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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase

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Abstract Fertile transgenic pearl millet plants expressing a phosphomannose isomerase (PMI) transgene under control of the maize ubiquitin constitutive promoter were obtained using the transformation system described here. Proliferating immature zygotic embryos were used as target tissue for bombardment using a particle inflow gun. Different culture and selection strategies were assessed in order to obtain an optimised mannose selection protocol. Stable integration of the *man*A gene into the genome of pearl millet was confirmed by PCR and Southern blot analysis. Stable integration of the *man*A transgene into the genome of pearl millet was demonstrated in T_1 and T_2 progeny of two independent transformation events with no more than four to ten copies of the transgene. Similar to results obtained from previous studies with maize and wheat, the *man*A gene was shown to be a superior selectable marker gene for improving transformation efficiencies when compared to antibiotic or herbicide selectable marker genes.

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Abbreviations *2,4-D:* 2,4-Diclorophenoxyacetic acid · *IAA:* Indole acetic acid · *ICRISAT:* International Crops Research Institute for the Semi-Arid Tropics · *IZEs:* Immature zygotic embryos

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br., formerly *P. americanum*] is a staple food for the world's poorest and most food-insecure people in Africa and India. It is grown largely for its ability to produce grain under hot, dry conditions on infertile soils of low water-holding capacity, where other crops generally fail. Thus, it is produced mainly in outlying areas peripheral to the major production and population centres of the developing world. Yearly, approximately 15 million tons of pearl millet is produced worldwide. The development of a reliable transformation protocol for this crop plant will form the basis for its future genetic enhancement by complimenting classical breeding programmes, which ultimately will benefit the people of India and sub-Saharan Africa.

Procedures for pearl millet transformation based on the use of the helium-driven PDS-1000/He or the particle inflow gun (PIG) using the *bar* herbicide-resistant gene or hygromycin phosphotransferase (*hph*) gene as selectable marker genes and/or *uid*A (GUS, β-glucuronidase) as the reporter gene in transgenic pearl millet have been published (Girgi et al. 2002; Goldman et al. 2003; Lambé et al. 1995, 1999). Apart from the work presented by Goldman and co-workers (2003) in which inflorescence-derived embryogenic tissue was targeted, pearl millet transformation is still limited by relatively low and erratic stable transformation efficiencies. Furthermore, our inadequate knowledge of just how antibiotic- or herbicide-resistant genes may affect the environment (gene transfer to the wild relatives, especially for pearl millet) and

human health, especially in food crops, has raised widespread public concern (Wang et al. 2000).

It is a well-known fact that in the genetic engineering of plants, only a minor fraction of the treated cells become transgenic, while the majority of the cells remain untransformed and need to be eliminated by selection (Joersbo and Okkels 1996). During negative selection, the majority of the cells in the cultured tissue die. These dying cells may release toxic substances (such as phenolics), which in turn may impair regeneration of the transformed cells. In addition, dying cells may form a barrier between the medium and the transgenic cells, thereby preventing or slowing the uptake of essential nutrients (Joersbo and Okkels 1996). Selection systems based on herbicide (Girgi et al. 2002) or antibiotic resistance (Zhang and Puonti-Kaerlas 2000) either allow the regeneration of escapes, even at a high selection pressure, or are deleterious to the regeneration process. In contrast, the mannose-positive selection system favours the regeneration and growth of the transgenic cells while the non-transgenic cells are starved but not killed. Therefore, untransformed tissue is separated from transgenic tissue by carbohydrate starvation of the untransformed cells.

The mannose selection system employs the phosphomannose isomerase (PMI)-expressing gene, *man*A, as the selectable marker gene, and mannose, which is converted to mannose-6-phosphate by endogenous hexokinase, as the selective agent (Joersbo et al. 1999). Transgenic PMI-expressing cells acquire the ability to convert mannose-6-phosphate to fructose-6-phosphate, while the non-transgenic cells accumulate mannose-6-phosphate. The phosphorylation of mannose triggers a signalling cascade that results in the repression of genes needed for germination (Pego et al. 1999) and energy depletion during seed germination (Wang et al. 2000). Mannose, readily taken up by the roots, inhibits the germination of seeds as a consequence of it being phosphorylated and not by the toxicity of the compound per se (Joersbo et al. 1998; Negrotto et al. 2000). Subsequently, the accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, thereby causing a block in glycolysis, and inhibits respiration by competitive inhibition of phosphoglucose isomerase (Goldsworthy and Street 1965). Pego and co-workers (1999) showed that the absence of germination in Arabidopsis seed in the presence of mannose was not due to ATP or phosphate depletion. These authors reported that mannoheptulose, a hexokinase-specific inhibitor, restores germination of these seeds and therefore concluded that the mannose-mediated repression of germination is the result of a hexokinase-mediated carbohydrate-induced gene regulation.

