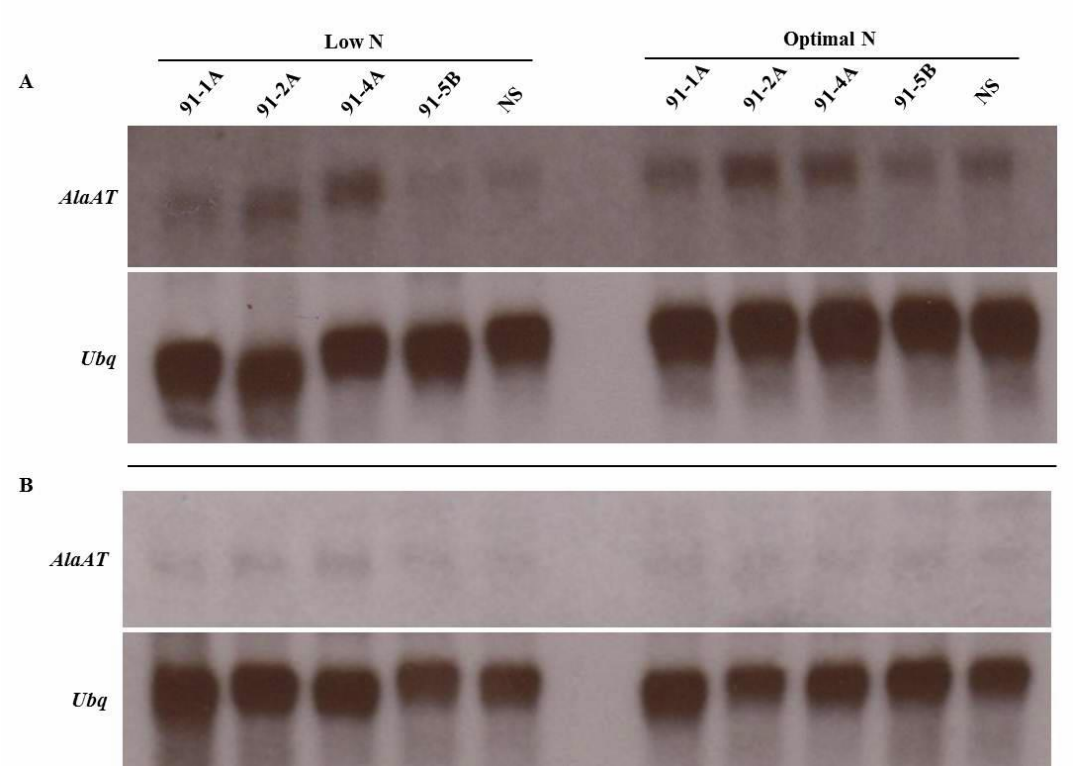

Figure 10 AlaAT transcript levels in LCT91 transgenic lines grown under low or optimal nitrogen conditions. **(A)** Northern blot analysis of RNA isolated from leaves of transgenic lines and a null segregant plant (NS), **(B)** Northern blot analysis of RNA isolated from AlaAT in roots of transgenic lines and a null segregant plant. Transcripts were detected by hybridization with ^{32}P-labeled probes corresponding to the CDS specific to each gene. Leaf and root samples contained 6 and 3 μg of RNA, respectively.

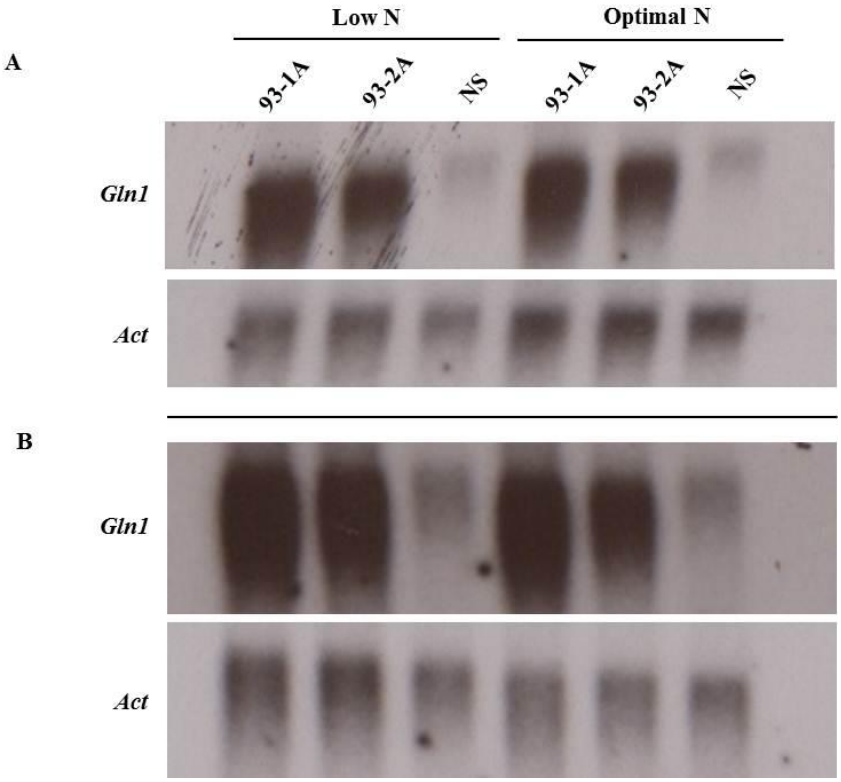

Figure 11 Level of transcripts for *Gln1* gene in LCT93 transgenic lines grown under low or optimal nitrogen conditions. **(A)** Northern blot analysis showing transcript levels of *Gln1* in leaves of transgenic lines and a null segregant plant (NS), **(B)** Northern blot analysis showing transcript levels of *Gln1* in roots of transgenic lines and a null segregant plant. Transcripts were detected by hybridization with ^{32}P-labeled probes corresponding to the CDS specific to each gene. Three μg of RNA were loaded in each lane.

