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Expression Pattern Conferred by a Glutamic Acid-Rich Protein Gene Promoter in Field-Grown Transgenic Cassava (*Manihot esculenta* Crantz)

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Short running title: A cassava promoter directs strong GUS expression in root and stem tissues

Abstract

A major constraint for incorporating new traits into cassava using biotechnology is the limited list of known/tested promoters that encourage the expression of transgenes in

cassava's starchy roots. Based on a previous report on the glutamic-acid-rich protein Pt2L4, indicating a preferential expression in roots, we cloned the corresponding gene including promoter sequence. A promoter fragment (CP2; 731bp) was evaluated for its potential to regulate the expression of the reporter gene *GUSPlus* in transgenic cassava plants grown in the field. Intense GUS staining was observed in storage roots and vascular stem tissues; less intense staining in leaves; and none in the pith. Consistent with determined mRNA levels of the *GUSPlus* gene, fluorometric analyses revealed equal activities in root pulp and stems, but 3.5 times less in leaves. In a second approach, the activity of a longer promoter fragment (CP1) including an intrinsic intron was evaluated in carrot plants. CP1 exhibited a pronounced tissue preference, conferring high expression in the secondary phloem and vascular cambium of roots, but 6 times lower expression levels in leaf vascular tissues. Thus, CP1 and CP2 may be useful tools to improve nutritional and agronomical traits of cassava by genetic engineering. To date, this is the first study presenting field data on the specificity and potential of promoters for transgenic cassava.

Key words

β-glucuronidase, Cassava, Carrot, Expression pattern, Promoter GUS fusion.

Abbreviations

CIAT, International Center for Tropical Agriculture; pCaMV35S, promoter of the 35S protein from Cauliflower Mosaic Virus; GUS, β-glucuronidase,

Introduction

Cassava (*Manihot esculenta* Crantz) roots comprise a major source of carbohydrates in the tropics, feeding more than 600 million people, mainly in Africa. Therefore, cassava is significant for the food safety of many depressed areas. In addition, cassava's highly efficient carbohydrate production predestines it to be a useful biomass for ethanol production (Amutha and Gunasekaran 2001). Because of its importance as a 'staple crop' and its economic potential, its genome has been sequenced (Cassava Genome Project 2009, http://www.phytozome.net/cassava). In addition, cassava has recently been subject to transcriptomic research for biotic and abiotic stresses (López et al. 2004; Reilly et al. 2007; Sakurai et al. 2007). In the last two decades, appropriate transgenic technologies have been developed to alleviate problems associated with pests and low micronutrient contents of this crop (Taylor et al. 2004).

As a strategy for crop improvement, genetic engineering of metabolic pathways requires specific promoters to confine transgene expression to a specific organ. In cassava, a major constraint is the limited availability of promoters with strong expression in roots and freedom from intellectual property claims. Indeed, the list of isolated, endogenous and exogenous promoters validated in cassava is very restricted. Today, we know that a widely used promoter, the constitutive Cauliflower 35S promoter (pCaMV35S), is unsuitable for directing strong expression of genes in cassava roots (Zhang et al. 2003a). It appears to lose its potency for directing the expression of the *GUS* reporter gene as cassava tissues mature, although expression is variable (Schopke et al. 1996; González et al. 1998; Sarria et al. 2000; Beltrán et al. 2009).

Among the few studies on the expression of genes in a specific organ of the cassava plant is that carried out by Ihemere et al. (2006). They determined that the potato class I patatin promoter (Kim et al. 1994) seems to be root specific. Generally, however, results derived from evaluations with a significant number of promoters for storage tissues are still not available for cassava as they are for other crops such as barley, wheat, and rice (Qu et al. 2008; Furtado et al. 2009).

de Souza et al. (2006) reported that hybridizations using Northern blots indicated that the glutamic-acid-rich protein (GARP) Pt2L4 is expressed in roots and stems but not in leaves of cassava. The authors also suggested that the gene *Mec1*coding for Pt2L4 may be implicated in the development and thickening of roots. At least two homologous genes coding for GARP exist in the cassava genome, according to Southern blot analyses (Zhang et al. 2003b; de Souza et al. 2006). Acquiring the promoters of these genes, which direct expression towards important organs such as roots and stems, would allow alternative regulatory sequences to express genes of interest in these organs.

During the preparation of this manuscript, de Souza et al. (2009) reported on the cloning of a genomic fragment containing a promoter sequence and part of the *Mec1* gene. The cloned promoter was shown to be functional by transient expression of a GUS-fusion in bean hypocotyledons (de Souza et al. 2009). In this work, we report, on the first evaluation of a promoter in transgenic cassava plants under field conditions. Based on the Pt2L4-cDNA sequence available, we cloned the whole *Mec1* gene including the promoter sequence. A promoter fragment (CP2) was fused with the *GUS* gene and introduced into cassava plants. The pattern of expression of the fusion CP2::*GUS* was determined by histochemical GUS staining and measuring

β-glucuronidase enzymatic activity in the organs of transgenic cassava plants grown in the field. Promoter CP2 was shown to be highly active, preferentially in stems and the storage tissues of roots, which makes it a good candidate for the genetic engineering of cassava. In a second approach, we evaluated a longer version of *Mec1* promoter (CP1) including an intrinsic intron in carrot plants, a model crop with storage roots and technically more feasible transformation system. Promoter CP1 could strongly express the *GUS* gene in roots, but only slightly in leaves, thus demonstrating its usefulness for expressing proteins in roots in heterologous systems and possibly preferential expression in cassava itself. CP2 and/or CP1 could be used, for example, to increase levels of iron, folate, pro-vitamin A and zinc of cassava to improve its nutritional value (Dellapenna 1999; Fregene and Puonti-Kaerlas 2002; Taylor et al. 2004; www.harvestplus.com).

