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# Tissue- and Organ-Specific Promoters for Expression of Heterologous Genes in Transgenic Cassava (*Manihot Esculenta Crantz*) Plants

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**Abstract**

Promoters are regions of DNA that initiates transcription of genes. A number of promoters have been identified that confer high level of expression of heterologous genes in transgenic plants. Some promoters have constitutive expression as they are active in all circumstances in the cell, while others are regulated, becoming active in certain cell or in response to specific stimuli. Despite the availability of tissue- and organ-specific promoters, most transgene expressions in Cassava are driven by constitutive cauliflower mosaic virus promoter. This paper examines the availability of promoters for transgene expression in plants, assesses the use of promoters for transgene expression in Cassava and establishes the need for tissue- and organ-specific promoters for expression of heterologous genes in Cassava.

**Keywords:** Cassava; Gene expression; Genetic transformation; Promoters

## The cassava crop

Cassava (*Manihot Esculenta* Crantz) is an important root tuber crop and serves as a source of dietary energy in most developing countries [1]. Cassava starch and by-products have found use in manufacturing of livestock feed, bio-ethanol, food additives, agrochemical and pharmaceutical industries [2,3]. Cassava is tolerant of moisture stress and soil acidity and gives high tuber yield on low fertile soils [3]. Young cassava leaves are consumed as vegetable in some African communities. Dried cassava stems are a source of firewood for cooking in some peri-urban areas of some developing countries. In Savannah belt of Africa, cassava stems are utilized as stakes in yam production. Cassava cultivation becomes more attractive because of flexibility in harvesting; processing and marketing as tubers can be stored in the soil for fairly long [4]. Depending on cultivar, about 65-91% of Cassava total root dry weight is made up of starch [5]. Cassava starch is being preferred to the conventional sources of starch as such wheat, maize, rice and potato, making global demand for Cassava starch to rise [2].

Numerous production constraints prevent utilization of cassava for achieving food security and economic growth. The most important are biotic and abiotic constraints such as diseases, pests, weeds, poor soil fertility and drought and these factors are militating against Cassava production [6,7]. Other problems facing cassava cultivation include postharvest physiological deterioration, high cyanide content, low protein content and fluctuating starch quality [7]. Limited success was recorded through the use of conventional breeding methods for improvement of Cassava against the biotic and abiotic constraints [4,6]. Conventional breeding of Cassava is challenging due to high heterozygosity, poor flowering, limited seed set and inbreeding depression of the crop [6,7]. Furthermore, an effective use of conventional breeding strategies for cassava improvement is curtailed by numerous metabolic pathways and gene networks involved in the crop's essential metabolism such as biosynthesis of starch and post-harvest physiological deterioration [3,7]. Therefore, there is need to use genetic engineering methods for Cassava improvement. Genetic engineering is suitable for cassava improvement against biotic and abiotic stresses because gene segregation through outcrossing is limited since cassava is vegetatively propagated by stem cuttings. For effective application of genetic engineering, availability of suitable promoters that are highly expressed in vital organs and cells of the crop becomes prime importance [8-11].

A promoter is a region of DNA that initiates transcription of a particular gene [12]. Promoters are located near the transcription start sites of genes, on the same strand and upstream on the DNA. Promoters can be about 100-3000 base pairs long. Generally, the basal or core promoter is located about 40 base pairs upstream of the start of transcription, and the upstream promoter region may extend as many as 200 base pairs farther upstream. Although initiation of transcription is dependent on sequences found in the core and upstream promoter region, many other DNA sequence motifs, which occur within the surrounding DNA, are also involved in the regulation of gene expression. The objectives of this paper are to (i) examine the available promoters for transgene expression in plants; (ii) assess the use of promoters for transgene expression in cassava; and (iii) establish the need for tissue- and organ-specific promoters for expression of heterologous genes in cassava.

## Available Promoters for Transgene Expression in Plants

A number of promoters have been identified that confer high level of expression of heterologous genes in transgenic plants including cassava. Some promoters have constitutive expression as they are active in all circumstances in the cell, while others are regulated, becoming active in the cell only in response to specific stimuli. Table 1 shows selected promoters for transgene expression in plants, their sources and type of expression driven by the promoters in plants. One of the most commonly used promoters for constitutive expression is the cauliflower mosaic (CaMV) 35S promoter [13]. The CaMV 35S promoter can drive high levels of transgene expression in both dicots and monocots. The CaMV 35S promoter is harvested from double-stranded DNA viral genomes which use host nuclear RNA polymerase and do not appear to depend on any transacting viral gene products. The 35S promoter from the Cauliflower mosaic virus (CaMV) in various configurations

