5. DISCUSSION

Sorghum plant has the ability to synthesize cyanogenic glycosides in vegetative tissue, which on injury enzymatically hydrolyze to release toxic cyanohydric acid (HCN). This ability of the plant is known as “cyanogenic potential”. Cyanogenic potential is a major limitation for the utilization of sorghum fodder. There are many economically important crops which are highly cyanogenic. Sorghum is one such crop. The most common cyanogen found in plants is glycoside of α-hydroxynitriles (i.e., cyanohydrin) (Moller and Puolton, 1993; Lechlenberg and Nahtedt, 1999; Moller and Seiger, 1999). The cyanogenic glycoside dhurrin is found in sorghum, which on breakdown produces HCN.

Different sorghum genotypes possess different levels of cyanogenic potential and this may decrease to different degrees with the advancing plant growth (Chaturvedi et al., 1994). The permissible/safe threshold limit for HCN in sorghum fodder is 200 mg/kg on dry weight basis and 500 mg/kg on fresh weight basis (AICSIP-Forage criteria; McBee et al., 1980). It has been observed that several high-yielding cultivars are not safe as fodder (Muthuswamy et al., 1976; Chaturvedi et al. 1994). Therefore, consumption of sorghum fodder has been fatal for the feeding cattle and ruminants, the sporadic cases of which are either not reported, or confined to local media. This situation warrants the development of sorghum cultivars with cyanogen-safe fodder.
Sorghum bicolor contains large quantities of the cyanogenic glucoside dhurrin. The Dhurrin biosynthesis pathway is well known (Koch et al., 1995). The CYP79A1 gene from sorghum encodes an enzyme that normally converts L-tyrosine to p-hydroxy phenylacetaldoxime in the biosynthesis of cyanogenic glycoside dhurrin. Being the first enzyme involved in dhurrin biosynthesis, down-regulation of CYP79A1 might result in the reduction of dhurrin synthesis and hence the cyanogenic potential. For this purpose, development of antisense technology is a valuable tool. Gene suppression to reduce the level of expression of a protein via transformation of plants with "anti-sense" RNA gene constructs, first reported in the late 1980s (Sandler et al., 1988), is now a well-established technique. Anti-sense technology exploits oligonucleotide analogs to bind to cognate RNA sequences through Watson-Crick hybridization, resulting in the destruction or disablement of the target RNA.

The anti-sense RNA extends the use of transgenic plants by providing a means of specifically inactivating a resident gene (native or foreign). The gene can be inactivated throughout the plant or in specific cell types by exploiting cell-specific promoters (Siritunga and Sayre, 2003).

The principle of the anti-sense RNA method is to transform to host cells or protoplasts, either directly or via Agrobacterium, with a partial or entire copy of the target structural gene fused in anti-sense orientation between a promoter and a terminator. In transgenic plants
derived from the transformed cells, the resident sense gene can be inactivated up to 100%. Precisely how anti-sense inactivation works is not yet clear, but is often suggested to depend on the binding of anti-sense mRNA to a complementary sense strand. This could be an interaction between antisense RNA and DNA blocking transcription, or between anti-sense and sense RNA in the nucleus affecting RNA processing, stability or transport into the cytoplasm, or an interaction in the cytoplasm blocking translation or affecting the stability of the sense mRNA (Weiss et al., 1999; Siritunga and Sayre, 2003).

Genetic transformation is now widely accepted as method of choice for alien gene transfer into selected cultivars for improving complex traits for which natural variation in the existing gene pool is inadequate. Compared to all other major crops, sorghum transformation is still in its infancy, and a lot more technical progress is yet to be achieved. Except for recent reports on Agrobacterium mediated transformation (Zhao et al., 2000; Gao et al., 2005 a & b, Nguyen et al., 2007; Gurel et al., 2009), the most favoured method of sorghum transformation is the particle bombardment technique (Casas et al., 1993; Able et al., 2001; Tadesse et al., 2003; Gray et al., 2004; Girijashanker et al., 2005).

The objective of this study was to construct and utilize antisense version of sorghum cytochrome P450 genes to regulate the cyanogenic dhurrin biosynthesis in fodder sorghum. Sorghum cytochrome P450 genes characterized earlier (Koch et al., 1995) can be suitably utilized
for the development of antisense RNA constructs. With this background, the following objectives were proposed for this study.


