Chapter 6

BIOTECHNOLOGY OF SORGHUM: PROSPECTS FOR IMPROVEMENT OF NUTRITIONAL AND BIOFUEL TRAITS

Gerardo Armando Aguado-Santacruz[1*], Blanca Moreno-Gómez[2], Luisa Marina Gómez-Torres[3], and José Luis Anaya-López[4]

[1]Plant Molecular Physiology Laboratory, CE Bajío-INIFAP. Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya Gto. 38010 México Tel. +52-461-6115323 ext. 122; Fax +52-461-6115323 ext. 141

[2]Plant Molecular Physiology Laboratory, CE Bajío-INIFAP. Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya Gto. México email: moreno.blanca@inifap.gob.mx

[3]Universidad Nacional de Colombia, Facultad de Ingeniería, Departamento de Ingeniería Química y Ambiental Kra 30 No. 45-03, Bogotá, D.C., Colombia. lmgomezt@gmail.com

[4]Biotechnology Unit, CE Bajío-INIFAP. Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya. Gto. México email: anaya.jose@inifap.gob.mx

ABSTRACT

An ideal food or biofuel crop should to be able to can adapt to several environments, tolerate extreme temperatures and diseases, use efficiently the unpredictable water available in marginal environments and have an adequate nutritional value and/or potential as biofuel. Although it is difficult to find a crop possessing all these characteristics, sorghum is one of the crops with superior tolerance to extreme abiotic or biotic factors and consequently widely extended all over the world. Sorghum is an important staple crop worldwide and more recently considered an important prospect for

* Corresponding author: gaguado@prodigy.net.mx; aguado.armando@inifap.gob.mx.

second generation biofuel crops. Although the progress in biotechnological management of sorghum has been relatively slow as compared to other cereals, modest achievements have been reached in manipulation of the lysine contents and protein digestibility of the sorghum grain, while engineering bioethanol potential has proved to be difficult because a trade off exists for maximizing the biomass production without affecting the quality of the material destined to bioethanol production. As compared to modifying the nutritional value of sorghum grain, improving the bioethanol potential of sweet sorghum seems to represent a more complicated challenge because little information is currently available about the precise genes involved in cell wall biogenesis as well as the precise functions of these multigenic families, so modifying the cell wall structure on the basis of the current knowledge is anticipated to result in the alteration of other important agronomic traits. Redesigning the genome of this crop for engineering a multipurpose sorghum cultivar possessing rich sugar/starch contents in stalk/grain and high biomass accumulation for production of lignocellulosic bioethanol will require a robust scientific framework and the development of efficient and reproducible systems for *in vitro* culture and genetic transformation.

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench, formerly *S. vulgare*) belongs to the family Poaceae, subfamily Panicoideae. The species *Sorghum bicolor* covers a wide range of varieties, from white and yellow to brown, red and almost black.

Sorghum is the fifth most important cereal crop grown in the world and a primary staple crop in several regions of the third world, principally in sub-Saharan Africa and India, where more than 300 million people rely on sorghum grain as a major staple food (Rooney, 2004; Laidlaw and Godwin, 2008). Likewise, this cereal is an important component of the grasslands of many tropical regions and an important food crop in Africa, South and Central America and South Asia.

In different parts of Africa this cereal is commonly known by names such as large millet, Guinea corn, kafir and hard, while in India and China is named as iowar or kaoliang, respectively.

It is difficult to determine where and when the domestication of sorghum took place although there is evidence that this crop is native from Africa, probably Ethiopia or Sudan. The last wild relatives of commercial sorghum are currently confined to Africa south of the Sahara indicating that its domestication took place there. Although rich finds of *S. bicolor* have been recovered from Qasr Ibrim in Egyptian Nubia, the wild materials have been dated to circa 800-600 BCE and the domesticated ones no earlier than CE 100.

There is controversy about how the geographical spread of the crop was produced. Nowadays, Durra cultivars extend continuously from Ethiopia and along the Nile to the Middle East and through India to Thailand. In Latin America, the knowledge of sorghum is relatively new. It was first introduced to America in 1857, and then used extensively to produce syrup in early 90's (Doggett, 1965).

In many developing countries, sorghum has a great popularity among farmers due to its greater adaptability and different forms of utilization (Pandey *et al*., 2010), as well as high yields, partially related to its C_4 photosynthetic pathway. In 2009, sorghum was harvested on 43,735,937 ha with an average yield of 14,198 kg/ha and a net production of 62,098,620 ton (FAOSTAT, 2009).

This multipurpose crop is used as flour in the preparation of breads and porridges, as a source of fibre, fuel and secondary products and it is also important as green fodder, stover, silage and hay to suit the diverse needs of farming systems including animal feed grain and forage (Baskaran *et al*., 2006).

In the early 1980s an estimated 39% of global production of sorghum was used as food and 54% for animal feed. By 1992-94, 42% of total utilization was for food and 48% for animal feed. Between 1992-94 and 2005, food sorghum consumption in developing countries (in Africa, Asia, Central America, Caribbean, and South America) is projected to increase from 26 million to over 30 million tons. Per capita consumption of sorghum is highest in Africa; per capita consumption is 90-100 kg/year in Burkina Faso and Sudan and this cereal provides over one third of the total calorie intake in these two countries (Léder, 2009).

In different parts of Africa, sorghum is an important component of the local beer making process, whereas in China a major use is for high alcohol beverage distillation. In developed countries this crop is usually grown as livestock feed and for industrial purposes such as ethanol production. In Mexico and other Latin-American countries sorghum is used principally as livestock feed and due to its high productive potential and versatility, the cultivated area, yield and uses have recently increased in this region of the world. The major producers of sorghum in the world are United States, India, Nigeria, China and México (Léder, 2009).

More recently sorghum is gaining importance as a bioenergy crop. As opposed to grain sorghum, some varieties containing high sugar contents are collectively known as sweet sorghum. These sorghum varieties are grown primarily for forage, silage, and syrup production.

SORGHUM AS A TARGET FOR GENETIC IMPROVEMENT

One of the main advantages of sorghum over other cereals such as rice, corn, wheat, oat, and barley, is related to a high resistance to unfavourable temperatures, water stress, salinity and diseases (Gnansounou *et al*., 2005, Rooney *et al*., 2007). This extreme tolerance to biotic and abiotic stresses, permits this crop to adapt easily to semi-arid tropical and subtropical regions and temperate regions all around the world, where rainfall is limiting. Its versatility as a multipurpose crop, ample distribution and easy adaption to different environments makes sorghum one of the most promising energy plants and an important target for improving nutritional traits.

Traditional breeding for superior agronomic traits has been carried out for years in sorghum improvement. However, this technology is limited to the current variation present in this species or in wild relatives with capacity to cross pollinate with sorghum. Furthermore, the low genetic pool present in sorghum limits the potential for improving this cereal by traditional breeding (Forsyth *et al*., 2003; Pandey *et al*., 2010).

Genetic engineering offers an alternative way to solve this problem and additionally allow the access to a vast pool of useful genes, from other cereals, plant species, or even from other kingdoms. Likewise, molecular breeding, which combines genotypic and phenotypic information, has emerged as a powerful approach and offers new perspectives for genetic improvement of sorghum without the necessity of transferring genes.

Genetic studies and molecular breeding approaches require basic genomic resources, such as molecular markers, genetic maps and sequence information. In the case of sorghum its genome sequence data have recently become available and revealed a 730-megabase genome (Paterson, 2009). It is expected that this information be instrumental in the efforts to correct some of the deficiencies of sorghum as a nutritional source and biofuel crop.

Biotechnological manipulation of sorghum has been slow because of its recalcitrance to tissue culture and genetic transformation, though certain advances have been reached in the culture of this crop under *in vitro* conditions (Emani *et al*., 2002). A prerequisite for genetic improvement of any plant species using modern biotechnology is the generation of totipotent material in sufficient amounts to allow the application of the different biotechnological tools such as genetic transformation, multiplication of valuable germplasm, recovery of somaclonal variants or production of pathogen-free plants, among others.

This starting material has to be generated and manipulated under *in vitro* conditions by tissue culture techniques. sorghum, like most of the cereals, is considered as a recalcitrant species to tissue culture. However, new achievements have permitted to overcome some of the obstacles commonly found when culturing grasses *in vitro*. Recognition of the determinant influence of plant genotype, type and physiological and developmental stage of explant, growth regulator combination, nutritional and vitamin balance, and general environmental growth conditions on the regenerative response of plants has played a fundamental role in overcoming the well-known recalcitrance of Poaceae to be grown under *in vitro* conditions (Aguado-Santacruz *et al*., 2007). Within the last 10 years, great advances have been achieved in the research of *in vitro* culture and genetic manipulation of cereals.

As a first step for developing reliable plant *in vitro* culture and genetic transformation systems, optimal and reproducible conditions for induction of suitable morphogenic responses have to be established. Later, a protocol for efficient regeneration and hardening of the regenerated plants is required. In this context the election of the type, physiological and developmental stage of the explant to be cultured plays a fundamental role in achieving successful regeneration systems.

The first sorghum embryogenic *in vitro* culture systems were developed by Masteller and Holden (1970) and Gamborg *et al*. (1977). Subsequently, plant regeneration systems for sorghum via somatic embryogenesis and organogenesis have been described for sorghum utilizing immature embryos (Gamborg *et al*., 1977; Thomas *et al*., 1977; Dunstan *et al*., 1978, 1979; Brar *et al*., 1979; Cai *et al*., 1987; Ma *et al*., 1987; Cai *et al*., 1990; Zhong *et al*., 1998; Elkonin, 2000; Gao *et al*., 2005; Girijashankar *et al*., 2005; Grootboom *et al*., 2008; Gurel *et al*., 2009), mature embryos (Thomas *et al*., 1977; Cai *et al*., 1987) immature inflorescences (Brettell *et al*., 1980; Boyes and Vasil, 1984; Cai and Butler, 1990; Kaeppler and Pederson, 1997), seedlings (Masteller and Holden, 1970; Brar *et al*., 1979; Davis and Kidd, 1980; Smith *et al*., 1983), leaf fragments (Wernicke and Brettell, 1980), anthers (Rose *et al*., 1986) and protoplasts (Wei and Xu, 1990).

Despite the relatively large number of studies carried out up to now, there are no reports related to maintenance of the callus lines for extended periods, which may be the result of the well-known recalcitrance of this crop. sorghum is one of the most difficult plant species to manipulate in tissue culture and consequently to transform genetically (Zhu *et al*., 1998; Shrawat and Lörz, 2006). Limited embryogenic callus production, excessive phenolic production and poor long-term plant regenerability are key limitations in sorghum tissue

culture (Wernicke and Brettell, 1982; Mackinnon *et al*., 1986; Rao *et al*., 1995; Sato *et al*., 2004; Sai *et al*., 2006).

Although immature embryos are probably the most viable source for generating the high quality material required for genetic transformation, there are some disadvantages to be considered when utilizing these explants. Immature embryos and immature inflorescences have a limited availability in time, while isolation of immature embryos implies a laborious work and difficulty for manipulation and propagation under *in vitro* conditions (Zhao *et al*., 2010a).

