Isolation and partial characterization of a root-specific promoter for stacking multiple traits into cassava (*Manihot esculenta* CRANTZ)

M.A. Gbadegesin[1] and J.R. Beeching[2]

[1]Department of Biochemistry, Molecular Biology Unit,
University of Ibadan, Ibadan, Nigeria
[2]Department of Biology and Biochemistry,
University of Bath, Bath, United Kingdom

Corresponding author: M.A. Gbadegesin
E-mail: magbadegesin@yahoo.com

ABSTRACT. Cassava can be cultivated on impoverished soils with minimum inputs, and its storage roots are a staple food for millions in Africa. However, these roots are low in bioavailable nutrients and in protein content, contain cyanogenic glycosides, and suffer from a very short post-harvest shelf-life, and the plant is susceptible to viral and bacterial diseases prevalent in Africa. The demand for improvement of cassava with respect to these traits comes from both farmers and national agricultural institutions. Genetic improvement of cassava cultivars by molecular biology techniques requires the availability of appropriate genes, a system to introduce these genes into cassava, and the use of suitable gene promoters. Cassava root-specific promoter for auxin-repressed protein was isolated using the gene walking approach, starting with a cDNA sequence. *In silico* analysis of promoter sequences revealed putative *cis*-acting regulatory elements, including root-specific elements, which may be required for gene expression in vascular tissues. Research on

the activities of this promoter is continuing, with the development of plant expression cassettes for transformation into major African elite lines and farmers' preferred cassava cultivars to enable testing of tissue-specific expression patterns in the field.

Key words: Cassava; Promoters; Gene walking; Gene expression

INTRODUCTION

The starchy storage roots of cassava provide the staple food for many millions of people in sub-Saharan Africa. In addition, they play a vital role as a famine reserve crop and are becoming increasingly important for processing into higher value products (Montagnac et al., 2009). Cassava can be cultivated on impoverished soils with minimum inputs and is therefore an excellent staple food crop for small hold farmers (Cock, 1985). However, it is far from being an ideal food and crop, as its roots are low in bioavailable nutrients and protein content, contain cyanogenic glycosides, and suffer from a very short post-harvest shelf-life, and the plant is susceptible to viral and bacterial diseases prevalent in Africa (Cock, 1985; Puonti-Kaerlas, 1998; Beeching et al., 1998; Restrepo et al., 2004; Verdier et al., 2004). The demand for the improvement of cassava with respect to these traits comes from both farmers and national agricultural institutions and is articulated through international organisations such as the Cassava Biotechnology Network, IITA and CIAT, together with national institutes, such as Kenya Agricultural Research Institute (KARI), Kenya, National Root Crop Research Institute (NRCRI), Nigeria, and National Agricultural Research Organisation (NARO), Uganda.

For cassava agriculture, it would be ideal to identify and produce solutions to these various problems and to introduce several or most of these improved traits into the major African elite lines and farmers' preferred cultivars. One possible solution is the use of genetic breeding to introduce traits of interest such as disease resistance, extended shelf life (post-harvest), improved protein and micronutrient (e.g., vitamin A, Zn, Fe) content and low cyanogenic content. However, this is largely not feasible due to the crop's clonal propagation, high heterozygosity and poor flowering ability (El-Sharkawy, 2004). Genetic manipulation through molecular biology techniques is an alternative means to introduce these traits. This approach has been adopted in other crop plants with success and offers opportunities for African crops (Thomson, 2008).

The pyramiding of multiple traits into the major African elite lines and farmers' preferred cassava cultivars may be divided into two aspects: first, how these multiple traits can be readily introduced into a range of different cassava cultivars and, second, how to ensure that all the introduced transgenes are expressed appropriately without interference. It is the second aspect that forms the problem tackled by this project. There is a critical need for root-specific promoters with a range of developmental and tissue specificities within the cassava storage root. Without access to such a toolkit, the transition from research to the release of cassava varieties improved for multiple traits will be delayed. It would be ideal to consider root-specific promoters from cassava, but unfortunately, these are currently largely unknown and uncharacterized. The objective of this project was to isolate and characterize suitable gene promoters from cassava, which could be used to target gene expression in this important food crop.

