

# Putative storage root specific promoters from cassava and yam: cloning and evaluation in transgenic carrots as a model system

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**Abstract** A prerequisite for biotechnological improvements of storage roots is the availability of tissue-specific promoters enabling high expression of transgenes. In this work, we cloned two genomic fragments, *pMe1* and *pDJ3S*, controlling the expression of a gene with unknown function from cassava (*Manihot esculenta*) and of the storage protein dioscorin 3 small subunit gene from yam (*Dioscorea japonica*), respectively. Using  $\beta$ -glucuronidase as a reporter, the activities of *pMe1* and *pDJ3S* were evaluated in independent transgenic carrot lines and compared to the constitutive *CaMV35S* and the previously described cassava *p15* promoters. Activities of *pMe1* and *pDJ3S* in storage roots were assessed using quantitative GUS assays that showed *pDJ3S* as the most active one. To determine organ specificities, *uidA* transcript levels in leaves, stems and roots were measured by real-time RT-PCR analyses showing highest storage root specificity for *pDJ3S*. Root cross sections revealed that *pMe1* was highly active in secondary xylem. In contrast, *pDJ3S* was active in all root tissues except for the central xylem. The expression

patterns caused by the cassava *p15* promoter in carrot storage roots were consistent with its previously described activities for the original storage organ. Our data demonstrate that the *pDJ3S* and, to a lesser extent, the *pMe1* regulatory sequences represent feasible candidates to drive high and preferential expression of genes in carrot storage roots.

**Keywords** Carrot · Cassava · Dioscorin · Promoters · Storage roots · Yam

## Abbreviations

CaMV	Cauliflower mosaic virus
DJ	<i>Dioscorea japonica</i>
GUS	$\beta$ -Glucuronidase
Me	<i>Manihot esculenta</i>
uidA	$\beta$ -Glucuronidase gene

## Introduction

Storage roots and tubers are important in human nutrition. According to the Food and Agriculture Organization of the United Nations (FAO 2000), almost 99% of the world production of tuber crops is provided by five species with potato (*Solanum tuberosum*) being the most important (45%) followed by cassava (*Manihot esculenta*; 26%), sweet potato (*Ipomoea batatas*; 20%), yam (*Dioscorea* spp.; 6%) and taro (*Colocasia esculenta*; 1%). In arid areas such as sub-Saharan Africa, cassava ranks first place thanks to its capability to grow on poor soils and to withstand drought. Worldwide, cassava provides food for more than 600 million people (Nweke et al. 2002).

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Storage roots and tubers are rich in starch; however, they contain low levels of other nutrients. For instance, on a dry weight basis, the total protein content of cassava storage roots is only about 1–2%, while it ranges in seeds from about 10% in cereals to about 40% in soybean (Shewry 2003). Cassava roots are also very poor in micronutrients. For instance, most cultivars possess extremely low levels of provitamin A, promoting the incidence of vitamin A deficiency diseases (VAD) in populations living predominantly on cassava-based diets. In addition, cassava roots are very poor in mineral content such as iron and zinc (Montagnac et al. 2009), adding to the burden of iron deficiency anaemia (Ariza-Nieto et al. 2006). Another problem in many cultivars of cassava is their toxicity due to the presence of cyanogenic glycosides. Furthermore, the phenomenon of post-harvest deterioration severely reduces shelf life, posing constraints to marketing (Booth 1976; Chávez et al. 2006; Montagnac et al. 2009). Thus, there are numerous good reasons to invest into the improvement of cassava; however, compared to rice, maize and wheat, investments have been very small.

Unlike many other crops, improvement through breeding is difficult with cassava. This problem relates to very long breeding cycles as well as to the large genetic load and the heterozygous nature of parents and consequently, progenies. Cassava is propagated vegetatively and has therefore never been pushed to produce inbreds from the segregating progenies of a given cross rendering varietal recovery an extremely difficult task (Ceballos et al. 2004). Due to these reasons and because of the multitude of possible options for improvement, cassava must be considered as a major target for genetic transformation. Ideally, target cultivars (landraces) would be transformed directly to produce the desired trait(s). However, genetic transformation requires a toolset of enabling technology, which is not sufficiently developed for cassava. One important component would be to have a suite of validated promoters providing storage root specificity of transgene expression to avoid unwanted side effects such as those that may arise upon enhancing contents of provitamin A carotenoids. For instance, the constitutive expression of the carotenogenic enzyme phytoene synthase often needed to achieve high  $\beta$ -carotene (provitamin A) levels (Fray et al. 1995; Shewmaker et al. 1999; Ye et al. 2000; Lindgren et al. 2003; Paine et al. 2005; Maass et al. 2009) resulted in dwarfism in tomato, most likely caused by redirection of the precursor geranylgeranyl diphosphate, which gibberellins and carotenoid pathways have in common (Fray et al. 1995). Similarly, constitutive expression of the bacterial carotene desaturase *CrtI* led to significant changes in the carotenoid pattern of rice and potato leaves, which may impact photosynthetic performance negatively (Al-Babili et al. 2006; Diretto et al. 2007).

