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Agrobacterium tumefaciens-mediated sorghum transformation using a mannose selection system

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Summary

A dual-marker plasmid containing the selectable marker gene, *manA*, and the reporter gene, *sgfp*, was used to transform immature sorghum embryos by employing an *Agrobacterium*-mediated system. Both genes were under the control of the *ubi1* promoter in a binary vector pPZP201. The *Escherichia coli* phosphomannose isomerase (PMI) gene, *pmi*, was used as the selectable marker gene and mannose was used as the selective agent. The *sgfp* gene encoding green fluorescence protein (GFP) was the reporter gene and served as a visual screening marker. A total of 167 transgenic plants were obtained from nine different embryogenic callus lines grown on a selection medium containing 1%–2% mannose. Embryoids and shoots regenerated via embryogenesis, that showed strong GFP fluorescence, were selected from two sorghum genotypes: C401, an inbred line, and Pioneer 8505, a commercial hybrid. The GFP accumulation in transgenic plants was observed with a dissecting stereomicroscope. The integration and expression of the *manA* gene was confirmed by Southern blot and Western blot analyses, and the feasibility of *manA* selection was demonstrated by the chlorophenol red (CPR) assay. Our results indicated that transgenes segregated in the Mendelian fashion in the T_1 generation. The conversion of mannose to a metabolizable fructose carbon source is beneficial to plants. In addition, except in soybean and a few legumes, no endogenous PMI activity has been detected in plant species, indicating that PMI is useful in the transformation of sorghum. In addition, PMI has no sequence homology to known allergens. Optimization of this selection system for sorghum transformation provides an efficient way to produce transgenic plants without using antibiotic or herbicidal agents as selectable markers, and our results showed that the transformation efficiency reached 2.88% for Pioneer 8505 and 3.30% for C401, both values higher than in previously published reports.

Keywords: *Agrobacterium tumefaciens*, green fluorescence protein (GFP), mannose selection, phosphomannose isomerase (PMI), sorghum.

Introduction

The transformation and regeneration of grain sorghum (*Sorghum bicolor* (L.) Moench) have been reported by a number of laboratories (Masteller and Holden, 1970; Cai *et al*., 1987; Ma *et al*., 1987; Cai and Butler, 1990; Hagio *et al*., 1991; Casas *et al*., 1993, 1997; Godwin and Chikwamba, 1994; Kaeppler and Pedersen, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002; Gao *et al*., 2005). One critical factor in these reports is the choice of selectable markers in the transformation experiments. Two selectable markers, the herbicide-resistant gene, *bar*, and the antibiotic-resistance gene, hygromycin phosphotransferase (*hph*), have been used for sorghum transformations, with the *bar* gene being successfully incorporated into transgenic plants by various institutions (Casas *et al*., 1993, 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002). The *bar* gene was isolated from the bacterium *Streptomyces hygroscopicus*, which encodes the enzyme phosphinothricin acetyl transferase (PAT) conferring resistance to the herbicide bialaphos, or Liberty.

The use of the *bar* gene has the advantage of allowing stringent selection of calli in culture to produce bialaphos-resistant plants. However, *bar* selection seems to be a leaky selection system resulting in many escapes; in addition, there is concern that the *bar* gene may be transmitted via pollen to wild relatives of sorghum, producing herbicide-resistant weeds such as Johnsongrass and shattercane. Other selectable markers in sorghum transformation consist of drug-resistance genes, which encode enzymes that inactivate antibiotics by modifying them to non-toxic forms. Bacterial genes that may serve as selectable markers in cereals include the *hph* gene (Weeks *et al*., 2000). The major concerns with regard to the use of antibiotic-resistance selectable markers include public acceptance and the possible migration of these genes to infectious bacteria, rendering the antibiotics ineffective for animal health applications (Balter, 1997). Thus, the commercial use of these genes may be limited, and the production of transgenic plants without the use of toxic compounds and the corresponding genes is highly desirable.

The engineering of genetically modified (GM) crops without the use of antibiotic-resistance genes should eliminate the potential risk of their transfer to the environment or to gut microbes. Several techniques to eliminate marker genes after incorporation have been developed to cope with this problem (Yoder and Goldsbrough, 1994), with the cotransformation systems seeming to be the simplest; in these systems, super binary vectors carrying two separate T-DNAs are used for transformation, and the segregation of the transgenes in the progeny allows the generation of marker-free plants (Komair *et al*., 1996; Jaiwal *et al*., 2002). The use of recombinase-mediated systems, in which *Cre-lox* mutants are employed to excise the newly introduced DNA marker (Ow, 2004), provides another method to remove the transgene. In addition, the deletor-gene system may offer another effective way to remove the selectable marker gene. The use of the green fluorescence protein (GFP) from the jellyfish *Aqueorea victoria* as a reporter gene for visual selection to produce putative fertile sorghum transgenic plants has recently been reported (Gao *et al*., 2005).