The structural gene, *man*A, from *Escherichia coli* has been used to successfully produce transgenic maize (Negrotto et al. 2000; Wang et al. 2000), cassava (Zhang and Puonti-Kaerlas 2000) and sugarbeet (Joersbo et al. 1998, 1999). The *man*A gene has been shown to be superior to antibiotic or herbicide (*pat* or *bar*) selectable marker genes for plant transformation in maize, wheat and sugar beet (Joersbo et al. 1998; Reed et al. 2001; Wright et al. 2001). Furthermore, a preliminary risk assessment done by Reed and co-workers (2001) indicated that with respect to the PMI protein in transgenic maize (1) it was readily digested in simulated mammalian gastric and intestinal fluids, (2) there was no detectable changes in glycoprotein profiles and (3) no statistically significant differences were obtained in yield and nutritional composition compared to untransformed maize. Furthermore, the database search revealed no significant homology of the *E. coli man*A gene product to any known toxin or allergen (Reed et al. 2001).

The aim of the investigation reported here was to use the positive mannose selectable marker gene technology to (1) limit the number of escapes, (2) improve the transformation efficiency and (3) avoid using antibiotic- or herbicide-resistant genes as selectable marker genes in pearl millet transformation.

Materials and methods

Seed material

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] seed, genotype 842B, was kindly provided by ICRISAT, Zimbabwe. Seedlings were planted in a soil mix consisting of red soil, rough sand and compost (1:1:1) and watered three times per week with a soluble fertiliser [Hortichem (N:P:K at 3:1:5), Ocean Agriculture, South Africa] until flowering. Covering the flowers with brown paper bags prevented cross-pollination.

Excision of IZEs

Greenhouse-grown florets of pearl millet containing IZEs (10–14 days post-pollination) were soaked in 70% (v/v) ethanol for 1 min and sterilised for 15 min in a 2.5% (v/v) sodium hypochlorite solution containing 0.1% (v/v) of the surfactant Tween 20 before being thoroughly rinsed with sterile distilled water. IZEs (0.5–1 mm in size) were aseptically excised from the florets using a dissecting microscope and placed with their axes in contact with the callus induction medium. All tissue culture procedures were performed under aseptic conditions.

Tissue culturing of IZEs

Callus induction medium A is described by Pinard and Chandrapalaiah (1991) and contains MS salts (Murashige and Skoog 1962), 2 mg l^{-1} 2,4 D, 30 g l^{-1} sucrose as carbon source and 8 g l^{-1} agar as solidifier. Cultures initiated on medium A induction medium were further cultured on regeneration medium as described by Pinard and Chandrapalaiah (1991), containing the hormones IAA (0.2 mg l^{-1}) and kinetin (0.5 mg l^{-1}), but the medium was modified by the addition 10 mg l^{-1} $AgNO_3$. These cultures were subsequently transferred to rooting medium, which was identical to the regeneration medium, except that both hormones and silver nitrate were omitted.

Callus induction medium, designated medium J in this study, is described by Gless and co-workers (1998) and contains L3 salts and vitamins as described by Jähne (1991), 2.5 mg l^{-1} 2,4-D, maltose as a carbon source, 4 g l^{-1} Gelrite as solidifier and modified by supplementing the medium with 20 mM L-proline. L-Proline and 2,4-D were omitted from the regeneration and rooting medium. Cultures initiated on medium J were matured on regeneration medium containing double the amount of carbohydrates for a

period of 2 weeks. The matured cultures were then regenerated and rooted on regeneration and rooting medium as described above.

Cultures on callus induction and regeneration media were incubated at 24–25°C under low-light conditions (1.8 μE m^{-2} s^{-1}), whereas regenerating shoots (≥1 cm) were incubated under dim light (18 μE m^{-2} s^{-1}).