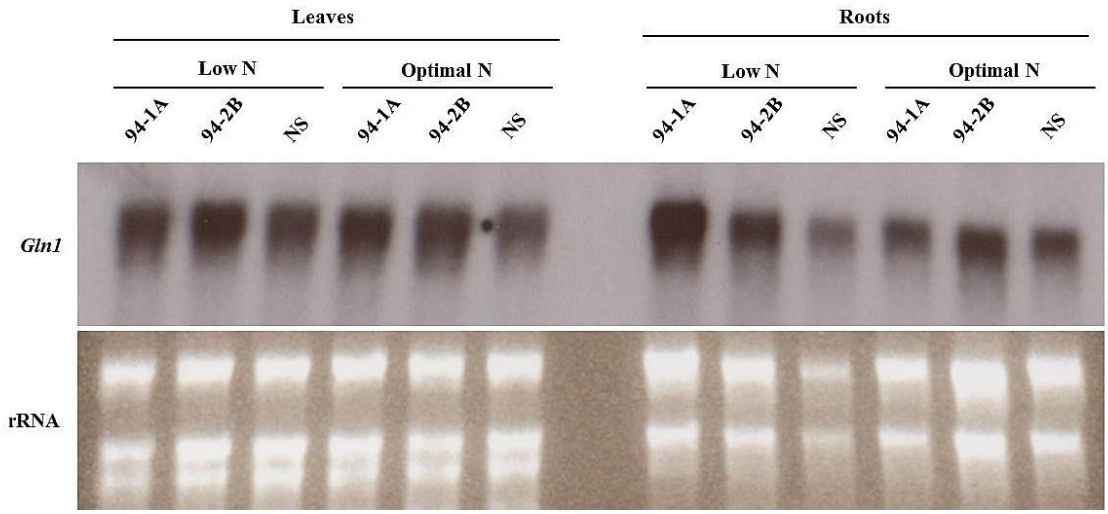

Figure 12 Level of transcripts for *Gln1* in leaf tissue from LCT94 transgenic lines and a null segregant (NS) plant grown under low or optimal nitrogen conditions. Transcripts were detected by hybridization with ^{32}P-labeled probes corresponding to the CDS specific to each gene. Seven µg of RNA were loaded in each lane.

separate experiment than that for lines 91-2A and 91-4A. No significant difference in AlaAT activity was detected in the leaves or roots from transgenic lines grown in either low or optimal N environments, in comparison to the null segregants (Figures 13A and 13B). On the other hand, GS1 activity was significantly higher in both leaves and roots of LCT93 plants than that of control plants grown under both low and high N conditions (Figure 14A). A 1.3 to 1.6 fold increase in GS activity was observed in the leaves of LCT93 transgenic plants, while a 1.3 to 2.2 fold was found in the roots. As opposed to LCT93 lines, LCT94 plants showed no significant difference in GS activity compared to the null segregants, in any of the tissues analyzed and under both the N treatments (Figure 14B).