Yam is the name applied to about 500 species of the genus *Dioscorea*, including widely grown species like *D. cayenensis*, *D. rotundata* and *D. alata*. In general, yam tubers contain dioscorins as the major storage protein accumulated in the vacuoles and accounting for about 85% of total protein (for review see Shewry 2003). Dioscorins are present as two classes, with about 70% similarity to each other and with significant homology to  $\alpha$ -carbonic anhydrase from various sources. This enzymatic activity was demonstrated for dioscorins purified from *D. batatas*, *D. alata* and *D. pseudojaponica* (for review see Shewry 2003). However, a comprehensive, more recent study on *D. batatas* tuber storage proteins and their enzymatic functions led to the isolation of four different dioscorins designated as DB1, DB2, DB3 and DB4, and revealed DB1 and DB3 as lectins binding mannose and maltose, respectively. In addition, it was shown that DB3 is composed of a large 66-kDa protein consisting of two 31-kDa subunits (DB3L, 242 amino acids) cross linked by disulfide bonds, and two small 31-kDa subunit (DB3S; 241 amino acids). The two subunits show a sequence identity of 72% to each other and exhibit high homology to dioscorin A and B from *D. alata* (Gaidamashvili et al. 2004).

Underground storage organs can originate from stems (potato, taro), hypocotyls (yam and sweet potato) and roots (cassava, carrot). The development of the storage root in cassava is initiated by rapid cambial activity along the root axis and by differentiation of secondary xylem towards the inside, with starch accumulation restricted to ray parenchyma cells, thereby replacing secondary xylem fibres (Lowe et al. 1982). The carrot storage root is formed mainly from secondary phloem containing storage parenchyma cells (Surlles et al. 2004). In contrast to carrot and cassava, the storage organ of sugar beet develops from both hypocotyl and primary root by secondary growth initiated by the generation of consecutive rings of secondary cambium producing xylem and phloem (Elliott and Weston 1993).

To date, the genetic transformation of cassava relies on heterologous promoters, such as the cauliflower mosaic virus *CaMV35S* promoter (González et al. 1998; Zhang et al. 2000; Jørgensen et al. 2005) and the cassava vein mosaic virus promoter (Verdaguer et al. 1996). Apart from these constitutive promoters, the tuber-specific potato class I patatin promoter was used to engineer starch synthesis (Ihemere et al. 2006). However, little is known about its tissue specificity in the cassava background. By differential screening of a storage root cDNA library of cassava Mcol1505, Zhang et al. (2003) identified the genes, *c15* and *c54*, encoding a cytochrome P<sub>450</sub> and a glutamic acid-rich protein, respectively. Using *uidA* as reporter gene, evaluation of the *p15* and *p54* promoters demonstrated that both of them were highly active in the starch-rich parenchyma

cells of cassava transgenic storage roots. However, both promoters were also active in phloem, cambium and xylem vessels of vascular tissues of leaves, stems and roots (Zhang et al. 2003).

To identify novel storage root specific promoters, we relied on GenBank data representing genes preferentially expressed in cassava roots. In addition, we considered candidate genes expressed in underground storage tissues homologous with the cassava root, i.e. yam tuber. As a first candidate, we cloned a promoter fragment (*pMe1*) from cassava controlling the expression of a gene with unknown function, showing preferential storage root expression and encoding a member of the  $\alpha$ -amylase inhibitors (AAI), lipid transfer (LT) and seed storage (SS) protein family (AAI\_LTSS). As a second candidate, we isolated a promoter fragment (*pDJ3S*) of the *D. japonica DJ3S* gene encoding the small subunit of the DJ3 dioscorin. In this work, the activities of these two promoters were assessed in carrots as a model system producing storage roots. This was deemed necessary because cassava transformation is very time consuming. As comparators, we used the cassava *p15* (Acc. no. AY217352) and the constitutive *CaMV35S* promoters. Our data demonstrate that the yam *pDJ3S* and, to lesser extent, the *pMe1* promoters represent suitable candidates to drive high and preferential expression of genes in carrot storage roots.

## Materials and methods

### Plant material and growth

Tubers of *Dioscorea japonica* were obtained from the local market and roots from *Manihot esculenta* Crantz were obtained from CIAT (landrace MPer 183). Wild white carrot seeds (*Daucus carota* subsp. *carota*, Queen Anne's Lace) were obtained from Richters Herbs (Goodwood, Canada). Carrot plants were grown in soil under long day conditions. Roots of 9-week-old carrot plants were used for histological dissection and RNA and protein extraction.