2. Genetic transformation of fodder sorghum using antisense constructs and molecular characterization of transgenics.

5.1 Construction of vectors with antisense CYP79A1

Based on the sequence of the CYP79A1 gene sequence available in the Genbank (Koch et al., 1995), 2.1 kb fragment was amplified from seven out of ten genotypes from the genomic DNA using gene specific primers. The amplified fragment (2.1 kb) was confirmed by sequencing. Further, these genotypes were utilized for CYP79A1 cDNA isolation for the development of the antisense construct.

Koch et al. (1995) have reported the isolation of a cDNA encoding the multifunctional N-hydroxylase, cytochrome P450tyr, catalyzing the first steps in the biosynthesis of the cyanogenic glucoside dhurrin in sorghum. The cDNA clone provides a tool for isolation of the homologous gene from other cyanogenic plants and for biotechnological approaches to tissue-specifically control and optimize the levels of cyanogenic glucosides in plants.

The CYP79A1 cDNA synthesized from total RNA by reverse transcription was amplified by PCR using ORF-specific primers. The 1.67 kb fragment was eluted and cloned into pTZ57R/T vector.
Restriction analysis using specific enzymes *viz.*, BamHI, HindIII and KpnI, indicated correspondence with reported earlier (Koch *et al.*, 1995). All the three enzymes indicated conformity of the position of restriction sites in both sequences. The orientation of the ORF with respect to the restriction sites on the pTZ57R/T plasmid was determined using the same enzymes which indicated sense orientation integration.

Anderson *et al.* (2000) screened a cDNA library from immature leaves and petioles of cassava with degenerate PCR primers specific for conserved sequences in three *CYP79* gene family members. Two full-length clones, *CYP79D1* and *CYP79D2*, were isolated. They were 85% identical and shared 54% similarity with *CYP79A1* of sorghum. Using an anti-sense strategy, Siritunga and Sayre (2003) introduced the 5′ ends (650 bp) of the *CYP79D1* and *CYP79D2* genes into cassava in reverse orientation by transformation.

Construction of gene with anti-sense orientation: The *CYP79A1* gene (ORF) was excised out from the pTZ57R/T vector and subcloned into pJS108 vector downstream to *Actin1* promoter, in place of the *UidA* (encoding GUS reporter) gene.

After transformation into *E.coli* the colonies were screened for the presence of the plasmid carrying the CYP79A1 cDNA using colony PCR. Further restriction analysis confirmed the presence and the anti-sense orientation of the *CYP79A1* gene in the plasmid designated pJS108-CYP79A1-AS, among the three clones obtained.
Siritunga and Sayre (2003) expressed the *CYP79D1* and *CYP79D2* genes in an anti-sense orientation in transgenic cassava under the control of the leaf-specific *Cab1* promoter. They demonstrated that both leaf and root levels of linamarin are reduced up to 94% and 99%, respectively, in *CYP79D1/CYP79D2* anti-sense plants.

### 5.2 Genetic transformation of sorghum

Microprojectile particle bombardment has been commonly used to assay transient transgene expression in plant tissues (Dennehey *et al.*, 1994; Ross *et al.*, 1995; Bower *et al.*, 1996; Chowdhury *et al.*, 1997). The particle inflow gun was deployed in the present investigation using the DNA of plasmid carrying the *CYP79A1* antisense strand under the control of *Actin1* promoter. Efficiency of bombardment parameters were verified by targeting the tissues with construct pJS108 containing the GUS marker gene. The bombardment conditions such as velocity of the particles and the distance of flight were optimized by testing the transformed cells for GUS expression.

