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Review

Harnessing sorghum and millet biotechnology for food and health

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Abstract

This review highlights recombinant DNA technology as a powerful tool to enhance the gene pools of sorghum and pearl millet crops regarded as jewels of Africa. Although important advances in the improvement of these species have been made by classical breeding and modern marker assisted selection, genetic manipulation and *in vitro* culture allows the gene pool to be broadened beyond that normally available for improvement by allowing the transfer of genes which control well-defined traits between species. The current state of sorghum and millet transformation technology is summarised and applications in the improvement of nutritional quality and the resistance to pathogens and pests for crops grown in Africa and Asia is discussed. Regulatory aspects including gene flow and future prospects are also discussed.

Keywords: Genetic engineering; Sorghum; Pearl millet; Biolistics; *Agrobacterium*

Contents

1. Introduction

Recent advances in the fields of genetics and genomics provide a more unified understanding of the biology of plants (Naylor et al., 2004). After more than a decade of investment powerful technologies have been developed for

Abbreviations: AK, aspartate kinase; CGIAR, Co-operative Group for International Agricultural Research; DHPS, dihydrodipicolinate synthase; GUS, *Escherichia coli* β-glucuronidase gene; IZEs, immature zygotic embryos; MAS, marker assisted selection; PIG, particle in flow gun; PMI, phosphomannose isomerase

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major cereal crops such as maize, wheat and rice, and more recently for the "orphan" crops, sorghum and pearl millet. DNA-based approaches have been applied in two broad areas of molecular breeding (1) the first step of molecular breeding entail genetic diversity studies and marker assisted selection (MAS) and (2) the second avenue involves the direct transfer of genes from one organism or genotype to another.

Grain sorghum (*Sorghum bicolor* L. Moench) and pearl millet [*Pennisetum glaucum* (L.) R. Br.] are staple foods that supply a major proportion of calories and protein to large segments of populations in the semi-arid tropical regions of Africa and Asia. The semi-arid tropics are characterised by unpredictable weather, limited and erratic rainfall and nutrient-poor soils and suffer from a host of agricultural constraints (Maqbool et al., 2001; Sharma and Ortiz, 2000). There is an urgent need to focus on improving crops relevant to the small farm holders and poor consumers in the developing countries of the humid and semi-arid tropics (Sharma et al., 2002). Traditional breeding has been for many years the main avenue for crop improvement in sorghum and pearl millet but is limited in that it only allows the exploitation of variation present in these species or in wild relatives with which they can be crossed. In contrast, genetic engineering offers direct access to a vast pool of useful genes, from other cereals, other plants or even from microbes or other organisms.

2. Advances in sorghum and millet biotechnology

Recombinant DNA technology has significantly augmented conventional crop improvement and offers great promise to assist plant breeders to meet the increased food demand predicted for the 21st century (Sharma et al., 2002). Efficient transformation systems, both biolistic- and *Agrobacterium*-mediated transformation, for important cereal crops such as maize (Armstrong and Songstad, 1993; Ishida et al., 1996), wheat (Cheng et al., 1997; Weeks et al., 1993), rice (Chan et al., 1992; Christou et al., 1991; Hiei et al., 1994), and barley (Tingay et al., 1997; Wan and Lemaux, 1994), have been established. Unfortunately, most cereal crops that have been transformed with genes of relevance are temperate crops, and particularly hybrid crops with high commercial value. The first Bt maize trial was already published in 1993 (Koziel et al., 1993) and subsequently various GM maize products have penetrated the commercial market (Brandt, 2003; Dunwell, 2000). Nevertheless, it is interesting to note that most of the groundwork for cereal transformation was made in sorghum and pearl millet. The first cereal embryogenic *in vitro* culture systems were established for sorghum (Gamborg et al., 1977; Masteller and Holden, 1970) and pearl millet (Vasil and Vasil, 1981). Furthermore, the first report on optimisation of transient expression of the reporter gene *uid*A (GUS) in scutellum cells of cultured immature zygotic embryos (IZEs), following microprojectile bombardment, was published for pearl millet (Taylor and Vasil, 1991). Nevertheless, no commercial transgenic sorghum or pearl millet product has reached the market.

Reliable and highly efficient regeneration systems for both sorghum and pearl millet breeding line(s) underpin the development of a reliable transformation system. Various explant sources were used in the past to initiate embryogenic tissue to facilitate stable transformation. Furthermore, regeneration systems for parental lines used for the production of hybrids have been established in the past for both sorghum and pearl millet (Harshavardhan et al., 2002; O'Kennedy et al., 2004a; Oldach et al., 2001).

