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GENETIC TRANSFORMATION AND HYBRIDIZATION

Biolistic-mediated genetic transformation of cowpea (*Vigna unguiculata*) and stable Mendelian inheritance of transgenes

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Abstract We describe a novel system of exploiting the biolistic process to generate stable transgenic cowpea (*Vigna unguiculata*) plants. The system is based on combining the use of the herbicide imazapyr to select transformed meristematic cells after physical introduction of the mutated *ahas* gene (coding for a mutated acetohydroxyacid synthase, under control of the *ahas* 5′ regulatory sequence) and a simple tissue culture protocol. The *gus* gene (under control of the *act2* promoter) was used as a reporter gene. The transformation frequency (defined as the total number of putative transgenic plants divided by the total number of embryonic axes bombarded) was 0.90%. Southern analyses showed the presence of both *ahas* and *gus* expression cassettes in all primary transgenic plants, and demonstrated one to three integrated copies of the transgenes into the genome. The progenies (first and second generations) of all self-fertilized transgenic lines revealed the presence of the transgenes (*gus* and *ahas*) co-segregated in a Mendelian fashion. Western blot analysis revealed that the GUS protein expressed in the transgenic plants had the same mass and isoelectric point as the bacterial native protein. This is the first report of biolistic-mediated cowpea transformation in which fertile transgenic plants transferred the foreign genes to next generations following Mendelian laws.

Keywords Gene expression · Gene transfer · Imazapyr · Transgene · Transgenic plant

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Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is widely grown in Africa, Latin America, Southeast Asia and southwestern regions of North America, with a world annual production of about 3.9 million metric tons (FAOSTAT 2006). For economic and cultural reasons, cowpea plays an important role in human nutrition since it is a rich source of protein, calories, certain minerals and vitamins (Obatolu 2003; Phillips et al. 2003).

Cowpea is highly susceptible to many viral diseases, such as Cowpea aphid borne mosaic virus, Cowpea severe mosaic virus, Blackeye cowpea mosaic virus and Cowpea yellow mosaic virus, and it is the host for a wide range of insect pests, notably flower bud thrips, pod borers, leaf beetles, pod suckers, aphids and leaf hoppers, which seriously limits the realization of its yield potential, estimated at 1.5–3.0 ton ha^{-1} (Murdock 1992; Aliyu 2007; Taiwo et al. 2007). Durable and adequate levels of resistance to these viruses and pests are lacking in the primary gene pool, but are available in distant wild species, which present barriers for gene transfer through conventional crossing techniques (Gomathinayagam et al. 1998). In addition, limited genetic diversity in cowpea breeding programs is of special concern because cowpea appears to have lower inherent genetic diversity than other cultivated crops as a result of a hypothesized single domestication event (Fang et al. 2007). Consequently, the transfer of virus

and insect resistance genes by genetic engineering could potentially aid plant breeders in overcoming these constraints.

During the last two decades, several investigations have attempted to develop efficient systems for the introduction of exogenous genes into the cowpea genome. However, the protocols published to date have failed to produce a system that is both simple to execute and variety independent. Garcia et al. (1986, 1987) were the first to investigate whether cowpea was susceptible to *Agrobacterium tumefaciens* infection and demonstrated that foreign genes could be stably introduced and expressed in cowpea cells. Although transgenic kanamycin-resistant calli were obtained, no plants could be regenerated. Penza et al. (1991) used mature cowpea embryos as a target for genetic transformation by *A. tumefaciens* and were able to regenerate putatively transgenic plants. However, genetic evidence of transgene integration was not presented. The first production of transgenic cowpea plants was reported by Muthukumar et al. (1996). These authors co-cultivated detached cotyledonary explants with *A. tumefaciens*, followed by transfer of the explants to a selective medium, and recovered hygromycin-resistant shoots that grew to maturity and set seed. Nevertheless, none of the seeds germinated and no evidence of transgene transmission to the progeny was obtained. Recently, two *Agrabacterium*-mediated transformation systems have been developed. Popelka et al. (2006) obtained transgenic plants with a frequency of transformation of 0.15%. Transgenic lines transmitted the transgene (*bar* gene) to their progenies following Mendelian laws (Popelka et al. 2006). Chaudhury et al. (2007) used cotyledonary node explants to generate fertile transgenic plants with an efficiency of 0.76%. Transgenic plants were selected with kanamycin and they inherited the transgenes in Mendelian fashion in the first generation. Nevertheless, these transformation systems are laborious, time consuming, likely to be genotype-dependent and present low frequency of germ line transformation.

Methods of cowpea transformation via direct DNA delivery have also been reported. The transient expression of reporter genes in cowpea seedlings following the electroporation of zygotic embryos with plasmid DNA harboring the chimeric *gus* gene has been demonstrated (Penza et al. 1992; Akella and Lurquin 1993), but so far no stably transformed plants have been obtained. Ikea et al. (2003) were able to generate transgenic cowpea plants after particle bombardment of embryonic axes. However, the transgenes were transmitted to only a small proportion of the progeny and no evidence for stable integration was presented. In addition, the tissue culture protocol used was time consuming, involving several treatments and medium transfers of the bombarded embryos, prior to achieving putative transgenic plantlets.