Transfer regime

IZE-derived calli were transferred to fresh medium every 2 weeks. White compact calli were produced within 4 weeks on callus induction medium A, but only after 6 weeks on medium J [callus induction (4 weeks) and maturation (2 weeks) medium]. After 4 or 6 weeks on the A- or J-based medium, respectively, calli were transferred to the appropriate regeneration media as described above and subcultured every 3 weeks to fresh media. Plantlets longer than 1 cm that had been produced on medium A were transferred to rooting medium. Rooted plantlets grown to 8–10 cm were hardened-off to a mist bed for approximately 2 weeks and then transferred to pots in the greenhouse. Rooted plantlets longer than 1 cm that had been produced on medium J remained on the regeneration medium described for this regime until they were hardened-off.

Plasmids

The construct pNOV3604 was obtained from Syngenta, and the ubiquitin promoter inserted upstream of the *man*A gene as a *Hind* III/*Bam* HI insert (Fig. 1A). Plasmid pNOV3604ubi DNA was extracted from overnight *Escherichia coli* cultures using a Qiagen (Southern Cross Biotechnologies) Maxiprep kit according to the supplier's recommendation.

Microprojectile bombardment

Pre-cultured IZEs (0–1 cm diameter) were placed in the middle of a 9-cm petri dish containing A or J induction media supplemented with an approximately 25% (m/v) increased amount of solidifying agent plus 0.2 *M* D-sorbitol and 0.2 *M* D-mannitol as described by Vain and co-workers (1993). A bombardment mixture was prepared by precipitating plasmid DNA on tungsten particles with $CaCl_2$ and spermidine-free base as described by O'Kennedy and co-workers (1998). All experiments were conducted with the particle inflow gun (PIG). Sixteen hours after bombardment, unless otherwise stated, bombarded tissue was transferred to the respective media omitting D-Sorbitol and D-mannitol. The DNA delivery parameters were as follows: 900–1,000 kPa, 0.16 μg DNA per shot, PIG positioned 17 cm from the target tissue and a 500-μm nylon mesh screen placed 8 cm above the target. A vacuum of approximately −87 kPa was applied, and the bombardment mix particles on the filter syringe were discharged when the helium was released following activation of the solenoid. The timer duration was 50 ms.

Selection and regeneration of transformants

Selection for *man*A containing and expressing pearl millet tissue was initiated 2–7 days following bombardment by placing the cultured IZEs on selection medium. Selection medium containing maltose (medium J) or sucrose (medium A) supplemented with mannose is described in the Results and discussion and in Tables 1 and 2 for each individual experiment. The concentration of mannose in the medium was kept constant throughout the selection period or stepwise increased within the range of 5 g l^{-1} to15 g l^{-1} while the content of sucrose or maltose was decreased. After 4–6 weeks on selection medium, cultured embryos that produced white compact calli, presumably somatic embryos, were transferred to regeneration selection medium. Regenerating putative transgenic plants were subcultured at 2- to 3-week intervals until they reached 8–10 cm in height. These were then hardened-off as described by O'Kennedy and co-workers (1998).