### Southern blot analysis

*Manihot esculenta* Crantz genomic DNA was isolated according to Sambrook and Russell (2001). 40  $\mu$ g of genomic DNA were digested with *EcoRI*, *HindIII*, *NcoI* and *XbaI* and fractionated electrophoretically through a 0.8% (w/v) agarose gel before capillary transfer and immobilization on nylon membrane (Hybond N<sup>+</sup>, Amersham Biosciences Europe, Freiburg, Germany). A probe covering the cloned genomic fragment was obtained from pMe1-Gen1 using the enzymes *BamHI* and *HindIII*. Radiolabeling,

blotting and hybridization were performed according to Sambrook and Russell (2001).

### Construction of binary vectors

To generate pCA-pMe1-uidA the *pMe1* promoter was amplified from pST-ext-gene using the primers Extgen1 and Extgen2 carrying a *SphI* and *XbaI* site, respectively. PCR was performed with the proofreading PWO DNA Polymerase (Peqlab, Erlangen, Germany), and the obtained promoter was digested with *SphI*, filled-in with T4 DNA polymerase and treated with *XbaI*. To replace the *CaMV35S* promoter driving the *uidA* expression in the binary pCAMBIA1305.2 (CAMBIA, Australia), the plasmid was digested with *NcoI*, filled-in with T4 DNA polymerase and treated with *XbaI*. After dephosphorylation, the plasmid was ligated with the *pMe1* fragment to yield pCA-pMe1-uidA.

To generate pCA-pDJ3S-uidA, the yam promoter was amplified from pYam-Gen using the *Pfx* DNA polymerase (Invitrogen, Paisley, UK) with the primers Yam G(i)1 and MutGUS that carries a *NcoI* site. The obtained product was then digested with *NcoI* and *EcoRV* and ligated into *NcoI* and *Ecl136II* treated pCA-CP1, a pCAMBIA1305.2 derivative, to yield pCA-pDJ3S-uidA. A 1 kb portion was then excised from the 5'-end region of the *pDJ3S* promoter of the pCA-pDJ3S-uidA plasmid using *EcoRI* and *Ecl136II* restriction sites. The plasmid was then religated to form pCA-pDJ3S-s-uidA.

pCA-p15-uidA binary vector was kindly provided by ETH (Zhang et al. 2003).

### Inverse PCR

To produce circular DNA fragments, 10  $\mu$ g of genomic DNA from cassava and yam were digested in a total volume of 100  $\mu$ l with *Clal* and *EcoRI*, respectively. Digested DNA was purified using GFX<sup>TM</sup> PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ) and eluted with 100  $\mu$ l of 60°C pre-warmed water. 40  $\mu$ l of purified genomic fragments were then ligated for 2 h at room temperature followed by 20 h at 16°C in a total volume of 300  $\mu$ l, using 100 U of T4 DNA ligase. The enzyme was then deactivated by heating for 10 min at 60°C, and circularised genomic DNA was precipitated with EtOH and resuspended in 100  $\mu$ l water. Inverse PCR was then performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using 1  $\mu$ l of the circularised DNA, 100  $\mu$ M dNTPs, 50 ng of each primer and 0.5  $\mu$ l Advantage<sup>®</sup> cDNA Polymerase Mix (DB Bioscience, CA, USA) in the buffer provided, as follows: 2 min initial denaturation at 94°C followed by 35 cycles (30 s 94°C, 30 s annealing, 5 min 68°C) and 10 min final polymerization at 68°C. For annealing, a temperature gradient of 1°C, ranging from 58 to 68°C, was applied.

### Promoter sequence analysis

To detect putative *cis*-acting regulatory elements in the promoters sequences cloned, the database PLACE was used (Higo et al. 1999). The database is accessible at <http://www.dna.affrc.go.jp/PLACE>.

### Carrot transformation

Transformation was carried out using pieces of cotyledons and hypocotyls of 1-week-old light-grown QAL seedlings and the *A. tumefaciens* GV3101 strain, according to Hardegger and Sturm (1998), with some modifications. Instead of claforan and geneticin, vancomycin (200 mg/l) and hygromycin (10 mg/l) were used to arrest bacterial growth and to select transgenic calli, respectively.

### GUS assays

$\beta$ -Glucuronidase (GUS) activity in carrot tap roots of primary transformants was determined as described by Jefferson (1987) and Jefferson et al. (1987). For the fluorometric GUS assay 100 mg tissue was homogenized in 1 ml GUS reaction buffer consisting of 50 mM NaPO<sub>4</sub>, pH 7.1 mM EDTA; 0.1% (v/v) Triton X-100; 0.1% (v/v) sodium sarcosine and 10 mM  $\beta$ -mercaptoethanol. After centrifugation, 10  $\mu$ l of the supernatant was added to 190  $\mu$ l GUS reaction buffer containing 0.56 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide (4-MUG) and incubated at 37°C. The amount of 4-methylumbelliferone (4-MU) produced was determined after 5 min, and the measurement was repeated five times in 15 min intervals. For this purpose, 20- $\mu$ l aliquots were mixed with 180  $\mu$ l water and measured in a luminescence spectrometer (LS 50, Perkin Elmer) at excitation 365 nm, emission 455 nm. 0.0267 fluorescent units corresponded to 1 pmol 4-MU. Protein content was determined with Bio-Rad protein assay (Bio-Rad, Munich, Germany), and enzyme activity was calculated as pmol 4-MU min<sup>-1</sup> ( $\mu$ g protein)<sup>-1</sup>.