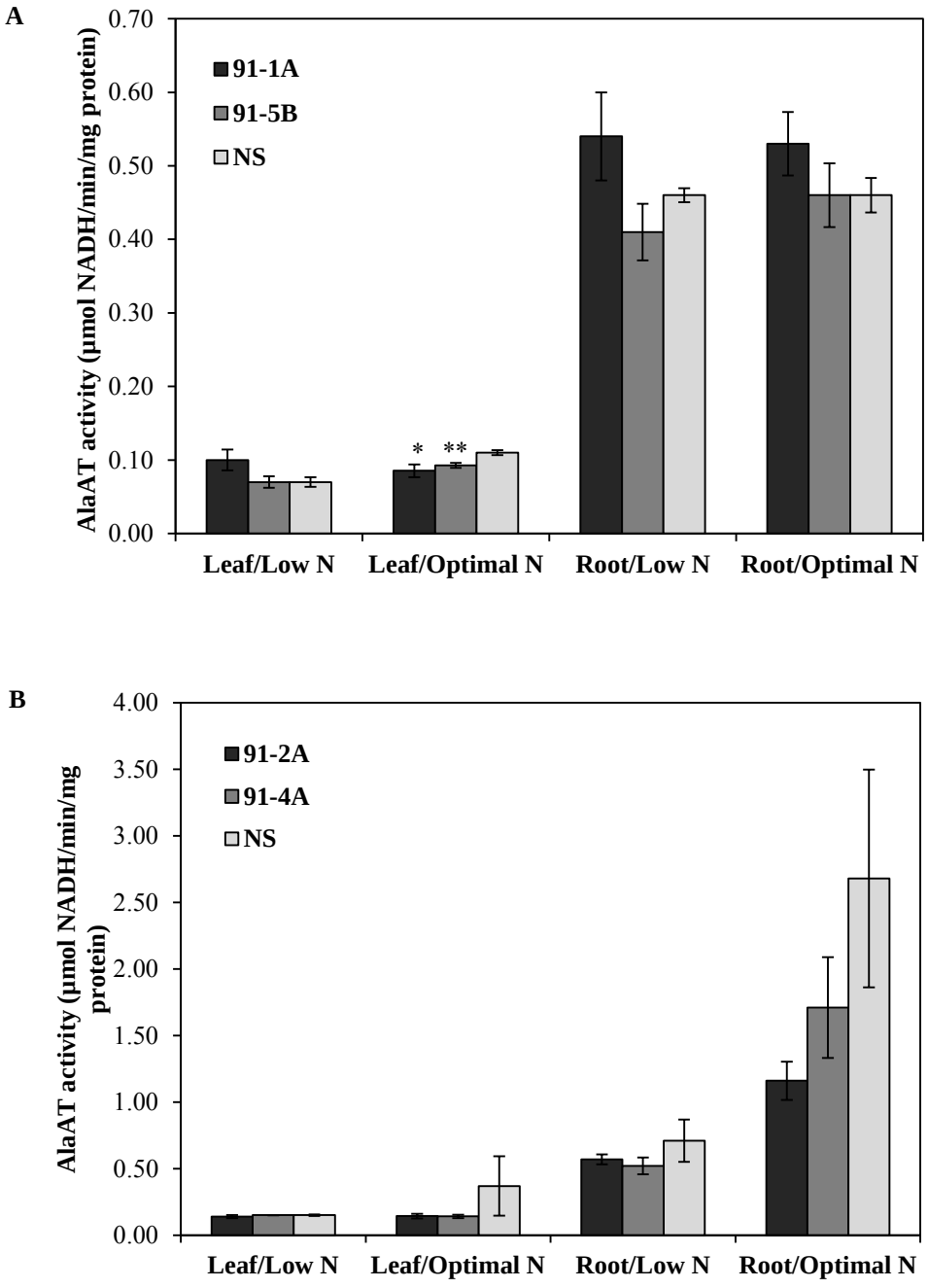

Figure 13 Activity of AlaAT enzyme in the leaves and roots of LCT91 transgenic plants grown under low or optimal nitrogen conditions. **(A)** AlaAT levels in lines 91-1A and 91-5B, and null segregants (NS), **(B)** AlaAT levels in lines 91-2A and 91-4A, and null segregants. AlaAT activities in lines 91-1A and 91-5B were analyzed in a separate experiment than that for lines 91-2A and 91-4A. Results shown are mean (±SEM) values from five individual plants. Asterisks indicate significant differences between the transgenic plants and NS controls; one asterisk (*) corresponds to $p < 0.05$ and two asterisks (**) correspond to $p < 0.01$.

