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Review

Applications of biotechnology for improvement of millet crops: Review of progress and future prospects

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Abstract Millets are small seeded grasses grown for food, feed or forage and cultivated mostly in less developed countries in poor soil and dry conditions. There are at least 10 genera and 14 species of millets belonging to the Poaceae (Gramineae) family. Tissue culture and plant regeneration occurring through different morphogenic pathways have been reported in great detail in millets. Gene transfer has been attempted using various methods, but so far transgenic plants have been developed only in Pearl millet and Bahiagrass. Not much work has been done on transgenesis in other millets. This is primarily because they have less economic value and are cultivated in poor countries, where research and development are also poor. In the present review we have attempted to provide available information on millet tissue culture and genetic transformation. We have underlined the importance of transgenesis in millet improvement and the role that biotechnology can play in the improvement of these crops grown in a variety of harsh conditions.

Key words: Cereals, micropropagation, millets.

Cereals and millets are members of the grass family: Poaceae (Gramineae), grown for their edible starchy seeds. The term 'millet' or minor cereals refers to small seeded cereals and forage grasses used for food, feed and forage. Millets are part of the diet of the people of China, Japan, Africa and India. In Western countries they are used mainly as birdseed. Millets embrace 10 genera and at least 14 species (Table 1). They are important because they are grown in poor soils with limited inputs and they constitute a major source of food for resource poor farmers of the areas of their cultivation. The projected food demand for 2025 (Borlaug 2002) will require the yield of millets to rise from 2.5 to 4.5 t ha^{-1}. This increase will largely come from improved varieties transgenetically modified for resistance to abiotic and biotic stress, using a tertiary gene pool.

The earliest attempt to culture cereal and millet dates back to the 1970s when successful callus formation and plant regeneration was reported in small millets (Rangan 1974, 1976). A major advance in cereal tissue culture was made largely through the efforts of Vasil and coworkers, Green and Phillips, and Potrykus and coworkers in the 1980s (see Morrish et al. 1987; Vasil 1987; Bhaskaran and Smith 1990). The visual identification of embryogenic callus and its selective propagation and use as a source for initiating suspension cultures and isolation of protoplasts from embryogenic suspension cultures became a crucial step in obtaining totipotent protoplasts (Vasil and Vasil 1980, 1992). Selection of proper donor explant and use of 2,4-dichlorophenoxy acetic acid made it possible to obtain embryogenic cultures in most of the cereals and millets (Kothari and Chandra 1995). The culture response is influenced by media composition, carbon source, genotype, explant source, growth conditions of the donor plant, other additives in the medium and the physical conditions of growth of the cultures (reviewed by Morrish et al. 1987; Vasil 1987).

Different explants have been used for raising regenerable cultures in millets. Immature embryos

Table 1. Millet species.

Species	Common name
Brachiaria ramosa	Browntop millet
Coix lachryma jobi	Job's tears
Digitaria exilis	Hungry rice
Digitaria ibura	Fonio or Hungry rice
Echinochloa colona	Jungle rice
Echinochloa decompositum	Australian millet
Echinochloa frumentacea	Japanese barnyard millet
Eleusine coracana	Finger millet
Eragrostis teff	Teff
Panicum miliaceum	Proso millet
Panicum miliare	Little millet
Paspalum notatum	Bahia grass
Paspalum scrobiculatum	Kodo millet
Pennisetum glaucum	Pearl millet
Setaria italica	Foxtail millet

Table 2. *In vitro* plant regeneration in millets.