For histochemical staining, carrot tap root dissections were incubated for 5 min at 37°C in X-Gluc buffer [1 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-gluc), 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.0, 0.01% (v/v) Triton X-100, 10 mM EDTA, 0.5 mM K<sub>4</sub>Fe(CN)<sub>6</sub> and 0.5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>]. After staining, dissections were treated for 10 s with 70% (v/v) ethanol.

### TaqMan real-time RT-PCR assay

RNA was isolated using the plant RNA isolation reagent (Invitrogen, Heidelberg, Germany). RNA purification and on-column DNaseI digestion were performed using the

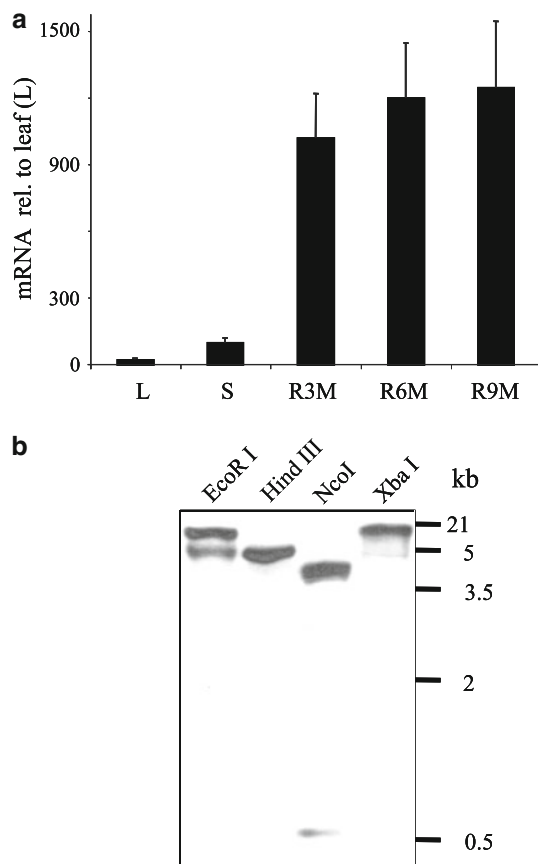
Qiagen RNeasy mini kit (Qiagen, Hilden, Germany). After first-strand cDNA synthesis, Taq-Man real-time RT-PCR assays were carried out with an ABI Prism 7000 (Applied Biosystems) using 18S rRNA levels for normalization. The relative quantity of the transcripts was calculated using the comparative threshold cycle method (Livak 1997). Data were first normalized to the corresponding 18S rRNA levels quantified using the eukaryotic 18S rRNA endogenous control kit (Applied Biosystems) and then calculated relative to a specific sample. Data represent averages of six transgenic lines per construct. Primers and 6FAM-labelled probes for *uidA* and *MeI* genes were designed using Primer Express software (Applied Biosystems). The measurements were performed with primer/probe combinations GUS-Fw, GUS-Rv/GUS-probe for *uidA* and Ext-Fw, Ext-Rv/Ext-probe for *MeI* (see Table S1 for primers/probes sequences).

## Results and discussion

Cassava *MeI* gene is encoded by a single copy gene preferentially expressed in storage roots

Nucleotide sequences with preferential root expression annotation reported in the GenBank of the NCBI (<http://www.ncbi.nlm.nih.gov/Genbank>) were selected and subsequently blasted against a collection of cassava ESTs described by Lopez et al. (2004). This process led to the identification of an EST (Acc. no. CK651140), here called *MeI*, preferentially expressed in storage roots and encoding a member of the AAI\_LTSS protein superfamily (see “Introduction”). According to the NCBI conserved domains database (Marchler-Bauer et al. 2009; <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>), the AAI\_LTSS protein family is characterized by a common pattern of eight cysteines forming four disulfide bridges.

To verify the *MeI* expression pattern, real-time RT-PCR analyses were performed. These demonstrated that the transcript levels of *MeI* are markedly higher in 3-month-old roots than in leaves or stems and steadily increased during storage root development (Fig. 1a). The copy number of the *MeI* gene was determined by Southern blot hybridization and revealed one band with the *Xba*I- and *Hind*III-digested DNA (Fig. 1b), indicating the presence of a single copy gene. This was confirmed by the presence of two fragments upon *Nco*I- and *Eco*RI-digestion (Fig. 1b), arising from corresponding restriction sites in the gene (Fig. S1a; Acc. no. GU324673). Taken together, the cassava *MeI* is represented by a single copy, which is preferentially expressed in storage roots. These features predict the *MeI* promoter as candidate to allow storage root specific expression of transgenes.