2.1. *In vitro culture of sorghum*

The choice of explant for *in vitro* cereal regeneration had been identified as one of the most important factors that determine regeneration capacity, together with the physiological and developmental state of the explants. Procedures for sorghum plant regeneration via somatic embryogenesis and organogenesis have been described for immature zygotic embryos (Brar et al., 1979; Cai et al., 1987; Dunstan et al., 1978, 1979; Gamborg et al., 1977; Girijashankar et al., 2005; Ma and Liang, 1987; Thomas et al., 1977; Zhong et al., 1998), mature embryos (Cai et al., 1987; Thomas et al., 1977), immature inflorescences (Boyes and Vasil, 1984; Brettell et al., 1980; Cai and Butler, 1990; Kaeppler and Pederson, 1997), seedlings (Brar et al., 1979; Davis and Kidd, 1980; Masteller and Holden, 1970; Smith et al., 1983), leaf fragments (Wernicke and Brettell, 1980) and anthers (Rose et al., 1986) used as explants. However, calli derived from IZEs have been the explant and tissue of choice for the production of transgenic plants (Able et al., 2001; Casas et al., 1993; Emani et al., 2002; Gao et al., 2005; Tadesse et al., 2003; Zhao et al., 2000; Zhu et al., 1998).

2.2. *In vitro culture of pearl millet*

Procedures for the regeneration of pearl millet plants via somatic embryogenesis have been described for IZEs (Goldman et al., 2003; Lambé et al., 1995, O'Kennedy et al., 2004a; Oldach et al., 2001; Vasil and Vasil, 1981), mature embryos (Botti and Vasil, 1983), immature inflorescences (Goldman et al., 2003; Pinard and Chandrapalaiah, 1991; Pius et al., 1993; Vasil and Vasil, 1981), shoot apices (Devi et al., 2000; Lambé et al., 1999, 2000) and apical meristems (Goldman et al., 2003). The addition of L-proline to the tissue culture induction medium resulted in a highly efficient embryogenic regeneration system for pearl millet, yielding on average 80 regenerants per immature zygotic embryo explant (O'Kennedy et al., 2004a).

2.3. *DNA delivery methods (Biolistic- and* Agrobacterium*-mediated transformation)*

Reliable transformation protocols for sorghum and pearl millet form the basis for genetic engineering of these staple food crops.

2.3.1. Transgenic sorghum

Both biolistic- and *Agrobacterium*-mediated transformation were employed in the past to produce transgenic sorghum (Table 1). The production of transgenic sorghum plants via particle bombardment of IZEs and inflorescences was reported for the first time by Casas et al. (1993, 1997), and was subsequently reported by Zhu et al. (1998), Able et al. (2001), Emani et al. (2002), Tadesse et al. (2000, 2003) and Gao et al. (2005), introducing mainly reporter and selectable marker genes. The first transgenic sorghum plants produced by *Agrobacterium*-mediated transformation using IZEs as explant were reported by Zhao et al. (2000). Subsequently, transformation efficiency was improved to 2.5%, using *Agrobacterium*, by Gao et al. (2005), who introduced the green fluorescence protein (*gfp*) reporter gene and a *man*A (see Section 2.3.2) positive selectable marker gene. In this study 40% of the transgenic plants contained only one copy of the transgene, and 24% two copies and no gene silencing occurred. *Agrobacterium*-based transformation is considered to have several advantages over direct DNA transfer methods, including higher transformation efficiencies, for example, 2.1% (Zhao et al., 2000) and 2.5% (Gao et al., 2005) compared to 0.08–0.33% (Casas et al., 1993, 1997), 0.18% (Emani et al., 2002), 1% (Zhu et al., 1998), and 1.3% (Tadesse et al., 2003) and 1.5% (Girijashankar et al., 2005) for biolistic transformation.

2.3.2. Transgenic pearl millet

A transformation protocol was established using the herbicide resistance selectable marker gene, *bar*, and the particle inflow gun (PIG) (Girgi et al., 2002). However, the transformation efficiency obtained was very low (0.02%) (Table 1). Subsequently, the *manA* (POSITECH,

Table 1
Summary of published work reporting the production of fertile sorghum and pearl millet transgenic plants