The positive selection system based on *Escherichia coli* phosphomannose isomerase (PMI) (Miles and Guest, 1984) as the selectable marker gene and mannose as the selective agent has been reported for the successful selection of transgenic sugar beet (Joersbo *et al*., 1998, 2000), maize (Negrotto *et al*., 2000; Wright *et al*., 2001), rice (Lucca *et al*., 2001) and wheat (Wright *et al*., 2001) plants. In this study, PMI was used for the first time in sorghum transformation, and appeared to have advantages over other selectable markers. Transgenic PMI-expressing cells have the ability to convert mannose-6-phosphate to the easily metabolizable fructose-6-phosphate, which can be used as a carbon source, improving the energy status of the transgenic cells and avoiding accumulation of the derivative selective agents (Joersbo *et al*., 1998). In addition, no endogenous PMI activity has been detected in plant cells, except in soybean and a few legumes, indicating that PMI selection should be useful in the transformation of sorghum or other cereal species. In addition, PMI has been evaluated for its potential allergenicity; purified PMI protein was readily digestible in a simulated gastric environment, has no sequence homology to known allergens, does not contain multiple disulphide bonds and has no *N*-glycosylation consensus sequences. There are no detectable changes in glycoprotein profiles in PMI-transformed plants when compared with non-transgenic controls. Such results indicate that PMI lacks many of the known attributes associated with known oral allergens (Privalle, 2002).

In this paper, we report the expression of the dual-marker consisting of *pmi* and *sgfp* genes and the successful selection using mannose as the selective agent after *Agrobacterium*-mediated transformation. This selection system provides an efficient way to produce transgenic sorghum plants without using antibiotic or herbicidal agents as selectable markers.

Results and discussion

Regeneration of transgenic plants

Tissue culture in sorghum is highly genotype dependent and only a group of selected genotypes produces calli and regenerates. In this experiment, we were limited to the use of only two genotypes for transformation research. Seed stocks of both C401 and Pioneer 8505 are available for other researchers by enquiry. Stable sorghum transformations were carried out and achieved by an *Agrobacterium*-mediated transformation procedure described by Zhao *et al*. (2000) and Gao *et al*. (2005). A total of 309 immature embryos from two sorghum genotypes were inoculated and cocultured with *A. tumefaciens* (strain EHA 101). The diameter of the calli (8.25–12.1 mm) derived from the control embryos was 1.5–2.2 times larger than that of the *A. tumefaciens*-infected embryos 10 days after they were cultured in a callus induction medium without the selective agent. In addition, 49.2% of the calli from the hybrid and 18.0% of calli from the inbred were necrotic and damaged by the bacterium and failed to propagate and regenerate. Tissue damaged by *A. tumefaciens* infection has been reported previously (Pu and Goodman, 1992; Hansen, 2000) and seems to be one of the major obstacles to *Agrobacterium*-mediated transformation. The embryo-derived calli were

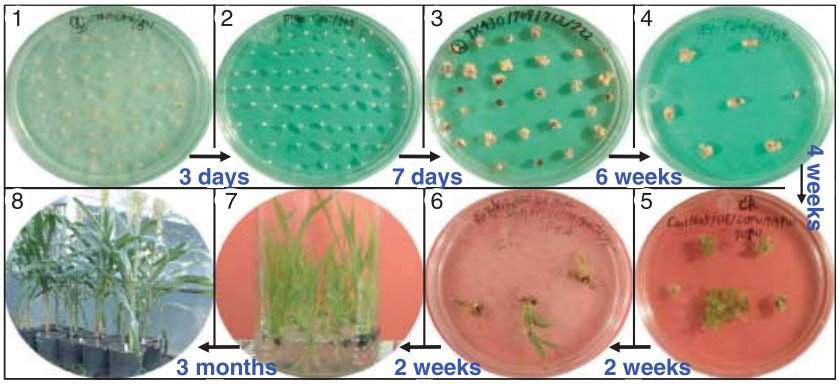

Figure 1 *Agrobacterium*-mediated sorghum transformation and plant regeneration: 1, inoculation and coculture; 2, callus induction; 3, 4, callus selection; 5, shoot regeneration; 6, rooting; 7, plantlets; 8, fertile plants.