Other explants such as mature embryos and shoot tips (Bhaskaran *et al*., 1987; Bhaskaran and Smith, 1988; Baskaran *et al*., 2006; Arulselvi and Krishnaveni, 2009) have been also utilized as the starting material for developing tissue culture systems of sorghum. Conversely to immature embryos, the mature seeds, and therefore mature embryos, are abundant and available year-round. However, mature embryos are considered as explants resulting in low regeneration frequencies and optimization of the media composition for callus induction and plant regeneration using this type of explant is commonly genotype-specific (Zhao *et al*., 2010a).

Immature embryos used for studying morphogenic responses of sorghum under *in vitro* conditions have been isolated between 12 to 20 days after pollination, while shoot tips have been commonly isolated from 3 to 7 day-old seedlings.

Only a few sorghum genotypes have shown potential for *in vitro* culture, situation that reflects not only the importance of considering the genetic background of the sorghum materials but also the necessity of optimizing the growing conditions of the sorghum explants and the balance of the media components as well as testing the effect of adding novel compounds.

Improvements and variations to the sorghum tissue culture systems have been always in the focus of the researchers. Media manipulation is still considered as the main experimental component for successful callus induction and plant regeneration from different sorghum cultivars. Macro and micronutrients levels used in most plant tissue culture media are based on MS medium developed by Murashige and Skoog (1962), while recent studies have shown that an increased level of copper and aluminium improve dramatically the sorghum plant regeneration efficiency (Anas and Yoshida, 2002; Gupta *et al*., 2002; Nirwan and Kothari, 2003).

An efficient transformation system for sorghum, or any crop, has to be based on reliable and highly reproducible tissue culture and regeneration systems. Immature embryos have been commonly considered as the best explant for the production of transgenic plants (Casas *et al*., 1993; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002; Tadesse *et al*., 2003; Gao *et al*., 2005). On the other hand, regeneration systems for parental lines used for the production of hybrids of sorghum have been established in the past (Oldach *et al*., 2001; Harshavardhan *et al*., 2002; O'Kennedy *et al*., 2006), which is important to facilitate the integration of transgenes within commercial lines.

PROSPECTS FOR IMPROVING THE NUTRITIONAL VALUE OF SORGHUM BY GENETIC ENGINEERING

Trends in both increase of the human population and pattern of consumption in developing countries imply that the global demand for food will continue to grow for the 21st century. The focus now is not only on increasing the food supply, but also on improving its quality, particularly with respect to nutritional value, especially in crops such as sorghum from which more than 500 million people around the world base their diet.

Although sorghum has a lot of advantages, for example a high tolerance to drought and heat, characteristics which make it an important foodstuff for Africa and other dry regions, its nutritional composition is far from the ideal one.

Nutritional Deficiencies of Sorghum

Sorghum has a similar chemical composition to maize (Léder, 2009). The majority of the carbohydrates in sorghum are in the form of starch, while soluble sugar, pentosans, cellulose, and hemicellulose are low. Regular endosperm sorghum types contain 23 to 30% amylose, but waxy varieties contain less than 5% amylose. Sorghum is a good source of fibre, mainly the insoluble (86.2%) fibre. The insoluble dietary fibre of sorghum and millet may decrease transit time and prevent gastrointestinal problems (Léder, 2009).

The main disadvantages of sorghum as a food come from its protein composition and digestibility, as well as from the presence of some compounds with anti-nutritional properties, which are discussed in the next sections.

Protein Composition and Digestibility of Sorghum

Although this cereal contains similar levels of starch and protein to other cereals, the proteinic component is of less quality and digestibility, especially when the meal or flour is wet cooked, process that reduces significantly its nutritional value (Wang *et al*., 2009).

Proteins of sorghum are classified into albumins (water soluble proteins), globulins (salt soluble), prolamins (alcohol soluble) and glutelins (protein soluble in dilute alkali) (Bansal *et al*., 2008). The protein content of sorghum is usually 11-13% but sometimes higher values are reported (Dendy, 1995); both protein content and composition varies due to genotype, and water availability, temperature, soil fertility and environmental conditions during grain development.

The grain storage proteins of maize, millets and sorghum are constituted by prolamins. These proteins are found in the seeds of cereal grains such as wheat (gliadin), barley (hordein), rye (secalin), corn (zein) and sorghum (kafirin) and as a minor protein, avenin in oats. Prolamins are characterized by high glutamine and proline contents and are generally soluble in strong alcohol solutions. Some prolamins, notably gliadin, and similar proteins found in the tribe Triticeae may induce coeliac disease in genetically predisposed individuals (Shewry and Halford, 2002).

Prolamins (kafirins) constitute the major protein fractions in sorghum, followed by glutelins. Lack of gluten is characteristic of protein composition, and traditionally the bread which cannot be baked from sorghum and millet is only cake bread.

Sorghum grain protein is notoriously deficient in the essential amino acid lysine (except for several new varieties of high lysine). *In vitro* studies and *in vivo* studies with livestock and

laboratory animals indicate that sorghum proteins are generally less digestible than those of other cereals; sorghum protein was shown to be 74.5%, compared to 78.5% of corn (Léder, 2009).

Deficiencies of the sorghum grain arise from the amino acid composition of the kafirins, which can account for up to 80% of the total grain proteins. sorghum grain prolamines are deficient in several essential amino acids such as threonine, tryptophan and methionine, but most notably lysine (Chamba *et al*., 2005).

Based on differences in solubility, molecular weight, structure and relationships to zeins revealed by the amino acid composition, sequences and immunochemical cross-reactions, the kafirins of sorghum are classified into α, β and γ kafirins (Taylor *et al*., 1984; Shull *et al*., 1991; Mazhar *et al*., 1993; Belton *et al*., 2006).

Extensive variability has been observed in the essential amino acid composition of the sorghum protein, which is related to environmental factors affecting the grain composition. Lysine content can vary from 71 to 212 mg per gram of nitrogen, while the methionine and tryptophan contents on average are 87 and 63 mg per gram of nitrogen, respectively (FAO, 1995).

Our ability to improve the nutritional quality and biofuel potential of sorghum grain protein by classical plant breeding is limited to the low genetic variation present in this species or in relatives with capacity to cross pollinate with this species.

Although improvement of the protein quality of sorghum has been attempted by traditional breeding only two mutant high lysine genes are currently available, the spontaneous mutant gene *hl* which was initially identified in an Ethiopian line and the *P721Q* gene, conferring an opaque phenotype (Mohan, 1975), which was originally induced by ethyl methane sulphonate (EMS). Ethiopian high-lysine sorghum lines IS11167 and IS11758, containing the *hl* gene, were selected among 9,000 different lines of sorghum (Singh and Axtell, 1973). Wild type lines contain between 2.9 and 3.3% lysine, and from 15.7 to 17.2% of protein (Singh and Axtell, 1973; Mohan, 1975). Despite that the incorporation of the mutant gene *hl* into new sorghum lines resulted in lysine contents increased between 45 and 67% relative to the control lines, these values were still lower to the 5.5% lysine content recommended by the World Health Organization (WHO; MacLean *et al*., 1981). Furthermore, it has been proved to be difficult to incorporate the high-lysine phenotype into varieties with high yield and acceptable agronomic performance and processing properties.

In addition, consumption of the IS11758 and P721Q lines by 30 to 60 month-old children was associated with weight loss and low concentrations of lysine and threonine. This finding was attributed to the low digestibility of the sorghum grain (MacLean *et al*., 1981).

At the end of 90's the new sorghum cultivar P851171, derived from *P721Q*, was cited as an improved sorghum variety with increased *in vitro* digestibility for cooked and non cooked protein (80 and 85%, respectively; Weaver *et al*., 1998). In spite of showing higher amino acid digestibility in comparison with "normal" sorghum and maize, the TME_n values found in this cultivar were lower and chickens fed with a protein-deficient diet did not show weight gain. This was explained by the presence of a partially vitreous endosperm containing less than 8% starch compared to line P721N from which it was derived (Elkin *et al*., 2002).

As a whole the information presented above suggests that an improvement of the protein quality of sorghum should take into account not only an increase of the lysine content above the levels recommended by WHO, but also of the protein digestibility.

Anti-Nutritional Components of Sorghum

Anti-nutritional compounds (protease inhibitors, galacto-oligosaccharides, lectins, ureases, phytates, tannins, phenolics, saponins and cyanogenic glycosides, among others) are plant constituents which play an important role in biological functions of plants. The effect of these compounds on human and animal organisms is partly negative because they can reduce the digestibility of nutrients and the absorption of minerals.

Some species of sorghum can contain levels of hydrogen cyanide, hordenine and nitrates lethal to grazing animals in the early stages of the plant's growth. Stressed plants, even at later stages of growth, can also contain toxic levels of cyanide. These compounds also inhibit growth as a result of their negative influence on the function of pancreases and the thyroid gland, and can cause pathological alterations in the liver.

All sorghums contain phenolic compounds, including phenolic acids and flavonoids. Some varieties contain condensed polyphenols, called tannins, in the layer under the seed coat but most cultivated sorghums do not contain any. These compounds can affect color, flavor and nutritional quality of the grain and products prepared from it. Tannins constitute an effective defense against the grain consumption by insects and birds.

The tannin content of seeds inhibits the activity of some enzymes and therefore adversely influences protein digestibility and cellulose breakdown. Animal tests have shown that tannins inhibit protein absorption, decreases utilization of minerals and results in some decrease of growth. Feeding pigs with fodder containing 4.21% tannin decreased protein digestibility by 5.6%. Before ripening, the tannin content of grain is always higher than after ripening. The tannin content of dark grains is always higher than that of pale grains (Léder, 2009). Some treatments are commonly utilized for removing polyphenolic compounds from the seed such as steeping, fermentation, malting, alkali or acid treatment, popping, roasting (dry or wet), parboiling and drying.

On the other hand, phytic acid and/or phytates complex with essential dietary minerals such as calcium, zinc, iron and magnesium and consequently become biologically unavailable for absorption. Sorghum bran contains the highest levels of phytates. Forty to fifty percent of phytate and total phosphorus can be partially removed by abrasive dehulling. However, iron absorption is still low in porridges made from low-extraction flours (Cook *et al*., 1997), because small amounts of phytate can inhibit iron absorption (Hurrell *et al*., 1992). Phytic acid in cereal foods can be degraded completely by phytases, enzymes that successively remove the phosphate groups from phytic acid until it no longer binds iron. Phytic acid has been completely degraded in weaning cereals by adding commercial exogenous phytases (Davidsson *et al*., 1997) or by activating the native phytases by a combination of soaking, germinating and fermenting (Marero *et al*., 1991).

Another important anti-nutritional component of sorghum is cyanogenic glycosides, which occur in most sorghum varieties. More than 2,600 plant species produce cyanogenic glycosides, one of the biggest and extensively studied classes of secondary metabolites (Ganjewala *et al*., 2010). Chemically cyanogenic glycosides are defined as glycosides of α-hydroxynitriles and plants store these compounds in vacuoles (Vetter, 2000). Large numbers of cyanogenic glycosides are produced in plants to mediate both and general and specialized functions. Although the primary role of these compounds is the organization of the chemical

defense system in plants and in plant-insect interactions (Zagrobelny *et al*., 2004), they also serve as nitrogen storage compounds (Busk and Moyer, 2002).