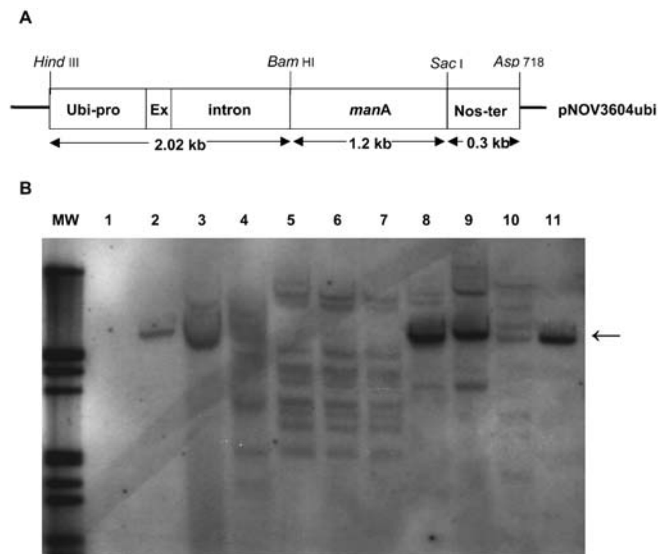

Fig. 1 **A** Diagram of construct pNOV3604ubi used for pearl millet transformation. Plasmid pNOV3604ubi (6,210 bp) contains the *man*A selectable marker gene under the control of the maize *Ubi1* promoter (*Ubi-pro*), first exon (*Ex*) and first intron and the nopaline synthase terminator (*Nos-ter*). **B** Southern blot analysis of independent T_0 transformation events of pearl millet genotype 842B. Genomic DNA was purified from plant leaf material, restricted with *Sac*I and the fragments separated on a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal fragment of the *man*A transgene. *Lanes*: *MW* DIG-labelled molecular weight marker III (Roche Diagnostics South Africa), *1* untransformed pearl millet DNA (line 842B), *2*, *11* untransformed pearl millet spiked with two and ten transgene copies, respectively, *3* Mann 1.31, *4* Mann 4.3, *5–7* clones of Mann 7.1 designated J1, M2 and M4, respectively, *8* Mann 11.6A, *9* Mann 11.6B, *10* Mann 11.7C. *Arrow* indicates the size (6.21 kb) of pNOV3604ubi linearised with *Sac*I

Germination of progeny on mannose-containing medium

Progeny of transgenic plants were germinated on half-strength MS medium containing half-strength MS salts, 1 g l^{-1} sucrose, 15 g l^{-1} mannose and solidified with 8 g l^{-1}agar.

DNA extraction

Genomic DNA was extracted from putative transgenic pearl millet leaf material using the mini extraction procedure of Dellaporta and co-workers (1983).

PCR analysis

manA (PMI forward: 5′-CGT TGA CTG AAC TTT ATG GTA TGG-3′; PMI as: CAC TCT GCT GGC TAA TGG TG-3′) specific primers were used to amplify a 965-kb fragment from genomic DNA preparations of putative transgenic pearl millet plantlets.

Southern blot analysis

Five micrograms of pearl millet genomic DNA digested with *Sac*I was separated on an agarose gel and analysed by Southern blotting as described by O'Kennedy (1998). The *man*A gene of pNO-V3604ubi was labelled with digoxigenin (DIG) by the PCR DIG probe synthesis kit as described by the supplier (Roche Diagnostics, South Africa).

Results and discussion

We supplemented the mannose-containing medium with sucrose, glucose or maltose in order to alleviate the toxic effect of mannose-6-phosphate. A dose-response curve was designed for pearl millet for testing increasing mannose content and decreasing maltose content (Table 1). The results showed that mannose inhibited callus production in a dose-dependent manner up to 20 g l^{-1}, a concentration at which growth was 95% suppressed, even when the medium was supplemented with 2 g l^{-1} maltose (Table 1). Results from previous studies indicated that the indirect toxic effect of mannose caused by the conversion to mannose-6-phosphate by endogenous hexokinase increases with decreasing sucrose concentration in the medium, suggesting an interaction between mannose and sucrose (Joersbo et al. 1998). The addition of sucrose, therefore, can alleviate the effect of mannose on growth and germination. Pego and co-workers (1999) reported that the addition of metabolisable sugars reversed the mannose-6-phosphate-mediated inhibition of germination of Arabidopsis seed. These researchers also reported that the phytotoxic effect of mannose-6-phosphate was strongly dependent on the nature and concentration of the saccharides added to the medium. In particular, glucose interacts strongly with the mannose-6-phosphate-caused phytotoxic effect (Goldsworthy and Street 1965; Joersbo et al. 1999). Glucose was found to be four- to sevenfold more potent than the other saccharides (sucrose, maltose and fructose) tested and was able to eliminate the mannose-6-phosphate toxicity at concentrations 15- to 25-fold higher than mannose (Joersbo et al. 1999).

We adjusted various parameters in order to optimise pearl millet transformation using the *man*A transgene: (1) tissue culture medium A or J, (2) preculture of IZEs for 4–10 days, (3) bombardment at a helium pressure of 900 kPa or 1,000 kPa, (4) selection initiated 2–7 days after bombardment, (5) single (160 ng per shot) or double bombardment (320 ng plasmid DNA in total) and (6) selection pressure ranging from 15 g l^{-1} sucrose or maltose supplemented with 5 g l^{-1} mannose to 20 mg l^{-1} glucose or maltose supplemented with 15 g l^{-1} mannose, for media regimes A or J, respectively (Table 2). However, the selection pressure was subsequently set for both media A and J at 15 g l^{-1} mannose, which is the osmotic equivalent of 30 g l^{-1} sucrose, supplemented with 2 g l^{-1} sucrose/maltose or 20 mg l^{-1} glucose/maltose, for media A/J, respectively.