Materials and methods

Inverse PCR

To produce circular DNA fragments, 10 µg of genomic DNA from cassava were digested with EcoRI in a total volume of 100 µl, purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) and eluted with 100 µl of 60°C pre-warmed water. 40 µl of purified genomic fragments were then ligated using 100 U of T4 DNA-ligase in a total volume of 300 µl. The ligation was performed for 2 h at room temperature, followed by 20 h at 16 °C. The ligase was then deactivated by heating for 10 min at 60°C, and circular genomic DNA was precipitated with EtOH and resuspended in 100 µl water. Inverse PCR was then performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany), using 1 µl of the circular DNA, 100 µM

dNTPs, 50 ng of the primers CAS II and CAS III (Table 1), and 0.5 µl Advantage® cDNA Polymerase Mix (DB Bioscience, CA, USA) in the buffer provided. Amplification profile was as follows: 2 min initial denaturation at 94℃ followed by 35 cycles (30 sec 94℃, 30 sec annealing, 5 min 68℃) and 10 min final polymerization at 68 ℃. For annealing, a temperature gradient of 1 ℃, ranging from 58 to 68℃, was applied. Sequences of the 5´ and 3´ ends of the ~2.2 Kb inverse-PCR products obtained were used to design the primers FinCas and CasP II (Table 1) for cloning of the *GAPR* gene. The amplification was performed with 200 ng genomic DNA, 100 ng of each primer, 250 µM dNTPs and *PfuUltra™* II Fusion HS DNA Polymerase (Stratagene Europe, Amsterdam, Netherlands) in the buffer provided. Amplification steps were 1 min initial denaturation at 95℃, 35 cycles of amplification (20 sec 95℃, 30 sec 55℃, 2 min 72℃) and 10 min final polymerization at 72℃. The obtained 2 Kb PCR product was purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, NJ, USA), and cloned into the pCR2.1®-TOPO® (Invitrogen, Paisley, UK) vector to yield pCR-CP. The integrity of the amplified gene was verified by sequencing.

Construction of binary vectors

To generate a *CP2*::*GUSPlus* cassette, a 731 bp promoter fragment was amplified from pCR-CP using the primers PCII and PCNI (Table 1) carrying a PstI and an NcoI site, respectively. The PCR was performed with the proofreading PWO DNA Polymerase (Peqlab, Erlangen, Germany). The amplified CP2 promoter fragment was digested with PstI and NcoI, and ligated to pCAMBIA1305.2 (Canberra, Australia) digested with the same two enzymes to yield pCP2. The *CP1::GUSPlus* cassette was obtained by amplifying a fragment carrying 1012 bp promoter sequence, followed by 18 bp of the coding sequence, the intron of *GAPR* gene and by further 9 bp. The PCR was

performed on the plasmid pCR-CP using the primers PCI and PCNII (Table 1) carrying a PstI and an NcoI site, respectively. The obtained fragment was digested and ligated into pCAMBIA1305.2 (Canberra, Australia), as described above, yielding pCP1.

Genetic transformation of cassava

The procedure for cassava transformation is described by Beltrán et al. (2009). Briefly, the plant material used for obtaining transgenic plants was friable embryogenic callus (FEC; Taylor et al. 1996) from the cassava genotype 60444 (M Nig 11). The genotype was transformed with *Agrobacterium tumefaciens* strain AGL1, containing plasmid pCP2. The plasmid's T-DNA region carried the gene *GUSPlus* under the direction of promoter CP2 and the gene *hpt*II under the promoter 35S which was the selective marker.

The tissue was inoculated with the bacterium (grown overnight), using 200 µl of suspension per gram of FEC. The inoculated FEC was then submitted to a vacuum pressure of 25″ of Hg (12.3 psi) per minute and co-cultivated for 48 h at 22 ℃ in darkness and at a relative humidity of 49%. To select transgenic tissue, hygromycin was used at 10 mg l^{-1} during the induction of somatic embryos (an early regeneration stage). Complete plants were then regenerated and preselected for the genes *GUSPlus* and *hpt*II, using amplification by PCR according to the methodology described by Beltrán et al. (2009).

Genetic transformation of carrot

For carrot transformation, we essentially used the protocol reported by Hardegger and Sturm (1998), using the variety Chantenay Red Core. Transgenic plants were selected, using amplification by PCR and the GUS test, and established in the greenhouse under controlled conditions.

Establishing transgenic lines in confinement fields

After regeneration, the transgenic cassava plants were propagated *in vitro* and transferred to the greenhouse where they were maintained for 2 months. They were then planted under confinement field conditions at the International Center for Tropical Agriculture (CIAT, its Spanish acronym). The planting plot for the transgenic plants complied with the following biosafety standards: (1) minimum separation of 500 m from the nearest plot planted to cassava, (2) planting of live barriers of elephant grass (*Pennisetum purpureum* Schum.), (3) removal of flowers before anthesis, (4) manual and chemical control of weeds during the experiment and of postharvest sprouting, and (5) incineration of plant residues.

To determine the pattern of expression of the *GUSPlus* gene as conferred by promoter CP2, samples of mature storage roots, stems, and leaves were collected and evaluated, using GUS staining and quantifying β-glucuronidase enzymatic activity. To determine finer differences in the expression of the fusion CP2::*GUSPlus*, the root cortex was analysed separately from the edible root pulp. The latter comprises mostly parenchyma and xylem, and is where starch accumulation occurs.

Obtaining nucleic acids

For the PCR tests, genomic DNA was extracted, using the QIAGEN DNeasy Plant Mini Kit (Maryland, USA), and starting with 300 mg of leaf tissue pulverized with liquid nitrogen. The DNA was quantified by absorbance and its quality confirmed by electrophoresis in 1% agarose gel and staining with ethidium bromide.

RNA was extracted, using an SV Total RNA Isolation Kit (Promega Corporation, Madison, WI, USA), visualized in 0.8% agarose gel to confirm its quality, and quantified by absorbance in a Tecan GENios fluorometer (Tecan Trading, Zurich, Switzerland). To rule out contamination with DNA, a standard PCR was carried out for gene *18S*. Where DNA residues had to be eliminated, the RNA was treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA, USA).

Southern blotting

We separated 10 µg of genomic DNA (digested by enzyme EcoRI) by electrophoresis in 1% agarose gels and transferred them to a nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). To hybridize the membrane, we used a DIG DNA Labelling Kit (Roche Molecular Biochemicals, Mannheim, Germany), following the manufacturer's instructions. The temperature was 42 ºC, and the *GUSPlus* probe was 200 bp long and marked with digoxigenin. EcoRI cuts at one site in the T-DNA region of the pCP2 plasmid, between cassettes *GUSPlus* and *hpt*II. Hence, the number of hybridization signals was interpreted as the number of copies integrated with the *GUS Plus* gene.

Histochemical GUS staining

The *in situ* activity of the β-glucuronidase enzyme of the transgenic cassava and carrot lines was determined, using the histochemical GUS staining test, according to Jefferson (1987). We used 7-month-old plants that had been grown in the field. Cross-sections of leaves, petioles, stems, and storage roots were left in stain for either 3 h or 12 h at 37 ℃, washed several times with sterilized distilled water, and, except for the root samples, immersed in 70% ethanol (v/v) to remove chlorophyll. The stain comprised NaH_2PO_4 50 mM, Na_2EDTA 10 mM, $K_4Fe(CN)_6$ 0.5 mM, $K_3Fe(CN)_6$ 0.5 mM, 0.1% Triton X-100 (v/v), $NaHPO_4$ for adjusting to pH 8.0, methanol, and 0.5 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc). The same tissues of nontransgenic plants were used as control.