Despite all efforts, the genetic manipulation of cowpea is still not trivial and regeneration and transformation systems need to be improved (Somers et al. 2003; Aragão and Campos 2007). Here we describe a novel system for biolistic-mediated genetic transformation of cowpea, which followed a transgene transmission through two generations. This system is based on the introduction of a mutant *ahas* gene (coding for acetolactate synthase) and selection with imazapyr, a herbicidal molecule that is capable of systemically translocating and concentrating in the apical meristematic region of the plant. We believe that this new technology should facilitate detailed genomics studies, as well as the development of transgenic cowpea varieties with improved agricultural characteristics.

Materials and methods

Electron microscopy

The apical region morphology of cultivars Paraguaçu, Gurguéia, MNC99-5417-8, Guariba, Vita 7, Pitiúba, Rouxinol, CE-315, CE-11 and MNC01-623-51 was studied under the scanning electron microscope. Embryonic axes were removed from seeds that had been soaked in distilled water for 16 h. Fifty embryonic axes for each cultivar were prepared as described by (Aragão and Rech 1997) and observed in a Zeiss DSM 962 SEM operating at 15 kV.

Determination of imazapyr selection dosage

Hand-harvested mature seeds (cv Paraguaçu, Pitiúba, Rouxinol and CE-11) were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min and then rinsed three times in sterile distilled water. The seeds were then soaked in distilled water for 18–20 h. The embryonic axes were excised from seeds, and the shoot apical meristems were exposed by removing the primary leaves and leaf primordia under a stereomicroscope. Explants were transferred to MS medium containing 3% glucose, 5 mg L^{-1} benzylaminopurine (BAP), 0.6% agar and 0, 50, 100, 150, 200, 300 or 400 n*M* imazapyr (2-[4.5-dihydro-4-methylethyl)-5-oxo-1H-imidazol-2-yl]-3-pyridinecarboxylic acid). The pH was adjusted to 5.7, prior to autoclaving. After 3 weeks, explants were analyzed for the presence of elongated shoots (3 cm in length). Each treatment containing ten embryonic axes was performed in triplicate. The experiment was repeated twice.

Cowpea transformation

Embryonic axes (cv. CE-11) were prepared as described above and positioned in the bombardment medium (MS basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7) with the shoot apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium. The bombardment was conducted as previously described (Aragão et al. 2000; Rech et al. 2008) with the plasmid pAG1, which contains the mutated *ahas* gene from *Arabidopsis thaliana* (Rech et al. 2008) and the *gus* gene under control of the *act2* promoter (*act2p*) (Fig. 1).

After bombardment, the embryonic axes were transferred to the selection and shoot induction medium (MS basal salts medium, supplemented with 5 mg L^{-1} BAP, 3% sucrose, 200 n*M* imazapyr and 0.6% agar, Sigma, pH 5.7) in baby-food jars containing 15 ml of the culture medium, cultured at 26°C in the dark for 2 days, and then transferred to light with a 16-h photoperiod (50 µmol m^{-2} s^{-1}). As soon as the shoots derived from the embryonic axes were 2–3 cm in length, a 1-mm-long section was removed from each leaf for analysis of GUS (*β*-glucoronidase) expression (Jefferson 1987). GUS-positive explants were transferred to MS medium supplemented with 3% sucrose, 0.6% agar and 3 g L^{-1} activated charcoal for rooting. Rooted explants were individually transferred to a plastic pot containing 0.2 dm^3 of an autoclaved mixture of fertilized soil:vermiculite (1:1), covered with a transparent plastic bag sealed with a rubber band and maintained in a greenhouse. After 1 week, the rubber band was removed. After an additional week, the plastic bag was also removed. As soon as the acclimatized plantlets reached approximately 10 cm in length, they were transferred to a pot containing 5 dm^3 of fertilized soil and allowed to set seeds.

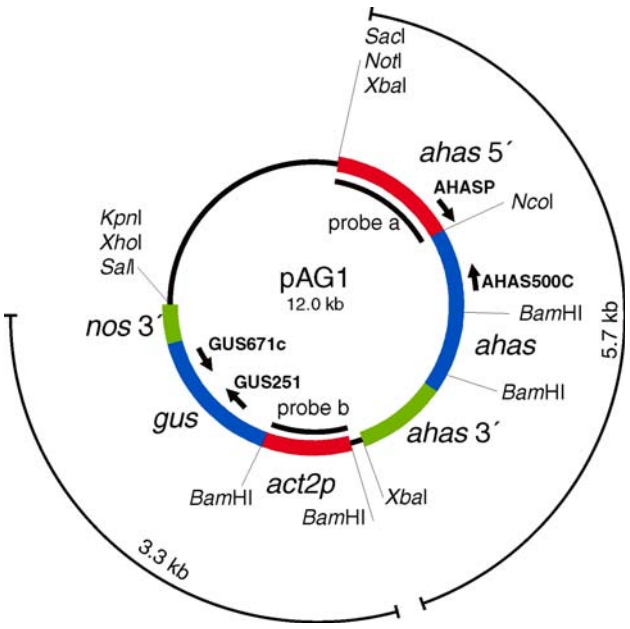

Fig. 1 Diagram of the plasmid vector pAG1 used for cowpea transformation. *Small arrows* indicate primers used for PCR screening. *Solid bars* represent probes used for Southern blot analysis

