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A *Cassava vein mosaic virus* promoter cassette induces high and stable gene expression in clonally propagated transgenic cassava (*Manihot esculenta* Crantz)

Olufemi O. Oyelakin [a,1], Jelili T. Opabode [b,*], Adebola A. Raji [a,1], Ivan L. Ingelbrecht [a,1]

[a] *Central Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria*
[b] *Department of Crop Production and Protection, Obafemi Awolowo University, Ile-Ife, Nigeria*

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ABSTRACT

The study described a T-DNA vector with a *Cassava vein mosaic virus* promoter cassette (pCsVMV) and a kanamycin selectable marker gene driven by the 35S *Cauliflower mosaic virus* promoter with a view to stably express transgenes over repeated cycles of clonal propagation. A *β-glucuronidase* reporter gene under control of pCsVMV (pCsVMV-GUS) was introduced into the cassava landrace 'Tokunbo' via *Agrobacterium*-mediated genetic transformation. Transgenic tobacco plants (*Nicotiana tabacum* SR1) with the same gene construct were also produced. In tobacco, the pCsVMV-GUS was highly expressed in all tissues tested such as leaf, stem, petiole, and roots. In transgenic cassava, the pCsVMV-GUS gene was highly expressed in all tissues and most cell types of in vitro plants including leaf, stem, petiole, and fibrous roots. The pCsVMV-GUS gene was also highly expressed in these tissues as well as in tubers of greenhouse grown cassava. High and stable pCsVMV-GUS gene expression was maintained over 3 cycles of ratooning under greenhouse conditions, thus showing the absence of undesired gene silencing effects after repeated in vitro subculturing and vegetative propagation. From the high constitutive levels of GUS activity observed, the study concluded that the CsVMV promoter cassette was useful for high-level expression in cassava over repeated cycles of clonal propagation.

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a root crop and serves as an important source of energy in the diet of 600 million people in tropical countries where food deficiency and malnutrition are common. In addition, cassava is used as raw material for industrial products such as starch, flour, and pharmaceuticals (Rickard et al., 1991). The increased cultivation of cassava is attributed to unique characteristics of cassava crop (Nweke et al., 2002). When growth conditions are adequate, it gives high tuber yield. Cassava is tolerant of drought and soil acidity and grows well in less fertile and marginal soils (El-Sharkawy, 2004). Mature cassava tubers can be left in situ in the field until favorable harvesting, processing and marketing conditions are available (Ceballos et al., 2004). There are several major problems that limit the production and use of cassava tubers by farmers and industry. For example, diseases and pests can significantly reduce yield if left uncontrolled. The storage roots of cassava are rich in starch (70–90% of their dry weight) but deficient in protein and other micronutrients (Cock, 1985). Moreover, once harvested, the roots are subjected to rapid postharvest physiological deterioration, which constrains their storage and marketing (Plumbley and Rickard, 1991).

Unlike many of the world's major crop plants, conventional breeding to solve major production problems aforementioned is difficult, long and cumbersome. Genetic improvement of cassava through sexual crosses is limited because many varieties rarely flower and seed production is often low. Also, cassava is highly heterozygous and suffers from high inbreeding depression (Ceballos et al., 2004). In the field, cassava is typically propagated clonally by stem cuttings. This propagation strategy is ideal for a transgenic approach to crop improvement as gene segregation through outcrossing is limited (Ihemere et al., 2006). The successful use of transgenic approach for improvement of cassava depends on the availability and effectiveness of promoters to drive transgene expression to sufficient levels in tissues of interest and on stable transgene expression after multiple cycles of vegetative propagation.

A number of promoters have been identified that confer high level of constitutive expression of heterologous genes in transgenic plants including cassava. The 35S promoter from the Cauliflower mosaic virus (CaMV) in various configurations has been the most widely used constitutive promoter in cassava (Li et al., 1996: Zhang et al., 2000). However,

Abbreviations: CsVMV, *Cassava vein mosaic virus*; GUS, β-glucuronidase.