Quantifying enzymatic activity

Protein was extracted according to Bao et al. (2000) and Bao and Lazarovits (2002), using 100 mg of samples from leaves, stems, and root cortex and pulp to which was added 1 ml of GUS extraction buffer [$NaHPO_4$ pH 7.0 50 mM, 2-mercaptoethanol 10 mM, Na_2EDTA pH 8.0 10 mM, 0.1% Sarkosyl® (w/v), and 0.1% Triton X-100 (v/v)]. The mixture was homogenized by vortexing and leaving on ice for 30 min to facilitate extraction. After centrifuging for 10 min at 10,000 rpm and 4 ℃, the supernatant was saved. Protein concentration was determined, using a Bradford microassay method (Bradford 1976) and a standard of bovine serum albumin (BSA; Sigma-Aldrich Corporation, St. Louis, MO, USA).

The fluorometric quantification of β-glucuronidase activity is based on the release, as a function of time, of the compound 4-methylumbelliferone (4-MU), a product of the enzyme's catalytic activity on the substrate 4-methylumbelliferyl β-D-glucuronide

hydrate (MUG) (Jefferson 1987). This reaction is triggered by dilutions of the protein extracts in the presence of MUG (final concentration 0.8 mM), and incubated for 10 min at 37 ℃. The fluorescence emitted was measured in a DyNA DNA Quant™ 200 fluorometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA, USA) with a spectrum of excitation of 356 nm and one of emission of 494 nm. The enzymatic activity was expressed as pmol 4-MU per minute per µg of protein.

Quantitative real-time RT-PCR

The primer pairs suitable for real-time PCR of the genes *GUSPlus*, *hpt*II, and *18S* were recently reported by Beltrán et al. (2009). SuperScript™ II First-Strand Synthesis System for RT-PCR was used with Ramdom Primers (Invitrogen Life Technologies, Carlsbad, CA, USA) to synthesize cDNA from 1 µg of total RNA.

For amplification, 1 µl of a 1:10 dilution of each synthesized cDNA was used, and a final volume of 20 µl, containing 10 µl of master mix from the DyNamo™ SYBR® Green qPCR Kit (Finnzymes Oy, Espoo, Finland), made. For the amplification reaction, 0.1 µM of each primer was used, and the program was: one cycle of 15 min at 94 ℃, followed by 40 cycles of 10 s at 94 ℃, 25 s at the annealing temperature of each primer pair, and 35 s at 72 ℃. The program finished with an amplification of melting curves, consisting of a sweeping of temperatures from 65 to 95 ℃, increasing by 0.2 ℃ each second.

The reactions were carried out in the continuous fluorescence detector (DNA Engine Opticon®, MJ Research, Waltham, MA, USA), using the OpticonMONITOR 2.0 software from the same company. The Tm value for each amplification was recorded to verify

the specificity of the amplified product, and the amplification compared with that obtained for plasmid pCP2 as the positive control.

To estimate the transcription levels, the method of relative quantification was used with correction of efficiency as described initially by Pfaffl (2001) and modified for cassava transgenes by Beltrán et al. (2009). To confirm the specificity of the amplified products, qPCR products from the genes *GUSPlus* and *18S* were sequenced, using a BigDye® Terminator Kit in an automatic sequencer (ABI PRISM® 3100, Applied Biosystems, Foster City, CA, USA). The sequences were analysed, using the BLAST algorithm (Altschul et al. 1997).

Results

Cloning of *Mec1* gene and characterizing the promoter

To clone the *Mec1* gene, inverse PCR on EcoRI-digested and circularized genomic DNA was performed using the primer pair CAS II/ CAS III deduced from the corresponding cDNA sequence (accession No: AY101376). A 1926 bp genomic fragment was then obtained by PCR on genomic DNA, using the primer pair FinCas/CasP II, which were designed based on the sequence of the inverse PCR product. As shown in Fig.1, the obtained genomic fragment contains 1012 bp promoter sequence followed by the coding region. Consisting with the previously published data of de Souza et al. (2009), the TATA box of the p*Mec1* was identified 103 bp upstream from the start ATG. According to the PLACE software (dna.affrc.go.jp/PLACE/signalup.html), the p*Mec1* promoter harbours a sucrose responsive element (SURE) conserved among genes regulated by sucrose, e.g. the

patatin I gene of potato, in addition to conserved motifs occurring in regulatory sequences of β-amylase genes from different species. The p*Mec1* promoter contains also motifs indicating putative regulation by light, biotic and abiotic stress, and the phytohormones gibberellins and auxin. Furthermore, the promoter includes several copies of both nodulin consensus sequences, NODCON1GM and NODCON2GM, which are present in nodule specific genes from soybean. A list of selected putative motifs is presented in Table 2. As previously reported, the *Mec*1 coding region contains an intron of 136bp (de Souza et al. 2009). The deduced cDNA (534 bp including stop codon) encodes a protein almost identical (97% identity) to that of the allergenic-related protein Pt2L4 (accession No: AAM55492) reported by de Souza et al. (2006). Comparison of the *Mec*1 shown here with the glutamic acid-rich protein C54 reported by Zhang et al. (2003a) revealed a sequence identity of about 60%, indicating that the two proteins might have different biological functions. Accordingly, the sequences of the corresponding promoters differ significantly, indicating differential regulation. For instance, *pC54* does not contain the sucrose responsive element (SURE) or ARF (auxin response factor) binding site, which are present in *pMec1*.

Generating transgenic cassava plants and their molecular characterization

For cassava transformation, a 731 bp fragment from the promoter of *Mec*1, from here onward called CP2 promoter, was translationally fused to the *GUSPlus* gene. The binary vector pCP2 was then employed to transform cassava embryogenic calli, using the *Agrobacterium tumefaciens* based FEC transformation system. Eight of the 103 hygromycin-resistant cell lines regenerated to plants, from which four were successfully established in the field. The transgenicity of the lines numbered 10, 22, 24, and 26 was confirmed by PCR-detection of the genes *GUS Plus* and *hpt*II (data not shown). In

addition, the four lines were identified as coming from different transformation events, as suggested by southern blot analysis (Figure 2).

Patterns of expression of CP2 in cassava tissues and organs

Histochemical analyses were carried out on different organs of 7-month-old cassava plants grown in the field. Although we could detect, throughout this research, the expression of transgenes in plants grown *in vitro* and in the greenhouse (data not shown), the objective of this study was to determine the activity of the promoter under field conditions, in plants ready for harvesting. The GUS staining pattern was examined for all samples of storage roots, stems, leaves, and petioles.