Optimization of physical and biological parameters can increase the efficiency of transformation processes (Birich and Bower, 1994). Several studies defined the optimum physical parameters of the bombardment process (Klein *et al.*, 1988b; Taylor and Vasil 1991; Russell *et al.*, 1992; Wang *et al.*, 1997). The protocols for producing transgenic sorghum are still being standardized using various methods of DNA transfer (Gurel *et al.*, 2009).
To optimize DNA delivery and minimize tissue damage, the parameters such as distance to the target tissue and velocity of the particles (dependent on helium gas pressure) were standardized. The maximum number of blue spots was observed when calli were bombarded at a distance of 8 cm from the nozzle, at 12 kg/cm² of helium pressure. Similar studies were conducted earlier in sorghum (Able et al., 2001). With the help of above optimized conditions, all the genetic transformation of the explants was carried out using tungsten particles (1 to 1.5 µm diameter) coated with the plasmid DNA under a partial vacuum (600 mm Hg). The helium pressures beyond 12 kg/cm² may result in tissue damage whereas at lower pressures there may not be enough tissue penetration (Sautter et al., 1991). The flight distance was another factor that affected transient expression. Transient expression was maximum when samples were placed at 8 cm from the filter unit. The lower efficiency observed in the tissues bombarded at short distance may be due to the possible excessive tissue damage caused (Kemper et al., 1996). Greater flight distances resulting in lower number of GUS positive spots observed in the present study agree with the results of Klein et al. (1988b), Oard et al. (1990), and Taylor and Vasil (1991). Therefore, minimizing the tissue damage and improving the tissue viability after bombardment are critical steps in obtaining successful transformants.

Casas et al. (1993), while optimizing bombardment parameters using sorghum immature embryo explants, found that the mean frequency of GUS expression was less than 20 foci per embryo.
Tadesse et al. (2003) optimized transformation conditions for the production of sorghum transgenics through microprojectile bombardment.

The advantage of adopting shoot meristem explants for the transformation lies in its year round availability unlike explants such as immature embryos and immature inflorescences (Zhong et al., 1998; Tadesse et al., 2003; Devi et al., 2004; Girijashanker et al. 2005). This system could be conveniently adapted for sorghum transformation as large number of multiple buds and somatic embryos capable of regeneration are obtained in a relatively short period. Heterogeneity of the explant is also minimized, as the shoot apices have very limited mother plant tissue, which also resulted in enhancing the rate of multiplication.

5.2.1 Selection and regeneration of transformants

In vitro selection against the selectable marker (bar) was initiated four weeks after bombardment. The four weeks incubation period allowed the recovery and proliferation of the cells transformed by bombardment, which may have been damaged during the process of delivering DNA. The meristems enlarge while keeping in the induction and maturation media. After two weeks, the meristematic masses containing multiple buds were allowed to develop into auxiliary plantlets on somatic embryo germination medium.

A two-step selection strategy using phosphinothricin (PPT) in the medium was adopted and was found useful. Only one-sixth of the
explants survived PPT selection and nearly one-sixth of the regenerated shoots were finally expressing the transgene. Nearly 30% of the rooted shoots were found to have been transformed. Initially for the first 2 weeks (first phase of selection), 1.5 mg/l PPT, was included in the medium. Transformed shoot forming sectors could be seen as green to yellow coloured growing tissue against a background of brown to black-coloured necrotic tissue (Plate 4.11 C & D). After two weeks, the live and growing portions were separated from necrotic tissue and were subcultured onto medium with 3.0 mg/l PPT (second phase of selection). Only the transformed sectors/shoots continued to grow and produce somatic embryos. The surviving shoots were transferred to hormone-free MS to set viable shoot and roots. Casas et al. (1993) used Bialophos and Zhong et al. (1998) used Basta as a selection agent in the rooting medium as well.

A total of 90 plants were regenerated and rooted, but only 45 survived till maturity and set seed. It has been observed by earlier studies that *in vitro* selection may be bit more stressful to the growing tissues, thus leading to loss of regenerative capacity of the tissues as observed in barley (Stiff *et al.*, 1995) and banana (Sagi *et al.*, 1995).

### 5.3 Molecular characterization of transgenics

Stable integration and expression of foreign genes are of critical importance for the successful application of genetic engineering in agricultural crops. A variety of molecular and biochemical tools including polymerase chain reaction (PCR), quantitative PCR, reverse transcription PCR (RT-PCR), Southern hybridization, Northern
hybridization, ELISA, and western immunoblots have been used to confirm the presence, integration and expression of transgenes. The most common and powerful ways of detecting the foreign DNA in a transgenic are PCR and DNA gel blot hybridization (Southern analysis).

5.3.1 PCR analysis of putative transgenics (T<sub>0</sub>)

PCR analysis was done to verify the presence of <i>bar</i> gene located on the antisense construct. Thirty out of 40 plants analyzed by PCR were found to contain the transgene. PCR could not be done using the antisense gene as the native DNA contains the CYP79A1 sense and antisense strands already. Transformed tissues with selective marker gene may be capable of detoxifying the selective agent so efficiently that non-transformed tissue in close proximity can also survive as reported by Christou <i>et al</i> (1991) and such a phenomena can lead to false positives during <i>in vitro</i> selection. This probably also explains why out of 30 plants that were PCR positive, only 25 showed transgene integration in southern analysis.