**Fig. 1** Characterization of *Me1* gene from cassava. **a** mRNA levels of the *Me1* gene were determined by real-time RT-PCR in leaves (*L*), stems (*S*) and storage roots from 3- (*R3M*), 6- (*R6M*) and 9- (*R9M*) month-old cassava plants. mRNA values are given relative to the levels determined in leaves (*L*). Bars represent the mean of 18S rRNA-normalized mRNA levels + SD of duplicate samples from three biological replicates, each. **b** Southern Blot analysis of *pMe1* gene using a *Me1* PCR fragment as a probe

#### Cloning of the cassava *pMe1* promoter

To clone the *Me1* promoter, inverse PCR on *Cla*I-digested and circularised genomic DNA was performed using the primer pair Ex-In1/Ex-In2 (for sequence, see Table S1) deduced from the corresponding EST sequence (Acc. no. CK651140). A 1,748-bp gene fragment (*Me1*) was then obtained by PCR on genomic DNA using the primer pair Ex-Prom1/Ext2 (for sequence, see Table S1), which were designed according to the sequence of the inverse PCR product. As shown in Fig. S1a, the obtained gene fragment *Me1* consists of 1,378 bp upstream of the start-ATG (*pMe1*), containing promoter sequence and unidentified 5' untranslated region (5' UTR), followed by 370 bp of the coding region (see Fig. S1a; Acc. no. GU324673). The TATA box of the *pMe1* is 120 bp upstream from the start-ATG. According to the PLACE software program (<http://www.dna.affrc.go.jp/PLACE>), the *pMe1* promoter contains

transcription factor binding sites reported for storage proteins, in addition to several putative motifs mediating hormone, biotic and abiotic stress responses (Table 1).

#### Cloning of a dioscorin *DJ3S* promoter from *D. japonica*

As first approach, we used genomic PCR to clone fragments corresponding to dioscorin mRNA sequences available in the NCBI database. Several sets of primers were designed based on the mRNAs of *Dioscorea batatas* DB3S storage protein (Gaidamashvili et al. 2004; Acc. no. AB178473); *Dioscorea alata* dioscorin B and A (Acc. no. AF243526 and AF245019) and of dioscorin from *D. cayenensis* (Acc. no. X76187). The usage of the primer pair Yam1/Yam3 (for sequence, see Table S1) led to the cloning of a ca. 1.7 kb genomic fragment encoding a *D. japonica* dioscorin 3 small subunit (*DJ3S*), as suggested by sequence comparisons with the *D. batata* DB3S and to the *D. japonica* 3 dioscorin sequences (Acc. no. AB178473 and AM849818, respectively). Inverse PCR was then performed on *Eco*RI-digested and circularised genomic DNA using the primer pair Y-Inv1/Y-Inv3 (for sequence, see Table S1) deduced from the genomic fragment. The sequences of the 5' and 3' ends of the ca. 3 kb inverse PCR product were used to design the primers Yam-G(I)1 and Yam-G(I)2 (for sequence, see Table S1), which allowed the cloning of a 2,309 bp genomic fragment consisting of 379 bp coding region and 1,930 bp upstream of the start-ATG (*pDJ3S*; see Fig. S1b; Acc. no. GU324672), containing promoter sequence and unidentified 5' UTR. A putative TATA box of the *pDJ3S* was identified 75 bp upstream from the start-ATG. According to the PLACE software, the *pDJ3S* promoter contains transcription factor binding sites reported for storage proteins and root-specific genes, in addition to several putative motifs mediating expression in root nodules and response to hormones, biotic and abiotic stress (Table 1). A short version *pDJ3S-s* (891 bp) was produced by cutting down 1,039 bp from the 5'-end of the 1930 promoter region (using *Ecl*136II restriction enzyme. See Fig. S1b).

#### Evaluation of the *pMe1* and the *pDJ3S* fragments in carrot

Since the transformation of carrot is more feasible and less time consuming than of cassava, we decided to test the activities of the fragments *pMe1*, the *pDJ3S* and its short version *pDJ3S-s* in the former species. To estimate the eligibility of carrot as a model system for testing storage root specificity, we also assessed the activity of the cassava *p15* promoter, which was previously evaluated in its respective storage tissue (Zhang et al. 2003). The

**Table 1** Putative *cis*-acting regulatory elements in the *pMe1* and *pDJ3S* genomic fragments from cassava and yam, respectively