Because roots comprise the organ of greatest interest in cassava, we focused our attention on identifying tissues stained with GUS. In root cross-sections, we could detect a differential pattern of expression of the *GUSPlus* gene in the three tissue systems that anatomically distinguish the cassava storage root: TSI (epidermis and cortical parenchyma), TSII (phloem and vascular cambium), and TSIII (secondary xylem with parenchyma cells that are highly specialized for storing starch) (de Souza et al. 2006).

The GUS staining analysis revealed strong and uniform expression across the three root tissue systems. However, the generalized pattern could be described as being strongest in the vascular tissue (central and secondary xylem); slightly less intense in the vascular cambium; but uniform throughout the parenchymatous tissue where starch accumulates (Fig. 3A, E).

In stem tissues (Fig. 3B), the expression of the *GUSPlus* gene was completely absent in the pith, explained in part by the presence of hollow sclerenchyma cells lined in xylem. Intense staining, however, was observed in the vascular bundles, including the xylem and phloem. The intensity of staining in the three outside layers declined gradually towards the pith (Fig. 3F). In petiole tissues (Fig. 3C), the pattern of expression was similar to that of stem tissues, showing no expression in the pith. Staining, however, was clearly less intense than in the stems. In leaf tissues (Fig. 3D), GUS staining seemed uniform, with an intensity that was comparable with that for petioles, but less than for roots and stems.

Quantifying GUS activity in cassava organs

Values of β-glucuronidase enzymatic activity were averaged across three different plants, with three replications of tissue per plant. The best expression was found in line 10, where levels of enzymatic activity were highest in the stems and root pulp, for which values were almost the same (17.2±2.1 and 17.5±1.6 pmol 4-MU per minute per µg protein, respectively; Fig. 4). An intermediate level of activity was recorded for TSI (epidermis and cortical parenchyma) at 11.9±1.5 4-MU per minute per µg protein. The lowest level of activity was recorded for leaves at 5±0.6 4-MU per minute per µg protein. The high level recorded for enzymatic activity in roots was reflected in the intense staining observed in cross-sections of this organ. The activity levels detected in leaves were also compatible with the light staining detected in leaf cross-sections.

Variations in mRNA levels of the *GUSPlus* gene and GUS activity in cassava roots

To refine the quantification of the potency of the *CP2* promoter fragment in directing gene expression in transgenic cassava roots, we evaluated levels of mRNA and enzymatic activity in four lines presenting variation in intensity of GUS staining (Fig. 5A). Results were classified within a quantitative range of enzymatic activity that fell into three categories: low (0.15±0.0), medium (1.27±0.04), and high (17.27±2.13 4-MU per minute per µg protein) (Fig. 5C). We point out that, in storage roots, high levels of enzymatic activity correlated with the intensity of histochemical GUS staining and the relative mRNA levels for the *GUSPlus* gene (Fig. 5A, B, C).

Patterns of expression and β-glucuronidase enzymatic activity in the organs and tissues of carrot transformed with CP1::*GUSPlus*

The carrot transformation system was used to evaluate, in a more expeditious way, the activity of a longer promoter fragment named CP1 (1012 bp long) fused to *GUSPlus* (Fig. 6A). As shown in Fig.1, the fragment CP1 included also an intrinsic intron, to account for possible regulatory roles exerted by this genetic element. Hence, 120 cotyledons from in vitro germinated seeds were transformed with *Agrobacterium*. They produced 228 calli from which 39 transgenic carrot plants were generated and transferred to the greenhouse. A weak GUS activity was visualized in leaves of several plants analyzed; it was restricted to the vascular tissue of leaf blades of plants expressing the *GUSPlus* gene. Results obtained with the best line are depicted in Fig. 6B.

In storage roots, β-glucuronidase activity appeared contrasting, showing intense and uniform GUS staining in secondary phloem (Fig. 6C). In contrast, GUS staining was noticeably absent in the central tissue, which comprised secondary xylem (Fig. 6C).

When the pattern of GUS staining was evaluated in whole *in vitro* plants, the promoter was observed to be stronger in roots than in leaves (not shown). We determined that levels of GUS enzymatic activity in roots and leaves to be 12.4±3.7 and 2.1±0.17 pmol 4-MU per minute per µg protein, respectively (Fig. 6D). This result indicated that, effectively, promoter activity was stronger in roots. Thus, the result confirmed that the CP1 promoter fragment had a pattern of differential expression, with a preference for roots, but restricted to the secondary phloem of this organ. This finding suggests that the CP1 promoter and the CP2 shorter version of the promoter pMec1, are both new candidates for the expression of genes of interest in storage roots.

Discussion

The lack of promoters that are suitable for the expression of genes in roots storing carbohydrates is a constraint in using cassava as a model for the expression of those genes of interest whose products accumulate in roots. To help improve this deficiency, we focused our attention on isolating and evaluating the expression of the promoter sequences of genes that code for GARPs whose expression is high in cassava stems and storage roots (de Souza et al. 2006).

To evaluate the promoter fragment CP2 in cassava, cross-sections of roots were examined. These showed strong GUS expression distributed uniformly throughout all tissues of this organ. The fact that the expression was also intense in parenchymatous tissues makes this promoter valuable. Starch accumulates in these tissues, which are usable for human and animal consumption, and for applications in the starch industry. However, staining demonstrated a more pronounced expression in the bundles of

xylem, phloem, and vascular cambium, in a manner that closely resembles the patterns conferred by the specific promoter of a major latex-like protein (*Mll*) in storage roots of sugar beet (*Beta vulgaris* L.; Oltmanns et al. 2006).

In addition, expression occurred in that region of the cortex that constitutes root peel, which is used in animal feed and which protects roots from soil diseases. That is, the proteins that control pathogens and insect pests such as the cassava burrower bug (*Cyrtomenus bergi* Froeschner*;* Bellotti et al. 1999) can be expressed through this promoter.

As is known, pCaMV35S still figures as one of the most heavily used promoters in genetic transformation of dicotyledonous and monocotyledonous for reaching high levels of constitutive expression (Gandhi et al. 1999). In cassava, this promoter has proved weak in root tissues, and is ruled out as the best candidate for expressing new, or improving existing traits in this organ (Zhang et al. 2003a). Sarria et al. (2000) pointed out that gene transcripts under its control may decline with maturity. Even though the pattern of expression of genes conferred by pCaMV35S is weak and heterogeneous in cassava roots, in leaves, it can direct high levels of variable GUS expression in greenhouse plants (Beltrán et al. 2009). Hence, this promoter continues to be heavily used for improving traits in this crop (Zhang et al. 2003b; Jørgensen et al. 2005).