The main cyanogenic glycoside of sorghum is dhurrin, which is found mainly in the leaves and germinating seeds of this grass and it can amount to 3-4% of the total dry seedling weight. In the course of processing germinating seeds, cyanide may be released. In the traditional food processing techniques (*e.g*. drying, malting) the cyanide level seemed to be lowered to zero or to well below that considered toxic (Léder, 2009).

Genetic engineering offers an opportunity to overcome some of the nutritional limitation of sorghum by introducing homologous genes or wild type or mutant genes from other organisms.

Genetic Manipulation of Sorghum for High Lysine Content and Increased Protein Digestibility

Deficiencies in the protein diet are a serious problem in Africa and other regions, where sorghum is a staple food. As stated before, the nutrient content of sorghum grain is generally similar to other cereals, while the lysine content is particularly low because the kafirin storage proteins are very low in this amino acid.

Relatively few studies have been carried out to improve the nutritional value of the sorghum grain by means of genetic engineering, though recent advances in the *in vitro* culture and genetic transformation of this cereal have open the possibility of modifying the essential amino acid content and the protein digestibility of the sorghum grain.

The first report of genetic transformation of sorghum described the introduction of DNA into protoplasts by electroporation and selection of transformed cells, without achieving plant regeneration (Battraw and Hall, 1991). Afterward, microprojectile bombardment was applied to obtain a transgenic non-regenerable cell suspension of this cereal (Hagio *et al*., 1991). As stated before, the limited progress in sorghum transformation is partly due to difficulties associated with its *in vitro* culture and lack of efficient and reliable protocols for genetic transformation.

Casas *et al*. (1993) obtained the first transgenic sorghum plants by bombardment of immature embryos and later on they obtained transgenic plants using immature inflorescences. The first reports of sorghum plants transformed with agronomically important genes were the production of transgenic plants containing the HT12 gene for higher grain lysine content (Zhao *et al*., 2003) and the cry1Ac gene for insect resistance (Girijashankar *et al*., 2005).

Although different studies have shown the feasibility of the sorghum transformation by bombardment of zygotic embryos (Casas *et al*., 1993), explants from immature inflorescences (Casas *et al*., 1997) and calli generated from immature embryos and inflorescences (Kononowicz *et al*., 1995), most studies still focus on improving transformation and regeneration frequencies. In this context, it is important to mention that the long periods of selection needed for the recovery and regeneration of putative transgenic plants often have hampered the optimization of conditions for sorghum transformation.

In a more recent work, Howe *et al.* (2006) reported that in only one of 17 experiments a 4.5% frequency (4 events in 89 explants) was achieved; frequencies in the other 16 ranged from 0.3 to 1.9%. These results emphasize the importance of continued work to find suitable

culture conditions for suitable morphogenic responses, as well as identify new genotypes of sorghum responsive to *in vitro* manipulation.

Even though most successful reports on sorghum transformation have relied upon a biolistic approach, *Agrobacterium*-mediated transformation has been accomplished recently (Zhao *et al*., 2000), so a long way remains to be walked in optimizing the conditions for successful transformation of sorghum with genes conferring improved agronomics traits, particularly increased nutritional value and biofuel potential.

Increasing the nutritional value of the sorghum grain by genetic engineering has been approached from different perspectives involving the expression of heterologous proteins and the regulation of endogenous proteins by over expressing or silencing the genes codifying them.

The heterologous expression strategy has included the transference of genes codifying proteins with a better balance of amino acids and high lysine content, proteins reducing the formation of disulfide bonds within the storage proteins and key proteins involved in the regulation of the lysine biosynthesis pathway.

An interesting biotechnological approach is currently underway to enhance the availability of iron and zinc and increase the vitamin content of sorghum by introducing genes for increased lysine and threonine contents, increased protein digestibility and reduction of phytic acid (inositol hexakisphosphate, IP6). These genes are combined in a single unit to behave as a single locus and are intended to be expressed in the seed endosperm (Hokanson *et al*., 2010).

Zhao *et al*. (2003) expressed a hordotionin mutant protein from barley (HT12) containing seven lysine residues additional to the five residues that normally contain this protein. Although this strategy resulted in transformed plants containing 50% more lysine than their wild counterparts, the digestibility of the sorghum protein grain was not evaluated and some problems are expected to arise because hordotionin is a cytotoxic peptide with antimicrobial activity and inhibitory activity of the α amylase (Florack and Stiekema, 1994; Pelegrini and Franco, 2005).

A similar strategy was patented by Cho *et al*. (2007) and consists in the expression of the thioredoxin h from barley in sorghum. This manipulation resulted in an increased digestibility of starch and kafirins. Thioredoxins are thermo stable proteins capable of experiencing reversible redox changes and to reduce the disulfide bonds, altering the tertiary structure and consequently the properties and biochemical activity of proteins containing CS-SC bonds. They are found in multiple cellular compartments of plants, animals and bacteria. Thioredoxin h specifically reduces the intramolecular bonds of the proteins gliadin and glutenin of wheat (Joye *et al*., 2009).

In relation to the expression of key proteins located within the lysine biosynthesis pathway, Tadesse and Jacobs (2002) expressed in sorghum plants the coding region of a mutant of the dihydrodipicolinate synthase gene (*dhdps*) from *Nicotiana sylvestris*, called *dhdps-rI*, which codifies a protein that is insensitive to feedback inhibition by lysine. The *dhdps* gene codifies the first enzyme regulating the lysine synthesis. The native DHDPS is inhibited by lysine feeback, so that the level of this amino acid is controlled by this mechanism. Thus, there is a special interest in expressing this insensitive DHDPS, as a mean to increase the nutritional value of different cereals. Using this approach the lysine content was increased 3.5 times in the leaves of transgenic sorghum plants compared to the wild type

counterparts. The lysine content of the grain was not reported probably because a constitutive promoter CaMV35S was utilized instead of a monocot endosperm-specific promoter.

Another alternative for increasing the protein quality is related to the strategy of silencing of certain proteins having an unfavorable amino acid balance by the interference RNA technology (RNAi). The first evidence of the viability of this approach in cereals was obtained in maize. Individual silencing of the M_r 19,000 and M_r 22,000 α zeins resulted in an opaque phenotype with Mendelian segregation (Segal *et al*., 2003; Huang *et al*., 2004), while the simultaneous silencing of both α zeins also produced maize lines possessing an opaque phenotype with 5.61 and 1.22% contents of lysine and tryptophan, respectively (Huang *et al*., 2006); these contents represented more than a 150% increase in these amino acids as compared to wild type maize lines.

Likewise, Jung (2008) patented the genetic silencing of the δ kafirin 2. This approach resulted in a 50% increase in the lysine content as compared to non transgenic grain. The patent also included the silencing of other related sequences such as α and γ kafirins, although no results were shown about this alternative sequence silencing. Because of their abundance and properties in grain, kafirins are key proteins for the global protein quality of the sorghum grain.

More recently Henley *et al*. (2010) developed the transgenic line ABS032 which showed 10 and 15% increases in protein and lysine contents, respectively, and a change from 0.19 to 45 in the Protein Digestibility Corrected Amino Acid Score (PDCAAS) as compared to the wild type sorghum cultivar P890812.

On the other hand, transformation of the P890812 cultivar with the pABS construct developed to silence the expression of the lysine ketoglutarate reductase (LKR), δ kafirin 2 and γ kafirin 1 and 2 by the co-suppression strategy using RNAi, resulted in 45.2 and 77.6% increases in the lysine content of whole grain and endosperm, respectively (Grootboom, 2010). It is interesting to stand out that using this construct, suppression of the LKR was only observed when the γ kafirin 1 and 2 were silenced; as expected, suppression of the δ kafirin 2 has no detectable effect and the expression of this protein could not be demonstrated despite the sequence of this gene is known (Belton *et al*., 2006).

Finally, it is important the significant efforts conducted by Gates Foundation with its program "African fortified sorghum" to improve the nutritional quality of sorghum. The objective of this project is the development of fortified sorghum grain by transferring a gene from barley codifying a high-lysine storage protein and increased levels of vitamin A, iron and zinc.

BIOTECHNOLOGICAL MANAGEMENT OF SORGHUM FOR IMPROVED BIOFUEL POTENTIAL

To replace the petroleum-derived energy sources and diminish the global carbon dioxide emissions, ethanol produced from plant sources (bioethanol), particularly lignocellulosic materials, is considered nowadays as an attractive alternative to fossil fuels. Bioethanol is currently produced from sugarcane and corn starch by a fermentative process in which

sucrose, glucose, fructose and other fermentable sugars are converted directly to ethanol by the yeast *Sacharomyces cerevisiae*.

Bioethanol is usually classified into three types depending on the type of raw material. The first one is bioethanol derived from sugar-based materials such as sugarcane and sugar beet. The second one is derived from starch-based materials such as grains of maize, wheat and sorghum, and root and tuber crops (*e.g.* sugar beet). The third one, the so-called cellulosic bioethanol or second generation bioethanol, is made from cellulosic materials including crop (*e.g.* rice straw and maize stover) and urban residues (*e.g.* waste paper) or woody materials (*e.g.* wood shavings). This lignocellulosic bioethanol is predicted to be used in the near future (Berndes *et al*., 2010).

The major bioethanol producers, USA and Brazil, have used mainly maize and sugarcane, respectively, as materials for bioethanol production. Bioethanol production from maize grain has rapidly increased in the USA with an annual increase rate of 12.9% from 1998 to 2006, while bioethanol production from sugarcane in Brazil during the same period increased at an annual rate of 1.7%. Since 1975 Brazil has launched a national effort to convert sugarcane into ethanol with a current production at 13.5 million Mg/year, whereas the United States has used maize starch for bioethanol at 16.5 million Mg/year (De Witt *et al*., 2010). In China, starch bioethanol is mainly produced from the decayed and aged maize, rice and wheat grains at 1.33 million Mg/year (Zhou and Thomson, 2009).

The promotion of bioethanol production in the USA has increased the demand for maize as material for bioethanol instead for food and forage. Because the amount of bioethanol production and the market grain prices increased synchronously, bioethanol was highly-publicized as the cause of the rise in grain prices. This is the issue of so-called "food-fuel competition" (Hattori and Morita, 2010). Some countries, like China, hold a long-term policy for food security by avoiding any competition from biomass-based applications. It determines all arable lands reserved for growing food crops rather than used for starch/sugar-based bioethanol and edible-oil-derived biodiesel products (Zhang *et al*., 2009; Zhou and Thomson, 2009). Despite these restrictions, bioethanol production in the world has been rapidly increasing from about 3,000 million kL in 2,000 to about 6,300 million kL in 2007 (Licht, 2007).