#	Regulatory element	Position	Motif	Reported for
<b><i>pMe1</i></b>				
1	−300CORE	897(−), 1199(+)	TGTAAAG	Endosperm storage proteins
2	AMYBOX2	587(−)	TATCCAT	α-Amylase, sugar starvation
3	CANBNNAPA	1248(+)	CNAACAC	Storage proteins, endosperm expression
4	CATATGGMSAUR	93(+), 502(+), 93(−), 502(−)	CATATG	Auxin response
5	CPBCSPOR	603(+), 1364(+), 1203(−)	TATTAG	Cytokinin response
6	DPBFCOREDCDC3	901(+), 1181(+)	ACACNNG	ABA-responsive elements
7	ELRECOREPCRPI	1058(+)	TTGACC	Elicitor response
8	ERELEE4	104(+)	AWTTCAAAA	Ethylene response
9	GT1GMSCAM4	712(+), 744(+), 1034(−)	GAAAAA	Pathogen and salt response
10	MYB2AT	917(−)	TAACTG	Water stress response
11	MYB2CONSENSUSAT	1075(+), 917(−)	YAACKG	ABA signalling
12	MYCATRD22	1182(+)	CACATG	Water stress; ABA
13	NTBBF1ARROLB	1341(+), 210(−), 561(−)	ACTTTA	Auxin induction
14	POLASIG2	420(+), 425(+), 446(−)	AATTTAA	α-Amylase
<b><i>pDJ3S</i></b>				
1	2SSEEDPROTBANAPA	1188(+), 1585(+)	CAAACAC	Storage proteins
2	BS1EGCCR	303(+)	AGCGGG	Vascular expression
3	CANBNNAPA	1188(+), 1585(+), 1025(−)	CNAACAC	Storage proteins, endosperm expression
4	CAREOSREP1	226(+), 1847(+)	CAACTC	Gibberellin response
5	CATATGGMSAUR	1267(+), 1267(−), 1267(−)	CATATG	Auxin response
6	GT1GMSCAM4	1237(+), 1285(+), 1399(+)	GAAAAA	Pathogen and salt response
7	HBOXCONSENSUS-PVCHS	1772(+)	CCTACCNN NNNNNCT	Wounding and abiotic stress
8	MYB2CONSENSUSAT	515(+)	YAACKG	ABA signalling
9	MYBGAHV	1373(−)	TAACAAA	Gibberellin response
10	NTBBF1ARROLB	880(+), 767(−)	ACTTTA	Auxin response
11	OSE1ROOTNODULE	1323(−), 1811(−)	AAAGAT	Expression in root nodules
12	PYRIMIDINEBOX- OSRAMY1A	335(−)	CCTTTT	α Amylase and sugar repression
13	SORLIP1AT	345(+), 651(+), 435(−) 470(−), 1272(−)	GCCAC	Root-specific genes

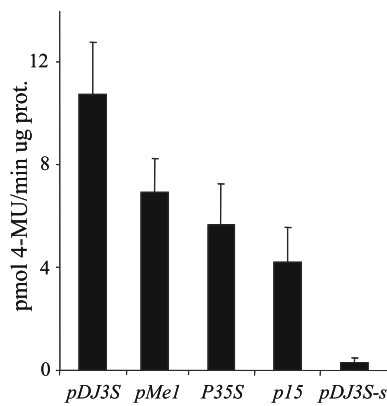
Regulatory elements for *pMe1* and *pDJ3S* fragments containing promoter sequences and 5′ UTRs of unknown lengths were identified using the PLACE database (Higo et al. 1999). Positions given are relative to the 5′-end of the fragments. The motifs orientation is marked (+, forward; −, reverse). Nucleotides indicated as follows: N for A, C, G or T; Y for C or T; K for G or T and W for A or T

constitutive *CaMV35S* promoter was employed as a constitutive comparator.

For this purpose, binary plasmids harbouring *pMe1::uidA* and *pDJ3S::uidA* fusions were constructed (junction sequences are shown in Fig. S2). At least six independent transgenic lines for each promoter were then generated and grown for 9 weeks to allow the formation of storage roots. The activities of *pMe1* and *pDJ3S* in transgenic carrot roots were measured by quantitative GUS assays. This revealed pronounced differences (Fig. 2) with the yam *pDJ3S* as the most active, followed by, the cassava

*pMe1*, the constitutive *CaMV35S*, the cassava *p15* and finally by the short *pDJ3S-s* fragment.

