Development of an Improved System to Transform Cassava

W. Msikita; D. Siritunga; R.T. Sayre. Department of Plant Cellular and Molecular Biology[3], Ohio State University, Columbus, 43210: Phone (614)292-8379; e-mail, msikita.1@osu.edu

Abstract

The development of a reliable methodology for genetic transformation of cassava is essential to the introduction of new traits into the crop. The current transformation protocols for cassava are long (about six months), amenable to somaclonal variation, and inefficient due to low integration of genes of interest into the genome. This study was undertaken to develop a somatic embyogenic-based system to transform cassava. Undifferentiated calli pieces and geminating somatic embryos for cassava cultivar M. col 2215 were inoculated with *Agrobacterium tumefaciens* plasmid carrying two antisense gene constructs targeted to suppress components of the starch biosynthetic pathway. Explants and the *Agrobacterium* containing the a binary T-DNA vector were co-cultured for forty-eight hours under continuous light on a shaker, and in amended shoot regeneration medium Following inoculation explants were incubated in the dark (1, 2, or 3 weeks) or transferred directly to a 12-hour light regime. Plants were regenerated following successive transfers of inoculated explants onto shoot induction, shoot regeneration, and shoot multiplication media. RT-PCR analyses of twelve putative transformants showed integration of the two genes of interest in all putants, but complete suppression of targeted genes in only four of the twelve putants. Results obtained are discussed with a view to developing a robust transformation system for cassava.

Introduction

Development of a reliable and genotype-independent methodology for the transformation of cassava is particularly important because of the crop's agricultural importance in many developing countries. Cassava has been transformed both by *Agrobacterium* and particle gun bombardment methods. Using particle bombardment methodology, DNA is delivered in multi-copies and with very low efficiency to the cells as compared to *Agrobacterium*-mediated transformation (Bidney et. al. 1992). Overall the efficiency of cassava transformation has remained very low (3-5% as compared to 10% in other agronomic crops - Raemekkers *et al.*, 1997). Cassava transformation is also long and amenable to somaclonal variation. There is need to develop a rapid, efficient and robust transformation system for cassava. The overall goal of this study was to improve cassava transformation protocol by modifying the inoculation protocol.

Materials and Methods

(i) Transformation

The transformation protocol is summarized in Figure 1. In Figure 2 the types of explant tissues used in transformation are shown. The media composition used in co-culturing *Agrobacterium* with the explant types and cassava tissue regeneration is shown in Table 1. Explants and the bacterial plasmid were co-cultured for forty-eight hours under continuous light on a shaker, and in amended shoot regeneration medium comprising 50% Murashige and Skoog basal salts, yeast extract (0.4 gm/L), sodium chloride (0.1 gm/L), glucose (1%, w/v), galactose (1%w/v), benzylaminopurine (1 mg/L), gibberellic acid (10 mg/L), thiamine-HCl (10 mg/l) and myo-inositol (100 mg/L), and pH adjusted to 5.5. Following inoculation explants were incubated in the dark (1, 2, or 3 weeks) or transferred directly to a 12-hour light regime. Plants were regenerated following successive transfers of inoculated explants onto shoot induction, shoot regeneration, and shoot multiplication media.

(ii) Transgene analysis

RNA was extracted from two leaves (100 mg) using the Invitrogen RNA kit method. RT-PCR amplifications were carried with primers corresponding to the 5' and 3' ends of genes Y and Z. The cDNA were synthesized according to the Supercript First Strand Synthesis® from Invitrogen. Verification of the success of the RT-PCR reaction was confirmed by amplification of CYP79D1 gene transcripts. RT-PCR experiments were carried out without cDNA (negative control), a positive control (PCR of plasmid DNA), and verification of the success of the RT-PCR reaction was confirmed by amplification of the CYP79D1 gene that encodes a cytochrome P450 enzyme which mediates linamarin synthesis (Anderssen et al., 2000).