transferred weekly to a new selection medium containing 1.5%–2.0% mannose, where mannose-resistant clones (30%) were obtained after 4–6 weeks of selection in darkness. The mannose-resistant embryogenic calli proliferated and formed green sectors, and then formed primary embryoids in regeneration medium containing 1.0%–1.5% mannose under a light culture (Figure 1), whereas the untransformed calli grew slowly and eventually lost vigor. In preliminary experiments, callus growth of sorghum was strongly arrested on medium containing 3.0% mannose combined with 2.0% sucrose and on medium containing 1.0% mannose alone in the initial phases. The concentration of mannose required for the efficient selection of sorghum was the same as that reported for maize (Wright *et al*., 2001), 10-fold higher than that used for sugar beet (Joersbo *et al*., 1998), twofold less than that used for rice (Lucca *et al*., 2001) and two-fold higher than that used for wheat selection (Wright *et al*., 2001). However, when using mannose selection (rather than the herbicide bialaphos or Liberty for *bar* gene selection; data not shown), necrotic calli were rarely observed. The resistant shoots grew longer roots than those of susceptible shoots on the rooting medium containing 1% mannose in 2 weeks. When plantlets reached a height of 5–8 cm, they were transferred to sterile soil pots in a glasshouse. Mannose selection continued through the full course of plantlet regeneration. Seven transgenic callus lines from the hybrid and two transgenic callus lines from the inbred were obtained. Together, a total of 167 fertile sorghum plants were successfully produced with a normal phenotype from different transgenic callus lines (Table 1).

Compared with the inbred, embryo-derived calli from the hybrid produced more pigments until plantlets were regenerated, with and without the selection agent. Relative to the controls, *A. tumefaciens* infection enhanced the pigment production for the embryo-derived calli. The toxicity of the phenolic compounds to cells was reduced by frequent subculture and the addition of 1% (w/v) PVPP (polyvinylpolypyrrolidone) in the medium, promoting fresh callus growth (Zhao *et al*., 2000; Gao *et al*., 2005).

Visual screening of transgenic plants

Before transferring the putative transgenic plantlets to the soil pots, the shoots/roots or young leaves in culture bottles were monitored for GFP and tested for PMI activity using the chlorophenol red (CPR) assay. The presence of GFP was monitored in nearly all developmental stages of the regenerated plants. Transient expression of GFP in *A. tumefaciens*-infected embryos was detectable as early as 2–3 days after cocultivation, and reached a maximum expression after 5–7 days. The average frequency of transient expression of GFP in the two genotypes was more than 40% at 7 days after infection. Individually, frequencies of stable GFP expression in the hybrid Pioneer 8505 and the inbred line C401 were 33.5% (range, 29%–37%) and 29.5% (range, 24%–39%), respectively

Table 1 Efficiency of *Agrobacterium*-mediated transformation of grain sorghum

Hybrid & inbred	Inoculated immature embryos	Necrotic calli (GFP⁻ & GFP⁺)		Resistant calli (GFP⁺)*		Transgenic calli lines		No. of transgenic plants†
		No.	%	No.	%	No.	%	
P8505	248	122	49.2	83	33.5	7	2.8	90 (1–72)
C401	61	11	18.0	18	29.5	2	3.3	77 (1–76)

*Resistant calli could grow on medium containing 2.0% mannose.

†Different transgenic callus lines produced various numbers of transgenic plants (variation, 1–76).