The discussion of the ideal plant species to be utilized for bioethanol production is still under controversy. To reach the bioenergy goal, biomass quality and quantity become crucial. In this context, selection of plant species with an ample adaptation to different environments, low input requirements (water, fertilizers and pesticides) and elevated tolerance to adverse environmental factors is fundamental for achieving high biomass production. Grass species with C_4 photosynthesis, such as miscanthus (*Miscanthus giganteus*), switchgrass (*Panicum virgatum*), elephant grass (*Pennisetum purpureum*), aleman grass (*Echinochloa polystachya*), fox tail millet (*Setaria italica*), sweet sorghum (*Sorghum bicolor*) and sugarcane (*Saccharum officinarum*) are ideal energy crops because they possess high conversion efficiency of light into biomass energy, high water use efficiency and high leaf level nitrogen use efficiency (Taylor *et al*., 2010), capacity to grow in marginal land areas, and a relatively high tolerance to soil constraints such as salinity and water-logging. Conversely, highly productive C_3 plants, such as poplar and willow, posses a relatively slower growth rate and have higher contents of lignin, making the polysaccharides less accessible, and consequently the biomass quality is lower.

The chemical energy contained within biomass may be harvested by conversion to bioethanol or by conversion to alternate fuels. In the context of bioethanol production, high quality biomass refers to a composition that can be easily and cheaply converted to liquid transport fuels. That is, the maximum accessible yield of firstly, monosaccharides and disaccharides, and secondly, easily extracted polysaccharides. Large quantities of polysaccharides, such as cellulose, contribute to biomass 'quantity' but biomass 'quality' is also important. Cellulose may be bound within lignin, and thus, inaccessible to processing. Lignin content, composition, and also the type of bonds between lignin, hemicellulose and cellulose are factors that influence biomass 'quality' (Byrt *et al*., 2011). Therefore, this biomass quantity/quality balance has to be always considered in selecting suitable biofuel crops and in research programs directed to improve the biofuel potential of crops.

Bioethanol production from sugar-rich biomasses, such as sugarcane and sugar beet, is the simplest process since the extracted sugar juices can be directly fermented to produce ethanol. Starch-derived bioethanol made from the grain (*e.g.* maize and wheat) or tuber biomass (*e.g.* cassava), require saccharification of starch to sugars before the fermentation process; in addition, the grains need to be pulverized and steamed to accelerate saccharification. Therefore, bioethanol made from starch generally requires more energy input than that made from sugar. The third type is cellulosic bioethanol in which even more energy input will be needed to soften cellulosic materials, such as by acidic hydrolysis, after the pulverization and steaming before the saccharification process. Processing lignocelluloses is expensive due the energy and or enzymatic costs involved in separating cellulose from lignin, and the enzymatic costs of hydrolysing the cellulose. Processes to separate lignin from cellulose include mechanical grinding; ammonia, carbon dioxide or steam explosion (autohydrolysis); acid or alkaline hydrolysis; oxidative delignification (with peroxide); high-temperature treatments (such as pyrolysis >300°C); lime treatment or treatment with fungi that produce lignin-degrading enzymes (such as peroxidases; Sun and Cheng, 2002; Jorgensen *et al*., 2007). More recently the use of ionic liquids to convert biomass has been investigated (Palkovits *et al*., 2010; Zhao *et al*., 2010b).

Currently, the cost of collecting and processing sugar cane juice and maize kernel starch, by crushing stems to extract juice and milling grain followed by saccharification, respectively, is relatively low compared to the cost of harvesting and processing lignocellulosic biomass.

Lignin, a main co-product in the conversion of cellulosic biomasses, is not useful as a carbon source for bioethanol production but can be burned to generate electricity and steam; combustion of lignin contributes to reduce the fossil energy input in the production of cellulosic bioethanol.

The sources of saccharides used to produce bioethanol by fermentation include soluble sugars, starch and structural sugars. The main soluble sugar harvested from plants is sucrose, and at present, the dominant crop grown for global sucrose production is sugarcane (approximately 75% of the world´s supply; Wu and Birch, 2007). The biggest source of starch for bioethanol production is maize kernel and grain from other cereals. Structural polysaccharides, cellulose and hemicellulose, may be hydrolysed to produce monosaccharides for fermentation. Cellulose (D-glucose linked by β−1,4-glucosidic bonds) is the most abundant source of polysaccharides on Earth (Bayer *et al*., 1998).

In general, bioenergy grasses have many advantages over other lignocellulosic crops, among them their shorter life cycle, which enables fast establishment (months vs. years),

grasses biomass offers flexibility to producers because it is produced on the existing farm equipment and support infrastructure from the agro-industry and has a greater potential for genetic improvement in the short term (Vermerris, 2011). Other advantages for using grasses is their different wall composition, specifically the high cellulose content and relatively lower lignin content which permits processing biomass, from sorghum or in general grasses, with much less energy (Parry and Jing, 2011; Vermerris, 2011).

Lignocellulosic biomass has long been recognized as a potential sustainable source of mixed sugars for fermentation to biofuels and other biomaterials. Several technologies have been developed during the past 80 years that allow this conversion process to occur. The natural resistance of plant cell walls to microbial and enzymatic deconstruction, collectively known as "biomass recalcitrance", is largely responsible for the high cost of lignocellulose conversion. To achieve sustainable energy production, it is necessary to overcome the chemical and structural properties that have evolved in biomass to prevent its disassembly.

Many promising energy crops appear to have been overlooked, in particular variants of sugarcane and sweet sorghum. Most of the literature about sorghum focuses on grain sorghum varieties, but the difference in potential energy yields between grain and sweet sorghum varieties is significant. Some sweet sorghum varieties have been reported to produce similar yields to sugarcane (Ratnavathi *et al*., 2010) and to compete well with miscanthus and switchgrass in regards to biomass yields (Propheter *et al*., 2010). Ethanol from sweet-stem sorghum appears to be a viable alternative to fossil fuels, especially for petroleum products as a cooking, lighting and automotive fuel. Sweet-stem sorghum is a multi-purpose crop, yielding food in the form of grain, fuel in the form of ethanol from its stem juice, and fodder from its leaves and bagasse. There are approximately 4,000 sweet sorghum cultivars distributed throughout the world (Grassi *et al*., 2004).

Sweet sorghums are sorghums that can reach heights of up to 6 m and that accumulate soluble sugars in their stems. After squeezing the stalks, these sugars can be fermented directly and conveniently to ethanol or other biofuels. The crushed stems (bagasse) can then be processed as lignocellulosic biomass. Sweet sorghum thus represents an ideal bridge between sugar-based and cellulosic fuels, and, given the rapid establishment of sweet sorghum, this species is expected to be of particular value in extending the processing window of sugarcane-based biorefineries (Vermerris, 2011).

Sorghum is considered as a promising source for next-generation biofuels because it possess several advantages over other bioethanol crops (Ananda *et al*., 2011; Chohnan *et al*., 2011; Davila-Gomez *et al*., 2011), including:

1. Ethanol can be obtained from grain (starch) and sweet stalks (sugars) and lignocellulosic raw material.
2. Sorghum possess an elevated tolerance to different biotic (diseases) and abiotic (drought, salinity, temperature) environmental factors.
3. Sorghum produces a large amount of biomass and fermentable sugars in stems and biomass with relatively low inputs (irrigation, fertilizer and pesticides).
4. Sorghum is a highly productive species and can produce large amounts of energy-dense biomass that can be processed efficiently.
5. The biomass production of this grass ranges from 58.3 to 80.5 tons of fresh stems per hectare with a faster growing period of about 4 to 5 months; it only needs 90 to 140 days to mature.

6. Sorghum is present in a wide range of harvest areas in semi-arid and colder zones; this grass can be grown in lands that are currently marginal for many other crops.
7. The water requirements of this grass are low: sorghum produces more biomass than corn, using 33% less water (Rooney *et al*., 2007).
8. Sorghum genome sequence data have recently become available and revealed a 730-megabase genome. The sorghum genome is appreciably smaller and less complex than the maize genome (Vermerris, 2011). It is expected that the current genomics and genetic research currently in process will allow the development of superior high yielding sorghum bioenergy cultivars.

Improvement of crops for biofuel production may be achieved by selective breeding for natural differences or by genetic modification, taking into account the biomass quantity/quality trade off mentioned before. The search for biofuel relevant traits and genes has been accelerated by the sequencing of the maize and sorghum genomes and subsequent verification of gene function by reverse and forward genetics.

Genomics-based plant breeding and biotechnology of sorghum offer the opportunity to make improvements to this crop related to produce more biomass for acre and more fuel or energy per ton. Using this technology it is possible to optimize yield-influencing traits, composition and conversion characteristics for ethanol production. These tools may allow the modelling of the ideal sorghum cultivar having rich sugar/starch contents in stalk/grain and high biomass accumulation for production of lignocellulosic bioethanol.

Elucidating the genetic basis of stem sugar and stem juice accumulation, modifying cell wall composition so that sorghum biomass can be processed more efficiently, maximizing biomass yield for a given geographic area and production system and understanding the different mechanisms underlying its elevated drought tolerance are the main focus areas among sorghum scientists that target bioenergy traits (Vermerris, 2011).

Increasing the Biomass Accumulation of Biofuel Crops

Biomass quantity may be increased by improving photosynthetic efficiency or increasing the tolerance of plants to adverse environmental conditions, particularly drought and salinity.

Investigations into the manipulation of the key photosynthetic enzymes, RUBISCO, pyruvate phosphate kinase (PPDK) and NADP malate dehydrogenase (NADP-MDH) in the C_4 dicotyledoneous species *Flaveria bidentis* have been reported (Furbank *et al*., 1997), whereas an alternative strategy to reduce photorespiration by manipulating catalase levels in tobacco has also been described (Brisson *et al*., 1998). Zhu *et al*. (2010) suggested improving leaf display to avoid light saturation and engineering carboxylases that are better adapted to forthcoming CO_2 concentrations. From these studies, control of photosynthetic flux in current air levels of CO_2 appears to be shared between PEP carboxylase, Rubisco and an enzyme involved in regenerating PEP for PEP carboxylase, pyruvate Pi-dikinase (Byrt *et al*., 2011). Increasing amounts of these enzymes or improving their kinetic properties to increase the catalytic rate are the most attractive targets (Matsuoka *et al*., 2001).

Several other approaches to yield improvements have been suggested. For example, it is claimed that improved yield can be achieved by manipulation of fructose-1,6-biphosphate aldolase (FDA), an enzyme that reversibly catalyses the reaction converting triosephosphate

to fructose-1,6-biphosphate. Leaves of transgenic plants which express the FDA from *Escherichia coli* in the chloroplast show significantly enhanced starch accumulation and lower sucrose concentration; they also had significantly higher root mass (Barry *et al*., 1998).

In addition to alterations in the activity of specific photosynthetic genes, a more general method of improving plant performance may be to modify plastid number (Osteryoung, 1998). Another strategy for increasing chlorophyll content, without modifying plastid number, is the expression of a hybrid protein comprising a yeast gene encoding 5-amino levulinic acid synthase (ALAS) and an N-terminal transit sequence for the small subunit of carboxydismutase. Similarly, manipulation of chlorophyll a/b binding genes has been used to modify chlorophyll accumulation, as well other characteristics. It was argued that overexpression of this protein leaded to increases in plastid proteins and also plant biomass.