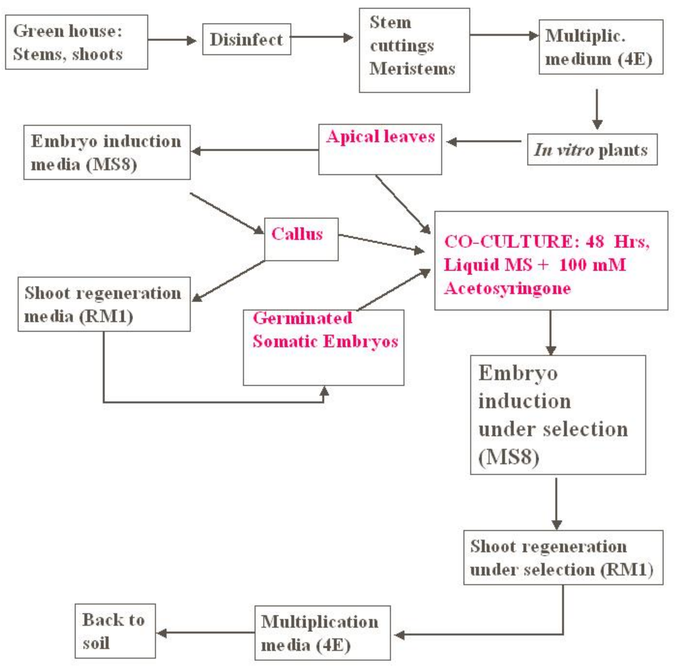

Figure 1. Scheme of cassava transformation.

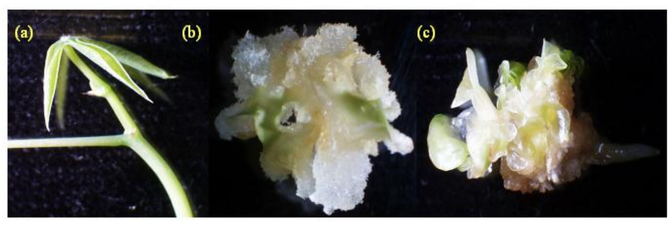

Figure 2. Types of explants used in cassava transformation: (a) apical leaf (b) undifferentiated callus (c) germinating somatic embyos

Table 1. Composition of different types of media used in cassava transformation

MS8 plates (500ml)

MS salts	2.15g
Sucrose	10g
2,4-D (0.5mg/ml)	8ml
B5 vitamins (100X)	5ml
$CuSO_4$ (1mM)	0.94ml
Casein	25mg

pH 5.7 with 1N KOH

RM1 plates (500ml)

MS salts	2.15g
Sucrose	10g
BAP (100ppm)	0.5mg
GA (100ppm)	5mg
Thiamine (100ppm)	5 mg
Myo-inositol (8000ppm)	50 mg

pH 5.7 with 1N KOH

YM (500 ml)

Yeast extract ----------0.2 gm
Mannitol ----------5 gm
NaCl ---------- 0.05 gm
$MgSO_4..7H_2O$ ---------0.1 gm
$K_2HPO_4.3H_2O$ -------0.25 gm
pH 7.0

Modified RM1 (500 ml)

MS salts ----------2.15 gms
Yeast extract ----------0.2 gms
Glucose ----------10 gms
NaCl ----------0.05 gm
BAP ---------- 0.5 mg
GA ---------- 5 mg
Thiamine-HCl ----- 5 mg
Myo-inositol -------- 50 mg
pH 5.5

Figure 3. Explant and light effects on recovery of putative transformed cassava plants. embryos; Udc=undifferentiated callus AL=apical leaves; GSE=germinating somatic.

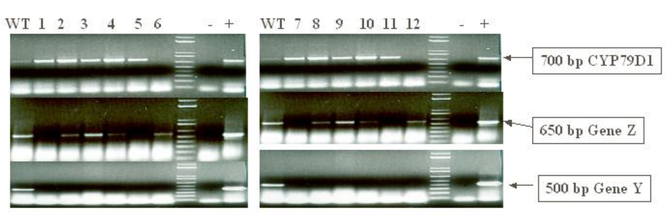

Figure 4. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis of genes Y and Z in putative cassava transformants.