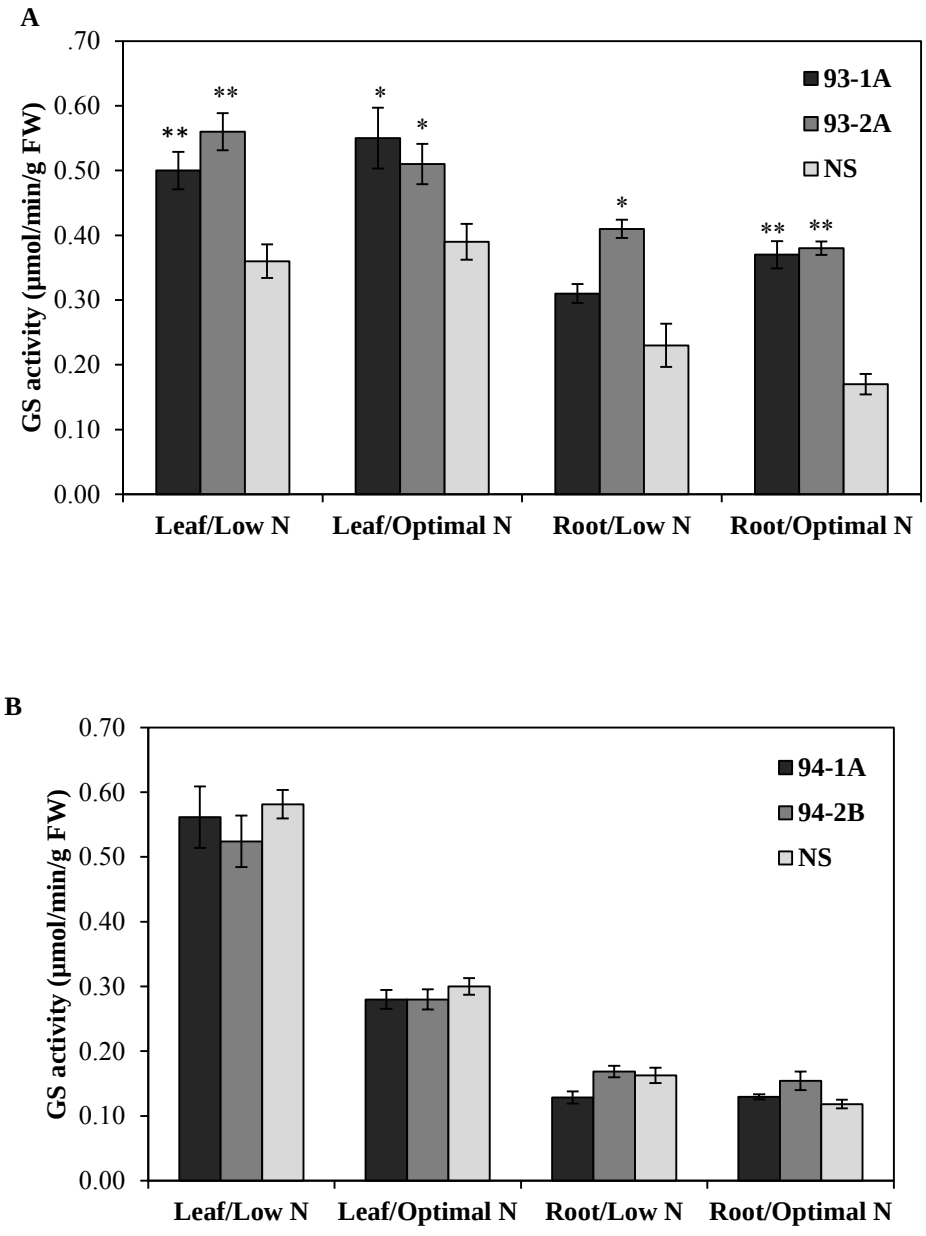

Figure 14 Activity of GS enzyme in leaves and roots of LCT93 and LCT94 transgenic plants grown under low or optimal nitrogen conditions. (A) GS levels in LCT93 lines and null segregants (NS), (B) GS levels in LCT94 lines and null segregant. Results shown are mean (±SEM) values from five individual plants. Asterisks indicate significant differences between the transgenic plants and NS controls, one asterisk (*) corresponds to $p < 0.05$ and two asterisks (**) correspond to $p < 0.01$.

4.4.3 Evaluation of growth, biomass production and grain yield of LCT93 transgenic plants

To determine the effect of *Gln1* overexpression on the growth and development of sorghum plants grown under low and optimal N conditions, biomass and grain production were evaluated in T_2 93-2A plants at the vegetative stage and after maturity in separate experiments. No significant difference in shoot or root DW was found in the 93-2A plants harvested at the five-leaf developmental stage compared to the wild-type counterparts (Table 7). However, at 35 dpa, 93-2A plants from the experiment started on October 3, 2013 (hereafter referred to as winter experiment), grown in optimal N concentrations and harvested in December 2012 showed significantly higher accumulation of biomass in shoot vegetative organs than controls (Table 8 and Figure 16). Interestingly, the 2.1-fold increase in shoot biomass was due to the formation of more secondary tillers compared to the null segregant plants (Figure 17). In contrast, no significant difference was observed in the DW of shoot vegetative parts between 93-2A plants and controls, grown under low N (Table 8 and Figure 16). Similarly, with regards to the grain yield, there were no significant differences between 93-2A and control plants grown under low N conditions. The situation under optimal N conditions was more complex. The primary tillers of 93-2A transgenic plants grown in optimal N concentration exhibited a significant reduction in seed number (Table 8). However, as mentioned earlier, the transformants had a tendency to produce a secondary tiller. The number of seeds in these secondary tillers was higher than that in the primary tillers of the transformant (Figure 15). Thus, at the termination of the experiment (35 dpa in the

Table 7 Biomass, ammonium and N content in 93-2A plants harvested at the vegetative stage

	Low N		Optimal N	
	93-2A	NS	93-2A	NS
Shoot DW (g)	0.19 ± 0.02	0.15 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Root DW (g)	0.34 ± 0.05	0.28 ± 0.05	0.15 ± 0.02	0.13 ± 0.04
NH_4^+ (μmol/g DW)	0.10 ± 0.01	0.11 ± 0.00	0.06 ± 0.00*	0.11 ± 0.00
Total N (%)	4.75 ± 0.06	4.64 ± 0.06	4.93 ± 0.18	4.59 ± 0.24

Ammonium and nitrogen levels were estimated in pooled samples of the three youngest leaves from transgenic and null segregant (NS) plants at the five-leaf stage. Results shown are mean (±SEM) values from five individual plants. Asterisks (*) indicate significant differences between the transgenic plants and NS controls ($p < 0.05$).

Table 8 Agronomic traits, N and ammonium content in 93-2A plants harvested at maturity (winter experiment)

	Low N		Optimal N	
	93-2A	NS	93-2A	NS
Flowering time (d)	64.57 ±0.97**	60.86 ± 0.46	60.43 ± 1.73	58.71 ± 0.81
Height of primary tiller (inch)	37.14 ± 1.01	38.43 ± 0.37	39.57 ± 1.71	39.00 ± 0.95
Grain number (primary tiller)	181.00 ± 15.97	175.71 ± 19.80	93.43 ± 20.31**	193.57 ± 12.55
Grain weight (50 seeds from primary tiller, g)	1.75 ± 0.04	1.82 ± 0.04	1.54 ± 0.07	1.62 ± 0.03
Grain number (secondary tiller)			154.86 ± 28.27	
DW of shoot vegetative organs (g)	6.63 ± 0.48	6.02 ± 0.43	27.78 ± 2.75**	13.37 ±1.00
NH_4^+ (μmol/g DW)	0.17 ± 0.02	0.19 ± 0.00	0.18 ± 0.00	0.19 ± 0.00
Total N (%)			2.95 ± 0.18	2.92 ± 0.27

For flowering time, height, grain weight and number, and DW of shoot vegetative organ, results shown are mean (±SEM) values from seven individual transgenic and null segregant (NS) plants. For ammonium and total N determination, results presented are mean (±SEM) values from five and four individual plants, respectively. Asterisks (**) indicate significant differences between the transgenic plants and NS controls ($p < 0.01$).