Other, non-photosynthetic, approaches to increasing yield of both shoot and root include overexpression of a cyclin gene, *cyc1a*, from *Arabidopsis* (Doerner and Lamb, 1998). Additionally, increased biomass production by enhancing the tillering rate of grasses has been reported by manipulating single genes such as *moc1* (Li *et al*., 2003) and *rolA* from *Agrobacterium rhizogenes* (Aguado-Santacruz *et al*., 2009). The role of strigolactones (compounds thought to be derived from carotenoids) in the control of shoot branching has also been recently recognized (Gomez-Roldan *et al*., 2008; Umehara *et al*., 2008).

In relation to the manipulation of plants for increased water stress, Aguado-Santacruz (2006) mentioned that practically all genetic modifications tested up to now, from the initial perception steps to the final response module, have resulted in an improved response of plants to water stress, with osmolyte pathway alteration being the most studied approach. Thus, the possibility of increasing the biomass of bioenergy crops by enhancing their drought tolerance is feasible, especially in marginal areas, where water availability is the major restrictive factor for production.

Improving the Quality of Bioethanol Crops

Genetic mapping and characterization of quantitative trait loci (QTLs) is considered a valuable tool for trait enhancement in sorghum. Quantitative trait loci associated with sugar concentration of the juice have been identified in a recombinant inbred line population derived from the sweet sorghum ‘Rio’ and the grain sorghum BTx623. 129 QTLs were identified controlling yield and composition of sugar in the stem as well yield and composition of starch and other components in the grain. One of the principal conclusions was that total non-structural carbohydrate yield can be increased by selecting for mayor QTLs from both grain and sweet sorghum types and suggested that increases in plant sinks may increase total energy production potential. From this research it was evident that novel germplasm with an overall higher sugar yield can be developed by combining QTLs (and ultimately loci) controlling juice volume and juice concentration (Murray *et al*., 2008).

Naturally occurring mutations have played a prominent role in genetics since the discipline was founded, and the ability to induce mutations with chemical (ethyl methanesulfonate, EMS; diethylsulfate, DES), physical (gamma rays, X-rays) and biological (transposons, T-DNA) agents, has propelled the field. Forward genetics is based on the identification of mutants with a phenotype of interest, with the ultimate goal the identification of novel genes involved in the process or pathway under study. Reverse genetics is an

approach to discover the function of a gene by analyzing the phenotypic effects of specific gene sequences obtained by DNA sequencing with the goal of better understanding the role of these genes in a process pathway. This investigative process proceeds in the opposite direction of so-called forward genetic screens of classical genetics. In other words, forward genetics seeks to find the genetic basis of a phenotype or trait, reverse genetics seeks to find what phenotypes arise as a result of particular genes. Nowadays, a reliable transposable element system for obtaining sorghum mutants that can be further exploited by forward and reverse genetics has not been developed (Vermerris, 2011).

Conversely to starch- and sucrose-derived bioethanol, lignocellulosic bioethanol is still under development because of its recalcitrance. Central to the "biomass recalcitrance" concept is the composition and structure of the plant cell walls. Crop biomass is principally made up of three components of cell walls: cellulose (30-45%), a β-1,4-glucan polymer that is crystalline, hemicelluloses (20-30%), branched polymers that are composed of mainly xylose and other five-carbon sugars; and lignins (25-35%), non-carbohydrates that interlink other polymers into a robust cell wall structure and architecture (Pauly and Keegstra, 2010). The properties of cellulose-crystallinity and lignin-crosslinking become a barrier that critically hinders biomass pretreatment and enzyme digestion (Abramson *et al*., 2010). Modification of plant cell wall structure, therefore, is the key step for improving biomass quality of energy crops.

Lignin significantly contributes to biomass recalcitrance. As stated before, greater energy or quantities of enzymes (cellulases) are needed to hydrolyse cellulose that is embedded within lignin. In addition to blocking the liberation of sugars from cellulose and adhering to hydrolytic enzymes, lignin may also release aromatic compounds that inhibit fermentation. Modifying lignin content, composition, hydrophobicity and cross-linking can improve the enzymatic hydrolysis of cell walls (Byrt *et al*., 2011).

Greater lignocellulosic ethanol yields may be achieved by modifying cell wall composition, for example, altering lignin content and composition. To retain fitness in future low-lignin crops, it may be necessary for lignin to be reduced in only specific tissues, or cell types (Shadle *et al*., 2007). Altering lignin in different cell types, such as retaining lignin in xylem cell walls but lowering lignin content in storage parenchyma cell walls may improve digestibility without compromising xylem function. By modifying cell wall composition in a tissue specific manor lignin in the sheath may still provide structural support and possibly defence against microbial attack, lignin in stomata may ensure efficient stomatal function and prevent unnecessary water loss, and lignin in xylem may contribute to normal xylem function, but stem parenchyma cell wall cellulose would be more accessible to degradation during processing (Byrt *et al*., 2011).

Lignin content and composition may vary due to natural mutation in the genes involved in the lignin biosynthesis pathway, such as observed for brown-midrib (*bmr*) mutant plants. *Bmr* mutant plants show a red-brown colour in the mid vein of the leaves and have significantly less lignin than normal plants (Oliver *et al*., 2005). In order to improve the biomass-to-fuel conversion of sorghum, brown midrib (*bmr*) mutants have been currently manipulated. Four independent loci were identified by Saballos *et al*. (2008), whereas additional *bmr* mutants were identified in the TILLING population of Xin *et al*. (2008). Several *bmr* mutants from both populations have shown to result in enhanced yields of fermentable sugars following enzymatic saccharification of sorghum biomass, even after thermochemical pretreatment (Dien *et al*., 2009). *Bmr6* and *bmr2* genes have been cloned.

The *bmr6* gene encodes the monolignol biosynthetic gene cinnamyl alcohol dehydrogenase (CAD) (Sattler *et al*., 2009), while *bmr2* gene also encodes a cell wall biosynthetic enzyme. Knowing the identity of the *bmr* genes and the nature of the mutations in these genes has enabled the development of allele specific markers that will allow more efficient use of these mutations in commercial breeding programs.

Selection of induced cell wall mutants is a practicable work for obtaining improved biofuel crops. This process includes three major steps: mutagenesis of the high-yield-grain crops, selection of the cell-wall-altered plants, and identification of the mutants that are of high grain yield and efficient biomass degradation. A selection of cell wall mutants has been carried out by screening of large mutagenesis pools of rice T-DNA knockout and maize transposon insertions. Additionally, chemical (ethyl methane sulfonate-induced) and physical (^{60}Cobalt irradiation) mutagenesis pools for other potentially typical mutants have also been generated. Distinct from the previously identified cell wall mutants that showed abnormal phenotypes as the dwarfism, irregular xylem and even lethality (Goubet *et al*., 2003; Desprez *et al*., 2007), the selected chemical and physical mutants displayed similar agronomic traits and grain yields to the wild type, but showed a remarkable alteration of cell wall composition; with various mild pretreatments, several mutants showed an increased rate of biomass degradation in comparison to the wild type (Xie and Peng, 2011).

Lignin may also be altered by selective plant breeding and transgenic approaches. Strategies to reduce the lignin contents of plants have targeted each of the steps in phenylpropanoid metabolism (Vanholme *et al*., 2010b). In general, alteration of the mRNA transcript levels of the genes involved in lignin biosynthesis reduces lignin content (Baucher *et al*., 2003). In many cases, altered regulation of lignin biosynthesis genes influences the ratios of p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) lignin monomers, and it may be possible to improve the digestibility of lignin by modifying the monomeric composition without the need to reduce the total lignin content (Simmons *et al*., 2010). However, some plant species may have mechanisms to compensate for changes in lignin content and composition, such as increasing the amount of cross-linking, which may increase recalcitrance.

Increasing the biosynthesis of ferulic acid, and its export to the cell wall, has been suggested as an alternative strategy to increase the value of bio-energy crops (Vanholme *et al*., 2010a). Transgenic poplar plants with defective cinnamoyl-CoA reductase showed increased ferulic acid levels which were associated with improved saccharification potential relative to wild type plants (Lepl´e *et al*., 2007). Incorporation of ferulic acid into the lignin monomer may lead to acetal bonds that are easily cleaved in pre-treatment processes to degrade lignin (Vanholme *et al*., 2010b).

A genetic engineering approach to modify the bioethanol potential of sorghum has been hampered by the tissue culture recalcitrance and poor reproducibility of transformation protocols of sweet (and grain) sorghum and deficient information about the underlying mechanisms associated to species-specific and developmental regulation of cell wall biogenesis. Fragmentary information is available about genes related to cell wall biogenesis, while their biochemical functions remains to be elucidated (Penning *et al*., 2009; Vermerris, 2011). Lack of this knowledge could unexpectedly lead to alteration of plant cell growth and development in transgenic plants intended for increased lignocellulosic biomass quality (Torney *et al*., 2007; Vega-Sánchez and Ronald, 2010).

Genome-based capabilities will be instrumental in identifying genes involved in the synthesis of cell-wall polymers and higher structures, analysing the cell wall design principles and determining the factors controlling the amounts, composition, and structure of polymers and polymer matrices.

A deeper understanding of the synthesis, deposition and hydrolysis of the distinctive cell walls of grasses will be crucial to properly manipulate the traits that contribute to biomass yield and quality of bioenergy crops (Himmel *et al*., 2007; Carpita and McCann, 2008), particularly sweet sorghum.

Finally, successful genetic design of improved bioethanol sorghum should embrace both a robust scientific framework related to gene function and wide application and reliable tissue culture and genetic transformation systems. Fortunately, the development of the first genetic transformation system for sweet sorghum (Raghawanshi and Birch, 2010) opens the possibility of testing new genes in the genetic context of an important bioenergy grass.

REFERENCES

Able, J. A., Rathus, C., Godwin, I. D. (2001). The investigation of optimal bombardment parameters for transient and stable transgene expression in sorghum. *In Vitro Cellular and Developmental Biology-Plant*, 37, 341-348.

Abramson, M., Shoseyov, O., Shani, Z. (2010). Plant cell wall reconstruction toward improved lignocellulosic production and processability. *Plant Science*, 178, 61-72.

Aguado-Santacruz, G. A. (2006). Genetic manipulation of plants for increased drought tolerance. In: R. G. Guevara-González, I. Torres-Pacheco (eds.), *Advances in Agricultural and Food Biotechnology*. (pp. 71-97). Kerala, India. Research Signpost.

Aguado-Santacruz, G. A., García-Moya, E., Aguilar-Acuña, J. L., Moreno-Gómez, B., Solís-Moya, E., Preciado-Ortiz, E. R., Jiménez-Bremont, J. F., Rascón-Cruz, Q. (2007). In vitro plant regeneration from quality protein maize (QPM). *In Vitro Cellular* and *Developmental Biology-Plant*, 43, 215-224.

Aguado-Santacruz, G. A., Rascón-Cruz, Q., Moreno-Gómez, B., Guevara-González, R. G., Guevara-Olvera, L., Jiménez-Bremont, J. F., Arévalo-Gallegos, S., García-Moya, E. (2009). Genetic transformation of blue grama grass with the rolA gene from Agrobacterium rhizogenes: regeneration of transgenic plants involves a "hairy embryo" stage. *In Vitro Cellular* and *Developmental Biology-Plant*, 45, 681-692.