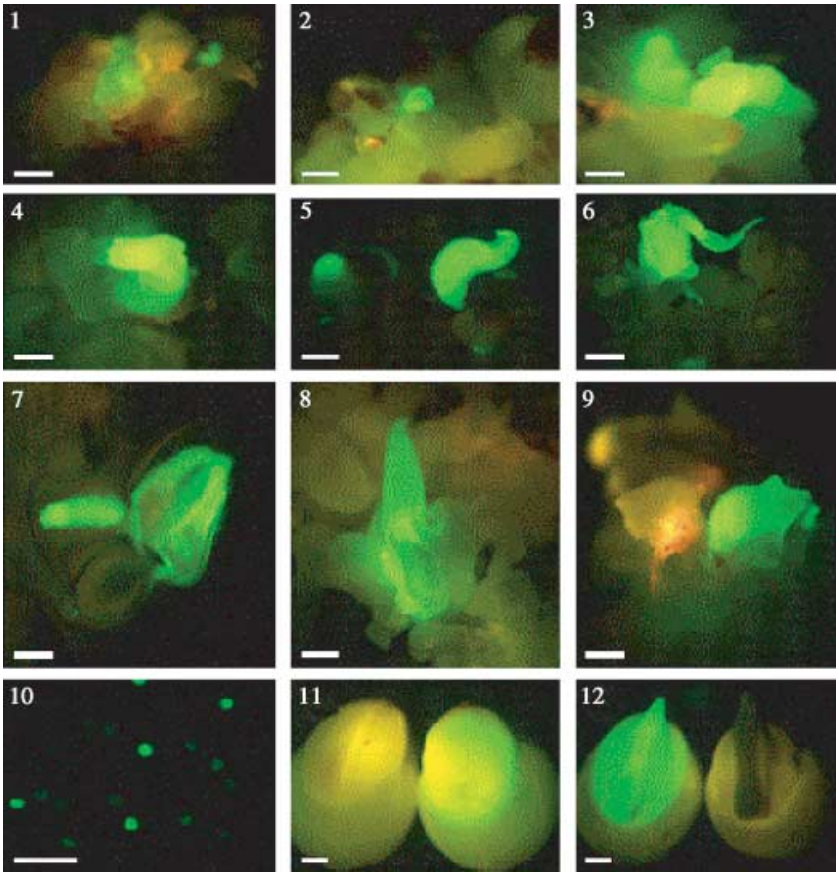

Figure 2 Green fluorescence protein (GFP) expression in different transgenic plant tissues and organs: 1–3, GFP expression in 5-week-old embryogenesis calli on 1.5% mannose selection medium; 4–6, GFP expression in embryoids formed from embryogenesis calli; 7–9, fluorescent embryoids with roots and shoots; 10, pollen grains (about 50% pollen grains show GFP fluorescence); 11–12, mature and germinated seed (non-transgenic seed shows no GFP fluorescence).

Table 2 Relative efficiency of sorghum transformation by different methods from previous reports

Method	Gene (efficiency %)	Author	Year
Biolistic	*hpt*, *Gus*, *Km*^{R} (–)	T. Hagio	1991
Biolistic	*Gus*, *bar* (0.08)	A. Casas	1993
	(0.33)	A. Casas	1997
Biolistic	*Chitinase*, *bar* (0.09)	H. Y. Zhu	1998
Biolistic	*GFP*, *bar* (1.00)	J. A. Able	2001
Biolistic	*Gus*, *bar* (0.18)	C. Emani	2002
Agrobacterium	*Gus* (–)	I. Godwin	1994
Agrobacterium	*Gus*, *bar* (2.10)	A. Zhao	2000
Agrobacterium	*GFP*, *TLP* (2.50)	Z. S. Gao	2005

(Table 1) and were highly reproducible. No green fluorescence was observed in control calli. These results suggest that *Agrobacterium*-mediated transfer of T-DNA to sorghum cells is highly efficient. Compared with previously published reports (Casas *et al*., 1997; Zhu *et al*., 1998; Zhao *et al*., 2000; Able *et al*., 2001; Emani *et al*., 2002), in which various reporter and selectable markers were used, this experiment appeared to have the highest transformation efficiency (Table 2).

GFP expression could be visually detected in tissues or organs with little or no chlorophyll, such as calli, embryoids, shoots, root tips, young leaves, anthers, pollen grains and immature, mature and germinating seed (Figure 2). Because of the strong GFP expression and ease of manipulation, root tips were used to identify the paired transgenic callus lines (positive and negative) and the transgenic plants derived from the same piece of callus. All the transgenic T_0 plantlets were routinely identified by using the root tips before they were transplanted to soil pots, and no escapes were observed. Albino plantlets and fully sterile transgenic plants were not observed.

The CPR assay included mannose in the medium to screen for transgenic plants. After the regeneration of plants, leaf tip tissues were used for CPR analysis. Acidification of the medium, as a result of the metabolic activity of living cells, leads to a colour change to yellow (Lucca *et al*., 2001). A representative CPR plate containing mannose in the wells with the leaf tissue is shown in Figure 3. Leaf tissues from all regenerated plants, transgenic and control, changed the medium colour to yellow in samples to which sucrose only was added to the medium as the carbon source. When the medium contained mannose, however, the leaf tissues from transgenic plants changed the medium colour to yellow, whereas the medium colour remained red for non-transgenic plants. The duration between initial visual selection and