MATERIAL AND METHODS

Plant material and DNA isolation

Cassava plants (cultivar CM 2177-2) were grown in the tropical glasshouse at the University of Bath at 22-28°C, relative humidity of 40-80% and a minimum light period of 12 h per day under daylight, supplemented with 400 W Phillips high-pressure sodium lights when necessary. High-quality genomic DNA was extracted from young leaf samples of this cassava cultivar by the method of Dellaporta et al. (1983).

Restriction enzyme digestion and adaptor ligation gene walker library construction

Separate aliquots (100 ng) of cassava DNA were completely digested with four different restriction enzymes that leave blunt ends, viz *Dra*I, *Eco*RV, *Stu*I, and *Pvu*II, in a total volume of 100 μL. Human genomic DNA aliquot was also digested with *Pvu*II to serve as a positive control. Each batch of digested genomic DNA was purified and then ligated separately to the GenomeWalker™ adaptor supplied with the GenomeWalker™ kit (Clontech Laboratories, Inc.). The digestion and ligation of DNA fragments were carried out under the conditions specified by the kit manufacturer. Ligation mixtures were then diluted as specified in the GenomeWalker™ kit instruction. Each adaptor-ligated, restriction enzyme-digested genomic DNA constitutes a "gene walker library".

Gene-specific primer design and procurement

Complementary DNA sequences for the cassava auxin repressed-like protein (ARP) gene were mined from the NCBI database (DB 924059). Gene-specific primers were designed from cDNA sequences using the PRIMERW program. The primers were supplied by Invitrogen, UK.

Polymerase chain reaction (PCR)-based gene walking technique

Two sets of PCR cycles, primary and secondary PCRs, were carried out in sequence. In the primary PCR, 1 μL derived from each library was amplified in a PTC 100 Programmable Thermal Controller (MJ Research Inc.), using a set of gene-specific primers (GSP1) and adaptor-specific primer (AP1). The latter was supplied with the GenomeWalker™ kit (Clontech Laboratories, Inc.) along with the Advantage 2 polymerase enzyme. In the secondary or nested PCR, the products of the primary PCR (1 μL 50-fold dilution) were used as templates using nested gene-specific primer (GSP2) and adaptor specific primer (AP2).

Cloning procedures

Amplified gene promoter was purified using the PCR purification kit (QIAquick) or gel extraction kit (Qiagen). The purified DNA was ligated into pGEM®-T Easy vector (Promega) and used to transform competent *Escherichia coli* DH5α according to standard procedures (Sambrook et al., 1989).

DNA sequencing and sequence analyses

PCR products or recombinant plasmid DNA molecules were sequenced on an ABI 377 automated dye primer sequencer (Applied Biosystems) using GSP2, AP2 or universal primers for the cloning vector. Initial confirmation of sequence identity was performed by BLASTN and TBLASTX searches against the GenBank non-redundant database using default parameters (Altschul et al., 1997). The database of the Plant *cis*-acting Regulatory DNA Elements (PLACE) (Higo et al., 1999) was used to determine plant *cis*-acting regulatory elements. Sequence data were assembled with the VECTOR NTI program (Lu and Moriyama, 2004). Where necessary, internal primers were designed and used for further fragment sequencing.