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has been the most widely used constitutive promoter in for some reasons: first, the CaMV 35S promoter is valuable to deliver high expression in virtually all regions of the transgenic plant; second, it is readily obtainable in research and academic settings; and third, it is available in plant transformation vector cassettes that allow for easy subcloning of the transgene of interest. Because of the success of the CaMV 35S promoter, other viral promoters have been developed for use. Many of these new virally derived promoters perform similarly or better than the CaMV 35S promoter, and drive high expression in both dicots and monocots. They include the Cassava vein mosaic virus (CsVMV) promoter, Australian banana streak virus (BSV) promoter, Mirabilis mosaic virus (MMV) promoter and Figwort mosaic virus (FMV) promoter [12]. It is noteworthy that a number of these strong constitutive promoters are derived from actin and ubiquitin genes in plants. Actin is a fundamental cytoskeletal component that is expressed in nearly every plant cell. The Act2 promoter was developed from the actin gene family in Arabidopsis [14]. Ubiquitin is one of the most highly conserved proteins known. It has been linked to many vital cellular processes including protein turnover, chromatin structure, and DNA repair. It is highly abundant in the cytoplasm of most every cell in the plant. Some are constitutively expressed while others also respond to stress [15].

Studies have established the interference in normal growth processes of transgenes expressed by constitutive promoters. As a result, research effort has been directed to isolation and development of tissue-specific promoters for transgenes expression. Targeted expression has become particularly important for the future development of value-added crops like cassava because the public may be more likely to accept 'less intrusive' expression of the transgene [12]. For example, confinement of an insecticidal transgene product to tissue attacked by insect pests instead of harvestable material could enhance public acceptability of the transgenic crops. Selected tissue-specific promoters that have been isolated from tubers, roots, pistils, anthers, leaves, pollens, seeds and nodules are listed in (Table 1.) Patatins, granule-bound starch synthase (GBSS), sporamin and beta-amylase promoters are the most characterised tuber and storage organ- specific promoters from genes involved in massive deposition of starch and the storage of highly abundant glycoproteins from potato and sweet potato [16]. Patatins are glycoproteins that account for approximately 40% of the total soluble protein found in the tuber. The potato class I patatin family members are B33 and PAT 21 which are highly expressed at early stages of tuber development in the vascular tissues as well as in later stages of development, in both parenchyma and vascular tissues. They are tuber-specific, but can be induced in the leaves by sucrose. There is substantial sequence homology between the B33 and PAT 21 promoters. In a novel use of the patatin promoter, the cytochrome P450 gene from rat (CYP1A1) was introduced into potatoes to enhance the detoxification of residual herbicides in the soil [17]. Developing tubers showed high levels of the CYP1A1 mRNA and protein and the concentrations of specific herbicides were much less than that of non-transformed tubers.

Starch in potato tubers consists of up to 25% amylose, and granule-bound starch synthase (GBSS) is the key enzyme in amylose biosynthesis. Visser et al. [18] successfully employed potato GBSS promoter fragment of 800 bp to drive high levels of reporter gene expression in both stolons and tubers, with little to no activity in leaves. Like patatin promoters, sugars can induce the GBSS promoter's expression in leaves, but not to levels as high as that of the patatin promoters. Furthermore, sporamin and amylase promoters are two well-characterized promoters from sweet potato [16]. Sporamin makes up 60-80% of total soluble proteins in the sweet potato storage organ.

| Sr. No. | Promoter | Source | Type of expression | Reference |
|---|---|---|---|---|
| 1. | *Act 2* | Arabidopsis | constitutive | [14] |
| 2. | *pUbi1* | maize | constitutive | [15] |
| 3. | *CaMV 35S* | virus | constitutive | [13] |
| 4. | *CsVMV* | virus | constitutive | [36] |
| 5. | *Potato wun1* | potato | inducible | [37] |
| 6. | *Patatin B33* | potato | Tuber/storage organ | [38] |
| 7. | *Sporamin* | Sweet potato | Tuber/storage organ | [16] |
| 8. | *Beta-phaseolin* | bean | Seed-specific | [39] |
| 9. | *Lat52* | tomato | Pollen-specific | [40] |
| 10. | *PsGNS2* | pea | Seed coat-specific | [41] |
| 11. | *TobRB7* | tobacco | Root-specific | [42] |
| 12. | *RA8* | rice | Anther-specific | [43] |
| 13. | *SK2* | potato | Pistil-specific | [44] |
| 14. | *CAB2* | arabidopsis | Green-tissue specific | [45] |
| 15. | *UEP1* | chrysanthemum | Floral-specific | [46] |
| 16. | *PsTL1* | pear | Pistil-specific | [47] |
| 17. | *Nvp30* | bean | Nodule-specific | [48] |
| 18. | *ZMCS* | maize | Pollen-specific | [49] |
| 19 | *C15* | cassava | Storage root-specific | [19] |
| 20 | *C54* | cassava | Storage root-specific | [19] |

**Table 1:** Selected promoters for transgene expression in plants, their source and type of expression driven by the promoters.