Ananda, N., Vadlania, P. V., Vara, Prasad, P. V. (2011). Evaluation of drought and heat stressed grain sorghum (*Sorghum bicolor*) for ethanol production. *Industrial Crops and Products*, 33, 779-782.

Anas, A., Yoshida, T. (2002). Genotypic difference of *Sorghum bicolor* in the callus formation and callus growth on aluminum-containing medium. *Plant Production Science*, 5, 242-247.

Arulselvi, I. P., Krishnaveni, S. (2009). Effect of hormones, explants and genotypes in in vitro culturing of Sorghum. *Journal of Biochemical Technology*, 1, 96-103

Bansal, S., Mishra, A., Tomar, A., Sharma, S., Khanna, V. K., Garg, G. K. (2008). Isolation and temporal endospermal expression of [gamma]-kafirin gene of grain sorghum (*Sorghum*

bicolor L. Moench) var. M 35-1 for introgression analysis of transgene. *Journal of Cereal Science*, 48, 808-815.

Barry, G. F., Cheikh, N., Kishore, G. M. (1998). Expression of fructose 1,6 bisphosphate aldolase in transgenic plants. Patent Application WO 98/58069.

Baskaran, P., Rajeswari, B. R., Jayabalan, N. (2006). Development of an in vitro regeneration system in sorghum [*Sorghum bicolor* (L.) Moench] using root transverse thin cell layers (tTCLs). *Turkish Journal of Botany*, 30, 1-9.

Battraw, M., Hall, T. C. (1991). Stable transformation of *Sorghum bicolor* protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes. *Theorical and Applied Genetics*, 82, 161-168.

Baucher, M., Halpin, C., Petit-Conil, M., Boerjan, W. (2003). Lignin, genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology*, 38, 305-350.

Bayer, E. A., Chanzy, H., Lamed, R., Shoham, Y. (1998). Cellulose, cellulases and cellusomes. *Current Opinion in Structural Biology*, 8, 548-557.

Belton, P. S., Delgadillo, I., Halford, N. G., Shewry, P. R. (2006). Kafirin structure and functionality. *Journal of Cereal Science*, 44, 272-286.

Berndes, G., Hansson, J., Egeskog, A., Johnsson, F. (2010). Strategies for 2nd generation biofuels in EU-Co-firing to stimulate feedstock supply development and process integration to improve energy efficiency and economic competitiveness. *Biomass and Bioenergy*, 34, 227-236.

Bhaskaran, S., Smith, R. J. (1988). Enhanced somatic embryogenesis in *Sorghum bicolor* from shoot tip culture. *In Vitro Cellular* and *Developmental Biology-Plant*, 24, 65-70.

Bhaskaran, S., Smith, R. H., Paliwal, S., Schertz, K. F. (1987). Somaclonal variation form *Sorghum bicolor* (L.) Moench cell culture. *Plant Cell, Tissue and Organ Culture*, 9, 189-196.

Boyes, C. J., Vasil, I. K. (1984). Plant regeneration by somatic embryogenesis from cultured young inflorescences of *Sorghum arundinaceum* (Desv.) Stapf. Var sudanense (sudan grass). *Plant Science Letters*, 35, 153-157.

Brar, D. S., Rambold, S., Gamborg, O., Constabel, F. (1979). Tissue culture of corn and sorghum. *Z. Pflanzenphysiology*, 95, 377-388.

Brettell, R. I., Wernicke, W., Thomas, E. (1980). Embryogenesis from cultured immature inflorescences of *Sorghum bicolor*. *Protoplasma*, 104, 141-148.

Brisson, L. F., Zelitch, I., Havir, E. A. (1998). Manipulation of catalase levels produces altered photosynthesis in transgenic tobacco plants. *Plant Physiology*, 116, 259-269.

Busk, P. K., Moller, B. L. (2002). Dhurrin synthesis in sorghum is regulated at the transcriptional liver and induced by nitrogen fertilization in older plants. *Plant Physiology*, 129, 1222-1231.

Byrt, C. S., Grof, C. P. L., Furbank, R. T. (2011). C_4 plants as biofuel feedstocks: optimising biomass production and feedstock quality from a loignocellulosic Perspective. *Journal of Integrative Plant Biology*, 53, 120-135.

Cai, T., Butler, L. (1990). Plant regeneration from embryogenic callus initiated from immature inflorescences of several high tannin sorghums. *Plant Cell, Tissue and Organ Culture*, 20, 101-110

Cai, T., Daly, B., Butler, L. (1987). Callus induction and plant regeneration from shoot portions of mature embryos of high-tannin sorghums. *Plant Cell, Tissue and Organ Culture*, 9, 245-252.

Cai, T., Ejeta, G., Aztell, J. D., Butler, L. G. (1990). Somaclonal variation in high tannin sorghums. *Theorical and Applied Genetics*, 79, 737-747.

Carpita, N. C., McCann, M. C. (2008). Maize and Sorghum: Genetic resources for bioenergy grasses. *Trends in Plant Science*, 13, 415-420.

Casas, A. M., Kononowicz, A. K., Haan, T. G., Zhang, L., Tomes, D. T., Bressan, R. A., Hasegawa, P. M. (1997). Transgenic sorghum plants obtained after microprojectile bombardment of immature inflorescences. *In Vitro Cellular and Developmental Biology-Plant*, 33, 92-100.

Casas, A. M., Kononowicz, A. K., Zehr, U. B., Tomes, D. T., Axtell, J. D., Butler, L. G., Bressan, R. A., Hasegawa, P. M. (1993). Transgenic sorghum plants via microprojectile bombardment. *Proceedings of the National Academy of Sciences*, 90, 11212-11216.

Chamba, E. B., Halford, N. G., Forsyth, J., Wilkinson, M., Shewry, P. R. (2005). Molecular cloning of [beta]-kafirin, a methionine-rich protein of sorghum grain. *Journal of Cereal Science*, 41, 381-383.

Cho, M., Wong, J. H., Lemaux, P. G., Buchanan, B. B. (2007). Transgenic plants with elevated thioredoxin levels. United States Patent 7179964.

Chohnan, S., Nakane, M. M., Rahman, H., Nitta, Y., Yoshiura, T., Ohta, H., Kurusu, Y. (2011). Fuel ethanol production from sweet sorghum using repeated-batch fermentation. *Journal of Bioscience and Bioengineering*, 111, 433-436.

Cook, J. D., Reddy, M. B., Burri, J., Juillerat, M. A., Hurrell, R. F. (1997). The influence of different cereal grains on iron absorption from infant cereal foods. *American Journal of Clinical Nutrition*, 65, 964-969.

Davidsson, L., Galan, P., Cherouvrier, F., Kastenmayer, P., Juillerat, M. A., Hercberg, S., Hurrell, R. F. (1997). Iron bioavailability from infant cereals by infants: the effect of dephytinization. *American Journal of Clinical Nutrition*, 65, 916-920.

Davila-Gomez, F. J., Chuck-Hernandez, C., Perez-Carrillo, E., Rooney, W. L. (2011). Evaluation of bioethanol production from five different varieties of sweet and forage sorghums (*Sorghum bicolor* (L.) Moench). *Industrial Crops and Products*, 33, 611-616.

Davis, M. E., Kidd, G. H. (1980). Optimization of sorghum primary callus growth. *Z. Pflanzenphysiol*, 98, 79-82.

De Witt, M., Junginger, M., Lensink, M. S., Londo, M., Faaij, A. (2010). Competition between biofuels: Modeling technological learning and cost reductions over time. *Biomass and Bioenergy*, 34, 203-217.

Dendy, D. A. V. (1995). Sorghum and Millets Chemistry and Technology. 406 pp. USA, St. Paul, Minnesota, American Association of Cereal Chemists, Inc.

Desprez, T., Juraniec, M., Crowell, E. F, Jouy, H., Pochylova, Z., Parcy, F., Höfte, H., Gonneau, M., Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in Arabidopsis thaliana. *Proceedings of the National Academy of Sciencies* USA, 104, 15572-15577.

Dien, B. S., Sarath, G., Pedersen, J. F., Sattler, S. E., Chen, H., Funnell-Harris, D. L., Nichols, N. N., Cotta, M. A. (2009). Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* (L.) Moench) lines with reduced lignin contents. *Bioenergy Research*, 2, 153-164.

Doerner, P. W., Lamb, C. J. (1998). Method of increasing growth and yield in plants. Patent Application WO 98/03631.

Doggett, H. (1965). Striga hermonthica on sorghum in East Africa. *The Journal of Agricultural Science*, 65, 183-194.

Dunstan, D. I., Short, K. C., Thomas, E. (1978). The anatomy of secondary morphogenesis in cultured scutellum tissues or *Sorghum bicolor*. *Protoplasma*, 97, 251-260.

Dunstan, D. I., Short, K. C., Dhaliwal, H., Thomas, E. (1979). Further studies on plantlet production from cultured tissues of *Sorghum bicolor*. *Protoplasma*, 101, 355-361.

Elkin, R. G., Arthur, E., Hamaker, B. R., Axtel, J. D., Douglas, M. W., Parsons, C. M. (2002). Nutritional value of highly digestible sorghum cultivar for meat-type chickens. *Journal of Agricultural Food Chemistry*, 50, 4146-4150.

Elkonin, L. A. (2000). Dominant male sterility mutation of sorghum induced in tissue culture and its inhibition. *International Sorghum and Millets Newsletter*, 41, 28-30.

Emani, C., Sunilkumar, G., Rathore, K. S. (2002). Transgene silencing and reactivation in sorghum. *Plant Science*, 162, 181-192.

FAO. Faostat database. http://www.fao.org/docrep/003/v6800s/v6800s05.htm#P3_81; 1995.

FAOSTA. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor; 2009.

Florack, D. E. A., Stiekema, W. J. (1994). Thionins: properties, possible biological roles and mechanisms of action. *Plant Molecular Biology*, 26, 25–37.

Forsyth, J. L., O´Kennedy, M. M., Grootboom, A., Shewry, P. R. (2003). Prospects for improving sorghum grain quality. In: P. S. Belton, , J. R. N. Taylor (Eds.), *Workshop on the proteins of shorgum and millets: enhancing nutritional and functional properties for Africa*. Pretoria, South Africa.

Furbank, R. T., Chitty, J. A., Jenkins, C. L. D., Taylor, W. C., Trevanion, S. J., Von Caemmerer, S., Ashton, A. R. (1997). Genetic manipulation of key photosynthetic enzymes in the C4 plant Flaveria bidentis. *Australian Journal of Plant Physiology*, 24, 477-485.

Gamborg, O. L., Shyluk, J. P., Brar, D. S., Constable, F. (1977). Morphogenesis and plant regeneration from callus of immature embryos of sorghum. *Plant Science Letters*, 10, 67-74.

Ganjewala, D., Kumar, S., Devi, S. A., Ambika, K. (2010). Advances in cyanogenic glycoside biosynthesis and detection in plants: A review. *Acta Biologica Szegediensis*, 54, 1-14.