Screening of transgenic plants by PCR

DNA was isolated from leaf disks according to Edwards et al. (1991). Each PCR reaction was carried out in 25 µl aliquots containing 10 mM TRIS–HCl (pH 8.4), 50 mM KCl, 2 mM MgCl2, 160 µM of each dNTP, 200 n*M* of each primer, 2 U of *Taq* polymerase (Phoneutria, Belo Horizonte, Brazil) and 20–30 ng of genomic DNA. The primers AHASP (5′-ACTAGAGATTCCAGCGTCAC-3′, within the *ahas* promoter) and AHAS500C (5′-GTGGCTATACAGATACCTGG-3′, within the *ahas* coding sequence) were utilized to amplify a 685 bp sequence. The primers GUS251 (5′-TTGGGCAGGCCAGCGTATCGT-3′) and GUS671c (5′-ATCACGCAGTTCAACGCTGAC-3′) were utilized to amplify a 420 bp sequence. The mixture was overlaid with mineral oil, denatured for 5 min at 95°C in an MJ thermal cycler (USA) and amplified for 35 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) with a final cycle of 7 min at 72°C. The reaction mixture was then loaded onto 1% agarose gel and visualized under UV light following ethidium bromide staining.

Southern blot analysis

Genomic DNA was isolated using the DNeasy Plant Mini Kit (Qiagen). Southern blotting was carried out as described by Sambrook and Russell (2001). Genomic DNA (15 µg) was digested with *Nco*I or *Xba*I, separated on 1% agarose gel and transferred to a nylon membrane (Hybond N+, Amershan Pharmacia Biotech). Hybridization was carried out using the 5′ region of the *ahas* gene (probe a) or the *act2* promoter (probe b), labeled with $^{\alpha}$32P dCTP (1.13×10^{14} Bq mol^{-1}) using a random primer DNA labeling kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The bands were visualized with a fluorescent image analyzer (FLA-3000) (FUJIFILM).

Protein extraction, two-dimensional electrophoresis and Immunoblotting analysis

Mature leaves from transformed and control plants were freeze-dried and a fine powder was obtained by grinding in a coffee mill and sieving through a 100-mesh metal sieve. Protein extraction was performed as described by Vasconcelos et al. (2005), and the evaluation of protein content in the extracts was assessed by the Bradford method (Bradford 1976). For two-dimensional electrophoresis, 11-centimeter Immobiline DryStrips, pH 4–7

(Amersham) were rehydrated overnight with rehydration buffer (7 M urea, 2 M thiourea, 1% triton X-100, 0.5% Pharmalyte 3–10, 65 mM DTT) containing the protein extract. Running was performed in a Multiphor II IEF system from Amersham Pharmacia Biotech. Electrical conditions were as described by the supplier. After the first-dimensional run, the IPG gel strips were sealed in plastic wrap and frozen at −80°C or incubated at room temperature to equilibrate the strips in 3 ml of equilibration buffer (50 mM Tris, 30%, 6 M urea, 2% SDS and traces of Bromophenol blue) containing 57.8 mg of DTT, prior to separation in the second dimension. The second dimension electrophoresis was performed in a vertical system with a uniform 15% separating gel (14 × 14 cm), at 25°C. After 2-DE, the proteins were transferred to a nitrocellulose membrane using a TE Series Transfor Electrophoresis Unit (Hoeffer Scientific Instruments, San Francisco, CA, USA). This membrane was probed with polyclonal antibodies raised against a synthetic peptide corresponding to amino acids 589–603 at the C-terminus of *E. coli* GUS, conjugated to KLH [produced by Sigma Aldrich (Product number G5545)], and detected with alkaline phosphatase-conjugated anti-goat IgG.

Progeny analysis

The analysis of the R1 generation was carried out by amplifying the introduced foreign *ahas* gene by PCR and by the GUS histochemical assay analysis of leaves of self-pollinated plants. The GUS histochemical assay was performed as described by Jefferson (1987). Chi-square (χ^2) analyses (Steel and Torrie 1980) were performed to determine if the observed segregation ratio was consistent with a Mendelian ratio in the R_1 generation.

Results

The morphology of the vegetative shoot of ten cowpea cultivars was analyzed. All revealed the apical meristematic region to be almost completely covered by the leaf primordia. Only a small area of the central region could be visualized (Fig. 2). Consequently, it was necessary to remove the primary and primordial leaves in order to expose the meristematic area to the DNA-coated microparticle (Fig. 2).