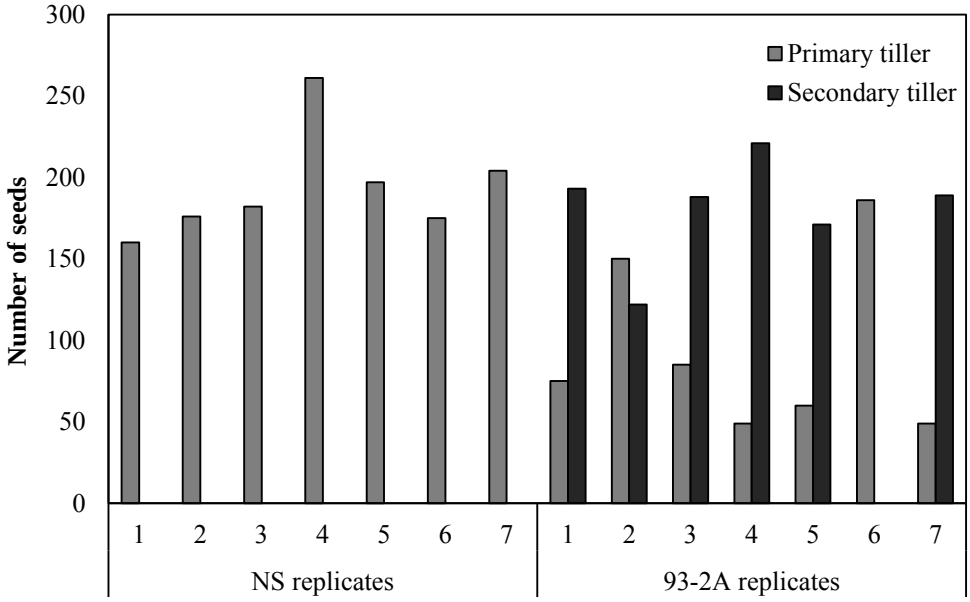

Figure 15 Number of seeds in the primary and secondary tillers from individual null segregants (NS) and T_2 plants from line 93-2A growing under optimal N conditions and harvested at 35 dpa (winter experiment).

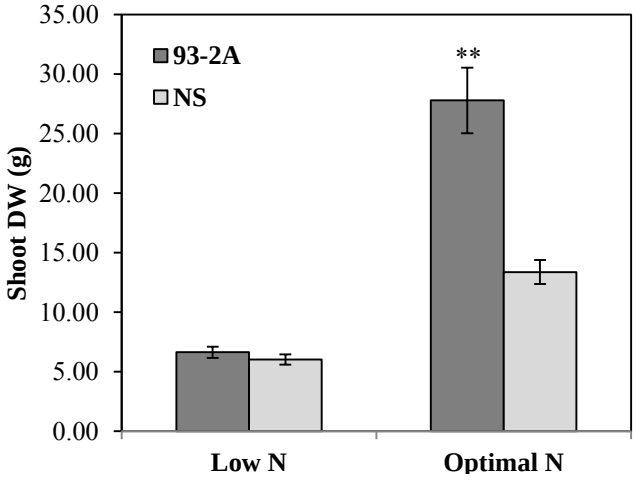

Figure 16 DW of vegetative shoot portion of LCT93-2A plants and null segregant (NS) plants (winter experiment). Plants were harvested 35 dpa. Results shown are mean (±SEM) values from seven individual plants. Asterisks (**) indicate significant differences between the transgenic plants and NS controls ($p < 0.01$).