Although data on the detection of transcripts for the fusion CP2::*GUSPlus* suggest that promoter fragment CP2 is active in leaves, the intensity of GUS staining and quantitative data on enzymatic activity reveal that CP2 is really much less active (by 3.5 times) in this tissue than in stems and roots. In the different organs of each transgenic

line, mRNA levels do not always reflect β-glucuronidase activity (data not shown). This phenomenon has not, until now, been reported in cassava, although it has already been demonstrated in other species such as strawberry (*Fragaria*) and *Agapanthus* (Schaart et al. 2002; Mori et al. 2007). The possibility has not been ruled out that the effects of position and/or post-transcriptional regulation may reduce the translation rate, inhibiting GUS enzymatic activity.

In biotechnology, to evaluate the range of concentration of transgenic protein is important, as transgenic plants with the desired levels of expression can be selected for specific applications (Furtado et al. 2009). The tendency is usually to select events with the highest levels of expression (more mRNA), but they do not always result in being the most adequate for expressing the desired trait. In some cases, such as in the modification of plant growth and development, controlled levels of expression of the transgenes involved may be more advisable (Phillips et al. 1992). In this study, we evaluated the expression of the β-glucuronidase protein, directed by the promoter fragment CP2, in different transgenic lines. According to the levels of expression found, we classified roots as having null, low, medium, or high enzymatic activity.

The number of copies of a transgene does not, in itself, seem to explain differences in enzymatic activity. Possibly, the effect of the position characterizing each integration into the genome and/or the post-transcriptional control was responsible. In the best cases, levels of enzymatic activity in roots surpassed those reported for the same type of tissue in beets, using the specific promoter *Mll* (Oltmanns et al. 2006). For future evaluations of transgenic events in the field, with new traits of agronomic interest, transgenic events will need to be evaluated with a broad range of expression to select

the most promising. With regard to plant morphology, all the lines analysed in the field had a normal appearance, producing vigorous roots similar to those of the control.

Results suggested that the CP2 promoter fragment would be useful for future biotechnological applications in cassava. For example, powerful promoters in stem and leaf phloem may be useful for conferring resistance to the cassava stem borer (*Chilomima clarkei* Amsel) (Bellotti 2002), or for controlling the cassava hornworm (*Erinnyis ello* L.; Bellottii 2002). Both lepidopteran pests cause serious problems in Latin America's cassava fields.

Considering the high level of activity in roots, the CP2 promoter fragment may also be useful for increasing the contents of micronutrients and/or proteins in this organ, or it could be used to modify starch characteristics among other traits of industrial importance. Thus, this promoter could be used to combine different traits in a single event that requires high levels of simultaneous expression in roots and vascular stem tissues. An example of such an event is producing varieties whose aerial parts resist pests and diseases, while roots exhibit improved nutritional qualities.

These results are pioneering in the establishment and analysis of transgenic lines in confined fields, following the biosafety standards established by CIAT and the Colombian government. These include live barriers that isolate the crop, or the emasculation or bagging of flowers, to mitigate pollen movement. Because cassava is heterozygous and propagates vegetatively, our experiments had to demonstrate stability of expression of transgenes across successive cycles of propagation, whether *in vitro* clonal, or in the greenhouse or field (Taylor et al. 2004).

According to de Souza et al. (2006), gene *mec1* finds expression in stems and different areas of storage roots. The authors also suggested that the GARP protein PtL2L4 is involved in the secondary growth of stems and storage roots. Our GUS staining evaluations (which reflected the activity of promoter CP2) demonstrated that these are the two organs where the most intense staining occurred (i.e. greatest enzymatic activity) and, hence, confirmed the authors' findings. Although de Souza et al. (2006) report that the Northern blot analyses did not detect signs of expression in leaves, we found high levels of transcription of the *GUS* gene under the CP2 promoter through real-time PCR (data not shown), even with the lowest levels of enzymatic activity.

These discrepancies could be attributed to either differences in the sensitivity of the techniques used to detect transcripts or the expression of gene *mec1* being under regulation (absent or low levels of expression) in cassava leaves. Because of the possible implication of promoter *pMec1* in stem and root growth, we conducted our tests on transgenic plants grown in the fields at CIAT where they were exposed to a conventional cassava cropping system.

The carrot transformation system permitted faster verification of the effectiveness of *the* larger promoter fragment CP1 in expressing genes in roots storing carbohydrates. Hence, in the GUS expression test, intense staining was observed particularly in the secondary phloem of roots, and a much lighter intensity in the leaf vascular system. The resulting GUS enzymatic activity in carrot showed that promoter CP1 was six times more active in roots than in leaves. This led us to test this finding with a gene for phytoene synthase (*crtB*) from *Pantoea ananatis* (previously *Erwinia uredovora*) to improve carotene content in cassava roots. Preliminary results demonstrated that,

effectively, the CP1 promoter directs the expression of *crtB* in cassava roots, producing a significant increase of carotenes in this organ (unpublished data).

With different promoters, including the constitutive, we observed considerable differences in the activity levels of β-glucuronidase in carrot on comparing transgenic plants grown *in vitro* with those grown in the greenhouse (Wally et al. 2008). In this study, we had aimed to minimize *in vitro* effects by analysing plants grown in the greenhouse. In quantitative terms, in leaves and roots, the CP1 promoter shows a very similar behaviour to UBQ3, which was recently suggested as ideal for expressing proteins in carrot tap roots. CP1 even surpassed the potency of, for example, promoters pCaMV35S, D35S, and *rol*D in carrot (Wally et al. 2008).

Finally, the results presented can be considered as pioneering in the evaluation of transgenic plants in the field, and as bringing this crop into the new era of seeking biotechnological products to benefit producers and consumers.

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Table 1 Primer sequences used to isolate and clone promoter pMec1 from cassava.

Primer	
CAS II	5´ TTG AAC CAA TGG GAA CTC ACC AC 3´
CAS III	5´ ACT GCT GGT GCT GCC TCT TCT GTT 3´
Fin-Cas I	5´ GAGGAGGAGGAGGAGGAGGGACT 3´
Cas-P II	5´CAAGCATCAACCAAGCACAATGTA 3´
PCI	5´ ATT CTG CAG GAG GAG GAG GAG GAG GAG 3´
PCII	5´ ATT CTG CAG CGT TGA CGG AAA GAA ACG 3´
PCNII	5´ CAG TCT CCA TGG CTG TTA CTA CCT A 3´

Table 2 Potential regulatory elements within the *Mec1* promoter from cassava (*Manihot esculenta*). The matches to known motifs have 100% similarity according to a search of the PLACE database. Positions given are relative to the 5´-end of the promoter. The orientation of the motifs is indicated (+, forward; -, reverse).