	Explant source	Genotypes	Transformation methodology and efficiency	Gene of interest	References
Sorghum	IZEs inflorescence	P898012	Biolistics 0.08–0.33%	35S-*bar* and 35S-*uid*A	Casas et al., 1993, 1997
		SRN39			
	IZEs	Tx430	Biolistics 1%	Ubi-*bar*: 35S-*chiII*	Zhu et al., 1998, Krishnaveni et al., 2001
	IZEs	SA281	Biolistics	Ubi-*gfp* and Ubi-*bar*	Able et al., 2001
	IZEs	RT430	Biolistics 0.18%	Act1D-*uid*A and Ubi-*bar*	Emani et al., 2002
	IZEs and shoot tips	214856	Biolistics 1.3%	35S-*uid*A; Adh1-*uid*A; Ubi-*uid*A; Act1D-*uid*A; *Ubi-bar;* Act-*neo*	Tadesse, 2000, Tadesse et al., 2003
	IZEs	P898012 PHI391	*Agrobacterium* 2.1%	Ubi-*bar* and Ubi-*uid*A Zein-*HT12*	Zhao et al., 2000, 2003
	Shoot apices	BT623	Biolistics 1.5%	35S-*bar* and Act1D-*uid*A *mpi*: Bt *cry*1Ac	Girijashankar et al., 2005
	IZEs	Pioneer 8505 C401	*Agrobacterium* 2.88–3.3%	Ubi-*man*A and Ubi-*sgfp*	Gao et al., 2005
Pearl millet	Embryogenic calli	N.E.	Biolistics	35S-*uid*A and 35S-*hph*	Lambé et al., 1995, 2000
	IZEs	7042, 842B	Biolistics 0.02–0.28%	Ubi-*bar* and Ubi-*uid*A 35S-*bar*	Girgi et al., 2002
		Manga Nara Bongo Nara			
	IZEs	842B	Biolistics 0.72%	Ubi-*man*A	O'Kennedy et al., 2004b
	IZEs	7042 Manga Nara	Biolistics 0.14%	35S-*bar and* Ubi-*afp*	Girgi et al., 2006
	Shoot-tip-derived embryogenic calli	ICMP 451	Biolistics	35S-*bar* and 35S-*pin*	Latha et al., 2006

Act1D, rice actin promoter.
*adh*1, maize alcohol dehydrogenase gene promoter.
bar, phosphinothricin acetyl transferase herbicide selectable marker gene.
hph, hygromycin phosphotransferase selectable marker gene.
*man*A, phosphomannose isomerase positive selectable marker gene.
mpi, maize protease inhibitor regulatory region promoter.35S, CaMV 35S cauliflower mosaic virus promoter.Ubi, Maize ubiquitin promoter.*uid*A, β-glucuronidase coding sequence.Zein, 27 kD maize gamma zein promoter.*Pin,* synthetic prawn antifungal protein encoding gene.

Syngenta) was used as selectable marker gene. The system employs the phosphomannose isomerase (PMI) expressing gene (*man*A) as a selectable marker gene and mannose, converted to mannose-6-phosphate by endogenous hexokinase, as selective agent. The mannose positive selection system favours the regeneration and growth of the transgenic cells while the non-transgenic cells are starved but not killed. Thus, untransformed tissue is separated from transgenic tissue by carbohydrate starvation of the untransformed cells. The use of *man*A selection limited the number of escapes to less than 10%. In contrast, using the *bar* gene and selecting with 3–5 mg l^{-1} bialaphos (the active ingredient of the herbicide) resulted in more than 90% non-transformed escapes. The *man*A selection system not only improved the transformation efficiency but also avoided the use of antibiotic or herbicide resistance genes as selectable markers in pearl millet transformation (O'Kennedy et al., 2004b). To date, no *Agrobacterium*-mediated transformation of pearl millet has been reported.

3. Nutritional quality improvement

We will focus on two aspects of nutritional quality which have been identified as priorities by major international funding programs (see Section 6). These are protein quality and the content of vitamins and minerals.

The reader is referred to several excellent reviews for detailed accounts of sorghum grain composition and nutritional quality (Dendy, 1995; Hulse et al., 1980; Taylor and Belton, 2002) while the properties of the prolamin storage proteins of sorghum and millets are discussed by Belton et al., 2006.

3.1. Protein quality

The major storage tissue in sorghum grain, in common with other cereals, is the endosperm which accounts for about 85% of the whole grain with the remainder being the germ and pericarp (accounting for about 9.55% and 6.5%, respectively) (Serna-Saldivar and Rooney, 1995). Hence, the composition of the endosperm cells largely determines the nutritional quality of the whole grain. With the exception of the single layer of aleurone cells, these cells are rich in starch (over 80%) and relatively poor in protein (approximately 10%) with less than 1% lipid (Serna-Saldivar and Rooney, 1995).

The major protein fraction in the starchy endosperm is the prolamin storage proteins (termed kafirins in sorghum) which account for about 80% of the total grain protein (Taylor et al., 1984). The prolamins are characterised by their low contents of essential amino acids, notably lysine which accounts for only 0.2% of the total amino acids in sorghum kafirin, less than 2% in the endosperm and less than 3% in the whole grain (all values are g/100 g and based on values cited in Serna-Saldivar and Rooney, 1995). This compares with a WHO recommended level of 5.5 g lysine/100 g protein (FAO, 1973).