Gao, Z., Jayaraj, J., Muthukrishnan, S., Claflin, L., Liang, G. H. (2005). Efficient genetic transformation of sorghum using a visual screening marker. *Genome*, 48, 321-333.

Girijashankar, V., Sharma, H. C., Sharma, K. K., Swathisree, V., Prasad, L. S., Bhat, B. V., Royer, M., Secundo, B. S., Narasu, M. L., Altosaar, I., Seetharama, N. (2005). Development of transgenic sorghum for insect resistance against the spotted stem borer (Chilo partellus). *Plant Cell Reports*, 24, 513-522.

Gnansounou, E., Dauriat, A., Wyman, C. E. (2005). Refining sweet sorghum to ethanol and sugar: economic trade-offs in the context of North China Biores. *Technology*, 96, 985-1002.

Gomez-Roldan, V., Fermas, S., Brewer, P. B., Puech-Pagès, V., Dun, E. A., Pillot, J., Letisse, F., Matusova, R., Danoun, S., Portais, J., Bouwmeester, H., Bécard, G., Beveridge, C. A., Rameau, C., Rochange, S. F. (2008). Strigolactone inhibition of shoot branching. *Nature*, 455, 189-194.

Goubet, F., Misrahi, A., Park, S. K., Zhang, Z., Twell, D., Dupree, P. (2003). AtCSLA7, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in *Arabidopsis*. *Plant Physiology*, 131, 547-557.

Grassi, G., Tondi, G., Helm, P. (2004). Small-sized Commercial Bioenergy Technologies as an Instrument of Rural Development. Biomass and Agriculture: Sustainability, Markets and Policies. (pp. 277-287). OECD Publication Service, Paris.

Grootboom, A. W. (2010). Effect of RNAi down-regulation of three lysine-deficient kafirins on seed lysine content of sorghum [*Sorghum bicolor* (L.) Moench]. Ph.D. thesis. Faculty of Natural and Agricultural Science, University of Pretoria, Department of Plant Science Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa.

Grootboom, A. W., O'Kennedy, M. M., Mkhonza, N. L., Kunert, K., Chakauya, E., Chikwamba, R. K. (2008). In vitro culture and plant regeneration of sorghum genotypes using immature zygotic embryos as explant source. *International Journal of Botany,* 4, 450-455.

Gupta, R., Ting, J. T., Sokolov, L. N., Johnson, S. A., Luan, S. (2002). A tumor suppressor homolog, AtPTEN1, is essential for pollen development in *Arabidopsis*. *Plant Cell*, 14, 2495-2507.

Gurel, S., Ekrem, G., Rajvinder, K., Joshua, W., Ling, M., Han, Q., Peggy, G. L. (2009). Efficient reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. *Plant Cell Reports*, 28, 429-444.

Hagio, T., Blowers, A. D., Earle, E. D. (1991). Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Reports*, 10, 260-264.

Harshavardhan, D., Rani, T. S., Ugalanathan, K., Seetharama, N. (2002). An improved protocol for regeneration of *Sorghum bicolor* from isolated shoot apices. *Plant Biotechnology*, 19, 163-171.

Hattori, T., Morita, S. (2010). Energy crops for sustainable bioethanol production; which, where and how?. *Plant Production Science*, 13, 221-234.

Henley, E. C., Taylor, J. R. N., Obukosia, S. D. (2010). The importance of dietary protein in human health: Combating protein deficiency in Sub-Saharan Africa through transgenic biofortified sorghum. *Advances in Food and Nutrition Research*, 60, 21-52.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., Foust, T. D. (2007). Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science*, 315, 804-807.

Hokanson, K. E., Ellstrand, N. C., Ouedraogo, J. T., Olweny, P. A., Schaal, P. A., Raybould, A. F. (2010). Biofortified Sorghum in Africa: using problem formulation to inform risk assessment. *Nature Biotechnology*, 28, 900-903.

Howe, A., Sato, S., Dweikat, I., Fromm, M., Clemente, T. (2006). Rapid and reproducible Agrobacterium-mediated transformation of sorghum. *Plant Cell Reports*, 25, 784-791.

Huang, S., Adams, W. R., Zhou, Q., Malloy, K .P., Voyles, D. A., Anthony, J., Kriz, A. L., Luethy, M. H. (2004). Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. *Journal of Agricultural and Food Chemistry*, 52, 1958-1964.

Huang, S., Frizzi, A., Florida, C. A., Kruger, D. E., Luethy, M. H. (2006). High lysine and high tryptophan maize resulting from the reduction of both 19- and 22-kD α-zeins. *Plant Molecular Biology*, 61, 525-535.

Hurrell, R. F., Juillerat, M. A., Reddy, M. B., Lynch, S. R., Dassenko, S. A., Cook, J. D. (1992). Soy protein, phytate and iron absorption in man. *American Journal of Clinical Nutrition*, 56, 573-578.

Jorgensen, H., Kristensen, J. B., Felby, C. (2007). Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioproducts and Biorefining,* 1, 119-134.

Joye, I. J., Lagrain, B., Delcour, J. A. (2009). Endogenous redox agents and enzymes that affect protein network formation during breadmaking – A review. *Journal of Cereal Science,* 50, 1-10.

Jung, R. (2008). Grain quality improvement through altered expression of seed proteins. United States Patent Application 20080134361.

Kaeppler, H. F., Pederson, J. F. (1997). Evaluation of 41 elite and exotic inbred sorghum genotypes for high quality callus production. *Plant Cell, Tissue and Organ Culture,* 48, 71-75.

Kononowicz, A. K., Casas, A. M., Tomes, D. T., Bressan, R. A., Hasegawa, P. M. (1995). New vistas are opened for Sorghum improvement by genetic transformation. *African Crop Science Journal,* 3, 171-180.

Laidlaw, H. K. C., Godwin, I. D. (2008). Sorghum. In: C. Kole, T. C. Hall (eds.), *Compendium of Transgenic Crop Plants: Transgenic Cereals and Forage Grasses.* (pp. 157-176). Oxford, UK. Blackwell Publishing.

Léder, I. (2009). Sorghum and millets. Cultivated plants, primarily as food sources. In: G. Fuleky. (ed.), Encyclopedia of Life Support Systems (EOLSS). UNESCO.

Lepl´e, J. C., Dauwe, R., Morreel, K., Storme, V., Lapierre, C., Pollet, B., Naumann, A., Kang, K. Y., Kim, H., Ruel, K., Lef´ebvre, A., Joseleau, J. P., Grima-Pettenati, J., De Rycke, R., Andersson-Gunner´s, S., Erban, A., Fehrle, I., Petit-Conil, M., Kopka, J., Polle, A., Messens, E., Sundberg, B., Mansfield, S. D., Ralph, J., Pilate, G., Boerjan, W. (2007). Downregulation of Cinnamoyl-Coenzyme A reductase in poplar: Multiple-level phenotyping reveals effects on cell wall polymer metabolism and structure. *Plant Cell,* 19, 3669-3691.

Li, X., Qian, Q., Fu, Z., Wang, Y., Xiong, G., Zeng, D., Wang, X., Liu, X., Teng, S., Hiroshi, F., Yuan, M., Luo, D., Han, B., Li, J. (2003). Control of tillering in rice. *Nature,* 422, 618-621.

Licht, F.O. (2007). World Ethanol and Biofuels Report. Vol.5. No. 17, pp. 354.

Ma, H., Gu, M., Liang, G. H. (1987). Plant regeneration from cultured immature embryos of *Sorghum bicolor* (L.) Moench. *Theoretical Applied Genetics,* 73, 389-394.

Mackinnon, C., Gunderson, G., Nabors, M. W. (1986). Plant regeneration by somatic embryogenesis from callus cultures of sweet sorghum. *Plant Cell Reports,* 5, 349-351.

MacLean, W. C., Lopez de Romaña, G., Placko, R. P., Graham, G. G. (1981). Protein quality and digestibility of sorghum in preschool children: Balance studies and plasma free amino acids. *Journal of Nutrition,* 111, 1928-1936.

Marero, L. M., Payumo, E. M., Aguinaldo, A. R., Matsumoto, I., Homma, S. (1991). The antinutritional factors in weaning foods prepared from germinated legumes and cereals. *Lebensmittel-Wissenschaft* und-*Technologie*, 24, 177-181.

Masteller, V. J., Holden, D. J. (1970). The growth of and organ formation from callus tissue of sorghum. *Plant Physiology*, 45, 362-364.

Matsuoka, M., Furbank, R. T., Fukayama, H., Miyao, M. (2001). Genetic Engineering of C4 photosynthesis. *Annual Review of Plant Physiology,* 52, 297-314.

Mazhar, H., Chandrashekar, A., Shetty, .H. S. (1993). Isolation and immunochemical characterisation of the alcohol extractable proteins (kafirins) of *Sorghum bicolor* (L.) Moench. *Journal of Cereal Science,* 17, 83-93.

Mohan, D. (1975). Chemically induced high lysine mutants in *Sorghum bicolor* (L.) moench. Ph.D. thesis, Purdue University, W. Lafayette, Indiana.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum,* 15, 473-497.

Murray, S. C., Sharma, A., Rooney, W. L., Klein, P. E., Mullet, J. E., Mitchell, S. E., Kresovich, S. (2008). Genetic improvement of sorghum as a biofuel feedstock In: quantitative loci for stem sugar and grain nonstructural carbohydrates. *Crop Science,* 48, 2165-2179.

Nirwan, R. S., Kothari, S. L. (2003). High copper levels improve callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. *In Vitro Cellular* and *Developmental Biology-Plant,* 39, 161-164.

O'Kennedy, M. M., Grootboom, A., Shewry, P. R. (2006). Harnessing sorghum and millet biotechnology for food and health. *Journal of Cereal Science,* 44, 224-235.

Oldach, K. H., Morgenstern, A., Rother, S., Girgi, M., Kennedy, O. M., Lorz, H. (2001). Efficient in vitro plant regeneration from immature zygotic embryos of pearl millet (*Pennisetum glaucum* (L.) R.Br.) and *Sorghum bicolor* (L.) Moench. *Plant Cell Reports,* 20, 416-421.

Oliver, A. L., Pedersen, J. F., Grant, R. J., Klopfenstein, T. J., Jose, H. D. (2005). Comparative effects of the sorghum bmr-6 and bmr-12 genes: II. Grain yield, stover yield, and stover quality in grain sorghum. *Crop Science,* 45, 2240-2245.

Osteryoung, K. W. (1998). Plant plastid division genes. Patent Application WO 98/00436.

Palkovits, R., Tajvidi, K., Procelewska, J., Rinaldi, R., Ruppert, A. (2010). Hydrogenolysis of cellulose combining mineral acids and hydrogenation catalysts. *Green Chemistry,* 12, 972-978.

Pandey, A. K., Venkatesh Bhat, B., Balakrishna, D., Seetharama, N. (2010). Genetic transformation of sorghum (*Sorghum bicolor* (L.) Moench.). *International Journal of Biotechnology and Biochemistry,* 6, 45-53.

Parry, M. A., Jing, H. C. (2011). Bioenergy plants: hopes, concerns and prospectives. *Journal of Integrative Plant Biology,* 53, 94-95.