Li-Chun Huang <i>et al</i>. (2007), in petunia, verified the integration of genes with an antisense orientation by PCR analyses of kanamycin-resistant regenerants. The expression of transgenes was confirmed by RT-PCR analysis.

5.3.2 Southern analysis

Southern blot hybridization was carried out using 20 µg of genomic DNA according to the standard protocol of Sambrook <i>et al</i>
To check the transgene integration, HindIII enzyme that releases the major fragment \( (1.3 \text{kb}) \) of \( CYP79A1 \) antisense gene was deployed. To estimate the number of sites of integration of transgenes, the enzyme XbaI that has a single restriction site in the plasmid was used. The membrane was probed with radioactively labelled \( \left( ^{32}\text{P} \text{dCTP} \right) \) probes.

The integration of transgene could be confirmed in 25 of the 36 transgenics tested as evident from the additional band in the HindIII restricted genomic DNA of the transgenics compared to the DNA from non-transformed control plant (Plate 4.15). Upper fragments that appear in all plants including control indicate the native \( CYP79A1 \) DNA. Similar results were obtained in cassava by Siritunga and Sayre (2003).

The Southern analysis for determining the number of sites of transgene integration was done in plants that were confirmed by HindIII restriction earlier (Plate 4.16). In this case, XbaI restriction enzyme was used to test the plants. It was observed that the plants exhibited two to five sites of integration as visualized by the fragments that showed up by hybridizing with the transgene bar probe (Plate 4.16). Though large proportion of plants exhibited two to three sites of transgene integration, some of them had upto five sites of integration. Interestingly, as the data of HCN content in \( T_1 \), \( T_2 \) and \( T_3 \) also reveal, plants with more sites of integration such as 41 and 5A (4 to 5 sites of integration) had lower HCN content.
Multiple copy of transgene have been reported by biolistic transformation. Datta et al. (2003) observed that in rice, higher copy number of transgenes led to its higher expression levels, where more β-carotene was produced. Similarly, transgenic rice with more than one copy of Bt gene performed better in field conditions suggesting effective levels of transgene expression (Tu et al., 2000; Ye et al., 2001). Also, observations of high transgene expression levels were reported in wheat transformed with multiple copies of reporter genes (Stoger et al., 1998). Transformants selected for producing higher levels of pharmaceuticals were found to contain three or more transgene copies (Nandi et al., 2002). Besides, events with a single copy, losing the expression of transgene in T1 and later generations has also been observed (Fu et al., 2000). Kohli et al. (1999) proposed that transgene “switching off” is largely due to the presence of one or more rearranged copies rather than multiple copies of the transgene.

However, on the contrary, multiple copies of transgenes were thought to result in gene silencing due to co-suppression (Matzke and Matzke, 1995). It was also believed that the expression of a multi-copy transgene may vary over generations (unstable) compared to a low copy number transgene event (Jones, 2005).

5.3.3 RT-PCR analysis of T0 transgenics

Reverse transcription PCR of T0 transgenics was done in transgenics tested by Southern analysis. One-step RT-PCR was done using bar primers, to detect the presence of the mRNA species of transgene. Primer specific to antisense CYP79A1 transcripts were
used to detect antisense transgene expression. Twenty-five out of 30 transgenics were found to express the transgene.

The important problem for the potential use of transgenic plants for crop improvement is the instability of transgene expression. Few of the T₀ regenerants in the present study failed to show up in RT-PCR, but were found positive in PCR and/ or Southern for transgene(s). Gene silencing and interactions between multiple copies of the same transgene or different transgenes result in unexpected expression pattern of foreign genes (Kumpatla et al., 1998). This transgene inactivation in T₀ plants may be because of the hemizygous state of the transgene as hypothesized by Zhu et al. (1998). Also several other factors such as transgene rearrangements and silencing (already discussed) may prevent the transgene expression.