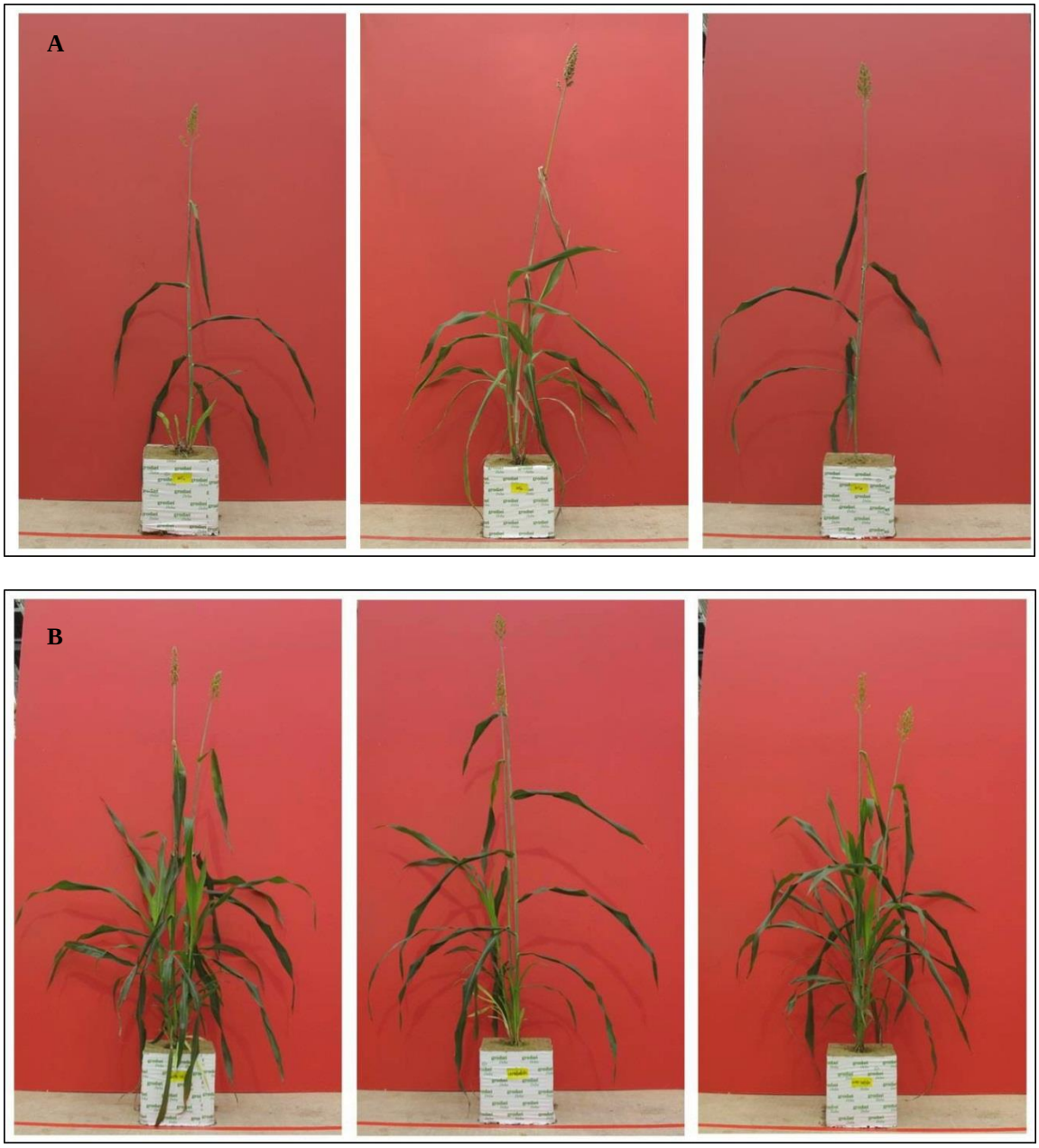

Figure 17 Image depicting the shoot phenotype of plants grown under optimal N conditions (winter experiment). **(A)** Null segregant plants, **(B)** 93-2A plants (35 dpa in primary tiller).

primary tiller), a significantly higher number of seeds was produced by the transformants compared to the null segregants. In contrast, no differences in growth or developmental parameters were observed between the transformants and null segregants grown under low nitrogen conditions. Additionally, no significant difference in height was observed between transgenic plants and null segregant controls, but a slight delay in flowering was detected in transgenic individuals grown under low N conditions.

In order to see if these results were reproducible, this experiment was repeated (initiated on February 11, 2013; spring experiment), and growth and development-related measurements were obtained on plants harvested on May 22, 2013. In contrast to plants harvested in the winter experiment, both, null segregant and transgenic plants from this second experiment had a tendency to produce secondary tillers that emerged from the stems of primary tillers (Figures 18A, 18B, 18C and 18D). At 35 dpa, 93-2A plants grown under optimal N conditions showed a significant 1.2-fold higher accumulation of the vegetative shoot biomass than the controls (Table 9). This increase in shoot biomass was due to the formation of more secondary tillers compared to the control plants. By contrast, no significant difference was observed between 93-2A plants and controls grown in low N conditions. Regarding grain yield, the primary tillers of 93-2A transgenic plants grown at both low and optimal N concentrations exhibited a significant reduction in seed number (Table 9). However, even though plants produced secondary tillers, at the termination of the experiment (35 dpa in the primary tiller) only few of them had flowered; thus, seed number could not be counted and taken into consideration for this study. Furthermore, no significant difference in height was found

between transgenics and null segregants, and a slight but significant decrease in flowering time was detected in transgenic individuals grown under both low and optimal N conditions.

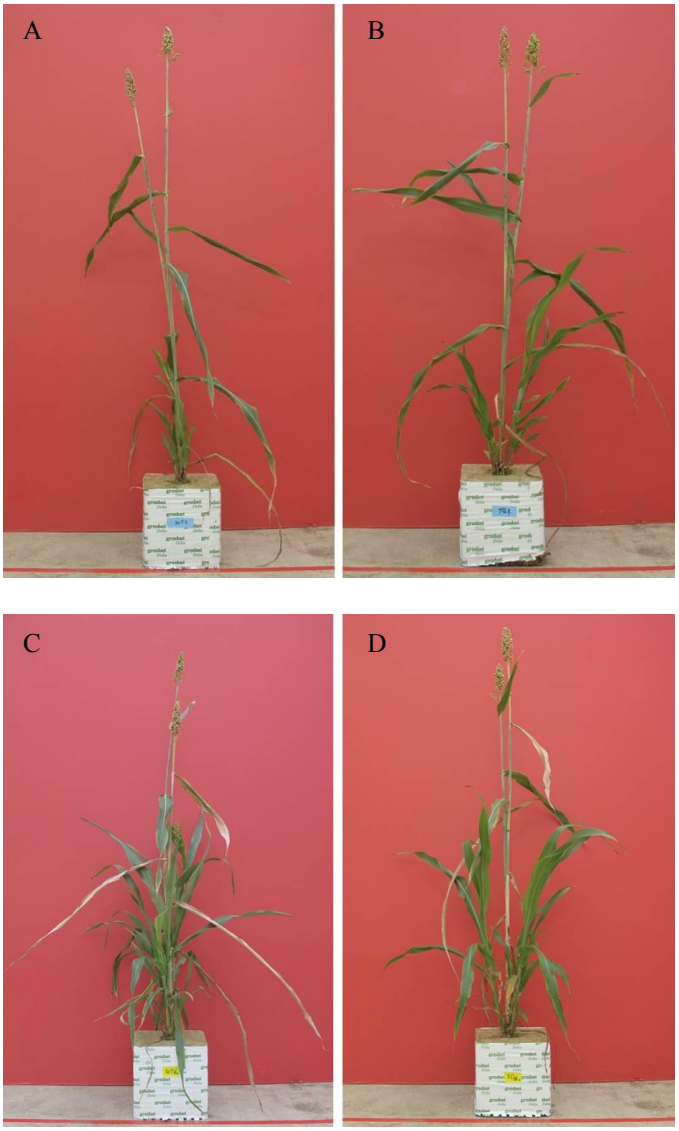

Figure 18 Image depicting the shoot phenotype of plants at 35 dpa (primary tiller) grown under low and optimal N conditions (spring experiment). **(A)** Null segregant plant under low N condition, **(B)** 93-2A plants under low N conditions. **(C)** Null segregant plant under optimal N. **(D)** 93-2A plant under optimal N condition.