Transgenic selection was carried out on mannose for the full period, as indicated in Table 2, until regenerated plantlets were hardened-off to the greenhouse. Thirteen transformation experiments using the full pNOV3604ubi construct containing the *man*A gene were performed (Table 2). Several combinations of mannose and sucrose or mannose and maltose in tissue culture media regimes A and J, respectively, were assessed using predominantly mannose. A constant high selection pressure of mannose was applied for experiments 1, 2, 7, 8 and 13, whereas a stepwise increase in the selection pressure was applied for experiments 3–6 and 9–12 (Table 2), until pearl millet plantlets were hardened-off to the greenhouse. Callus tissue, within the first 2 months (encompassing four transfers), failed to proliferate further and became watery and browned when cultured on a high selection pressure of 2/15 (2 g l^{-1} sucrose or maltose and 15 g l^{-1} mannose) or 0.02/15 (20 mg l^{-1} glucose or maltose and 15 g l^{-1} mannose). Nevertheless, seven out of the eight independent transformation events arose on this high selection pressure (Table 2). The total period in tissue culture, from the excision of IZEs until when plantlets were hardened-off to the greenhouse, was 12 weeks.

Primary transgenic plantlets were identified by PCR and subsequent Southern blot analysis of primary transformants and their progeny (Figs. 1, 2). Genomic DNA was digested with *Sac*I, a unique restriction site in the construct. Of the 23 putative transgenic plants, 18 contained the transgene *man*A (Table 2). Escapees were therefore limited to approximately 20%, which resulted in a major reduction in the time and facilities necessary for the molecular analysis of untransformed plants. Only 8 of the 18 transformation events had a unique integration pattern of the transgene *man*A (Figs. 1, 2). Preliminary results show that although high-copy number integration was occasionally obtained (Fig. 1), the transgenic plants producing T_1 and T_2 progeny had an estimated low-copy number integration of the *man*A transgene (a maximum of four to ten copies in tandem; Fig. 2B, lane 2 represents two copies). Furthermore, the integration pattern was maintained from one generation to the next. The Southern

Table 1 Response of IZEs cultured on mannose-containing medium J supplemented with maltose. Twelve embryos per plate were cultured, three replicates per treatment

Mannose (g l^{-1})	Maltose (g l^{-1})	Average fresh weight (g)	Percentage inhibition
0	30	3.65±0.74	0
2.5	20	2.41±0.38	34
2.5	12	2.26±0.49	38
5	10	1.75±0.26	52
5	5	1.10±0.15	70
10	2	0.57±0.20	84
20	2	0.17±0.02	95

Table 2 Conditions for bombardment of pearl millet IZEs of genotype 842B, with plasmid DNA of pNOV3604ubi (160 ng per shot) after a sorbitol and mannitol osmoticum treatment or 120 g/l sucrose (medium A) or maltose (medium J) osmotic treatment (sucrose or maltose osmotic treatment in italics). Parameters that lead to the production of stable transgenic plants are underlined