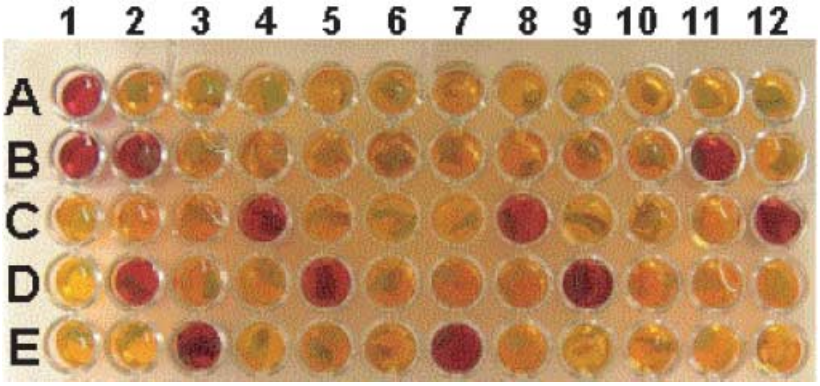

Figure 3 Chlorophenol red (CPR) assay of phosphomannose isomerase (PMI) activity in transgenic plants: (A) sucrose; (B–E) mannose; (A1, B1) blank; (A2, B2) negative control; The samples in lines A and B are from the same plants. The samples inlines C, D and E are from different plants. The colour reaction recording was observed after 3 days of incubation at 28 °C and a red colour shows non-transgenic plants.

formation of transgenic plantlets ranged from 6 to 10 weeks. Compared with Liberty herbicide selective agent for the *bar* gene or cyanamide for the *cah* gene, the mannose selection system had less negative effects on plant regeneration (data not shown). One of the transgenic callus lines produced 76 plantlets during a 6-month period. We did not observe any albinos using the GFP/PMI system, and all except one transgenic plant derived from various callus lines were normal with regard to morphology and fertility after they had been transplanted and grown in soil pots in glasshouses and in the field.

Molecular analysis of T_0 and T_1 plants

Because all transgenics were identified using visual GFP/PMI-based selection, the genomic DNA of transformants and control were digested and subjected to Southern blot analysis to confirm the integration of T-DNA into the plants. Leaf samples were collected from 48 T_0 and T_1 plants representing six single embryo-derived callus lines for the hybrid Pioneer 8505 and the inbred line C401. All transformants showed the integration of the *sgfp* gene, *pmi* gene and the maize promoter *ubi1* in their genome. To test the independence of DNA integration events and the number of integrated DNA copies, plant DNA samples were digested by the restriction enzyme *Bam*HI or *Hin*dIII and were probed with the *sgfp* coding region. Different integration patterns of T-DNA and insertion numbers were found in T_0 and T_1 plants (Figure 4). There was complete correspondence between positive GFP and Southern blot showing the *pmi* gene; 33.3% had a single copy and 66.7% had two or more copies. One callus line showed patterns of two insertion events. Two callus lines that produced more than 72 plants showed one to two copies of integrated T-DNA. One of these Southern blots is shown in Figure 4C, in which the DNA was digested by *Hin*dIII and probed with the *pmi* gene; a signal of a 3423-bp band was observed representing the integration of the complete PMI DNA sequence in transgenic plants. The non-transgenic control plants did not hybridize with the *pmi* coding region, confirming that the transformation process was successful.

Some GFP-positive T_0 and T_1 plants were analysed for *pmi* gene expression by Western blot; all plants showed the expected 45-kDa protein band. In contrast, no corresponding band was detected in the negative control, a seed-derived sorghum plant (Figure 5). The positive results demonstrated that the incorporated gene was actively transcribed in the transgenic plants and correlated well with the CPR analysis.

The high correlation between Southern and Western blot analyses and the precise correspondence between GFP expression and PMI assay strongly suggest that, either alone or in combination, GFP and PMI may be used effectively as the selection system for sorghum transformation.

Segregation in progeny

To confirm that the transgene was transmitted to the next generation, four T_0 plants representing two independent events were selfed and their T_1 seedlings were examined for GFP expression. Four transgenic families from single-copy or two-copy events showed the expected 3 : 1 Mendelian segregation ratio for insertion in a single locus (Table 3).

Table 3 Expression and segregation of green fluorescence protein (GFP) in T_1 progeny of transgenic sorghum

Transgenic line*	Observed†		Expected		χ^2	*P* value
	GFP$^+$	GFP$^-$	GFP$^+$	GFP$^-$		
P3-3	223	59	211.50	70.50	2.501	0.25–0.10
P3-4	530	129	494.25	164.75	10.343	0.005–0.001
C1-1	327	117	333.00	111.00	0.432	0.75–0.50
C1-2	129	42	128.25	42.75	0.017	0.90–0.80

*P, Pioneer 8505; C, C401.