Paterson, A. H. (2009). The *Sorghum bicolor* genome and the diversification of grasses. *Nature,* 457, 551-556.

Pauly, M., Keegstra, K. (2010). Plant cell wall polymers as precursors for biofuels. *Current Opinion in Plant Biology,* 13, 305-312.

Pelegrini, P. B., Franco, O. L. (2005). Plant γ–thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. *The International Journal of Biochemistry and Cell Biology,* 37, 2239-2253.

Penning, B. W., Hunter, C. T., Tayengwa, R., Eveland, A. L., Dugard, C. K., Olek, A. T., Vermerris, W., Koch, K. E., McCarty, D. R., Davis, M. F., Thomas, S. R., McCann, M.C., Carpita, N. C. (2009). Genetic resources for Maize cell wall biology. *Plant Physiology,* 151, 1703-1728.

Propheter, J. L., Staggenborg, S. A., Wu, X., Wang, D. (2010). Performance of annual and perennial biofuel crops: yield during the first two years. *Agronomy Journal,* 102, 806-814.

Raghawanshi, A., Birch, R. G. (2010). Genetic transformation of sweet sorghum. *Plant Cell Reports*, 29, 997-1005.

Rao, A. M., Sree, K. P., Kishor, P. B. K. (1995). Enhanced plant regeneration in grain and sweet sorghum by asparagine, proline and cefotaxime. *Plant Cell Reports*, 15, 72-75.

Ratnavathi, C. V., Suresh, K., Vijay Kumar, B. S., Pallavi, M., Komala, V. V., Seetharama, N. (2010). Study on genotypic variation for ethanol production from sweet sorghum juice. *Biomass and Bioenergy*, 34, 947-952.

Rooney, W. L. (2004). Sorghum improvement-integrating traditional and new technology to produce improved genotypes. In: L. S. Donald (ed.), *Advances in Agronomy*, (Vol. 83, pp. 37-109). Academic Press. Great Britain. Elsevier.

Rooney, W. L., Blumenthal, J., Bean, B., Mullet, J. E. (2007). Designing sorghum as a dedicated bioenergy feedstock. *Biofuels, Bioproducts and Biorefining*, 1, 147-157.

Rose, J. B., Dunwell, J. M., Sunderland, N. (1986). Anther culture of *Sorghum bicolor* (L.) Moench. *Plant Cell, Tissue and Organ Culture*, 6, 15-22.

Saballos, A., Vermerris, W., Rivera, L., Ejeta, G. (2008). Allelic association, chemical characterization and saccharification properties of brown midrib mutants of sorghum (*Sorghum bicolor* (L.) Moench). *Bioenergy Research*, 2, 193-204.

Sai, N. S., Visarada, K., Lakshmi, Y. A., Pashupatinath, E., Rao, S. V., Seetharama, N. (2006). In vitro culture methods in sorghum with shoot tip as the explant material. *Plant Cell Reports*, 25, 174-182.

Sato, S., Clemente, T., Dweikat, I. (2004). Identification of an elite sorghum genotype with high in vitro performance capacity. *In Vitro Cellular and Developmental Biology-Plant*, 40, 57-60.

Sattler, S., Saathoff, A. J., Haas, E. J., Palmer, N. A., Funnell-Harris, D. L., Sarath, G., Pedersen, J. F. (2009). A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum brown midrib 6 phenotype. *Plant Physiology*, 150, 584-595.

Segal, G., Song, R., Messing, J. (2003). A new opaque variant of Maize by single dominant RNA-interference-inducing transgene. *Genetics*, 165, 387-397.

Shadle, G., Chen, F., Reddy, M. S. S., Jackson, L., Nakashima, J., Dixon, R. A. (2007). Down-regulation of hydroxycinnamoyl CoA: Shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. *Phytochemistry*, 68, 1521-1529.

Shewry, P. R., Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany*, 53, 947-958.

Shrawat, A. K., Lorz, H. (2006). *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. *Plant Biotechnology Journal*, 4, 575-603.

Shull, J. M., Watterson, J. J., Kirleis, A. W. (1991). Proposed nomenclature for the alcohol-soluble proteins (kafirins) of *Sorghum bicolor* (L. Moench) based on molecular weight, solubility, and structure. *Journal of Agricultural and Food Chemistry*, 39, 83-87.

Simmons, B. A., Loqu´e, D., Ralph, J. (2010). Advances in modifying lignin for enhanced biofuel production. *Current Opinion in Plant Biology*, 13, 1-8.

Singh, R., Axtell, J. D. (1973). High lysine mutant gene (hl) that improves protein quality and biological value of grain sorghum. *Crop Science*, 13, 535-539.

Smith, R. H., Bhaskaran, S., Schertz, K. (1983). Sorghum plant regeneration from aluminum selection media. *Plant Cell Reports*, 2, 129-132

Sun, Y., Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, 83, 1-11.

Tadesse, Y., Jacobs, M. (2002). Transgenic sorghum with improved nutritional quality. In: G. G. Khachatourians, A. McHughen, R. Scorza, W. K. Nip, Y. H. Haui (Eds.), *Transgenic plants and crops.* (pp. 801-812). New York.

Tadesse, Y., Sagi, L., Swennen, R., Jacobs, M. (2003). Optimization of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. *Plant Cell, Tissue and Organ Culture*, 75, 1-18.

Taylor, J. R. N., Schüssler, L., Van der Walt, W. H. (1984). Fractionation of proteins from low-tannin sorghum grain. *Journal of Agricultural and Food Chemistry*, 32,149-154.

Taylor, S. H., Hulme, S. P., Rees, M., Ripley, B. S., Woodward, F. I., Osborne, C. P. (2010). Ecophysiological traits in C3 and C4 grasses: a phylogenetically controlled screening experiment. *New Phytologist*, 185, 780-791.

Thomas, E., King, P. J., Potrykus, I. (1977). Shoot and embryo like structure formation from cultured tissues of *Sorghum bicolor*. *Naturwissenschaften*, 64, 587-590.

Torney, F., Moeller, L., Scarpa, A., Wang, K. (2007). Genetic engineering approaches to improve bioethanol production from Maize. *Current Opinion in Biotechnology*, 18, 193-199.

Umehara, M., Hanada, A., Yoshida, S., Akiyama, K., Arite, T., Takeda-Kamiya, N., Magome, H., Kamiya, Y., Shirasu, K., Yoneyama, K., Kyozuka, J., Yamaguchi, S. (2008). Inhibition of shoot branching by new terpenoid plant hormones. *Nature*, 455, 195-200.

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W. (2010a). Lignin biosynthesis and structure. *Plant Physiology*, 153, 895-905.

Vanholme, R., Van Acker, R., Boerjan, W. (2010b). Potential of Arabidopsis systems biology to advance the biofuel field. *Trends in Biotechnology*, 8, 543-547.

Vega-Sánchez, M. E., Ronald, P. C. (2010). Genetic and biotechnological approaches for biofuel crop improvement. *Current Opinion in Biotechnology*, 21, 218-224.

Vermerris, W. (2011). Survey of genomics approaches to improve bioenergy traits in Maize, Sorghum and Sugarcane. *Journal of Integrative Plant Biology*, 53, 105-119.

Vetter, J. (2000). Plant cyanogenic glycosides. *Toxicon*, 38, 11-36.

Wang, H., Zhang, M., Cai, Y. (2009). Problems, challenges, and strategic options of grain security in China. In: L. S. Donald (ed.), *Advances in Agronomy*, (Vol. 103, pp. 101-147). Great Britain. Elsevier Academic Press.

Weaver, C. A., Hamaker, B. R., Axtell, J. D. (1998). Discovery of grain sorghum germplasm with high uncooked and cooked in vitro protein digestibilities. *Cereal Chemistry*, 75, 665-670.

Wei, Z. M., Xu, Z. H. (1990). Plant regeneration from protoplasts of immature cotyledons of Glycine soja Sieb. et Zucc. *Plant Science*, 70, 101-104.

Wernicke, W., Brettell, R. (1980). Somatic embryogenesis from *Sorghum bicolor* leaves. *Nature*, 287, 138-139.

Wernicke, W., Brettell, R. I. S. (1982). Morphogenesis from cultured leaf tissue of *Sorghum bicolor* culture initiation. *Protoplasma*, 111, 19-27.

Wu, L., Birch, R. G. (2007). Doubled sugar content in sugarcane plants modified to produce a sucrose isomer. *Plant Biotechnology Journal*, 5, 109-117.

Xie, G., Peng, L. (2011). Genetic engineering of energy crops: A strategy for biofuel production in China. *Journal of Integrative Plant Biology*, 53, 143-150.

Xin, Z., Wang, M. L., Barkley, N. A., Burow, G., Franks, C., Pederson, G., Burke, J. (2008). Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology*, 8, 103.

Zagrobelny, M., Bak, S., Rasmussen, A. V., Jørgensen, B., Naumann, C. M., Møller, B. L. (2004). Cyanogenic glucosides and plant-insect interactions. *Phytochemistry*, 65, 293-306.

Zhang, P. D., Yan, Y. L., Tian, Y. S., Yang, X. T., Zhang, Y. K. (2009). Bioenergy industries development in China: Dilemma and solution. *Renewable, Sustainable Energy Reviews*, 13, 2571-2579.

Zhao, H., Baker, G. A., Cowins, J. V. (2010b). Fast enzymatic saccharification of switchgrass after pretreatment with ionic liquids. *Biotechnology Progress*, 26, 127-133.

Zhao, L., Shujun, L., Songquan, S. (2010a). Optimization of callus induction and plant regeneration from germinating seeds of sweet sorghum (*Sorghum bicolor* Moench). *African Journal of Biotechnology*, 9, 2367-2374.

Zhao, Z. Y., Cai, T., Tagliani, L., Miller, M., Wang, N., Pang, H., Rudert, R., Schroder, S., Hondred, D., Seltzer, J., Pierce, D. (2000). Agrobacterium-mediated sorghum transformation. *Plant Molecular Biology*, 44, 789-798.

Zhao, Z. Y., Glassman, K., Sewalt, V., Wang, N., Miller, M., Chang, S., Thompson, T., Catron, S., Wu, E., Bidney, D., Kedebe, Y., Jung, R. (2003). Nutritionally improved transgenic sorghum. In: I. K. Vasil (ed.), *Plant Biotechnology 2002 and Beyond*, (pp. 413-416). Dordrecht, Netherlands. Kluwer Academic Publishers.

Zhong, H., Wang, W., Sticklen, M. (1998). In vitro regeneration of *Sorghum bicolor* (L.) Moench: efficient plant regeneration from shoot apices. *Journal of Plant Physiology*, 153, 719-726.

Zhou, A., Thomson, E. (2009). The development of biofuels in Asia. *Applied Energy*, 86, S11-S20.

Zhu, H., Muthukrishnan, S., Krishnaveni, S., Wilde, G., Jeoung, J. M., Liang, G. H. (1998). Biolistic transformation of sorghum using a rice chitinase gene. *Journal of Genetics and Breeding*, 52, 243-252.

Zhu, X. G., Long, S. P., Ort, D. R. (2010). Improving photosynthetic efficiency for greater yield. *Annual Review of Plant Biology*, 61, 235-261.