To evaluate organ specificity of expression, the corresponding leaves and stems were also subjected to GUS assays. However, the GUS enzymatic activities determined in leaves were negligibly low (data not shown), also in the *CaMV35S::uidA* transformants. Such low GUS enzymatic activities might be caused by interfering secondary metabolites or by the instability of the enzyme in these carrot tissues. Similar difficulties have been reported in carrot old leaves (Hardegger and Sturm 1998) and wheat where GUS

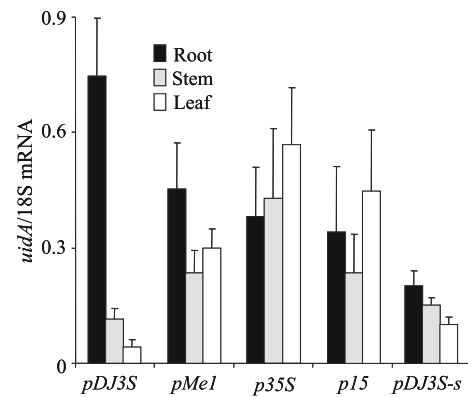


**Fig. 2** Quantitative GUS assays with transgenic carrot roots. GUS assays were performed using 4-methylumbelliferyl- $\beta$ -D-glucuronide (4-MUG) as substrate with storage roots from transgenic carrots transformed with constructs harbouring the various promoter::*uidA* fusions investigated *pDJ3S* and its short version (*pDJ3S-s*) from yam, *pMe1* from cassava, *p15* from cassava and *CaMV35S*. Bars represent the mean + SD of the specific activities of six different transgenic lines per construct

activities in transgenic leaf and root tissues were supposed to be inhibited by an unidentified non-proteinaceous compound (Bahieldin et al. 2005). In carrot roots where GUS activity could be determined, the rank-order given in Fig. 2 showed highest activity with the yam *pDJ3S*.

Because of the problems given above, we resorted to transcript levels to assess the organ specificities of the promoters tested. For this purpose, real-time RT-PCR analyses were carried out using RNA isolated from roots, stems and leaves of six transgenic lines for each promoter (Fig. 3). With the exception of the previously described cassava promoter *p15* that showed comparatively higher activity in leaves, all tested regulatory sequences exhibited better storage root specificity when compared to the constitutive *CaMV35S* promoter. The highest expression levels were again obtained with the *pDJ3S* from yam, as seen with the GUS enzymatic assays. In addition, the *pDJ3S* from yam provided the best storage root specificity. The cassava *pMe1* showed intermediate root specificity expression levels in the carrot system.

In Fig. 4, cross sections of the GUS-stained storage roots from the diverse promoter::*uidA* transgenic carrot are shown. The constitutive *CaMV35S* promoter (Fig. 4a) did not stain the residual primary xylem and was not active in large parts of the secondary xylem. Similar to previously published data (Hardegger and Sturm 1998), GUS staining was mainly observed in the younger xylem and phloem ray cells and in adjacent parenchymatic cell. In contrast to the *CaMV35S* promoter, the cassava *p15* promoter, used as a comparator in this study, led to very intense GUS staining of the vascular cambium; staining of the xylem and phloem rays was restricted to the youngest cells (Fig. 4b). This is in agreement with the results obtained in cassava roots where