†GFP expression was observed from germinating seed.

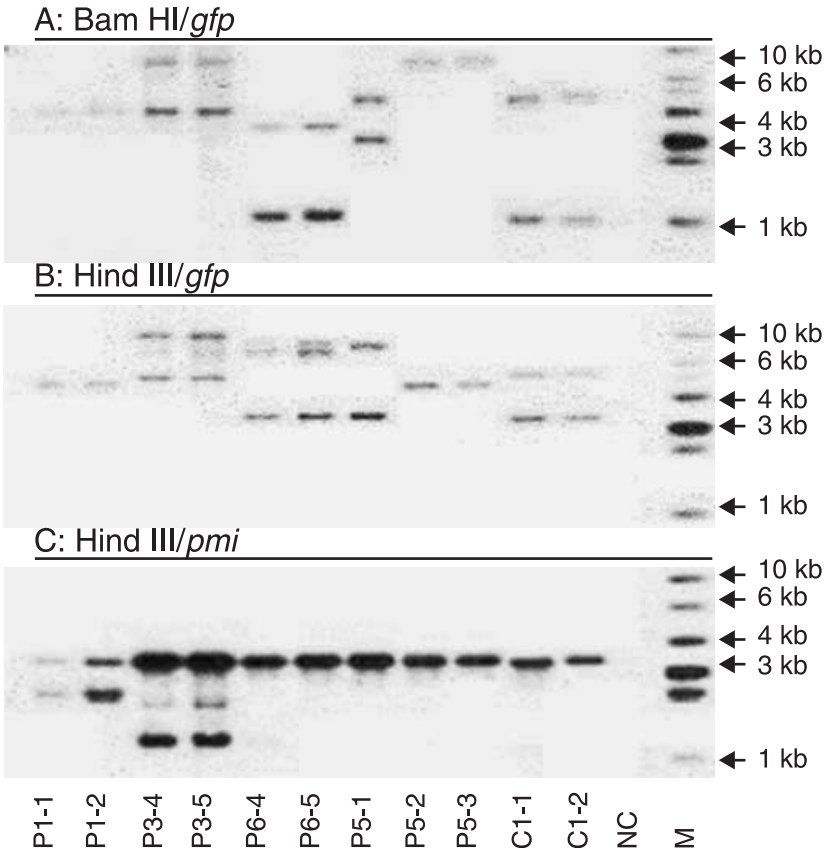

Figure 4 Southern blot analysis of molecular evidence of integration in transgenic plants: (A) plant DNA was digested by *Bam*HI and probed with the green fluorescence protein (GFP) coding region; (B, C) plant DNA was digested by *Hin*dIII and probed with GFP and the *Escherichia coli manA* coding region, respectively; NC, negative control DNA digested with corresponding enzyme in (A)–(C); M, 1-kb DNA ladder; P, P8505; C, C401.

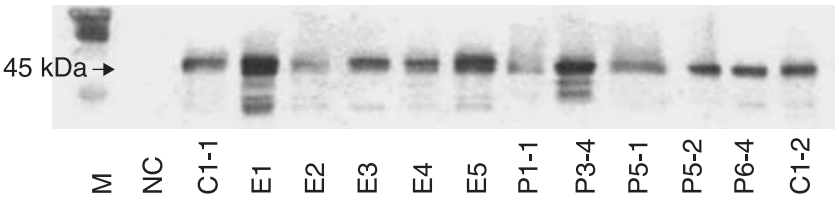

Figure 5 Western blot analysis of phosphomannose isomerase (PMI) expression in transgenic plants: M, molecular weight marker of RPN755; NC, negative control; C, T_0 plants of C401; P, T_0 plants of P8505; E, T_1 plants of C401.

Experimental procedures

Agrobacterium strain and vector

A. tumefaciens (strain EHA 101) carrying a binary vector pPZP201 was used for transformation. *Hin*dIII fragments carrying the *ubi*1 promoter-PMI cassette from pNOV 2819 were recovered and inserted into the unique *Hin*dIII site of the plasmid pPZP201-GFP (Hajdukiewicz *et al*., 1994). The plasmid pPZP201-GFP-PMI contained the *sgfp* gene encoding for GFP (Chiu *et al*., 1996) and the *pmi* gene encoding for PMI. Both genes were under the control of the maize ubiquitin *ubi*1 (Christensen *et al*., 1992) (Figure 6). The *pmi* gene came from Novartis (Syngenta, St Louis, MO) and we signed an agreement to use it for research purposes only. The *sgfp* expression matrix (*Ubi*-1::*sgfp*::*nos3*=) was constructed from the *sgfp* gene and was initially loaded into the T-DNA region of the binary vector pPZP201 to generate the plasmid pUGFP (*Ubi*-1::*sgfp*). To create the dual