Table 9 Agronomic traits, N and ammonium content in 93-2A plants harvested at maturity (spring experiment)

	Low N		Optimal N	
	93-2A	NS	93-2A	NS
Flowering time (d)	58.86 ± 0.38*	59.86 ± 0.32	56.50 ± 0.42**	58.14 ± 0.13
Height of primary tiller (inch)	38.93 ± 0.77	39.86 ± 0.48	39.38 ± 0.71	38.21 ± 1.48
Grain number (primary tiller)	153.57 ± 22.82*	217.57 ± 21.66	107.88 ± 25.08**	327.14 ± 22.01
Grain weight (50 seeds from primary tiller, g)	1.81 ± 0.03	1.79 ± 0.02	1.61 ± 0.05	1.60 ± 0.04
DW of shoot vegetative organs (g)	29.50 ± 2.34	30.18 ± 1.90	33.62 ± 1.37**	28.48 ± 0.57
Number of tillers	4.57 ± 0.45	4.57 ± 0.28	7.50 ± 0.27**	5.71 ± 0.34

Results shown are mean (±SEM) values from eight individual transgenic and null segregant (NS) plants. Asterisks indicate significant differences between the transgenic plants and NS controls; one asterisk (*) corresponds to $p < 0.05$ and two asterisks (**) correspond to $p < 0.01$.

4.4.4 Ammonium and nitrogen levels in LCT93 transgenic plants

To estimate the impact of *Gln1* overexpression on N metabolism in sorghum plants grown under low and optimal N conditions, the ammonium and total N contents were determined in T_2 93-2A plants at the vegetative stage and at maturity (Tables 7 and 8). There was no significant difference in nitrogen levels in the leaves from young plants grown in either low or optimal N concentrations in comparison to controls. However, there was a significant decrease in ammonium content in young leaves from 93-2A plants grown under optimal N conditions, compared to null the segregants.

4.5 Discussion

Using a transgenic approach, we attempted to investigate the effect of the overexpression of a barley AlaAT gene on sorghum NUE. Two studies have

demonstrated that canola (Good et al., 2007) and rice (Shrawat et al., 2008) transgenic plants overexpressing a barley AlaAT gene exhibited higher NUE under low and optimal N availability, respectively. Generally, AlaAT has not been considered to be a major checkpoint in N metabolism; however, it seems that alanine plays an important role in the storage and transport of N in plants, and it has been observed that its levels tend to increase during N stress conditions (Good et al., 2004). In this study, the AlaAT gene was driven by an Ald1 gene promoter from brachypodium. Although the activity of this promoter has not been characterized in transgenic sorghum, previous reports have suggested that aldehyde dehydrogenase promoters from other species, such as canola and rice, are highly active in the root tissue (Good et al., 2007; Shrawat et al., 2008) and under drought, cold, heat and salinity stress (Stroeher et al., 1995). In this case, northern blot analyses (Figure 10) and alanine aminotransferase enzyme assays (Figures 13A and 13B) revealed that the brachypodium Ald1 promoter failed to direct strong expression of the barley AlaAT gene in both leaves and roots of transgenic sorghum plants grown under low or optimal N conditions, in comparison to the null segregant controls. One possible reason for this low level of transgene expression may be that some of the trans-acting elements necessary for the activation of the brachypodium Ald1 promoter or to control its strength are absent in sorghum. These results reaffirm the importance of performing promoter-analysis in which the promoter that is intended to be used for plant transformation is first examined for its strength, as well as temporal and spatial activity in the target organism prior to using it to drive the transgene of interest.

The effect of the overexpression of *Gln1* in sorghum NUE was also investigated. To that end, transgenic lines carrying a sorghum *Gln1* gene under the control of the CaMV 35S promoter or the maize ubiquitin promoter were generated. Northern blot analyses and glutamine synthetase enzyme assays revealed that the CaMV 35SP::*Gln1* transformants did not show higher expression of the *Gln1* gene in either leaves or roots of the sorghum plants grown under low or optimal N conditions (Figures 12 and 14B). Although the CaMV 35S promoter is a very strong constitutive promoter, it is also known to have lower activity in monocotyledonous plants. Nevertheless, this promoter has been used successfully to drive transgenes in monocots and indeed the selectable marker gene, *hpt*, used to select sorghum transformants in this study was under the control of CaMV 35S promoter. It will require extensive investigation to ascertain why this set of transgenic lines did not express the *Gln1* gene. In contrast, the maize ubiquitin promoter did direct strong expression of the transgene in both tissues (leaves and roots) examined and under both N treatments tested (Figures 11 and 13A). In fact, UbqP::*Gln1* transformants grown under optimal N conditions and harvested at the vegetative stage showed lower levels of ammonium than controls, which could be due to the higher GS1 activity in these plants (Oliveira et al., 2002; Martin et al., 2006). Intriguingly, no difference in ammonium levels was observed between young transgenic and control plants growing under low N availability or between transgenic and controls harvested at maturity (Tables 7 and 8).

Overexpression of the *Gln1* gene in LCT93 lines didn't affect the growth of transgenic plants at the early stages of development (Table 7), but had a significant

effect at maturity (Table 8). In the winter experiment, initiated on October 3, 2012, *Gln1* overexpressing lines showed a significant increase in shoot vegetative biomass, in comparison to the null segregant controls grown under optimal N conditions but not under low N conditions (Table 8 and Figure 16). These findings suggest that the overexpression of GS1 had an effect on the physiological response of sorghum to N availability. In the spring experiment, initiated on February 11, 2013, transgenic lines also exhibited a higher shoot vegetative biomass accumulation than the null segregants when they were grown under optimal N conditions but not under low N conditions (Table 9). Although the increased biomass of transformants, observed in the spring experiment was significantly higher compared to controls, it was not as high as that in the winter experiment. This discrepancy might be a result of a seasonal effect caused by the difference in day length, and, thus, the amount of daylight to which the plants were exposed during both the winter and spring experiments. The plants may have also experienced different temperatures in the two experiments.