Similar data have been reported for millets, although there are fewer studies. For example, pearl millet grain contains about 3 g lysine/100 g protein compared with 1.4 g/100 g in the endosperm and 1% or less in the prolamins (pennisetins) (Abdelrahman et al., 1984; Nwasike et al., 1979). Prolamin fractions from other millets have also been reported to have low contents of lysine. Parameswaran and Thayumanavan (1995) reported lysine contents ranging between 0.65 and 1.85 mol% for prolamin fractions from five minor millets with the highest value being for foxtail millet (*Setaria italica*). Unlike the situation with legume seeds, methionine is not generally limiting in sorghum and millets and methionine-rich prolamin components have been reported in foxtail millet (α- and β-setarins) (Naren and Virupaksha, 1990a, b) and in fonio (*Digitaria exilis*) (de Lumen et al., 1993). In fact, fonio grains are unusually rich in methionine (4.8 g/100 g protein) (de Lumen et al., 1986) and may be suitable for supplementing low methionine diets.

The poor nutritional quality of the kafirins is compounded by the fact that they are difficult to digest and that their digestibility decreases on cooking (Duodu et al., 2003). This does not apply to the prolamins of millets or the related zeins of maize.

The structures of the kafirins and the basis for their poor digestibility are discussed by Belton et al., 2006. The focus is therefore on approaches to nutritional improvement that aim to replace or supplement the kafirins with proteins of high nutritional quality or with increased amounts of the essential amino acids.

Early attempts to improve sorghum nutritional quality focused on the identification of "high lysine" mutants, based on the identification of several such lines in maize (Mertz et al., 1964; Nelson et al., 1965). Two mutants were identified in sorghum, the *hl* gene in an Ethiopian line (Singh and Axtell, 1973) and the *P721 opaque* gene which was induced with the chemical mutagen diethylsulphate (Axtell et al., 1979). Both lines are "low prolamin" mutants in which the proportion of kafirin is reduced by about 50% with compensatory increases in other more lysine-rich proteins and free amino acids. This results in increases in lysine of about 40–60% but is associated with deleterious effects on seed weight and yield. Oria et al. (2000) reported the identification of a novel line with high protein digestibility from a cross involving the high lysine *P721 opaque* mutant. Nevertheless, the limited success achieved in developing cultivars incorporating high lysine genes of maize, despite considerable investment over 40 years, indicates that commercial exploitation of high lysine sorghum lines will be difficult. Similarly, although Huang et al. (2004) have shown that antisense suppression of α-zein synthesis leads to a similar "low prolamin/high lysine" phenotype in maize, the agronomic performance of these lines remains to be established.

Although most of the amino acids in seeds are in the form of proteins small pools of free amino acids are also

present. These typically account for 1% or less of the total and their amounts are strictly regulated by feedback inhibition of the enzymes that catalyse their biosynthesis. It is therefore necessary to modify this feedback regulation if free amino acids are to accumulate at sufficient levels to contribute to the nutritional quality of the whole seed. In plants, the synthesis of lysine, threonine and methionine follows the same initial pathway from aspartic acid. The entry into this pathway and the branch point to lysine are controlled by two feedback-regulated enzymes, aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS), respectively. Feedback-insensitive forms of these enzymes in bacteria are relatively easy to identify, so many workers have isolated genes encoding these enzymes and expressed them in transgenic plants to increase the pools of free amino acids.

Mazur et al. (1999) expressed a feedback-insensitive DHPS from *Corynebacterium* in the aleurone and embryo of maize, resulting in up to two-fold increases in total grain lysine, but expression of the same gene in the starchy endosperm had no effect as increased breakdown of the lysine also occurred. Similar increased degradation has been reported in soybean and canola (oilseed rape) (Falco et al., 1995; Mazur et al., 1999) although this was eliminated in *Arabidopsis* by knocking out the genes of lysine catabolism (Zhu and Galili, 2003). Brinch-Pedersen et al. (1996) reported that expression of a feedback-insensitive form of DHPS from *E. coli* in barley under the control of the "constitutive" CaMV 35S promoter resulted in a two-fold increase in free lysine in the grain.

The expression of bacterial genes in plants while an excellent strategy for "proof of concept" is unlikely to be as acceptable to consumers and regulatory authorities as would be the expression of plant-derived genes. It is therefore significant that Lee et al. (2001) have achieved some success by expressing a DHPS gene from maize in transgenic rice. This gene was mutated *in vitro* to make a single amino acid substitution which had been shown previously to result in lysine-insensitivity. Expression in rice under control of the CaMV 35S promoter resulted in increases in free lysine of two-fold or greater, despite increased lysine catabolism. However, the increases in free lysine reported by Brinch-Pedersen et al. (1996) and Lee et al. (2001) would not be expected to have a significant impact on the proportion of lysine in the whole grain.