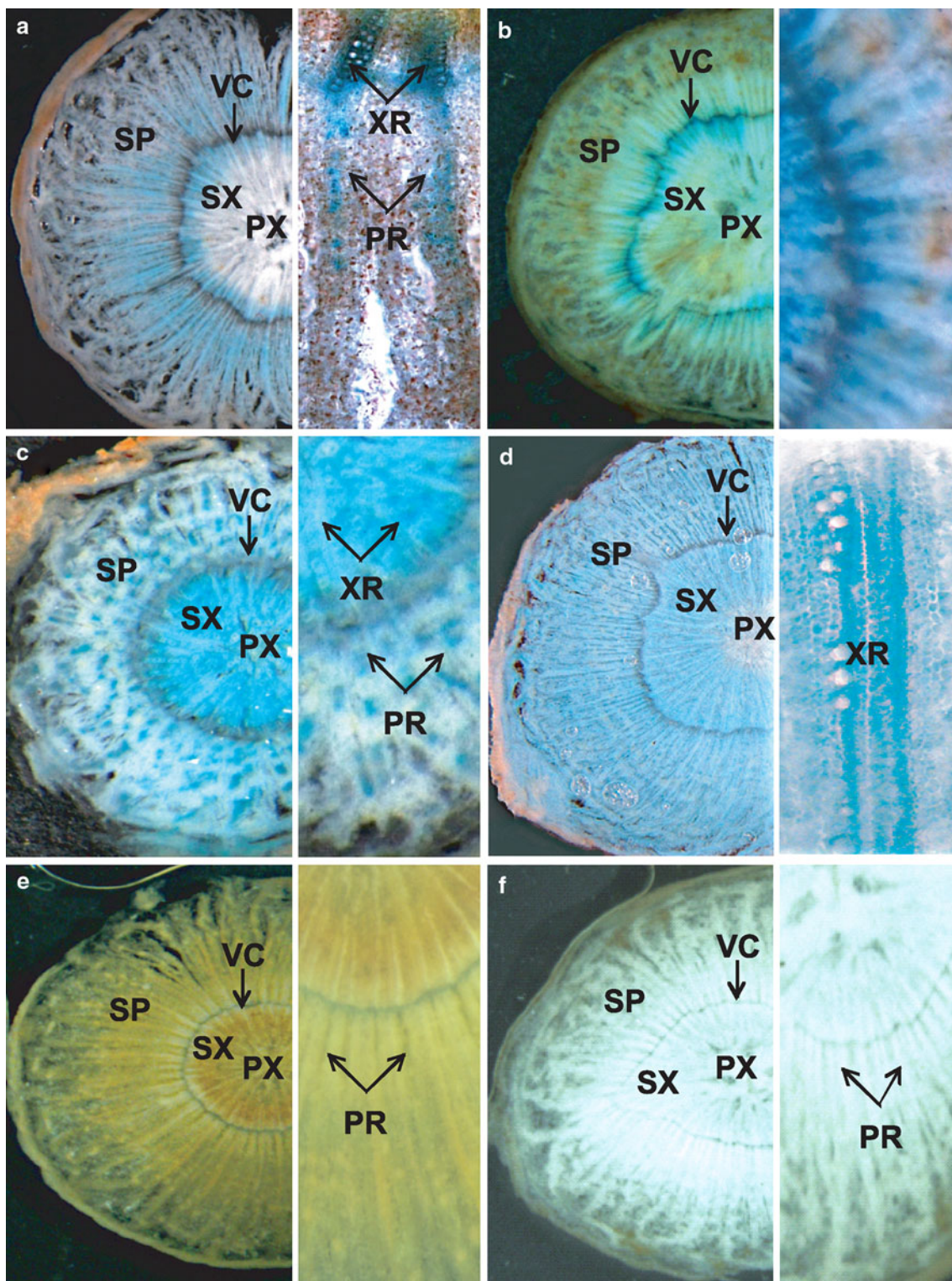
**Fig. 3** Expression levels of reporter *uidA* gene in different organs of transgenic carrots with various promoter::*uidA* constructs. Total RNA from 9-week-old roots, stems and leaves from transgenic carrots were used for real-time RT-PCR. Transcript levels are expressed after normalization to 18S rRNA concentrations of the corresponding samples. Bars represent the mean + SD of six different transgenic lines

the strongest GUS activity was observed in the cambium (Zhang et al. 2003). A completely different expression pattern was obtained with the cloned *pMe1* from cassava (Fig. 4c), showing the highest expression in the secondary xylem. This appears favourable since the cassava storage root develops mainly from this tissue. Another difference to the *p15* pattern is that both xylem and phloem rays were not stained rather appearing as “negatively stained”. Instead, the *pMe1* activity was restricted to areas within the functional secondary xylem and phloem leading to a “spotted” appearance.

The *pDJ3S* fragment from yam (Fig. 4d) was active in all root tissues except in the central xylem. The secondary xylem appeared somewhat stronger coloured in most cases. Strongest expression was, however, observed for those parenchyma cells aligning the xylem and phloem rays (seen at higher magnification in Fig. 4d) leading to highly parallel appearance of the stain at the given plane of focus, feinting ray multiplication. The short version of this fragment (*pDJ3S-s*) abolished this specificity and caused a staining that was similar to the one observed with the *CaMV35S* promoter (not shown).

To further confirm its tissue specificity, the *pDJ3S* fragment was fused to the bacterial *CriB* gene (from *Pantoea ananatis* coding for phytoene synthase) known to be able to drive carotenoid expression in white carrot roots (Maass et al. 2009), thereby obtaining a second reporter gene construct. This led to a similar pattern of activity visualized by carotenoid deposition with strongly labelled rays and a predominance of colour in the secondary xylem (Fig. 4e and f).

Based on these studies and assuming that the carrot model system holds for cassava, one would recommend the complete *pDJ3S* fragment from yam for use in biotechnological



**Fig. 4** Histochemical localization of GUS activity in carrot storage roots. The *smaller pictures* show selected details (see text). Roots of 9-week-old transgenic carrots (diameter ca. 2 cm) were used to produce 360  $\mu\text{m}$  cryo-sections. **a** *CaMV35S* promoter, **b** *p15* promoter from cassava, **c** *pMe1* from cassava, **d** *pDJ3S* from yam,

**e** carotenoid formation driven by *CrtB* (phytoene synthase) under control of *pDJ3S*, **f** untransformed white root control from **e**. *PX* primary xylem, *SX* secondary xylem, *SX* secondary phloem, *VC* vascular cambium, *PR* phloem ray, *XR* xylem ray



approaches aiming at improving traits of storage roots. It showed the highest levels of expression, the best storage root specificity and activity in the secondary xylem constituting by large the main tissue of cassava roots, albeit being less active and less storage root specific. Since the *CrtB* gene is capable of driving carotenoid formation in white cassava roots (unpublished data), fusions with this *pDJ3S* will be made and tested in stably transformed cassava plants.

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