In the winter experiment it was evident that the increase in biomass in the transgenic plants grown under optimal N availability was a result of the formation of secondary tillers (Figure 17). Interestingly, in the spring experiment, both null segregant and transgenic plants grown under any of the two N treatments produced multiple secondary tillers (Figure 18). However, statistical analyses showed that in the spring experiment, there was, indeed, formation of more tillers in the transgenic plants than in the controls grown under optimal N conditions (Table 9). The difference, in terms of

tillering, between the two experiments could have been due to the differences in day length, light quantity and intensity between fall and spring seasons.

As a matter of fact, other authors have also observed a light dependent growth phenotype in transgenic plants overexpressing *Gln1* genes. In a study conducted by Oliveira et al. (2002)**,** tobacco plants transformed with a pea (*Pisum sativum* L.) *Gln1* gene and grown under different N concentrations, exhibited slightly higher growth rate compared to the controls under low light (50 μmol cm^{-2} s^{-1}) than under high light (200 μmol cm^{-2} s^{-1}) conditions. Brauer et al. (2011) observed that *Gln1* transgenic rice plants exhibited increased spikelet yield when they were grown in growth chambers but not under greenhouse conditions. They suggested that these differences might be attributed to different light intensities in each of the environments, being higher in the greenhouse during the months when their experiment was conducted. Moreover, Finnemann and Schjoerring (2000) proposed a model for the mechanism of post-translational regulation of GS1 in canola (*Brassica napus* L.), in which the activity of the GS1 enzyme is controlled by phosphorylation that is affected by light (Miflin and Habash, 2002). According to their model, in the dark, the plant ATP/AMP ratio is higher than in the light, and this favors the phosphorylation of GS1, protecting the enzyme from degradation. In contrast, under light, GS1 does not undergo phosphorylation and, thereby, may be more unstable and prone to degradation. If a similar mechanism is responsible for the post-translational regulation of GS1 in sorghum, it could explain the differences observed in tillering and vegetative biomass increase in the transgenic plants

between the winter and spring experiments. Taken together, these results indicate that light plays an important role in GS1-mediated improvement in plant biomass and yield.

It is interesting that in the winter experiment, the vegetative biomass of null segregant plants was twice as high under optimal N conditions compared to the low N conditions (Tables 8). On the other hand, there was no significant difference in the vegetative biomass of control plants grown under the different N levels in the spring experiment (Table 9). This result clearly shows that N availability affects the vegetative growth in a significant manner depending on the environmental conditions experienced by the plants in the greenhouse.

Furthermore, in the winter experiment, transformants showed a tendency to produce a secondary tiller soon after the first one under optimal N levels, therefore resulting in higher number of seeds per plant, overall, at the time of termination of the experiment (Table 8). However, this increase in tillering was accompanied by a reduction in the seed number in the primary tiller. It is possible that N remobilization within the transgenic plants occurred in a way that impaired grain set, but favored bud outgrowth and tillering since endogenous factors involved in branching are regulated by the availability of nutrients, including nitrogen (Kebrom et al., 2013). Nonetheless, tillering in monocots is a complex phenomenon controlled by multiple hormonal, developmental and environmental factors; therefore, more extensive research needs to be conducted in order to understand in detail the role that GS1 plays in branching in these transgenic lines. In contrast to the winter experiment, where we observed higher overall seed yields in the transformants under optimal N conditions, in the spring experiment,

the transgenic plants had significantly lower number of seeds under both N levels (Table 9). This is a surprising and unexpected result and points to the complexity of the interaction between climatic conditions and plant growth and development, especially in relation to N metabolism.

Overall, this study suggests that it is possible to enhance biomass production in sorghum through the overexpression of *Gln1* genes under certain conditions. A significant increase in biomass and yield was obtained in the transformants during the winter months in the greenhouse and that too under optimal N levels. However, in the spring experiment, while there was a small but significant increase in biomass and tillering in the transformants, their seed yield were lower than the null segregant controls. This indicates that growth and development depend on a complex interaction between N availability, GS activity and environmental conditions. Thus, more extensive research is necessary to obtain higher biomass or yield in plants by means of genetic engineering. Further work is needed to investigate the use of tissue-specific promoters and the use of gene stacking strategies that involve the insertion of *Gln1* genes in conjunction with one or more genes responsible for N uptake and utilization. Although our strategy to improve NUE through manipulation of a single gene did not succeed under all conditions, potential exists to improve growth, development or yield of sorghum, ideally under low N conditions.

5. CONCLUSIONS

The conclusions drawn from this dissertation as described as:

1. The 1.8 kbp long sequence of the rice *GluA-2* promoter examined in this study directs endosperm-specific transgene expression in sorghum.
2. Transgene expression controlled by the rice *GluA-2* promoter in sorghum started at approximately 14 dpa and increased thereafter until around 21 dpa.
3. The rice *GluA-2 p*romoter can serve as a useful tool in introducing novel traits to the sorghum seed by means of genetic engineering.
4. It is possible to improve biomass production in sorghum by the overexpression of *Gln1* genes under certain conditions.
5. Overexpression of a *Gln1* gene resulted in increased biomass and yield in transgenic plants grown under optimal N levels during the winter months in the greenhouse.
6. The overexpression of the *Gln1* gene had an effect on the physiological response of sorghum to N availability and also led to a light dependent growth phenotype.

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