These studies on other cereals indicate that it should also be possible to engineer sorghum to increase the levels of free lysine to have a significant impact on nutritional quality.

The second approach to improving protein quality is to transform sorghum to express additional "nutritionally-enhanced" proteins. Much of the work on this topic has focused on methionine-rich proteins for expression in legume seeds in which the main amino acid deficiency is in the sulphur-containing amino acids (cysteine and methionine) (reviewed by Tabe and Higgins, 1998). Less work has been performed on lysine-rich proteins and these also occur less widely in nature than methionine-rich proteins, possibly because lysine is positively charged at cellular pHs and high proportions are less readily accommodated in proteins.

Studies of lysine-rich proteins have been pioneered by Rao and colleagues at Pioneer Hibred (Des Moines, USA) who have used two naturally occurring high lysine proteins as a basis for extensive protein engineering studies. Hordothionin is a seed protein from barley which carries five lysines out of 45 residues in total. Rao et al. (1994) used molecular modelling to design mutated forms containing up to 12 lysine residues (HT12) and confirmed the acceptability of these mutations by synthesising and characterising the proteins.

A sequence encoding the HT12 form of hordothionin has been used to transform sorghum, under control of the maize gamma-zein promoter which would be expected to confer strong expression in the starchy endosperm of the grain (Zhao et al., 2003). The plants were co-transformed with two *Agrobacterium* vectors containing the *bar* and *HT12* genes, respectively, allowing them to be separated by segregation in subsequent generations. Five lines were obtained which were transformed with both genes and three of these expressed high levels of HT12 in their grain. Furthermore, the *HT12* gene segregated from *bar* in at least one of these lines allowing the production of high lysine progeny which lacked herbicide resistance. The expression of HT12 was confirmed by Western blotting and ELISA assays while amino acid analysis showed that the grain lysine content was increased by about 50% compared with the wild type grain.

This is an impressive demonstration of the potential to improve nutritional quality of sorghum by genetic engineering. However, it is possible that the use of a modified hordothionin will raise some concern with consumers and regulatory authorities. This is because thionins have well-documented toxicity *in vitro* against a wide range of micro-organisms (bacteria, fungi, yeasts), invertebrates and animal cells (Florack and Stiekema, 1994).

Similar studies have since been performed on barley chymotrypsin inhibitor 2 (CI-2). CI-2 was initially identified as one of four "lysine-rich" proteins which were present in elevated amounts in the high lysine barley line Hiproly (Hejgaard and Boisen, 1980) and contains eight lysines out of 83 residues. Roesler and Rao (1999, 2000) extensively mutagenised this protein, generating forms containing up to 25 mol% lysine and confirming their structures and stabilities by analysis of recombinant proteins expressed in *E. coli*. In our similar approach, as part of an EU-funded project aimed at improving sorghum grain quality, a form of modified CI-2 containing three additional lysine residues (13.1 mol% lysine) (Forsyth et al., 2005) has been incorporated into vectors for sorghum transformation. Although CI-2 inhibits chymotrypsin there is no evidence that it has anti-nutritional effects when fed as cereal grain to animals. Nevertheless,

the mutant forms discussed here have all been shown to have reduced or no chymotrypsin inhibitory activity.

Whereas hordothionin and CI-2 are both small proteins which may have protective functions, Liu et al. (1997) have identified a pollen-specific protein from a diploid potato species (*Solanum berthaultii*) which comprises 240 amino acids including 40 lysines (16.7 mol%). The function of this protein (*sb401*) is unknown but homology with a mouse protein suggests that it may be involved in cytoskeletal organisation. Nevertheless expression of *sb401* in maize grain resulted in increases in both the grain protein content and grain lysine content (by up to about 50%) (Yu et al., 2004).

A final and novel approach to increasing grain lysine has been demonstrated by Wu et al. (2003). They exploited the fact that natural errors occur in protein synthesis by transforming rice to express $tRNA^{lys}$ species that introduce lysine at alternative codons (to replace Gln, Asn and Glu) during protein synthesis. This resulted in increases in the lysine contents of the grain prolamins by up to 75% and of the rice grain by up to 6.6%. The authors suggested that targeting the substitutions to residues which tend to occur on the protein surface should allow higher levels of lysine enrichment to be achieved without effects on the grain growth and development.

However, it must be borne in mind that any changes in the composition of the grain that are achieved should not adversely affect seed functionality, agronomic performance, grain yield or compromise the properties for processing, particularly for traditional foods which are highly valued by African consumers.