It is composed of two multigene subfamilies, A and B, which contain approximately 10 total members. Analysis indicates that sporamin was expressed almost exclusively in the storage tuber, with a small amount of expression in stems (1-4.5% soluble protein). Recently, Zhang et al. [19] isolated two promoters, c15 and c54, from cassava that are related to vascular expression and secondary growth of storage roots. A 1,465-bp fragment of c15 and 1,081-bp fragment of c54 were translational fused to the uidA reporter gene and introduced into Cassava and Arabidopsis. The expression patterns in transgenic plants showed that both promoters are predominantly active in phloem, cambium and xylem vessels of vascular tissues from leaves, stems and roots.

Chlorophyll-containing tissue supports the expression of a number of well-characterized, light-inducible genes. The best-characterized light-inducible genes in plants are members of the rbcS multigene family encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. Analysis of transgenic tomato plants expressing an rbcS-promoter/GUS fusion gene confirmed that promoter fragments ranging from 0.6 to 3.0 kb of rbcS1, rbcS2, and rbcS3A genes were sufficient to confer the temporal and organ-specific expression pattern [20]. In these genes, the I-box and G-box are located within -600 to -100 bp upstream of the transcription initiation site. The rbcS gene has been successfully used to confer resistance against insect in some crops. For examples, a synthetic truncated Cry1Ac gene was linked to the rice rbcS promoter and its transit peptide sequence (tp), and was transformed in rice using the Agrobacterium-mediated transformation method. Use of the rbcS-tp sequence increased the Cry1Ac transcript and protein levels by 25- and 100-fold, respectively, with the accumulated protein in chloroplasts comprising up to 2% of the total soluble proteins. The high level of Cry1Ac expression resulted in high levels of plant resistance to three common rice pests; rice leaf folder, rice green caterpillar and rice skipper, as evidenced by insect feeding assays. Transgenic plants were also evaluated for resistance to natural infestations by rice leaf folder under field conditions. Throughout the entire period of plant growth, the transgenic plants showed no symptoms of damage, whereas non-transgenic control plants were severely damaged by rice leaf folders [21].

Similarly, a rbcS promoter was isolated from Gossypium arboreum

Var. 786. The promoter was fused with an insecticidal gene Cry1Ac to confer resistance in cotton (Gossypium hirsutum) against lepidopteran pests, especially the American boll worm. A local cotton variety, NIAB-846, was transformed using this construct via the Agrobacterium tumefaciens strain LB4404. The same cotton variety was transformed with another construct pk2Ac harboring Cry1Ac under the 35S promoter. The comparative study for insecticidal gene expression in Rb-Ac plants (transformed with Cry1Ac driven by rbcS promoter) and pk2Ac plants (transformed with Cry1Ac driven by 35S promoter) showed that rbcS is an efficient promoter to drive the expression of Cry1Ac gene consistent in the green parts of cotton plants as compared to 35S promoter [22].

A second, highly expressed, green tissue gene family is the chlorophyll a/b-binding (cab) protein genes. The cab proteins are associated with the light-harvesting complex proteins to form the light-harvesting complex (Lhc). Like the rbcS proteins, it is also one of the most abundant proteins found in the leaves of all green plants. However, the expression pattern of the cab genes in plants is different from that of the rbcS genes. The cab promoters respond to light and diurnal or circadian rhythms [23].

A valuable root-specific promoter that has been used for many genetic engineering objectives is the TobRB7 promoter from tobacco [24]. TobRB7 is a putative membrane channel aquaporin, which is expressed in a root-specific manner. A promoter:GUS deletion series showed that the highest activity was directed by the D0.6 promoter, which included 636 bp 5'flanking [24]. Root-specific activity was shown as early as 2 d post-germination in tobacco transgenics and was strongest in the meristem and central cylinder. The DO.3 promoter:GUS construct of TobRB7 (299 bp 5'flanking) is responsive to root-knot nematode-directed expression [25], although it appears to express at some basal level in root tissue. Using the D0.3 promoter construct, Shen et al. [26] fused the hrmA gene from Pseudomonas syringae to convert a compatible plant-pathogen interaction into an incompatible interaction. The transgenic tobacco in this study also displayed high levels of resistance to multiple other pathogens, including viral, fungal, and bacterial [26]. These results suggest that expression of bacterial avr genes using controlled low-level expression in the roots could generate broad-spectrum resistance to any type of bacterial, fungal, or viral root pathogen.