Experiment number[a]	Number of embryos	Preculture period (in days)[b]	Helium pressure (kPa)	Osmoticum treatment (h) +osm/-osm	Selection[c]	Mannose selection history[d]	Number of putative regenerants	Transgenic T_0 plants designated
1	27	6 or 7	900	16/56	3	ind 15/5→reg 15/5	0	
	34					ind 5/15→reg 5/15	0	
2	48	6 or 7	1,000	16/56	3	Ind 15/5→reg 15/5	0	
	38					ind 5/15→reg 5/15	0	
	36					ind 2/15→reg 2/15	1	Man 2.24
3	80	4 or 5	900	16/56	3	Ind 15/5→reg 1/15	0	
4	25	10	900	16/32	2	Ind 15/5→reg 15/5→reg 1/15	0	
5	27	6	900	16/56	3	Ind 5/15→reg 5/15→reg 1/15	0	
	27		1,000				0	
6	40	7	1,000	16/152	7		0	
	40	5		168/0		Init 0.02/15→reg 0/15	0	
	40			***168/0***			0	
	20			16/152			0	
	20			168/0			0	
	20			***168/0***				
	522						**1**	**1**
7	19	6 or 7	900	16/56	3	ind 15/5→reg 40/10→reg 15/5	0	
	58					ind 2–5/15→reg 4/20→reg 2–5/15	1	Man 1.31
8	44	6 or 7	1,000	16/56	3	Ind 15/5→reg 40/10→reg 15/5	0	
	49					Ind 5/15→reg 20/20→reg 5/15	0	
	44					Ind 2/15→reg 4/20→reg 2/15	0	
9	96	4 or 5	900	16/56	3	Ind 15/5→reg 40/10→reg 1/15	1	Man 4.3
10	68	10	900	16/32	2	15/5→40/10→1/15	0	
11	31	6	900	16/56		Ind 5/15→reg 5/25→reg 5/15→reg 1/15	2	Man 7.1 J1 & J3
	36		1,000				3	Man 7.1 KK1, 2, 4
							6	Man 7.1 M1–4, O1
12	60	7	1000	16/152	7		0	
	40	5		168/0		Ind 0.02/15→reg 0.02/30→reg 0/15	1	
	40			***168/0***			0	
	20			16/152			0	
	20			168/0			0	
	20			***168/0***			0	
13	58[e]	7	900	120/48	7		0	
	88	5		120/48		Ind 0.02/15→reg 0.02/30→reg 0.02/15	2	Man 11.6 A & B
	57			16/152			3	Man 11.7 A—C
	57			***168/0***			0	
	45			120/48			1	
	26			16/152			1	
	976						**22**	**17**

[a] Cultures on medium A (experiments 1–6) or medium J (experiments 7–13)
[b] Pre-culture period of IZE before bombardment
[c] Mannose selection initiated after bombardment, indicated by the number of days
[d] Sucrose/mannose (mg l^{-1}) (medium A) or maltose/mannose (mg l^{-1}) (medium J) selection during callus induction (ind, month 1) and regeneration (reg, months 2–6); glucose was used instead of sucrose for low selection (0.02 mg l^{-1}) for medium A
[e] Double bombardment, 160 ng per shot; therefore 320 ng introduced in total

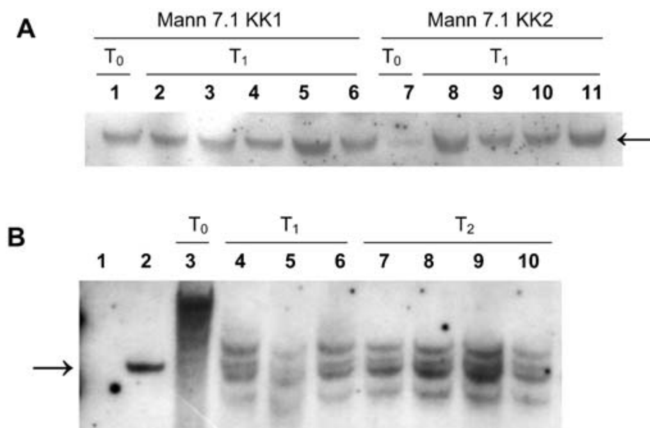

Fig. 2A, B Southern blot analysis of the progeny of transgenic pearl millet genotype 842B. **A** Two clones of a transformation event designated Mann 7.1 KK1 and KK2, **B** transformation event designated Mann 2.24. Genomic DNA was purified from plant leaf material, restricted with *Sac*I and the fragments separated on a 0.8% agarose gel, transferred to a nylon membrane, then probed with an internal fragment of the *man*A transgene. **A** *Lanes*: *1* Mann 7.1 KK1 T_0, *2–6* individual Mann 7.1 KK1 T_1 progeny, *7* Mann 7.1 KK2 T_0, *8–11* individual Mann 7.1 KK2 T_1 progeny. **B** *Lanes*: *1* Untransformed pearl millet DNA, genotype 842B, *2* untransformed pearl millet spiked with two transgene copies, *3* undigested genomic DNA of Mann 2.24 T_0, *4–6* individual Mann 2.24 T_1 progeny, *7–10* individual Mann 2.24 T_2 progeny. *Arrow* indicates the size (6.21 kb) of pNOV3604ubi linearised with *Sac*I

blot of transformation event clones Mann 7.1 KK1 and KK2 clearly shows an intact band of 6.21 kb that corresponds to the linear pNOV3604ubi construct of an identical size and indicates the intactness of the *man*A gene (Fig. 2).