3.2. Minerals and vitamins

Sorghum and millets are important sources of some minerals, particularly iron and zinc, but all except finger millet and tef are low in calcium (Serna-Saldivar and Rooney, 1995). However, these minerals are concentrated in the pericarp, aleurone and germ and hence are removed by decortication resulting in deficiency in the endosperm flour. Furthermore, the minerals in the aleurone layer are largely in the form of phytates, which are mixed salts of phytic acid (*myo*-inositol-(1,2,3,4,5,6)-hexa*kis* phosphate). These salts also account for over 70% of the total phosphate in cereal grains and are poorly digested leading to mineral deficiency even when whole grains are consumed.

Similarly, although sorghum and millets are important sources of B vitamins (except B12) these are also concentrated in the germ and aleurone and removed by decortication (Serna-Saldivar and Rooney, 1995).

Consequently, simply increasing the amounts of vitamins and minerals in the grain may not be sufficient to improve diets of consumers of decorticated grain products as it is also necessary to increase their concentrations in the starchy endosperm and, in the case of minerals, their availability.

The pathway of phytate from *myo*-inositol is well understood and it is possible to screen mutant populations for reduced phytate accumulation. This has led to the identification of low phytate mutants in maize, barley, wheat and rice (Larson and Raboy, 1999; Larson et al., 2000; Shi et al., 2003) and similar mutations could presumably be selected in sorghum and millets and incorporated into breeding programs.

An alternative strategy to increase mineral availability is to express a fungal phytase in the developing seed to digest the phytate when the grain is consumed. This approach has been shown to result in benefits in terms of reduced phosphate excretion or increased growth when transgenic soybean and canola seeds were fed to pigs and chickens (Denbow et al., 1998; Zhang et al., 2000a, b).

Fungal phytase has also been expressed in seeds of wheat and rice and current work on these crops is focussing on the expression of heat stable forms of the enzyme to reduce denaturation during cooking or food processing (Brinch-Pedersen et al., 2000, 2002, 2003; Lucca et al., 2001).

Genetic engineering can also be used to increase the accumulation of minerals and vitamins in the starchy endosperm of the cereal seed. For example, expression of soybean ferritin (an iron binding protein) in developing seeds of rice has resulted in two- to three-fold increases in the iron content of the endosperm. However, more recent studies of lines expressing soybean ferritin under the control of a stronger promoter showed that the level of iron accumulation was lower than would have been expected based on the expression level of the ferritin protein, indicating that iron uptake and transport may limit accumulation (Qu et al., 2005). Similarly, the well-publicised "golden rice" is particularly important because the increased content of pro-vitamin A is targeted to the starchy endosperm rather than restricted to the aleurone (Datta et al., 2003; Paine et al., 2005; Ye et al., 2000). Our increasing knowledge of the biosynthesis and regulation of other key nutrients, such as folate (Hossain et al., 2004), should therefore allow nutritional traits to be expressed in the developing cereal endosperm.

The importance of vitamin A and minerals (iron and zinc) in the diets of those who consume sorghum and millets is recognised in the HarvestPlus program (www.harvestplus.org). This is a ten year Challenge Program of the CGIAR focused on increasing the amounts of these nutrients in a range of crops including millets and sorghum. The analysis of 84 sorghum lines including the parental lines of popular hybrids, varieties and germplasm accessions was reported by Reddy et al. (2005). This showed significant differences in the amounts of Fe (mean 28.0 ± 0.9 ppm, range 20.1–37.0 ppm), Zn (mean 19.0 ± 0.8 ppm, range 13.4–31.0 ppm) and phytates (mean 7.6 ± 0.1 mg g^{-1}, range 3.8–13.5 mg g^{-1}). The authors also reported differences in β-carotene content, with only traces being present in non-yellow lines but 0.56–1.13 ppm in lines with yellow endosperm. This variation provides a valuable

basis for increasing pro-vitamin A, iron and zinc by conventional plant breeding.

The "orphan" status of sorghum and millets means that little work has been performed so far on the nutritional enhancement of their grains. However, work on major cereals (wheat, rice, maize) and on other crops has demonstrated that substantial improvements can be made, particularly by using genetic engineering approaches. The new internationally funded Africa Harvest program (http://www.abbfi.org/) will focus on many of the targets discussed here (protein digestibility, essential amino acid composition, mineral and vitamin availability) in sorghum and it is hoped that the success achieved will then be applied to the millets.

Furthermore, funding from the Bill and Melinda Gates Foundation has recently been awarded to develop transgenic sorghum, with elevated levels of the essential amino acids, lysine, threonine and tryptophan; vitamins A and E, and iron and zinc, all of which are deficient in sorghum. The project is entitled: "Nutritionally enhanced sorghum for the arid and semi-arid tropical areas of Africa".