## The Use of Promoters for Transgene Expression in Cassava Genetic Transformation

Since the first two reports of successful genetic transformation of cassava were published simultaneously from two laboratories in 1996, several genetic modification events in cassava has been reported. (Table 2) showed a selected published genetic transformation studies in Cassava with emphasis on promoters, plasmids, transgenes and gene transfer methods used for the studies. In one of earlier efforts to produce transgenic Cassava lines, Gonzalez et al. [27] employed CaMV35S promoter to drive expression of intron-interrupted uidA gene in cassava cultivar TMS 60444 using pILTAB plasmid with NPT II as selectable marker by Agrobacterium-mediated gene transfer method. In the study, selection of transformed tissue with paromomycin resulted in the establishment of antibiotic-resistant, β-glucuronidase-expressing lines of friable embryogenic callus from which embryos and subsequently plants were regenerated. Southern blot analysis demonstrated stable integration of the uidA gene into the cassava genome in five lines of transformed embryogenic suspension cultures and in two plant lines. Similarly, in the first report of the bar gene conferring herbicide resistance to cassava plants by Sarria et al. [28], transgenic plants of cassava resistant to the herbicide Basta were obtained through Agrobacterium-mediated transformation. The expression of bar, UidA and NPT II gene were under the control of CaMV35S promoter. Greenhouse tests of resistance to Basta (Hoechst) showed three plant lines with different levels of tolerance to the herbicide. Based on Southern tests of transgenesis, the transformation efficiency was 1%.

Zhang et al. [29] engineered cassava cultivar TMS 60444 plants with increased African cassava mosaic virus resistance driven by CaMV35S promoter using improved asRNA technology in which the DNA for the viral asRNA gene was fused to the 3′UTR of the HPT gene to create the transgenes. Transgenic African cassava mosaic virus resistance-resistant plants had significantly reduced viral DNA accumulation in their infected leaves. Likewise, Hankoua et al. [30] used plasmid pCAMBIA carrying the hygromycin selectable marker gene (hpt) and the uidA visual marker gene within its T-DNA, each under the control of the CaMV35S promoter to demonstrate the first successful establishment of cassava regeneration and transformation capacity in Africa via organogenesis, somatic embryogenesis and friable embryogenic callus (FEC). The first reported use of promoter other than CaMV35S to drive transgenes expression was made by Ihemere et al. [31]. Ihemere et al. [31] generated transgenic cultivar TMS 71173 lines by expressing a modified form of the bacterial glgC gene under the control of a Class I patatin promoter. AGPase catalyses the rate-limiting step in starch biosynthesis, and therefore the expression of a more active bacterial form of the enzyme was expected to increase starch production. Transgenic plants (three) expressing the glgC gene had up to 70% higher AGPase activity than control plants when assayed under conditions optimal for plant and not bacterial AGPase activity. Recently, Zainuddin et al. [32] employed *Agrobacterium* tumefaciens LBA4404 harboring CAMBIA1301 plasmid which contains the hpt II gene for resistance to hygromycin and the UidA reporter gene driven by the constitutive CaMV35S promoter to generate transgenic cassava cultivar TMS60444 plants. Early this year, Oyelakin et al. [11] constructed and tested a T-DNA vector with pCsVMV-GUS and CaMV 35S-NPTII cassettes transcribing in opposite direction in cassava transgenic plants. They further evaluated the activity, level and