Species	Growth regulators used for callus induction/ plant regeneration	References
Echinochloa colona	2,4-D, BAP, NAA, Kn	Samantaray et al. 1995, 1996, 2001; Rout et al. 1997
E. frumentacea	2,4-D, Kn	Talwar and Rashid 1989; Sankhla et al. 1992
Eleusine coracana	2,4-D, NAA, IBA, BAP, Kn, Pic, GA3	Thiru and MohanRam, 1980; Mohanty et al. 1985; Wakizuka and Yamaguchi 1987; Eapen and George 1989; Sivadas et al. 1990; Vishnoi and Kothari 1995; Poddar et al. 1997; Kumar et al. 2001; Kothari et al. 2004
E. indica	2,4-D, Kn, GA_3	Kumar et al. 2001; Yemets et al. 2003
Panicum bisulcatum	2,4-D	Fladung and Hasselbach 1986; Akashi and Adachi 1991
P. maxicum	2,4-D, NAA	Lu and Vasil 1981,1982; Kothari et al. 1994
P. miliaceum	2,4-D, 2,4,5-T, BAP	Nabors et al. 1983; Jain et al. 2001
P. milioides	2,4-D	Fladung and Hasselbach 1986
Paspalum dilatatum	2,4-D	Akashi and Adachi 1992
P. notatum	2,4-D, BAP	Marousky and West 1987, 1990; Bovo and Mroginsky 1989; Akashi et al. 1993; Chen et al. 2001
P. scrobiculatum	2,4-D, Kn, NAA, BAP	Rangan 1976; Nayak and Sen 1989; Kavi Kishor et al. 1992; Vikant and Rashi 2001; Arockiasamy et al. 2001; Vikrant and Rashid 2002a, b; Kaur and Kothari 2003, 2004; Vikrant and Rashid 2003
P. simplex	2,4-D, Kn, NAA, BAP	Molinari et al. 2003
P. vaginatum	2,4-D	Cardona and Duncan 1997
Pennisetum americanum	2,4-D, IAA	Vasil and Vasil 1981a, b; Botti and Vasil 1983, 1984; Taylor and Vasil 1995, 1996
P. glaucum	2,4-D, pCPA, Kn, BAP, NAA	Mythili et al. 1997, 2001; Devi et al. 2000; Devi and Sticklen 2001; Oldach et al. 2001; Srivastav and Kothari 2002
P. typhoides	2,4-D, 2,4,5-T, IAA	Nabors et al. 1983
Setaria italica	2,4-D, 2,4,5-T, Kn, BAP, Zeatin, NAA	Xu et al. 1984; Rao et al. 1988; Reddy et al. 1988, 1990; Kavi Kishor et al. 1992; Osuna-Avilla et al. 1995; Vishnoi and Kothari 1996

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; 2,4,5-T, 2,4,5-trichlorophenoxy acetic acid; NAA, α-naphthalene acetic acid; BAP, 6-benzylamino purine; Kn, Kinetin; IAA, Indole-3-acetic acid; *p*CPA, *para* chlorophenoxy acetic acid; Pic, 4-amino-3,5-6-trichloropicolinic acid; TDZ, Thidiazuron; IBA, Indole-3-butyric acid; GA_3, Gibberellic acid.

with scutellum at milk stage provide the best starting material. But mature embryos, whole seeds, immature inflorescence, seedling leaf bases and roots have all been used for initiating cultures (Table 2). The cultured explants form callus, and then plant regeneration occurs through either somatic embryogenesis or organogenesis (Figure 1). In some cases such as cultured embryos of *Eleusine coracana*, first an enlarged apical dome forms and then shoot buds get differentiated on the entire surface of the dome. (Wakizuka and Yamaguchi 1987; Kumar et al. 2001). Microtillering has also been noted in some cases. There are numerous reviews available on plant regeneration in millets (Vasil 1987; Bhaskaran and Smith 1990; Kothari and Chandra 1995; Repellin et al. 2001). The present review will therefore, focus mainly on the studies on genetic transformation in millets.

Transformation of millets

Improvement of millets using biotechnology has been overlooked due to economic or regional considerations. The objectives of improvement include better utilization of natural resources, development of resistance against biotic and abiotic stresses and improvement of quality for wider consumer acceptance.