Another important aspect to transgene expression is the promoter control. The monocot plant gene promoters such as Actin1 gene promoter of rice and ubiquitin1 gene promoter of maize show high constitutive activity in monocots like sorghum (McElroy and Brettell, 1994). These two promoter constructs have one native intron incorporated in their transcription unit, which resulted in elevated mRNA abundance and enhanced transgene expression in transformed cells of cereals (Callis et al., 1987; Luehen and Walbot, 1991). In the present study, Actin1 promoter was used whose expression in sorghum was already demonstrated by earlier workers (Able et al., 2001; Jeoung et al., 2002; Tadesse et al., 2003). Transgene silencing often correlates with DNA methylation, and it is of particular interest
to identify sequences in plant transgenes that induce methylation and result in unstable expression. Methylation can be induced by interactions between homologous transgene copies or may reflect genomic position effects (Christou et al., 1991). The gene silencing may occur at transcriptional (Meyer et al., 1993) and post-transcriptional level (Smith et al., 1994). Kumpatla et al. (1998) reported a functional role for methylation in gene silencing.

5.4 Estimation of HCN content in T₀ transgenics

The HCN content was determined in T₀ transgenics 60 days after transferring them to pots, by picric acid method. The HCN content was reduced in the T₀ plants which ranged from 17.25 to 178.66 ppm with a mean of 104.06 ppm as compared to 192.08 ppm in the non-transformed control. A total of 14 transgenics had less than 100 ppm, which was remarkable. He et al. (2003) used antisense sorghum O-methyltransferase gene (omt) to down-regulate maize OMT and reduced lignin in maize. Twenty-eight T₀ plants regenerated from 17 herbicide-resistant callus lines from 13 independent bombardments expressed the brown midrib (low lignin) phenotype. Siritunga and Sayre (2003) developed transgenic cassava with reduced levels of CYP79D1 and CYP79D2 enzymes resulting in the inhibition of cyanogen production by anti-sense technology. In sorghum, Gray et al. (2004) obtained insect resistance plant by microprojectile bombardment of shoot meristems with cry1Ab and cry1Ac. Devi et al., (2004) obtained drought tolerance by bombarding shoot meristems isolated from germinating seedlings with HVA1 gene.
André D’Aoust (1999) developed transgenic tomato plants with reduced sucrose synthase (SuSy) activity in fruit by expressing an antisense RNA fragment of the TOMSSF gene under the control of the cauliflower mosaic virus 35S promoter.

The transgenic citrus lines that produce higher level (over expression) of antisense ACS RNA were found to repress the increase of ACC content following the chilling treatment (Wong et al., 2001).

5.5 Cyanogenic potential of non-transformed control (CSV 15)

The control CSV 15 had higher HCN level upto 70 days thus it was an appropriate candidate for the down-regulation of dhurrin biosynthesis. As per the classification of McBee et al. (1980) and Chaturvedi et al. (1994), CSV 15 is classified as “unsafe” for feeding to cattle. The Sudan grass derivative SSG 59-3 had HCN within permissible limits. This agrees with the results of Xian-rong et al. (1989) who found that Sudan grass lines had lowest HCN and sweet sorghum lines had the highest HCN content.

5.6 Estimation of HCN content in T1, T2 & T3 progenies of transgenics

Progenies of 37 T0 events were characterized by PCR (for bar transgene) and were tested for HCN content in leaves 45 days after sowing in containment glasshouse with regular irrigations. Events with a number of plants with substantially reduced HCN content (17 events) were observed (Table 4.7). The plants tested positive for transgene in PCR and promising in terms of lowered HCN content.
were identified. The HCN content in these plants varied from 5.1 to 149.8 ppm as against the mean HCN content of non-transformed control of 211.5 ppm. Thus, the reduced cyanogen potential of T₀ plants was inherited stably in these progenies.

The progenies (T₂) of the promising 43 T₁ plants were characterized by PCR and assayed for HCN as earlier. The plants with reduced HCN consistently across the generations were identified (Plate 4.24). T₃ generation is an advanced stage where lines are expected to be relatively stable for inheritance and transgene expression. Hence, 7 T₂ plant progenies from two T₀ plants (5A and 41) that were consistently positive in molecular analyses and possessed lowered HCN content across generations were advanced to T₃. The T₃ progenies were all positive for presence of transgene as indicated by PCR in almost all cases (Plate 4.25) and showed consistently low HCN levels (Plate 4.26). This suggested that advancing of generations with selection and selfing had effectively eliminated the non-transgenic segregants. The HCN content in T₃ generation was highly reduced (mean of 62.9 ppm in event 41 & 76.2 in event 5A) compared to control (mean of 221.4 ppm). It is suggested that a total of 3 to 5 progenies with lowest HCN content from each event may be advanced to next generation to obtain the most promising lines with consistently lower cyanogenic potential.