Results

There were significant differences in number of putative transformants recovered due to explant type, and light treatment (Figure 3). Using undifferentiated calli (Udc), one-week incubation in the dark produced the highest mean plant recovery (5). Using germinated somatic embryos (GSE), the highest plant recovery (mean of 3) was obtained at two weeks of dark incubation. No putative transformants were recovered when Udc explants were not incubated in the dark. On the other hand, putative transformants were recovered from GSE regardless of light treatment regimes with the best recovery obtained when explants were incubated in the dark for two weeks. From apical leaves, a mean of two putative transformants were recovered only at one and two week dark incubation of the explants.

RT-PCR analysis was carried out to find out the putative transformants that were expressing the transgene. The transformed plants had complete or partial transcript reductions (650 and 500 bp respectively for genes Z and Y) while the wild type consistently showed bands (Figure 4). Antisense constructs of genes Y and Z were expressed in the leaf under control of the 2X35 S promoter. Amplification of cDNA from leaf tissues of wild type and transgenic plants using CYP79D1 primers was positive and indicates that the promoter used was effective in the leaf tissue used to analyze for transgene.

Discussion

The goal of transformation is to introduce foreign DNA into the plant genome without altering the desirable characteristics of the genotype that is being transformed. This study has produced cassava plants with complete or partial reduction of expression of the Y and/or Z genes. It is apparent from the observations reported here that by using liquid co-culture inoculation system, introducing foreign DNA into cassava has not been the problem. The problem has been to get the introduced DNA correctly expressed to produce the desired phenotype. The cassava genome has been estimated to be 760 Mbp (Arumuganathan and Earle, 1991), and foreign DNA introduced will randomly integrate into the genome. DNA introduced into the genome by non-targeted method is likely integrated into the genome by non-homologous recombination method (Gallego et. al. 2003) and may or may not produce the desired phenotypic effect.

Fregene and Pounti-Kaerlas (2002) have summarized the various types of explant tissues that have been used in cassava transformation, and these include: somatic embryo cotyledons, young leaf lobes, and friable embryogenic callus. Among the variable tissues used to transform cassava, friable embyogenic callus has been reported (Raemakers, 1997) to be the most prolific, but it also tends to produce high degree of somaclonal variants. The influence of explant and light on cassava transformation has been reported (Msikita et. al. 2002) and this study is in concurrence with earlier observations.

The overall goal of this study was to increase the number of plants transformed. Co-culture of *Agrobacterium* carrying the plasmid of interest with the explant in liquid medium appears to increase T-DNA integration per transformation event, but does not always produce the desired phenotype. The duration of the transformation using this protocol ranges from 12-18 weeks which is long, and may thus may be amenable to somaclonal variation though no obvious variation has been observed in the transformants obtained.

Literature cited

1. Andessen M., Bush P., Svendsen, I. and Moller B. 2000. Cytochrome P450 from cassava catalyzing the first steps in the biosynthesis of the cyanogenic glycosides linamarin and lotaustralin. J. Biol Chem. *275*: 1966-1975.

2. Arumuganathan, K. and Earle, E.D. 1991. Nuclear DNA Content of Some Important Plant Species. *Plant Molecular Biology Reporter* 9(3): 211-215.

3. Bidney, D., Seelonge, C., Martich, J., Burrus, M., Sims, L. and Huffman, G. 1992. Microprojectile bombardment of plant tissues increases transformation frequency by Agrobacterium tumefaciens. Plant Molecular Biology, 18: 301-313.

4. Fregene M. and Puonti-Kaerlas J. 2002. Cassava Biotechnology. In: Cassava Biology, Production and Utilization eds. Hillocks RJ , Thresh JM and Bellotti AC CABI Publishing, Oxon, New York

5. Gallego, M.E., Bleuyard, J.Y., Daoudal-Cotterell, S, Jallut, N., and White, C.I. 2003. Ku80 plays a role in non-homologous recombination but is not required for T-DNA integration in *Arabidopsis*. Plant J. 35:557-565.

6. Msikita, W., R.T. Sayre, V. White, and J. Marks. 2001. Influence of explant source and light on efficiency of *Agrobacterium*-mediated transformation of cassava. Fifth International Meeting of the Cassava Biotechnology Network. St. Louis, MO. Nov. 4-9.

7. Raemakers C.J.J.M., Sofiari E., Jacobsen E., and Visser R.G.F. 1997. Regeneration and transformation of cassava. Euphytica 96: 153-161.