4. Resistance to pathogens and pests

Zhu et al. (1998) were the first to introduce an agronomically important gene, encoding a rice chitinase (*chill*) into the genome of sorghum. Transgenic progeny were tested by injection of *Fusarium thapsinum* into the stalk and root-dip inoculation for resistance to stalk rot but only 45–50% of the transgenic seedlings were moderately resistant to the fungus (Krishnaveni et al., 2001). Gene silencing was restricted to the chitinase gene driven by the CaMV 35S promoter whereas the selectable marker gene, bar, driven by the ubiquitin promoter was fully expressed in all transgenic progeny of all events. The authors are currently introducing combinations of PR-genes driven by the maize ubiquitin-intron to minimise transgene silencing effects (Krishnaveni et al., 2001).

The most important applications of biotechnology for plant protection amongst the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) mandate crops, especially in Africa, include downy mildew in pearl millet (Sharma and Ortiz, 2000). *Trichoderma atroviride* is a well-known biological control agent, which can be used in combination with *Bacillus* spp. to combat *Sclerospora graminicola*, causal agent of downy mildew in pearl millet (Shetty and Kumar, 2000). Previous studies showed that a 78 kDa $(1\rightarrow3)$-β-glucanase from *T. atroviride* exhibited potent antimicrobial activity to the oomycetous pseudofungal pathogen *Phytophthora* (Fogliano et al., 2002). The gluc78 gene (Donzelli et al., 2001) from *T. atroviride* which degrades $(1\rightarrow3)$-β-glucan in the cell walls of the pathogen, was placed down-stream of the potato proteinase inhibitor IIK wound inducible promoter followed by the rice *Act*1 intron, and also downstream of the maize ubiquitin constitutive promoter and introduced into the genome of pearl millet (O'Kennedy et al., unpublished). Pathogenicity trials are currently underway.

The antimicrobial protein gene *afp* from the mould *Aspergillus giganteus* was introduced into two pearl millet genotypes by particle bombardment (Girgi et al., 2006). Stable integration and expression of the *afp* gene was confirmed in two independent transgenic T_0 plants and their progeny using Southern blot and RT-PCR analysis. *In vitro* infection of detached leaves and *in vivo* inoculation of whole plants with the basidomycete *Puccinia substriata*, the causal agent of rust disease, and the oomycete *S. graminicola*, causal agent of downy mildew, resulted in a significant reduction of disease symptoms in comparison to wild type control plants. The disease resistance of pearl millet was increased by up to 90% when infected with two diverse, economically important pathogens. Disease resistance against *S. graminicola* was also obtained by expressing the PIN protein encoded by the synthetic prawn antifungal gene in the downy mildew susceptible genotype ICMP451 (Latha et al., 2006). These are the first published reports of genetic engineering for resistance of pearl millet against infections by plant microbes.

The complexity of plant defence response mechanisms against pathogen invasion (McDowell and Woffenden, 2003; Somssich and Hahlbrock, 1998), the rapid development of new virulent forms of phytopathogens (Johnson, 2000; Kamoun et al., 1999) and the failure to accumulate the desired gene product at the expected level in the transgenic plant have hindered the commercial development of transgenics that are resistant to crop pathogens. Strategies for increasing host-plant resistance in sorghum and millets and concerns regarding the use of genes encoding for pathogen-related proteins are discussed by Chandrashekar and Satyanarayana (2006).

The first paper reporting the development of transgenic sorghum expressing insect resistance was published by in 2005. Transgenic sorghum plants expressing a synthetic *Bt cry1Ac* gene under the control of a wound-inducible promoter from a maize protease inhibitor gene (*mpi*) were produced via particle bombardment of shoot apices (Girijashankar et al., 2005). Although reductions in leaf damage (60%), larval mortality (40%) and larval weight (36%) were recorded when compared with control plants, larval mortality was less than 25% and surviving larvae tunnelled into young shoots. The levels of Bt proteins produced (1–8 ng per gram of leaf tissue) were far below the lethal dose required to give complete protection against neonate larvae of *Chilo partellus*. Girijashandar and co-workers are studying the constitutive expression of the same protein.