The transformation efficiency using the particle inflow gun and the herbicide resistance gene, *bar,* was improved from 0.02% (Girgi et al. 2002) on medium regime A to 0.19% and 0.72% with *man*A as the selectable marker gene on media regime A and J, respectively. However, an individual experiment gave a frequency of 3% (Table 2, experiment 11). The highest percentage of transformation efficiency was obtained for experiment 11, where a stepwise increase of the selection pressure was applied. This translated to one transformation event per plate; on average, there were 31–35 pre-cultured IZEs per plate. Of the 23 *man*A putative transgenic plants surviving the mannose selection pressure, 18 were transgenic, as confirmed by Southern blot analysis (Table 2). Of these 18 transgenic plants, eight had a unique integration pattern, whereas the other ten were clones. Although the transformation efficiency is still low, the mannose-positive system is effective in selecting almost only transgenic tissue and, eventually, transgenic plants. In contrast, 455 putative transgenic plants were regenerated on the bialaphos (1–2 mg l^{-1} bialaphos, which is the active ingredient of the herbicide Basta) selection system, of which only two stable transgenic plants—clones—contained the *bar* transgene (Girgi et al. 2002). Therefore, using the *man*A selectable marker gene for pearl millet transformation does not only increase the transformation efficiency but also provides an effective selection system that eliminates the labour-intensive tissue culture selection and molecular analysis of putative transgenics.

The germination of progeny of transgenic pearl millet seeds on mannose-containing medium provided a rapid and easy screening procedure for eliminating non-transgenic material since T_0 and T_1 plants often produced a large number of seeds (Table 3). Although T_0 plants were often stunted and produced a limited number of T_1 seeds (sometimes only five seeds), T_1 plants were phenotypically normal and produced numerous seeds. Mendelian segregation was determined by germinating some of the seeds obtained from transgenic plants on media containing 1 g l^{-1} sucrose and 15 g l^{-1} mannose, as described in Materials and methods. Untransformed pearl millet seed resulted in zero regenerants per 1,157 seeds (Man 11.6B) or less than 2% germination (untransformed 842B control seed) (Table 3). Although stable integration up to the first or second generation was obtained for transformation events Mann 7.1 KK1 and KK2 and Mann 2.24, a Mendelian segregation was obtained only in the second (Mann 2.24) or third (Mann 7.1 KK1) generation. The reason for this lack of a Mendelian segregation of seed produced by the transgenic pearl millet can most possibly be contributed to the small population size germinated on

Table 3 Segregation of *man*A gene activity in T_1 and T_2 progeny of transgenic pearl millet plants germinating on medium containing 1 g l^{-1} sucrose and 15 g l^{-1} mannose as described in the Materials and methods. The plants were self-pollinated

Transformation event	T_1 germination (+:−)	T_2 germination (+:−)	T_3 germination (+:−)
Man 2.24	29:37	201:122	
Man 7.1 J1	1:132		
Man 7.1 J3	0:5		
Man 7.1 M1	4:54		
Man 7.1 M3	0:48		
Man 7.1 M5	2:91		
Man 7.1 KK1	137:146	12:185	288:43
Man 7.1 KK2	65:173	38:218	94:97
Man 7.1 KK4	0:707		
Man 11.6 B	0:1157		
Man 11.7 A	0:140		
Man 11.7 B	2:105		
Man 11.7 C	0:147		
842B untransformed	3:180		

mannose selection medium, as genotype 842B produces on average 1,000 seeds per inflorescence.

Finally, in order for *man*A to be a useful, selection system the protocol should be applicable to a range of genotypes. It would be interesting to assess the transformability of other pearl millet genotypes using the *man*A gene as selectable marker gene.

Conclusion

In the investigation reported here, an effective transformation system was established that routinely produces transgenic pearl millet plants. The protocol provides an efficient transformation system for introducing into the genome of pearl millet genes of agricultural interest combined with the *man*A selectable marker gene. In addition, particle bombardment of pearl millet gave rise to transformation events with a relatively low-copy integration of the transgene *man*A. Finally, the mannose selection system has no potential risk to animals, humans or environmental safety.

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