Explants and transgene delivery method

Historically protoplasts were considered attractive targets for transformation as introduction of DNA was feasible by electroporation or chemical methods (Hauptmann et al. 1987), but regeneration from protoplasts has always been a problem due to recalcitrance or genotype dependence (Potrykus 1990). After the development of the particle gun it became possible to introduce DNA into intact cells and tissues and this became the preferred mode of gene transfer. Immature embryos and embryogenic cultures were bombarded with DNA coated particles to obtain transgenic plants. Particle delivery devices such as PDS 1000/He (Du Pont or Biorad, Munich, Germany) or the particle inflow gun (PIG) (Finer et al. 1992) were used to introduce gold/tungston particles coated with desired vectors into the cells. Osmotic treatment of explant during the bombardment was reported to be helpful for the transformation (Girgi et al. 1992; Goldman et al. 2003).

Vectors

Promoter, enhancer, introns and polyadenylation regions are known to affect the expression of transgenes in transgenic plants (Birch 1997). Lambé et al. (1995, 2000) tested several vectors having *gus, hph, bar* or *nptII* regions in various combinations of CaMV 35s, *Adh1* and *Emu* intron (maize *Adh1*) and terminators (CaMV 35s,

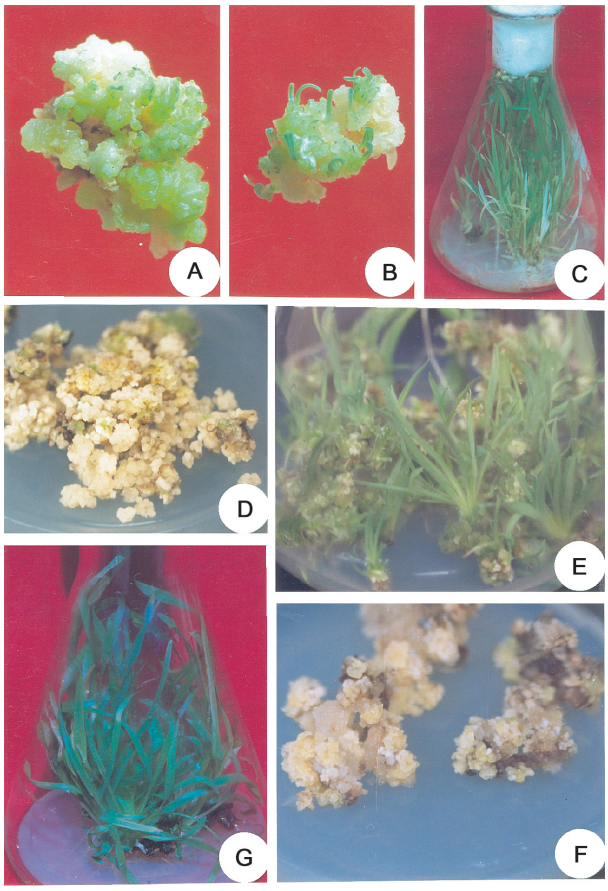

Figure 1. A. Maintenance of immature embryo derived regenerative callus in *Eleusine coracana*. B. Early stage of plant regeneration from callus cultures in *E. coracana*. C. Regenerated plants of *E. coracana*. D. Embryogenic callus cultures of *Paspalum scrobiculatum*. E. Plant regeneration in *P. scrobiculatum*. F. Long term maintained embryogenic callus of *Pennisetum glaucum*. G. Plant regeneration in *P. glaucum*.

ocs and *nos*). They reported highest transient expression of *gus* gene by using the plasmid having *gus* gene with recombinant *Emu* promotor with *Adh1* intron and *nos* terminator in their experiments on Pearl millet transformation. Use of hygromycin *b* was found useful in selecting the transformed calli and transgenic plants.