In order to evaluate the effect of imazapyr on shoot formation and elongation, a dose-response curve was calculated. We observed that imazapyr inhibited shoot elongation in a dose-dependent manner up to approximately 200 nM, when shoot elongation was completely suppressed in all of the cultivars which were tested (cv Paraguaçu, Pitiúba, Rouxinol and CE-11) (Fig. 3). In concentrations of 300 nM or 400 nM no elongated shoots were observed (data not shown).

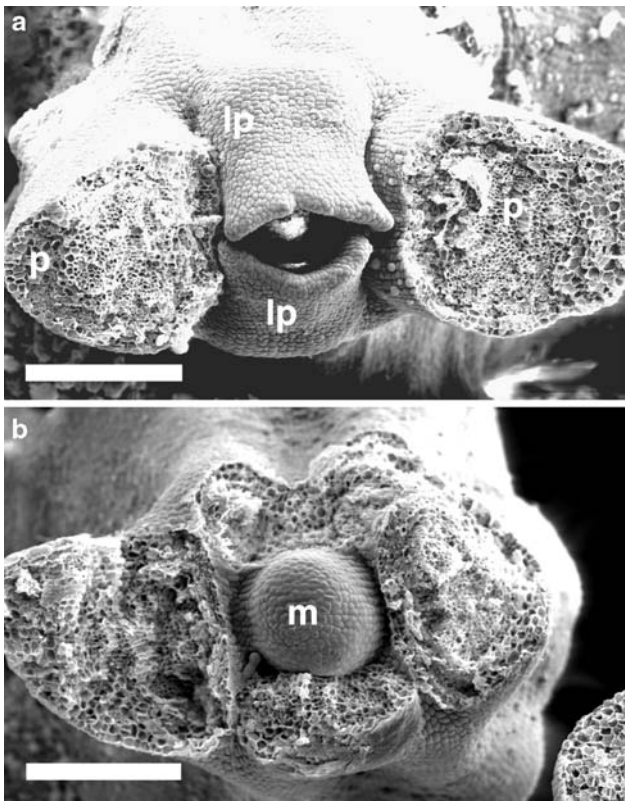

Fig. 2 Scanning electron micrographs showing the morphology of the cowpea embryonic apex. **a** The apical region after the removal of the primary leaves (*p*). **b** The leaf primordia (*lp*) are removed to expose the apical meristematic region (*m*). *Bars* represent 200 μm

Apical regions of cowpea embryonic axes (cv. CE-11) were bombarded with the plasmid pAG1 (Fig. 1). Nine independent bombardment experiments were carried out, in which 552 embryonic axes were bombarded. Following bombardment, embryonic axes were cultured in selection and multiple shoot induction medium. Twenty-four hours after bombardment, the majority of the embryonic axes analyzed (ca. 20) showed extensive *gus* gene expression within the apical region (Fig. 4). After 2 weeks in culture under selection with 200 nM imazapyr, elongated shoots (about 2 cm long) were observed. Typically, a total of 3–4 shoots were induced from each bombarded embryonic axis, but only 2% elongated. Out of 13 elongated shoots, PCR and GUS histochemical analyses confirmed the presence of both *ahas* and *gus* transgenes in five elongated shoots, generated from independent explants. About 61% of elongated shoots were escapes. However, no escapes were observed under higher selection pressure (≥300 nM imazapyr), but the number of plants obtained was much lower and no transgenic plants were observed (data not shown). The transformation frequency (defined as the total number

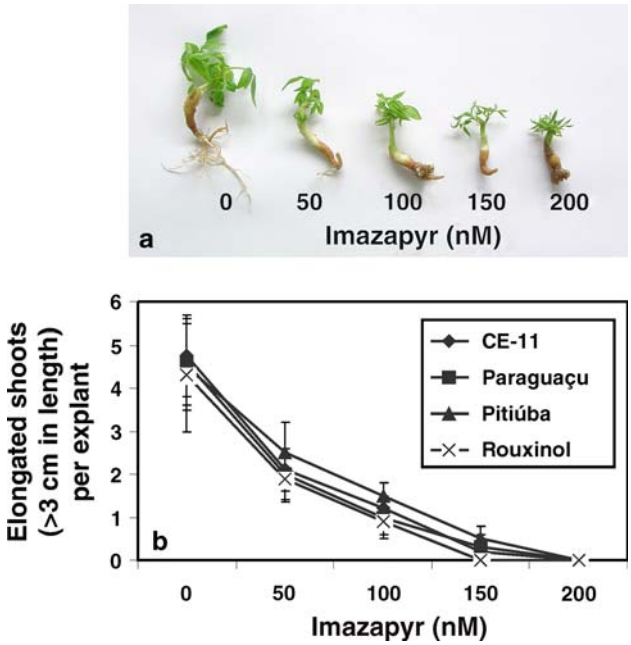

Fig. 3 Effect of imazapyr on shoot formation and elongation in the apical region of cowpea embryonic axes, after 3 weeks cultivation on MS medium containing 3% glucose and 5 mg L^{-1}1 BAP. **a** Shoot formation and elongation suppression observed for cv. CE-11. **b** Effect of imazapyr on shoot survival and elongation for cv. Paraguaçu, Pitiúba, Rouxinol and CE-11. Each *point* represents the mean of $n = 90$ samples, *error bars* represent SEM