| Sr. No. | Promoter used | Plasmid | gene | Gene transfer method | Reference |
|---|---|---|---|---|---|
| 1. | CaMV35S | *pJIT100, pJIT64* | Luciferase | Particle bombardment | [50] |
| 2. | CaMV35S | *pILTAB* | *Uid A* | *Agrobacterium* | [17] |
| 3. | CaMV35S | *pGV1040* | *Uid A* | *Agrobacterium* | [18] |
| 4. | CaMV35S | *pHMG* | *Uid A* | *Agrobacterium* | [51] |
| 5. | CaMV35S | *pTOK233* | *Uid A* | *Agrobacterium* | [52] |
| 6. | CaMV35S | *pPZPIII* | *CYP79D1, CYP79D2, GUS* | *Agrobacterium* | [53] |
| 7 | CaMV35S | *pPZP100* | *asAC1,asAC2, asAC3* | *Agrobacterium* | [19] |
| 8. | CaMV35S | *pGBSS-as2,pGBSS-as7* | Luciferase,GBSS1 cDNA | Particle bombardment | [54] |
| 9. | CaMV35S | *pCAMBIA1301* | *Uid A* | *Agrobacterium* | [30] |
| 10. | Class 1 potato patatin | *p3D* | *glgC (336D)* | *Agrobacterium* | [31] |
| 11 | CaMV35S | *pCAMBIA1305.1* | *Uid A* | *Agrobacterium* | [54] |
| 12. | CaMV35S | *pCAMBIA1301* | *Uid A* | *Agrobacterium* | [32] |
| 13. | CsVMV/ CaVMV35S | *pOYE135* | *Uid A* | *Agrobacterium* | [11] |

CaVMV35S – cauliflower mosaic virus CsVMV – **cassava** vein mosaic virus

**Table 2:** Selected promoters, plasmids, transgenes and gene transfer methods used in **cassava.**

pattern of expression of pCsVMV-GUS in various organs and tissues of clonally propagated transgenic cassava plants. Analysis of transgenic cassava plants indicates that pCsVMV-GUS is active in all organs and various cell types. The pCsVMV-GUS drives strong and constitutive expression in vascular tissues of petiole, stem and tuberous root and in leaf mesophyll tissues and vascular stele of roots of transgenic cassava plants.

## The Need for Tissue-and Organ-Specific Plant Derived Promoters for Cassava Transformation

Two factors have made deployment of tissue-and organ-specific plant derived promoters for cassava genetic modification compelling: inherent properties and expression patterns of constitutive promoters, and the tissue and organ specific nature of most cassava production problems and improvement needs. A prominent shortcoming associated with heterologous expressions of genes by constitutive promoters of viral origin is the controversies generated over consumption of genetically modified foods could be traced to perception of risk to human health with the use of transgenes made with genes of infective viruses. In addition, constitutive transgene expression can become a problem if a specific transgene is overexpressed at the wrong time in tissues where it is not normally expressed resulting in unexpected consequences on plant growth and development and the environment [12]. Hence, plant gene promoters that are activated precisely when and where they are needed would be ideal for genetic engineering strategies for enhanced biotic and abiotic stresses tolerance in cassava.

Molecular studies have established that genes involve in starch biosynthesis are expressed in storage roots and leaves [31]. Cassava starch requires improvement in amylose/amylopectin ratio to expand its application for food and industrial purposes. High expression of antisense or RNA interference genes could be achieved in storage roots and leaves of cassava by patatins, Granule-Bound Starch Synthase (GBSS), sporamin and beta-amylase tuber specific promoters to obtain modified starches with enhanced functionality. Similarly, root-specific promoters could be suitable for improvement of nutritional value of cassava storage roots to drive genes such as gene encoding storage protein rich in essential amino acids [33]. Furthermore, there are several insect pests affecting cassavas foliage and/ or stems, particularly Lepidoptera, Diptera and Hemiptera. There is little or no genetic resistance to these pests and their management is commonly achieved through biological control [6]. Foliage and stem specific promoters such as rcbS and cab genes are suitable in transgenic approach to drive strong expression of cry genes encoding insect-specific endotoxin (Bt toxins) from Bacillus thuringinensis for protection against this insect pests. Another potential use of organ-specific could be in the control of post-harvest physiological deterioration of cassava tubers which starts from root xylem and spread to adjacent storage parenchyma. Several tuber deterioration genes have been cloned in cassava, including catalase [34]. Inhibition of the activity of the tuber deterioration genes could be achieved by strong expression of their antisense genes under tuber-specific promoter. African mosaic virus and brown streak mosaic virus diseases are major constraints to cassava production. The viruses are transported through the phloem of vascular tissues during long-distance transport [35]. Therefore, strong expression of anti-viral genes under the control of vascular tissues specific promoters such as c15 and c54 promoters from cassava in phloem cells might inhibit the systemic spread of the viruses.

In conclusion, a large numbers of promoters suitable for constitutive and organ-specific expression of heterologous genes in plants have been identified, isolated and characterized. A limited number of these tissue-specific promoters have been used for genetic transformation in cassava. Therefore, there is need to expand the use of promoters for cassava transformation as a result of the limitations of constitutive promoters which promotes unintended and negative effects on the transgenic plants and environments. The use of organ- or tissue-specific promoters should be incorporated into cassava genetic improvement programmes since many biotic constraints manifested at various organ (Table 1).

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