Selection and plant regeneration

In an early report, Hauptmann et al. (1988) used hygromycin, kanamycin and methotrexate to test their efficacy for protoplast cultures of *T. monococcum*, *Panicum maximum*, *Saccharum officinarum* and a tri-specific hybrid of *Pennisetum* species, transformed with resistance genes. Protoplast cultures of *Panicum maximum* transformed with a vector containing DHFR gene expression cassette were selected using methotrexate. Resistant colonies were selected and gene integration was confirmed by Southern analysis. All tested cell lines showed resistance to high levels of kanamycin. However, no plants were regenerated. Lambé et al. (1995) reported maintenance of hygromycin resistant transformed callus of Pearl millet for two years without loss of resistance phenotype, but plants were not regenerated.

In a later study on Pearl millet, Lambé et al. (2000) reported regeneration of hygromycin resistant plants obtained from type II callus.

In an important publication on Pearl millet, Girgi et al. (2002) reported transgenic plants using *bar* and *gus* genes. Four breeding lines of pearl millet were bombarded with *gus* reporter and *bar* marker genes. The Ti progeny of regenerated plants showed co-segregation of both marker genes indicating that both genes integrated at the same locus in the Pearl millet genotype.

Goldman et al. (2003) selected the transgenic tissues bombarded with *bar* gene on a medium containing $15\,mg\,l^{-1}$ phosphinothricin but later on the embryogenic cultures were transferred to a phosphinothricin free medium. The germinated somatic embryos were then exposed to $3–10\,mg\,l^{-1}$ phosphinothricin.

In a more recent report O'Kennedy et al. (2004) reported production of fertile transgenic Pearl millet plants expressing a phosphomannose isomerase gene under the maize ubiquitin promoter. Immature zygotic embryos were used for particle bombardment Integration and stable expression of *man* A gene was demonstrated in T_1 and T_2 progeny. The *man* A gene was reported to be superior gene for improving transformation efficiency as compared to the *bar* gene.

Bahia grass (*Paspalum notatum*) is a major subtropical grass species grown in the USA, Mexico and Argentina in the areas affected by severe drought and overgrazing. In a report on genetic transformation of *Paspalum notatum*, Smith et al. (2002) bombarded embryogenic callus with *bar* gene and obtained transgenic plants, which showed presence of *bar* gene in PCR and Southern analysis.

Integration, expression and inheritance of the transgene

Transgenic colonies of *Panicum maximum* transformed with DHLF gene showed homology to nick translated vector DNA (Hauptmann et al. 1988). The copy number of inserted plasmid ranged from 1–10 in independent cell lines.

In a study by Lambé et al. (1995) also, the number of inserted plasmid copies ranged from 1–10 in DNA from selected hygromycin resistant callus lines when hybridized with *hph* probe. The number of copies was 1–4 for co-transformed *gus* gene on specific Southern analysis. Silencing of the *gus* gene was noted after one year. However, reactivation of the *gus*-gene was observed within two weeks when these calli were cultured on medium supplemented with $10\,\mu M$ 5-azacytidine (aza C). This loss of gene activity was attributed to the methylation of *gus* gene as presence of its copies was

Table 3. Transformation of millet species.

Plant	Explant	Vector with reporter/selectable marker gene	Result	References
Echinochloa crusgalli	Leaf	*uidA*	TE	Gupta et al. 2001
Eleusine coracana	Callus, leaf	*uidA*	TE	Gupta et al. 2001
Panicum maximum	Protoplasts	cat, *nptII*, *dhfr*, *hph*	TE	Hauptmann et al. 1987, 1988
Paspalum notatum	Callus	*bar*	TP	Smith et al. 2002
Pennisetum americanum	Protoplsts	cat, *nptII*, *dhfr*, *hph*	TE	Hauptmann et al. 1987, 1988
P. glaucum	Embryo/cell culture	*uidA*	TE	Taylor and Vasil 1991; Taylor et al. 1993
P. glaucum	Callus	*uidA*, *hph*, *bar*, *nptII*	TP	Lambé et al. 1995, 2000
P. glaucum	Scutellum	*uidA*, *bar*	TP	Girgi et al. 2002
P. glaucum	Embryogenic tissue	*uidA*, *bar*, *gfp*	TP	Goldman et al. 2003
P. glaucum	Embryo	*manA*	TP	O'Kennedy et al. 2004

Abbreviations: TE, Transient Expression; TP, Transgenic Plants.

shown by Southern analysis of maintained calli. The methylation pattern of introduced reporter gene is known to affect its expression level in barley and wheat (Graham and Larkin 1995; Rogers and Rogers 1995).