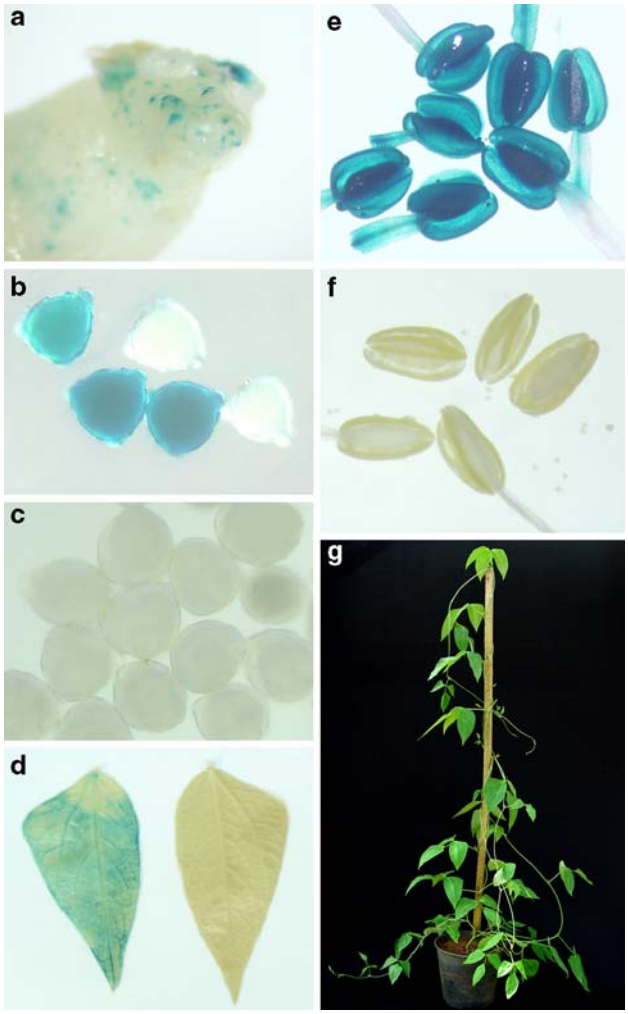

Fig. 4 Cowpea transformation. *a* Embryonic axis expressing the *gus* gene in the meristematic region 24 h after bombardment; **b** microspores from a transgenic line showing *gus* gene segregation; **c** microspores from a non-transgenic line; **d** leaves from control (*right*) and transgenic (*left*) plants expressing the *gus* gene; **e** anthers from transgenic and **f** non-transgenic plant; **g** a primary transgenic plant after acclimatization

of putative transgenic plants divided by the total number of embryonic axes bombarded) was 0.90%. All plantlets that developed vigorous roots were acclimatized and transferred to soil. Histochemical GUS assays performed on leaf and floral tissues from all transgenic plants revealed intense *β*-glucuronidase activity (Fig. 4).

Southern blot analyses of genomic DNA isolated from the T_0 generation transgenic cowpea plants were carried out to evaluate the integration of the introduced *ahas* and *gus* cassettes. Southern analyses with the genomic DNA digested with *Xba*I followed by hybridization to *ahas*5′ fragment (probe a) showed the presence of a complete *ahas* cassette (denoted by the 5.7 kb fragment) in all T_0 generation lines analyzed (Fig. 5). Since the plasmid pAG1 has a unique *Nco*I restriction site (Fig. 1), Southern analysis carried out with genomic DNA digested with *Nco*I followed by hybridization to probe 'a' allowed us to estimate that the copy number of *ahas* cassette was one for lines 5.1, 5.2 and 5.5, three for line 5.3 and two for line 5.4 (Fig. 5). Additionally, Southern analyses was carried out with the genomic DNA digested with *Xba*I and *Nco*I, and probed with the *act2p* fragment (probe b). Results showed that line 5.3 presented one incomplete *gus* expression cassette because a fragment smaller than 3.3 kb was observed (which is the size of the *gus* cassette) for the DNA digested with *Xba*I (Fig. 5). Additionally, the fact that only two bands were observed for line 5.3 and only one band was found for line 5.4, suggests that these lines have only one functional *gus* cassette (Fig. 5). DNA isolated from non-transformed plants did not hybridize with the 5′*ahas* probe (Fig. 5).

A Western blot analysis was carried out with line 5.5 to detect the GUS protein within the total proteins isolated from leaf and fractionated by 2D gel electrophoresis. The results (Fig. 6) showed the presence of a protein that reacts with the GUS antibodies. This protein has the same molecular mass and isoelectric point as that of the bacterial GUS protein (Fig. 6). Non-transgenic plants presented no signal.