Motif	No. of motif	Sequence	Position	Function
ACGTTBOX	1	AACGTT	393(+)	T-box binding of bZIP Proteins
ARFAT	1	TGTCTC	952(+)	ARF (auxin response factor) binding site
ASF1MOTIFCAMV	1	TGACG	31(+)	ASF-1 binding site present in CaMV35S promoter; transcriptional activation by auxin and/or salicylic acid
CBFHV	1	RYCGAC	611(+)	Binding site of barley CBF1; dehydration responsive elements
CCAATBOX1	1	CCAAT	485(-)	Present in promoters of heat shock proteins
CTRMCAMV35S	1	TCTCTCTCT	940(+)	Enhancing gene expression, found in the CaMV35S promoter
EBOXBNNAPA	1	CANNTG	709(+)	E-box, a *cis*-acting element of napA storage-protein gene of *Brassica napus*
ELRECOREPCRP1	1	TTGACC	822(-)	EIRE, an elicitor responsive element of parsley PR1 genes
GAREAT	2	TAACAAR	429(-), 616(-)	GARE, GA-responsive element
GT1GMSCAM4	3	GAAAAA	190(+), 381(+), 564(+)	GT-1 motif found in promoter of soybean CaM isoform-4 involved in pathogen- and salt-induced response
IBOX	4	GATAAG	45(+), 106(+), 167(+), 297(+)	Conserved sequence upstream of light-regulated genes
LTRECOREATCOR15	1	CCGAC	612(+)	Core of low temperature responsive element (LTRE) of cor15a gene in Arabidopsis
MYBCORE	1	CNGTTR	709(+)	Binding of ATMYB1 and ATMYB2 from Arabidopsis; water stress response
MYBST1	1	GGATA	446(-)	Core motif of MybSt1 (a potato MYB homolog) binding site
NODCON1GM	5	AAAGAT	72(+),133(+), 194(+),259(+), 324(+)	Putative nodulin consensus sequences
NODCON2GM	7	CTCTT	511(+),789(+), 840(+),867(+), 914(+),945(+), 535(-),	Putative nodulin consensus sequences
SEBFCONSSTPR10A	1	YTGTCWC	951 (+)	Binding site of the potato silencing element binding factor (SEBF) gene found in promoter of pathogenesis-related gene (PR-10a)
SEF4MOTIFGM7S	1	RTTTTTR	406(+)	SEF4 binding site; consensus sequence found in promoter of soybean 7S globulin gene
SURE1STPAT21	1	AATAGAAAA	528(+)	Sucrose responsive element (SURE) conserved among genes regulated by sucrose.
SP8BFIBSP8BIB	2	TACTATT	672(+), 438(-)	SPBF binding site (SP8b) present in sweet potato sporamin (gSPO-B1) and beta-amylase genes.
WBBOXPCWRKY1	1	TTTGACY	822 (-)	Binding of WRKY proteins, present in amylase genes from different species.
WBOXNTCHN48		CTGACY	1007 (+)	A W box identified in tobacco class I basic chitinase gene CHN48, binding to NtWRKYs possibly involved in elicitor-response

Figure Legends

Fig. 1 Sequence of the cloned *Mec1* gene. The cloned gene includes 1012 bp promoter sequence (uppercase letters) followed by 670 bp coding region (lowercase letters,) including an intrinsic intron (136 bp, shaded) and finally by 244 bp 3´-non coding sequence (uppercase letters). Initiation-, stop-codon and TATA-box are presented bold and italic. Sequence of the CP2 fragment is underlined. Sequence of the CP1 fragment including the intrinsic intron and 27 bp coding sequence is shown in italic

Fig. 2 (A) Representation of the T-DNA region with promoter CP2 fused with gene *GUSPlus* and *hpt*II under promoter 35S, used as selective marker. T is the terminator region. (B) Selection of transgenic plants through the PCR of a 191-bp-long fragment of the *GUSPlus* gene and 182 bp of the *hpt*II gene. (C) Independent transgenic events (10, 22, 26, and 24) are distinguished by Southern blotting. NT is non-transgenic; P is the plasmid

Fig. 3 Histochemical detection of the expression of the fusion CP2::*GUSPlus* in tissues of 7-month-old transgenic cassava plants (line 10) grown in the field. Cross-sections of (A) roots, (B) stems, (C) petioles, and (D) leaves. (E) Inset of root section delineated in (A). TS refers to tissue system, of which there are three: I, II, and III. (F) Inset of stem section delineated in (B). (Symbols: pp = phelogen/pheloderm, e = epidermis, mp = palisade parenchyma, ms = spongy parenchyma, p = parenchyma, pt = pith, px = primary xylem, sx = secondary xylem, vc = vascular cambium, x = xylem.)

Fig. 4 Quantification of GUS enzymatic activity in extracts of different organs of transgenic cassava (Line # 10) containing the fusion CP2::*GUSPlus* and in non-transgenic cassava (NT). Root P is root pulp, i.e. TSII and TSIII; Root C is root cortex, i.e. TS

Fig. 5 Variability of the expression of the fusion CP2::*GUSPlus* in mature roots (7 months old) of transgenic cassava grown in confined fields. (A) Patterns of histochemical GUS staining for the non-transgenic control (NT) and lines with null, low, medium, and high expression. (B) The corresponding value in real-time PCR of quantification of messenger RNA levels for the fusion CP2::*GUSPlus*. (C) Quantification of β-glucuronidase enzymatic activity in the same tissues

Fig. 6 Evaluation of the expression of the fusion CP1::*GUSPlus* in leaves and roots of the best transgenic carrot line. (A) Schematic representation of the T-DNA used in the transformation. T is the terminator region. (B) GUS staining in a mature leaf. p = parenchyma and v = vascular tissues. (C) GUS staining in mature storage root. sx = secondary xylem; vc = vascular cambium; sp = secondary phloem. (D) Quantification of enzymatic activity in roots and leaves of the same transgenic line and a non-transgenic control (NT)