* Corresponding author at: Crop Production and Protection Department, Obafemi Awolowo University, Ile-Ife, Nigeria. Tel.: +2348034172865.
E-mail address: jopabode@yahoo.com (J.T. Opabode).
[1] c/o Lambourn Ltd, Carolyn House, 26 Dingwall Road, Croydon CR9 3EE, United Kingdom.

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the repeated (duplication) inclusion of CaMV 35S promoter sequence in plant transformation vectors has been implicated in unwanted gene silencing effects. For instance, a 35S-driven transgene expressing the *Arabidopsis* constitutive photomorphogenesis1 (COP1) gene protein as a fusion to β-glucuronidase (GUS) or green fluorescent protein (GFP) results in the homology-dependent transcriptional silencing of the endogenous *COP1* gene, as evident by a characteristic *COP1* mutant phenotype at the adult vegetative stage (Qin et al., 2003). Similarly, in gentian plant, transcriptional gene silencing by methylation in the promoter sequences of transgenic plants carrying a T-DNA construct consisting of a tandem repeat of two 35S promoter is reported to be homology-dependent gene silencing involving the promoter region (Mishiba et al., 2005). Thus, there is a need to construct transformation vectors with different strong promoters to reduce incidences of gene silencing triggered by repeated promoter sequences. One of such suitable promoters that confer high level of gene expression in transgenic plants is the cassava vein mosaic virus (CsVMV) promoter (Verdaguer et al., 1996). Previous studies showed that the levels of activity and differential expression pattern of a "constitutive" promoter can vary substantially depending on the plant species and tissue type. For example, earlier transformation study on CsVMV promoter in tobacco and rice plants reported that the promoter was active in all plant organs tested and in a variety of cell types, suggesting a near constitutive pattern of expression (Verdaguer et al., 1996). However, in grape, the CsVMV promoter was highly active in somatic embryo tissues but produced low level of expression in roots through all developmental stages (Li et al., 2001). By comparison, the CsVMV promoter drives higher transgene expression than CaMV 35S promoter in the leaves, roots, and nodules of transgenic alfalfa (Samac et al., 2004) and matured soybean root nodules (Govindarajulu et al., 2008). Although several studies examined promoter expression in cassava (Gonzalez et al., 1998; Zhang et al., 2000), the CsVMV promoter has not been examined in stably transformed cassava plants. Additional knowledge on the functionality of alternative promoter like CsVMV in cassava is essential to improve the efficacy of transgene expression in this economically important crop.

This study sets out to: (i) construct a T-DNA vector with a CsVMV promoter cassette to drive expression of a gene of interest in combination with a 35S CaMV promoter driving a kanamycin selectable marker gene; (ii) examine the expression level and pattern of the CsVMV promoter in stably transformed cassava tissues and organs; and (iii) evaluate the expression level of this CsVMV promoter cassette in transgenic cassava plants after repeated cycles of vegetative propagation.

2. Materials and methods

2.1. Plant materials and growth conditions

Cassava cultivar TME 12 plantlets were obtained from the in vitro germplasm collection of Tissue Culture Laboratory, IITA, Ibadan. The plantlets were maintained on MS medium (Murashige and Skoog, 1962; Sigma) supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and adjusted to pH 5.8 before autoclaving for 20 min at 121 °C at 25 ± 2 °C under a 16-h photoperiod (90 μmol m^{-2} s^{-1}) and subcultured at 4-week interval. Tobacco (*Nicotiana tabaccum* cv SRI) were grown from seeds. Growth media and conditions used for tobacco plants were as described by Oyelakin (2009). All somatic embryo cultures were kept in the dark at 25 ± 2 °C and subcultures were made at 4-week interval. Prior to transformation, immature cyclic somatic embryos were incubated under light to generate green somatic embryos. In vitro transformed cassava and tobacco plantlets with well-developed roots were rinsed with water to wash off the agar medium and transplanted to peat pellet (AS Jiffy Products Ltd, Norway) in plastic pots that were covered to maintain high humidity. The plants were grown at 22–26 °C in a containment facility for 3 weeks and transferred to the greenhouse where they were grown to maturity.