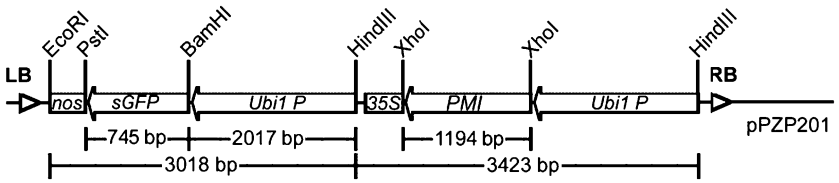

Figure 6 Plasmid construction map: RB, LB, T-DNA borders; *Ubi*, ubiquitin promoter; *35S* and *nos*, termination region; numbers in parentheses, physical distances in base pairs.

marker *manA-sgfp*, the *E. coli manA* expression cassette (*Ubi*-1::*manA*::*nos3*=) on the plasmid vector pRT104 was released by the *Hin*dIII restriction enzyme and subcloned upstream of the *sgfp* expression matrix in the plasmid pUGFP. The orientation of the insert was confirmed by digestion of the recombinant plasmid and gel analysis. The derived pPMI-GFP plasmid (Figure 6) was mobilized into the *A. tumefaciens* strain EHA 101 (Hood *et al*., 1986) by electroporation (Mozo and Hooykaas, 1991). Gene constructs and plasmids are available on request.

Plant material and transformation

Two sorghum genotypes were used: a commercial hybrid, Pioneer 8505, grown on a farm under irrigation in east Manhattan, KS, USA, and an inbred line, C401 (from China; available on request), grown in a glasshouse under a day/night temperature of 22/20 °C. Caryopses were collected from the plants at 10–14 days after anthesis, sterilized in 70% ethanol for 30 s and 12% Clorox (0.6% NaOCl) for 20 min, and then rinsed twice with sterilized distilled water. Seeds were aseptically dissected to remove and collect immature zygotic embryos.

A. tumefaciens (strain EHA 101; available on request) was grown overnight at 28 °C on Luria–Bertani (LB) medium supplemented with 50 mg/L kanamycin and 100 mg/L spectinomycin with shaking at 200 r.p.m. The bacterial cells were collected and suspended in Murashige & Skoog (MS) inoculation medium [4.4 g/L MS salts, 0.1 g/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1 mg/L thiamine HCl, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 68.5 g/L sucrose, 36 g/L glucose, pH 5.6; add 100 μM acetosyringone before using], diluted to A_{600} = 0.2 and suspended in 100 μM acetosyringone. The embryos were cultured with the scutellum side up and were subcultured once every 2 weeks. If the immature embryos produced phenolic pigments, as observed for Pioneer 8505, the subculture interval was reduced to 1 week and 1% polyvinylpyrrolidone (PVPP) (w/v) was added to the culture medium [MS salts, myo-inositol, vitamins and growth regulators as for MS inoculation medium, 20 g/L sucrose, 10 g/L glucose, 0.5 g/L 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer, 0.7 g/L L-proline, 10 mg/L ascorbic acid, 100 μM acetosyringone and 2.2 g/L phytagel, pH 5.8] (Zhao *et al*., 2000; Gao *et al*., 2005). After coculture, calli were moved to callus-inducing medium (MS salts, myo-inositol, vitamins and growth regulators as for MS inoculation medium, 30 g/L sucrose, 100 mg/L carbenicillin, 1% PVPP, 2.2 g/L phytagel, pH 5.8) containing 100 mg/L of carbenicillin to inhibit further growth of *Agrobacterium* during recovery. Selection was performed for 2 weeks on a medium containing 3.0% sucrose and 2.0% mannose, for 2 weeks on a medium containing 2.0% sucrose and 1.5% mannose and for a further 2 weeks or longer than 2 weeks on medium containing 1.5% sucrose plus 1.0% mannose (concentrations of 0.5%, 1.0% 1.5%, 2.0% and 3.0% mannose combined with 1.0%, 2.0% and 3.0% sucrose and 0.5%, 1.0% and 1.5% mannose alone were used in initial calli selection experiments); 1% mannose was also included in the regeneration and rooting medium. All selection media contained 100 mg/L of carbenicillin to eliminate *Agrobacterium* contamination. After 4–6 weeks of selection in darkness to produce embryogenic calli, these were transferred on to regeneration medium [MS salts, myo-inositol, vitamins as for MS inoculation medium, no 2,4-D, with 0.2 mg/L indole-3-acetic acid (IAA), 0.5 mg/L kinetin, 700 mg/L proline] under a light intensity of 85 mol/s/m^2 and a photoperiod of 16 h. The surviving embryogenic calli and regenerated shoots all showed green fluorescence under the fluorescence microscope. The rooting medium included 1/2 MS salts, 0.25 mg/L nicotinic acid, 0.25 mg/L pyridoxine HCl, 0.5 mg/L thiamine HCl, 0.05 g/L myo-inositol, 0.2 mg/L NAA (1-naphthaleneacetic acid), 0.2 mg/L IBA (Indole-3-butyric acid), 0.2 mg/L IAA, 100 mg/L carbenicillin, 2.2 g/L phytagel, pH 5.8. The plantlets showing green fluorescence and good root systems were transferred to sterile soil pots and grown in a glasshouse with a photoperiod of 16 h and a day/night temperature of 25/20 °C.