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   1  GAGGAGGA GGAGGAGG AGGGACTA TTTCGTTG ACGGAAAG AAACGATA AGAACATT TTAATAGA
  65  TGTAAGAA AAGATAGG GACTATTT CGTTGACG GAAAGAAA CGATAAGG ACATTTTA ATAGATGT
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 193  AAAAGATA AAGACGTT TTAATATA TTTATGAA AATATAGA GAGGGACG ATTTCGTT GACGGAAA
 257  GAAAAGAT GAGGAGGG ACTATTTC ATTGACGG AAAGAAAC GATAAGGA CGTTTTAA TAGATATG
 321  AGGAAAGA TAGGGACT ATTTCATT GACGGAAA GAAACAAT AAGGACGT TTTAATAG ATATGAAA
 385  AAGGTAAA AACGTTTT AATAGATT TTTGAAAA TGTAGGGA CTAACTTG TTAATAAT AGTAATAT
 449  CCAAAAAC TAAATAAA GGGTTTTA ATTGAGGG TAAAATTG GATTTTAA ACATTTTC TCTCTCCT
 513  CTTTTATT TAATTTTA ATAGAAAA GAGGACGG AAGGACTA TTTCGTTG ACGGAAAA AAAAATAT
 577  AAGGACGT TTTAATAG GTTTCTAA AAATATAG GGACCGAC TTGTTAAT AATGGCAA TACTCAGA
 641  GACTAAAT TATAAATC TCCCAAAT ATATATTT ACTATTTA GAAAACAT TAATATAT TATATATT