The progeny of the five self-fertilized transgenic plants were screened by GUS histochemical assay and PCR

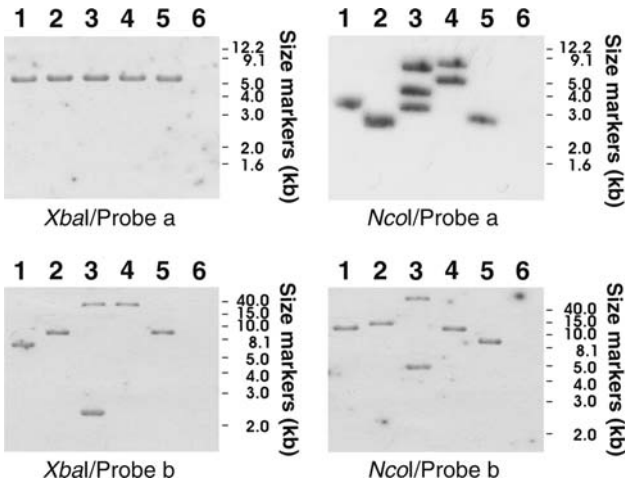

Fig. 5 Southern blot analysis of putative transformed lines (R_0 generation). Genomic DNAs were digested with *Xba*I or *Nco*I, transferred to a nylon membrane and hybridized with probe 'a' or probe 'b'. *Lanes 1–5*: Independently transformed lines (line 5.1, line 5.2, line 5.3, line 5.4, line 5.5, respectively). *Lane 6* Non-transformed plant. Molecular size markers are indicated on the *right*

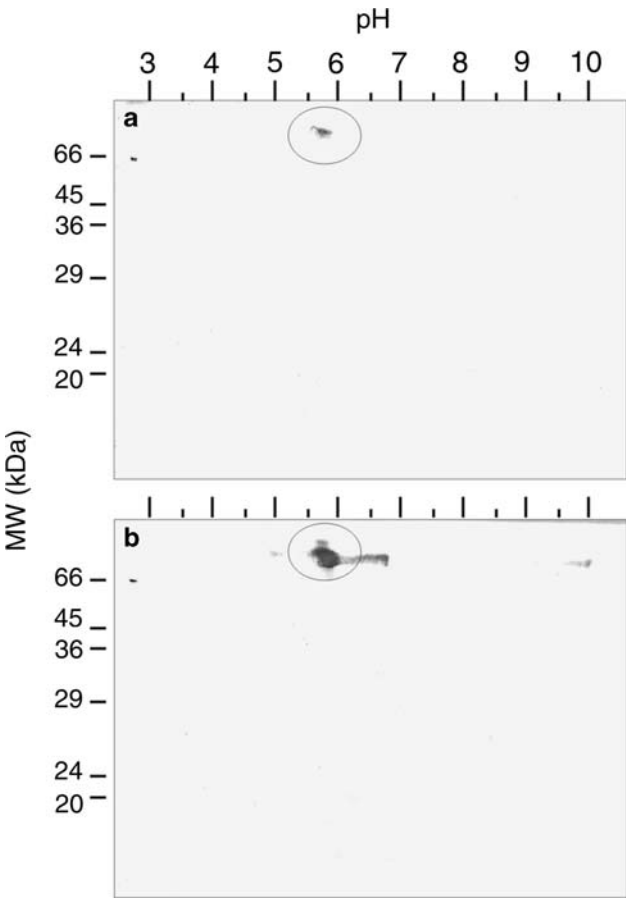

Fig. 6 Western blot analysis of proteins isolated from leaves of transgenic cowpea plants. Total proteins from transgenic plants (**a**) were fractionated by 2D-electrophoresis and transferred to PVDF membranes; after transfer, membranes were incubated with polyclonal antibodies raised against a synthetic peptide corresponding to amino acids 589–603 at the C-terminus of *E. coli* GUS protein, followed by incubation with secondary antibodies labeled with alkaline phosphatase. A western blot analysis of a GUS preparation purchased from Sigma-Aldrich is shown in (**b**)

analysis for the presence of the *gus* and *ahas* transgenes (Fig. 7). Data revealed that both *gus* and *ahas* genes co-segregated in a Mendelian ratio in all transgenic lines. Southern blot analysis confirmed the presence of the *ahas* expression cassette in the T_1 generation (Fig. 7). Four lines presented a ratio of 3:1 (102 positives: 30 negatives, χ^2 = 0.36, P = 0.54, df = 1, for line 5.1; 233 positives: 76 negatives, χ^2 = 0.01, P = 0.86, df = 1, for line 5.2; 167 positives: 51 negatives, χ^2 = 0.29, P = 0.58, df = 1, for line 5.4, and 345 positives: 123 negatives, χ^2 = 0.41, P = 0.52, df = 1, for line 5.5). One line presented a ratio of 15:1 (243 positives: 17 negatives, χ^2 = 0.04, P = 0.84, df = 1, for line 5.3). Transgenic seeds from T_1 generation were sown and plants were grown to maturity to collect T_2 seeds. T_2 generation exhibited a segregation of 1:2:1 with a homozygous sub-line (data not shown).

In order to verify the presence of chimerism in primary transgenic plants, the position and distribution of the T_1 seeds was recorded. Transgenic seeds were randomly distributed in the whole plants and were found in all pods (Fig. 8).