2.2. Construction of plant transformation vector

An expression cassette was assembled in silico by merging a 515-bp promoter fragment from CsVMV corresponding to position 7171–7675 of the CsVMV genome (Calvert et al., 1995) to a 265-bp fragment encompassing the 3′end of the *nopaline synthase* gene (Yanisch-Perron et al., 1985) with in between a 45-bp multiple cloning site. This sequence was flanked by *Eco*RI and *Hin*dIII sites at the 5′ and 3′ end respectively. This 837 bp module was synthetized (Genscript, NY, USA) and subcloned into the *Eco*RI and *Hin*dIII sites of pCAMBIA2300 (Jefferson et al., 1987) yielding pING71 (Fig. 1a).

Next, the *Uid*A coding sequence was isolated from pATAG3 (Goossens et al., 1999) via PCR using OLIV3: 5′-TCAAGGTACCAA CC**ATG**GTCCGTCCTGTAGAAAC (start codon in bold) and OLIV16: 5′-TACATATACTGCAG**TCA**TTGTTTGCCTCCCTGCTG (stop codon in bold) and the resulting 2.0-kb fragment was subcloned into *Kpn*I/*Pst*I digested pING71, resulting in pOYE153 (Fig. 1b). The transformation vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method as described by Burrow et al. (1990).

2.3. Transformation of cassava and tobacco

Primary and secondary somatic embryos were produced as described by Jorgensen et al. (2005). Primary somatic embryos were obtained by isolating enlarged shoot meristem, taken from 4-week old in-vitro plantlets of cassava cultivar TME 12, from incubated nodal stem segment on MS medium (Murashige and Skoog, 1962; Sigma) supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and 6-benzylaminopurine (10 mg/l) for seven days. The isolated meristems were incubated on MS medium supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and 10 mg/l picloram. After 2 weeks, primary somatic embryos were induced. The globular-and-torpedo- shaped embryo matured on MS medium supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and 6-benzylaminopurine (0.1 mg/l). When embryos reached the cotyledonary stage, the cotyledons were harvested and placed on MS medium supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and 2,4-dichlorophenoxyacetic acid (6 mg/l) to induce formation of secondary embryos. Mature (green) somatic embryos were produced by transferring cotyledons of secondary somatic embryos on MS medium supplemented with 2% sucrose, 0.8% agar, 2 μM $CuSO_4$ and 2, 4-dichlorophenoxyacetic acid (6 mg/l) to induce embryos

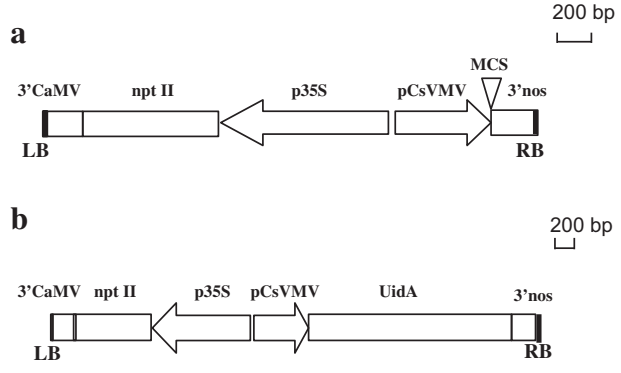

Fig. 1. Physical maps of T-DNA vectors used for transformation. (a) pING71. (b) pOYE135. 3′ UTRCaMV — 3′ untranslated region of cauliflower mosaic virus promoter; NPT II — coding region of neomycin phosphotransferase II gene from *Escherichia coli*; CaMV 35S — coding sequence of 35S promoter from cauliflower mosaic virus. CsVMV — coding region from cassava vein mosaic virus promoter; UidA-protein — coding region for β-glucuronidase from *E. coli*; 3′NOS — polyadenylation signal of the gene for nophaline synthase in the Ti plasmid; LB and RB — left and right borders of T-DNA transformation vector.