The phenomenon of transgene silencing appears to be a major obstacle in the transformation of sorghum. Emani et al. (2002) reported methylation-based transgene silencing of reporter gene, *uid*A and the herbicide resistance gene, *bar*, in transgenic sorghum. Girijashankar et al. (2005) obtained low levels of protein *cry1Ac* transgene

expression but not complete gene silencing. Expression of the target gene could potentially be enhanced by biasing the codons towards those typical of sorghum genes or by exploiting regulatory regions such as nuclear *Adh* matrix attachment regions sequences (Able et al., 2004). Comparison of the efficiencies of different promoters specifically for the trait of interest or the identification and isolation of novel constitutive promoters might further enhance the expression of selected traits (Hill-Ambroz and Weeks, 2001; Tadesse et al., 2003). The strength of four heterologous promoters was determined by both histochemical β-glucuronidase activity staining and fluorometric enzymatic activity assay in IZEs and shoot tips (Tadesse et al., 2003). The maize ubiquitin-1 (*ubi1*) promoter was superior to the rice actin-1 (*act1D*), maize alcohol dehydrogenase-1 (*adh1*) and cauliflower mosaic virus CaMV 35S promoters in the order given (Tadesse et al., 2003), but the dual 35S promoter are superior to *act1D* and *adh1* promoters (Hill-Ambroz and Weeks, 2001). Nevertheless, the expression conferred by the *ubi1* promoter in sorghum is still low in comparison to gene expression in wheat (Hill-Ambroz and Weeks, 2001) and novel constitutive promoters might be of significant benefit.

5. Pollen-mediated gene flow in crops indigenous to Africa

It is essential to reduce direct pollen-mediated gene flow from genetically modified (GM) sorghum and pearl millet to non-GM plants, as both sorghum and pearl millet are indigenous to Africa. Sorghum pollen-mediated gene flow was shown to occur at frequencies of 2.54% at a distance of 13 m, 1% at 26 m and decreasing to 0.06% at 158 m but will depend on flowering period and weather conditions especially the wind (Schmidt and Bothma, 2006). To adhere to biosafety regulations and minimise inadvertent introgression of transgenes to non-GM sorghum and pearl millet varieties, the several strategies have potential to be applied to transgenic sorghum and pearl millet. Firstly, the removal of antibiotic or herbicidal selectable marker genes which confer resistance to antibiotics or herbicides (Scutt et al., 2002) and secondly, the use of a positive selectable marker gene, such as *man*A (O'Kennedy et al., 2004b) will be desirable. Thirdly, cytoplasmic-nuclear male sterility (CMS) can be used to severely restrict but not eliminate pollen-mediated gene flow, since fractional restoration of fertility in sorghum A_3 cytoplasm occurs at a rate of only approximately 0.4% (Pedersen et al., 2003). Lastly, the introduction of transgenes for nutritional quality improvement that confer no agronomical or competitive advantage to weedy species and non-GM varieties should not only be a safe technology but also it would help meet the acute need for nutritionally enhanced staple food crops in Africa (www.supersorghum.org, accessed July 2006).

6. Concluding remarks and future prospects

In spite of the substantial introduction of new sorghum and pearl millet cultivars in semiarid Sub-Saharan Africa during recent decades (Mgonja et al., 2005; Monyo, 2002) and even hybrid cultivars in Botswana (BSH1) and Sudan (Hageen Dura-1) (Ejeta, 1988), inorganic fertilisers and improved water management are essential for large yield increases (Ahmed et al., 2000). Government policies, transportation infrastructure and market development of the target African countries and crops also need to be addressed for conventional and/or transformation-based crop cultivar improvement to significantly contribute to food supply.

New lines of sorghum and millets containing transgenes will need to be tested at least as stringently as any other introduced or improved cultivars of these crops. A thorough assessment of their allergenic potential and the monitoring of any unintended effects on food composition will provide a solid basis for food safety assessment. The Food and Agricultural Organisation/World Health Organisation (WHO/FAO) have provided decision trees for a rigorous assessment and testing for GM foods (Halsberger, 2003) which would be applicable to transgenic sorghum and pearl millet expressing the genes of interest. Furthermore, the phenotypes of transgenic sorghum and pearl millet plants produced and the milling and processing qualities of the transgenic seed need to be assessed.

Finally, the gains in food production provided by the Green Revolution have reached their ceiling while the world population continues to rise (Wisniewski et al., 2002). A new Green Revolution will necessitate the application of recent advances in plant breeding, including new tissue culture techniques, marker-aided selection, mutagenesis and genetic modification (Wisniewski et al., 2002) to meet our increasing requirement for food, feed, fodder and fuel, with cereal grains playing a pivotal role (Hoisington et al., 1999). Whereas the affluent nations can afford to adopt elitist positions and pay more for food produced by the so-called natural methods; the one billion chronically poor and hungry people of this world cannot (Wisniewski et al., 2002). Therefore, despite the diverse and widespread potential for beneficial applications of transgenic products in agriculture, there remains a critical need to present these benefits to the general public in a real and understandable way that stimulates an unbiased and responsible public debate (Sharma et al., 2002) and pro-GM government policies.

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