Antisense mediated down-regulation of OMT in maize resulted in significantly lower O-methyltransferase activity in T₁ transgenics
compared with controls, with some plants showing a 60% reduction (He et al., 2003).

5.7 PPT leaf painting assay in T₁ generation transgenics

Use of dominant selectable markers is an integral part of the transformation strategies. The sensitivity of plant cells to a selection marker depends on the genotype, the physiological condition, explant type, and tissue culture conditions. Identification of the functionality of the transgene in the transgenic plants and tracking the inheritance of the transgenes in their progeny using a simple method and without destructive sampling with PPT leaf painting assay came handy. Most of the other procedures are usually time-consuming, laborious and expensive. Direct in-planta assays for selectable marker gene activity, such as spraying whole plants or leaf painting with herbicide (Datta et al., 1992, Harshavardhan et al., 2002), germination of seed on selective media (Hiei et al., 1994) or in vitro leaf disc assays (Wang and Waterhouse. 1997) are known in the literature.

A total of 32 T₁ transgenic seeds along with non-transformed controls were tested by PPT leaf painting assay. A range of scorching damage levels (PPT tolerance) were observed in the progenies. Of the 32 events screened, 21 events were without chlorosis in at least 50% plants. The progenies of T₀ plants that showed low HCN content also showed resistance to PPT indicating the expression of both bar and CYP79A1 antisense transgenes in these plants. The assay was also successfully used by Datta et al. (1992) in indica rice. The method
adopted in the present study is according to Harshavardhan et al. (2002), which was demonstrated to be genotype-independent.

**5.8 Inheritance studies**

The progenies of at least seven \( T_0 \) plants indicated inheritance of the transgene in Mendelian fashion (Table 4.9). The hemizygous transgene segregated in 3:1 ratio (high: low) for HCN content. Earlier researchers have indicated that with regard to inheritance and expression of the transgenes, hemizygous nature of transgenics and the transgene segregation in 3:1 ratio of dominant: recessive in selfed progeny are common (Kim et al., 1999; Maqbool and Christou 1999). In backcross experiments with non-transformed plants, 1:1 segregation is expected. There are also reports, which mention of deviations in the inheritance ratios both within individual lines as well as the experiment as such (Sachs et al., 1996). The segregation in rest of the plants in this study was deviating from the Mendelian ratio. One of the reasons for this could be the smaller number of progenies in these plants. Transgene expression heterogeneity may also be due to the influence of factors like position effects, gene rearrangement, gene silencing and co-suppression (Zhu et al., 1998, Maqbool and Christou, 1999; Iyer et al., 2000).

In the present study, the antisense RNA approach was used to down-regulate dhurrin synthesis. Other robust tools of RNA interference-mediated down-regulation technologies (Kusaba, 2004) were not deployed since it was not intended to completely block the dhurrin biosynthesis pathway. The synthesis of a small quantity of
the dhurrin may be desirable as a defense against insects (Woodhead and Bernays, 1977; Tattersall et al., 2001). Antisense technology is known to substantially down-regulate but not block the target gene completely (He et al., 2003; Siritunga and Sayre, 2003).

The present study effectively demonstrated that

1. The antisense CYP79A1 strategy was effective in producing sorghum plants with lower cyanogenic potential.
2. The particle bombardment method of transformation remains a useful tool for obtaining transgenic sorghum plants.
3. The frequency and inheritance of the transgene improved with the advancement of generations due to elimination of non-carrier plants of transgene by molecular and HCN analyses.
4. The actin1 promoter may be adequately driving the antisense CYP79A1 expression in the leaf tissues of sorghum.

Absence of other unintended agronomic variations in the transgenics indicated that the transgenics thus obtained would be useful as cultivars once cleared by the regulatory processes and in-depth studies of non-obvious changes and their effects on the cattle and the environment should be done. The antisense transgene can be passed onto other forage sorghum cultivars by marker-assisted backcross breeding.