Presence of co-transformed *gus* gene was observed on Southern hybridization in 60% of the hygromycin resistant clones by Lambé et al. (2000). 82% of all the analyzed transformants contained the intact *hph* gene expression cassette and all of them had the intact *gus* gene expression unit. Copy number for *hph* gene was between 5–20 in 40% of the lines in comparison to 10% for *gus* gene. Restriction of DNA that releases the two expression cassettes and numerous hybridizing patterns with higher and lower molecular weight were detected showing the recombination of the gene unit. Southern analysis using the enzymes that cut only once in the plasmid also showed integration of several plasmid copies at the same locus. Presence of *hph* gene activity was confirmed by hygromycin assay. All the selected lines showed the *hph* gene activity, and this activity was maintained over a long period. Activity for the *gus* gene detected by the histochemical assay, however, decreased over time. After one year 40% of the positive calli no longer responded to the *gus* assay. Loss of *gus* activity was attributed to the methylation and it could be restored by culturing the calli in the presence of 5-azacytidine, which is known to inhibit methylation (Jones 2003; Klaas et al. 1989). Expression of the *hph* gene was also observed in all selected plants, though absence of *gus* gene expression was observed in some plants despite the presence of *gus* expression unit. Also *gus* gene expression was observed in the plants regenerated from the calli in which *gus* expression was restored after 5-azacytidine treatment, showing methylation being the cause of gene silencing. Phenotypically, the majority of the plants were normal but some of them were dwarf and some others showed anomalies such as curling of leaves. Since such anomalies were not present in any non-transformed control plants but were present in plants regenerated from the explants, which were bombarded without DNA, this result was attributed to the bombardment process itself. Seed set varied from a few seed to nearly full. Seeds from the 8 self-fertile plants were germinated on non-selective media for 3–5 days and then transferred to the medium with $30\,mg\,l^{-1}$ hygromycin B to test for the *hph* gene expression. In seeds from three plants no germination was observed and in the others it ranged from 37–86%. Three R_0 plants segregated resistant and sensitive F_1-plants. Two plants segregated only sensitive plants. Presence of *hph* gene expression unit was confirmed by Southern hybridization in these sensitive plants and low expression of the *hph* gene was attributed to their sensitivity. However *hph* gene expression was again observed in the mature plants indicating that the *hph* gene expression was rather developmental stage dependent. Deviation from normal inheritance ratio and expression was also observed in the progenies.

Girgi et al. (2002) reported 4–7 copies of *bar* gene and 2–7 copies of *gus* gene in plants co-transformed with both genes by PDS method. In a single plant obtained by the PIG method a dozen copies of the *bar* gene were observed. A 1 : 1 segregation ratio for *gus* gene and 3 : 1 ratio for *bar* gene segregation indicated the insertion of multiple copies of genes at the same loci, therefore segregation took place as a single unit. Transgenic plants were phenotypically normal and fully fertile.

In the study by Goldman et al. (2003) no transgenic plants were obtained if no selection was carried out and expression of co-transferred *gfp*-gene was also during the early stage of callusing. In presence of phosphinothricin *gfp* gene expressing calli were observed after three weeks and transgenic plants were obtained from these sectors. In some co-transformation experiments GFP expression was not observed in the herbicide resistant plants. Segregation study of these plants revealed that these plants gave rise to progeny expressing either only PPT resistance or with both PPT resistance and GFP expression. GFP expression was visible in roots, immature embryos, callus, shoots