Discussion

We have developed a novel system to obtain fertile transgenic cowpea plants, which are able to transfer the transgenes in a stable manner to the following generations. It is not time consuming and requires 7–9 months from explant preparation to harvested seeds from the first generation. The system is based on the bombardment of DNA-coated microparticles to introduce foreign genes into apical meristematic cells and selection of the transgenic cells with a herbicide molecule. The herbicide molecule belongs to the imidazolinone class (imazapyr), which is capable of systemically translocating and concentrating in the apical meristematic region of the plant. This selection, coupled with a multiple shooting induction strategy, allowed for the recovery of transgenic cowpea lines at a frequency of germ line transformation that is about sixfold higher than that obtained by Popelka et al. (2006), using an *Agrobacterium*-mediated transformation protocol. Moreover, in our protocol, all explants with elongated shoots were rooted on hormone-free medium. When compared with the *Agrobacterium*-mediated protocol described by Chaudhury et al. (2007), the system described here gives a slightly higher frequency of transformation. However, our system is based on a simpler tissue culture protocol that does not require

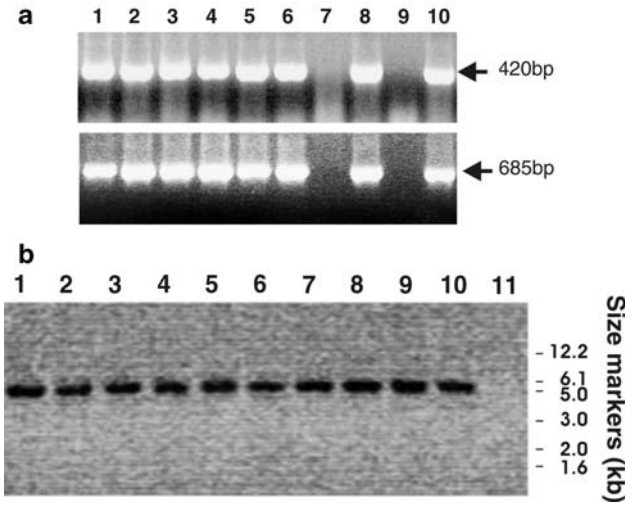

Fig. 7 **a** PCR analysis of cowpea plants from the T_1 generation. *Lanes 1–6* and *8* Transformed plants. *Lane 7* Non-transformed segregating plant. *Lane 9* non-transformed plant (negative control). *Lane 10* positive control (plasmid pAG1). *Arrows* indicate the expected fragments from *gus* (*upper lanes*) and *ahas* (*lower lanes*) genes. **b** Southern blot analysis of transformed lines in the R_1 generation. Genomic DNA was digested with *Xba*I, transferred to a nylon membrane and probed with the *ahas* promoter (probe a). *Lanes 1* and *2* Plants from line 5.1; *Lanes 3* and *4* plants from line 5.2; *Lanes 5* and *6* Plants from line 5.3; *Lanes 7* and *8* plants from line 5.4; *Lanes 9* and *10* Plants from line 5.5). *Lane 11* Non-transformed plant. Molecular size markers are indicated on the *right*

several cultivation steps as in the existent systems (Ikea et al. 2003; Popelka et al. 2006; Chaudhury et al. 2007) and utilizes mature seeds that do not require elaborate treatments. Moreover, although only the cultivar CE-11 was used for transformation, there is nothing intrinsic to the protocol that would limit its application to other cowpea varieties. In principle, it could be utilized with any cultivar for which a multiple shooting induction has been optimized. Indeed, similar systems have proved their use for several cultivars of soybean, common bean and cotton (Aragão et al. 2000, 2005; Rech et al. 2008). However, to test this hypothesis, other cowpea cultivars should be studied further.

Although no comparative studies have been carried out, it seems that selection with imazapir was more efficient that with other selective agents previously used for cowpea transformation, such as phosphinothricin, kanamycin, geneticin and hygromycin (Penza et al. 1991; Ikea et al. 2003; Popelka et al. 2006). About 38% of elongated shoots were not transgenic. However, when imazapyr concentration was increased to reduce the number of escapes, no elongated shoots were obtained or the number of transgenic plants was drastically reduced. Similar results were observed for soybean, pea and lentil under selection with herbicide-selective molecules (Aragão et al. 2000; Polowick et al. 2000, Gulati et al. 2002).