Visual screening of transgenic plants

Fluorescence visual screening of transgenics

Fluorescence visual screening was performed using an epifluorescent Nikon SMZ800 stereomicroscope with different filter sets suitable for the spectrum of 450–490 nm excitation of GFP. Expression of the *sgfp* gene was observed at 2–7 days after *A. tumefaciens* infection of the calli until the regeneration of plantlets. GFP expression was visible in all young tissues and organs with small amounts of chlorophyll, including calli, embryoids, shoots, roots, leaves, anthers, pollen, immature seed, mature seed and germinating seed. Because stronger autofluorescence was detected on the radicles and seedlings, the final identification of T_0 transgenic plantlets and the segregation in T_1 progeny were based on GFP expression of young roots and seedlings.

CPR assay

A CPR assay provides rapid visual screening of the transformed plants expressing the *pmi* gene (Kramer *et al*., 1993;

Wright *et al*., 1996; Haensch *et al*., 1998; Lucca *et al*., 2001; Wright *et al*., 2001). The transgenic tissue was able to metabolize the selection agent, mannose, to acidify the medium and change its colour from red to yellow, whereas the non-transgenics and those transgenics with a silenced *pmi* gene were not able to use the mannose, and the medium remained red. Young leaf tip pieces, approximately 0.5 cm in length, were taken from regenerated plantlets in sterile containers. The leaf tissue samples were immersed in a solution containing MS medium with either 2% mannose (sample wells) or 2% sucrose (control wells) at pH 5.8 in separate multiwell plates. After keeping the samples in darkness for 3–4 days, they were evaluated for colour change.

Molecular analysis

Western blot analysis

Protein extracts, sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis were carried out as described by Winston *et al*. (1987) and Chen *et al*. (1998). After electrophoresis, the proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a semidry blotting apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The membranes were blocked with 2.5% gelatin for 1 h and soaked overnight in the PMI bean and rabbit antibody diluted 1 : 4000 in 1 × TBST (10 mM Tris, pH 7.5, 140 mM NaCl and 0.5 mL/L Tween 20). The blots were washed with 1 × TBST three times for a period of 5 min each time. Affinity-purified goat anti-rabbit IgG(H + L) horseradish peroxidase (HRP) conjugate (Bio-Rad) was used as the second antibody at a dilution of 1 : 2000 in 1 × TBST. The blots were incubated in the second antibody for 2–4 h. Protein band complexes with the antibody were visualized using the HRP colour development reagent (4-chloro-1-naphthol) (Bio-Rad).

Southern blot analysis

Sorghum genomic DNA was isolated from the leaves of T_0 and T_1 plants using the SDS-DNA extraction method (Chen *et al*., 1998) and Southern blotting was performed using the standard procedure. Aliquots of 10 μg of total genomic DNA were digested with a restriction enzyme, *Hin*dIII or *Bam*HI, for *sgfp* integration analysis, and Southern blotting was performed using ^{32}P-labelled coding fragments of *ubi1* promoter/*pmi*/*sgfp* genes. To identify the transgenic events, 48 plants with GFP fluorescence were randomly sampled from nine independent callus lines and analysed.

Test of progeny

The self-pollinated progeny were germinated and grown for 3–7 days in the dark and were then examined for the presence or absence of GFP fluorescence under a fluorescence dissecting microscope. Seedlings expressing the *sgfp* gene and those that did not show the GFP protein were recorded to determine the segregation ratio of the transgene.

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