 705  TTCACAGT TGATAATA ATTGATGA CGAAGAAA TCTCATGG ATCTAGCT ACAAGATC AACTTGTT
 769  TAACATTA GTATCAAC CATTTTGC CATTTCTC TTTGATTT CAGTGAGA TGAGGGGT CAAATCCC
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 897  ATTCTCTA TATAAGCC ACTCTTAT TCATCCTC TCTCTGCA CCATCTCT CTCTTTCT GTCTCTCC
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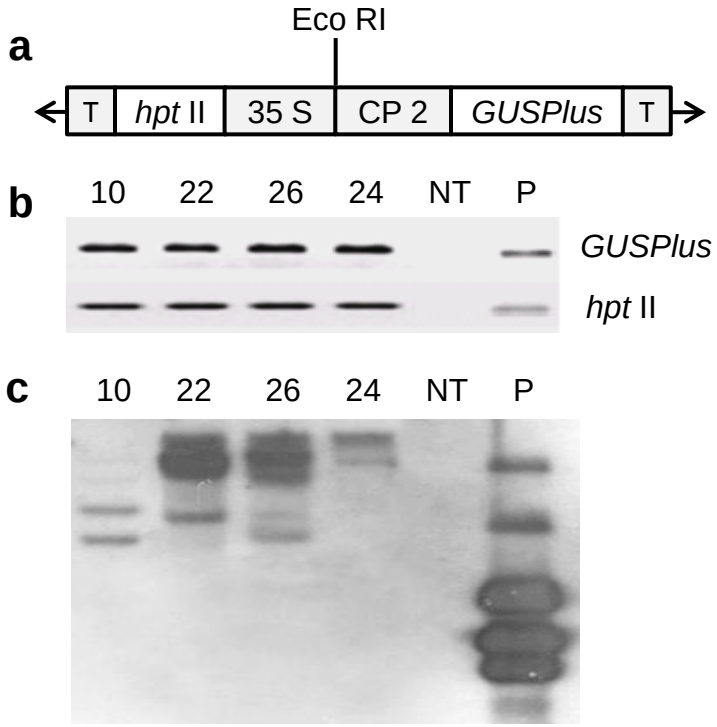
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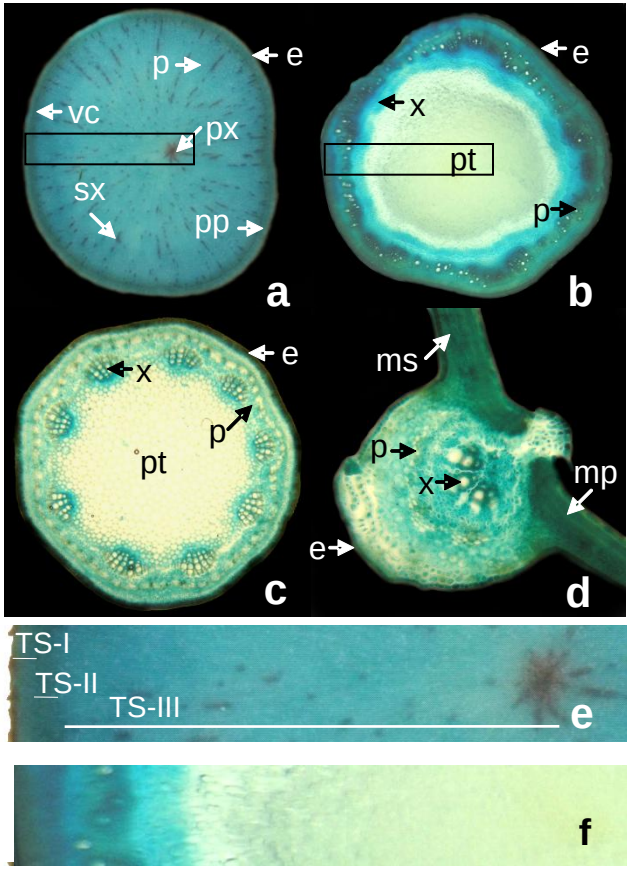
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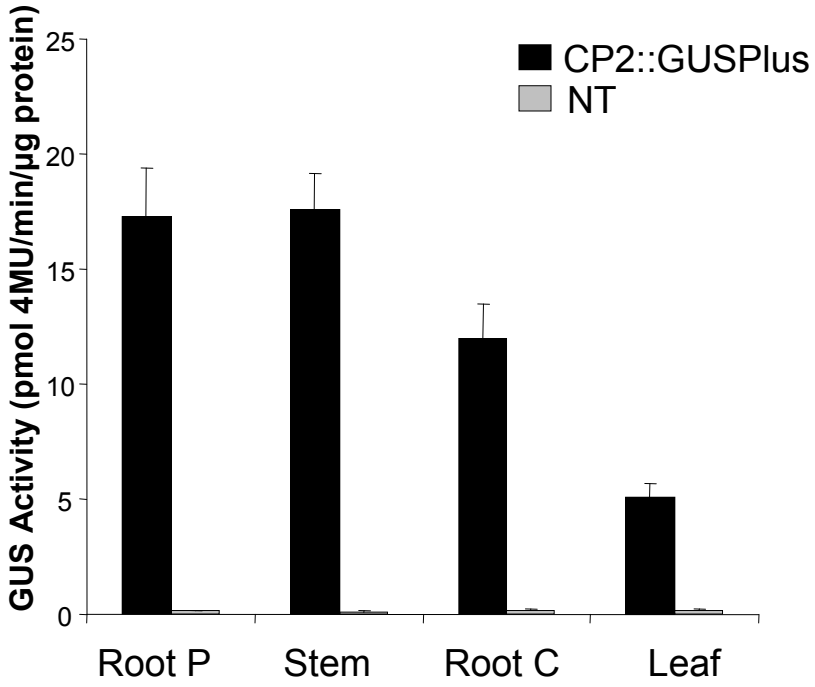
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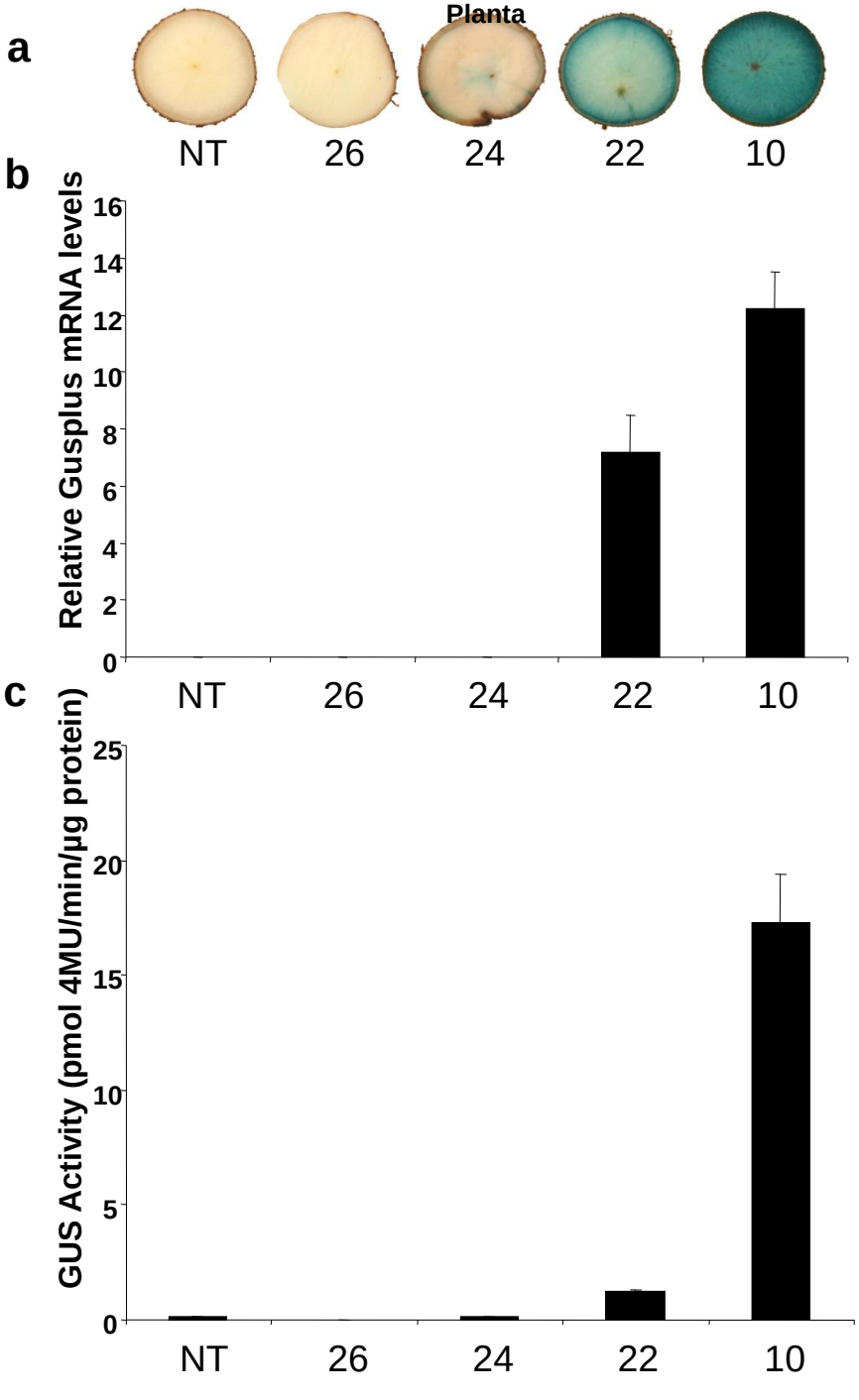
Root P
Stem
Root C
Leaf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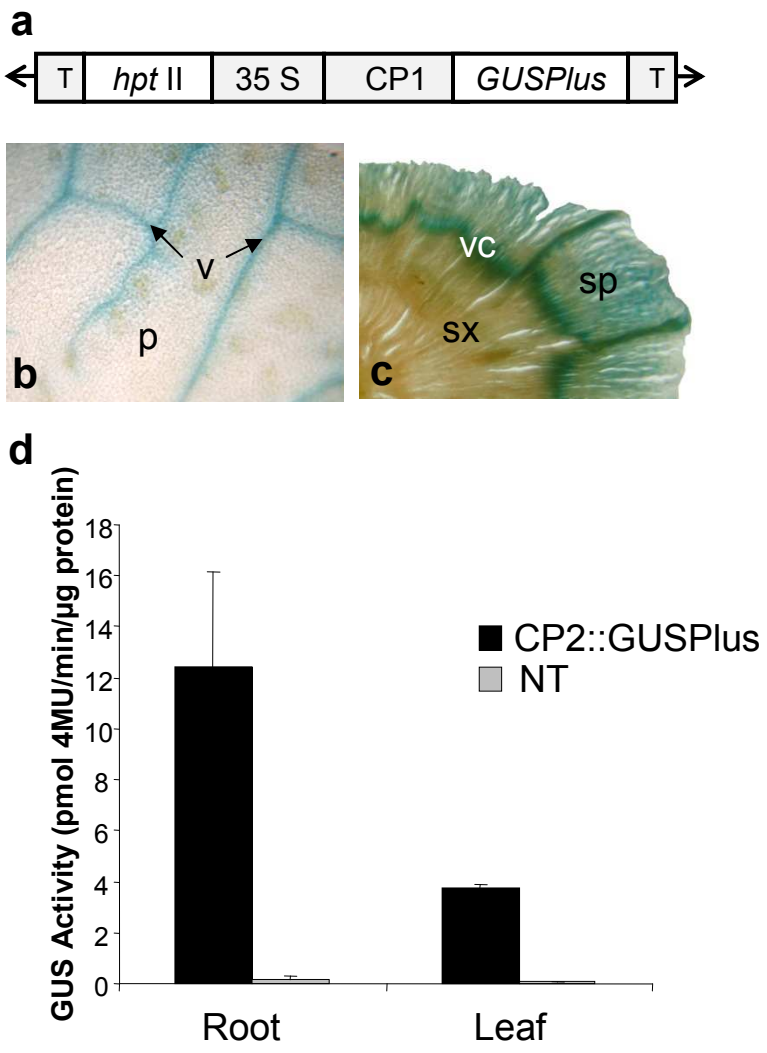
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