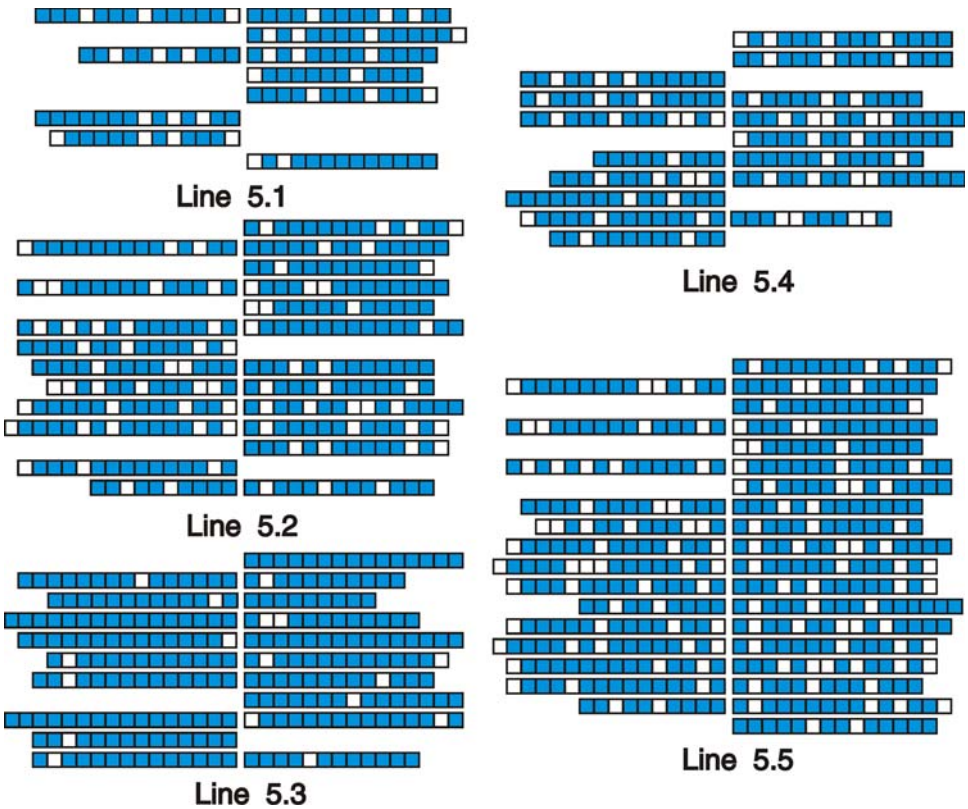

Fig. 8 Schematic representation of the distribution of transgenic (*solid boxes*) and non-transgenic (*white boxes*) plants (T1 generation) in the transgenic mother plant. The *small boxes* represent the seeds and a group of *boxes* represents a pod positioned in the plant

Since our transformation system is based on the bombardment of the apical meristem, it is mandatory to have the meristematic cells exposed to the accelerated DNA-coated microparticles. In all of the embryonic axes of the cultivars analyzed, the apical meristematic region was almost completely covered by the leaf primordia, reducing drastically the number of cells that could be reached by the microparticles, thus impairing the efficiency of transformation. We have faced a similar situation with the common bean, but it was possible to circumvent this drawback by identifying varieties, which had more suitable shoot apex morphologies (Aragão and Rech 1997, 1996). In the present study, as we were unable to find a cowpea cultivar displaying such characteristics, we removed both primary and primordial leaves under a light stereomicroscope to increase the exposure of the shoot apical meristem. However, in order to avoid manipulations such as those reported, we consider it necessary to perform a wider screening in the cowpea germoplasm, to identify a cultivar with a shoot apical meristem region that is more suitable for microparticle bombardment experiments.

The integration of the *ahas* and *gus* gene expression cassettes in the cowpea genome was confirmed by Southern blot analysis, which also indicated that most of the transgenic plants harbored a low number of copies of the transgenes. Fragments smaller than 12.0 kb were observed in the Southern analysis with genomic DNA digested with *Nco*I and hybridized with the *ahas*5′ region. This suggests that for most transgenic lines the circularized vector was broken in the region between the sites for *Kpn*I and *Sac*I during integration. Although the vector is expected to randomly break for integration, it could be explained by the fact that transgenic lines were selected based on concomitant tolerance to imazapyr and *gus* gene expression. Consequently, lines presenting only the *ahas* or *gus* cassette would be eliminated during plant selection. Further analysis, involving the sequencing of the complete integrated *loci* and probing for different regions, would provide more detailed information on the transgene integration and organization of target sites in the cowpea genome.

Progeny analysis of self-pollinated T_1 generation transgenic plants revealed that the segregation occurred according to the laws of Mendel, in a ratio of 3:1 or 15:1. These data are in agreement with the Southern blot analysis. Lines 5.1, 5.2 and 5.5, which exhibited a segregation ratio of 3:1, presented only one copy of the *ahas* transgene integrated into the plant genome. Line 5.3, which presented three copies of the transgenes, showed a segregation ratio of 15:1, suggesting that the copies are distributed in two *loci*.

In order to verify the presence of chimerism in the primary transgenic plants, the distribution of the T_1 seeds was recorded. Transgenic seeds were randomly distributed in all the plants and were present in all pods. This suggests that primary transgenic cowpea lines were not chimeric. We have previously showed that selection with imazapyr would reduce the possibility of chimerism in soybean and cotton plants (Aragão et al. 2000, 2005; Rech et al. 2008).

Biotechnological tools to complement traditional breeding may facilitate the generation of new varieties carrying genes with desired agronomic traits that are difficult to find in primary or secondary genepools. We have established a higher-frequency genetic transformation system for cowpea that will be of particular importance for functional genomic studies as well as for the practical application of genetic engineering to introduce important traits such as pest resistance into this important legume.

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