germinating from callus and stems from mature plants. In one plant *gfp* gene was transmitted only by the female gamete on self-pollination. Southern blot analysis confirmed the presence of *bar* from pAHC25 and *Hind*III fragment. In the *Hind*III digest beside the 5.5 kb *bar*-containing *Hind*III fragment, smaller and larger bands were observed. These bands were attributed to the rearrangement events and multiple copies at a single locus or at multiple loci. A similar pattern in the progeny plants indicated integration at a single locus. Digestion with *Sac*I also indicated that most plants contained multiple copies and possibly more than one unique insertion site. Integration of *gfp* gene was also confirmed by Southern hybridization. Fertility of transgenic plants varied between lines. Seeds were harvested from 69 of the 110 transgenic plants. Most of the plants were female fertile when cross-pollinated. The green house environment was supposed to have a negative effect on pollen viability and fertilization and may explain why some self- and cross- pollination with the same plant produced different fertility levels. Some of the plants were fully sterile. Transmission and segregation was observed with transgenic plants from both the diploid and tetraploid primary transformants. Most ratios fit a 1 : 1 (resistant to sensitive) segregation ratio expected for a test cross with the transgene from a single locus. Some self-pollination events led to a 3 : 1 segregation.

In the study by O'Kennedy et al. (2004), ten of eighteen transgenic lines had a unique integration pattern for the transgene in the Southern blot, thus showing the independent transformation events. Though a high copy number of the transgene was inserted into some lines, only transgenic lines with a maximum of 4–10 copies produced the next progenies. Primary transformants were often stunted and produced few seeds, but progeny plants were found to be phenotypically normal and produced a number of seeds. Progeny seeds were selected on a medium containing 15 g l^{-1} mannose and 1 g l^{-1} glucose. None of the seeds from the non-transgenic lines germinated on this medium. Mendelian segregation for the transgene was observed only in the second or third generation. Only a small population was available in the early progenies.

Future prospects

Although *in vitro* culture techniques were developed quite early for all the millet species (Vasil 1987) transformation of millets has so far lagged behind in comparison to major cereals (wheat, rice, maize and barley). One of the main reasons is that many of the millets are not of economic importance to developed countries and therefore scarcity of research funding has always been a problem. Also major labs have concentrated their research efforts on improvement of major cereals and many of these cereals have a quite developed transformation system (Vasil 1994; Repellin 2001). Genetically modified maize, wheat and rice are either under field evaluation or are being grown by farmers in large areas (Birch 1997). The impact of genetically modified crops on society has been discussed in several publications (Boulter 1997; Khachatourians 2002). Millets are still not very responsive to transformation protocols. There are no model cultivars which can be transformed at an efficient rate for any of the millet species. The *Agrobacterium* transformation system is becoming the main mode of transformation for major cereals (Komari and Kubo 1999; Koichi et al. 2002). Though initially they were thought to be out of the host range, this system is important because of its usually giving high transformation efficiency, simple integration pattern and simple handling. At present protocols are not available to infect millet explants with the *Agrobacterium*. Also the *Agrobacterium* transformation system is highly cultivar dependent and it is important to look for millet cultivars that can be transformed with the *Agrobacterium*.

Major growing regions of millets are in the under developed and developing world, where the main goal is still to increase production rather than to improve nutritional value. Increase in production is mainly possible by conventional breeding methods of selection and controlled hybrids. Many of the cultivars with natural resistance against biotic and abiotic resistance are already available. This is also one of the reasons why millets have been overlooked so far for improvement for novel traits.

All the major cereals crops including rice, wheat, maize, barley, *Avena* and *Tritordeum* have been genetically transformed using the particle gun, protoplasts or Agrobacterium mediated gene transfers (for reviews see Repellin et al. 2001; Koichi et al. 2002). But, production of transgenic plants in millets remain restricted only to pearl millet (Lambé et al. 2000; Girgi et al. 2002) and bahia grass (Smith et al. 2002). Other small millets have been overlooked due to economic or regional considerations. The genetic transformation protocols for millets are important to bring the tertiary gene pool into the improved cultivated varieties.

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