RESULTS AND DISCUSSION

PCR-amplified cassava promoter regions

Cassava root-specific promoters required for pyramiding of multiple traits into the major cassava lines and farmers' preferred cassava cultivars are currently largely unknown and uncharacterized. Gene-specific primers were designed using cassava cDNA sequences as template in PCR-based upstream gene walking experiments. Putative cassava gene promoter regions were amplified by primary and secondary PCRs (as described in the Material and Methods section). Figure 1 represents the amplification of the promoter region of a putative ARP gene. The primary PCR produced many DNA bands, which were reduced to few or even a single band in the secondary PCR. In this way, gene specific amplifications were favored. Amplified fragments in the size range of 1.5-2.0 kb were sequenced. Sometimes, smaller fragments were also sequenced for comparison with the sequence from larger fragments in multiple sequence alignments. Figure 2 shows the assembly of ARP gene fragment sequences.
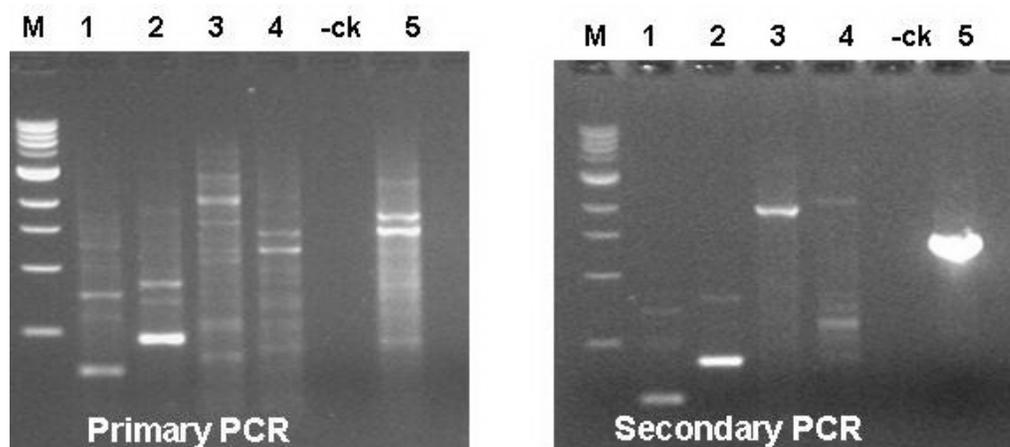
Figure 1. PCR amplification of cassava DNA (promoter regions) for the auxin repressed-like protein gene. M = 1-kb DNA size marker; *lane 1* = *Dra*I; *lane 2* = *Eco*RV; *lane 3* = *Stu*I; *lane 4* = *Pvu*II; *lane 5* = human/*Pvu*II libraries; -ck = negative control with no DNA template.
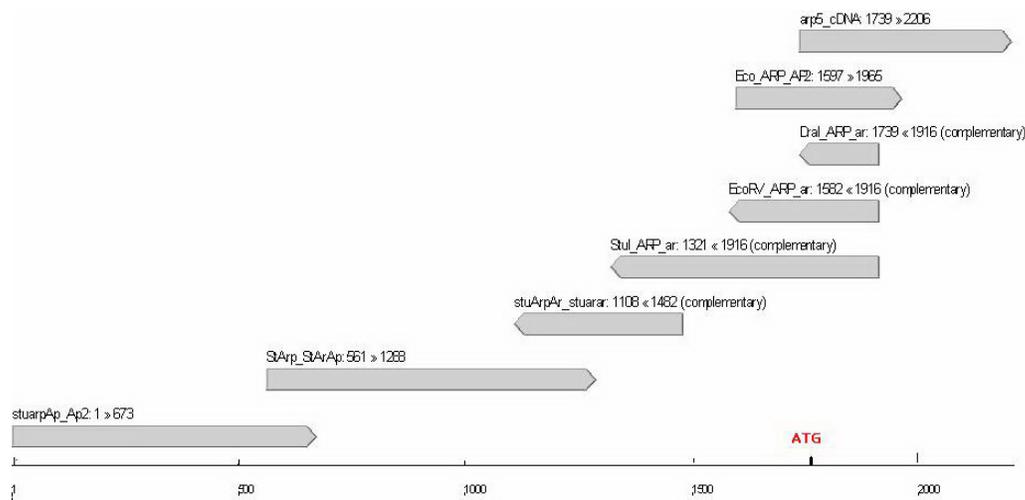
Figure 2. Scheme for the assembly of DNA sequence for auxin repressed-like protein gene. Gene sequence fragments are represented by arrowed head bar showing the 5 → 3 DNA fragment sequence direction. The name of a sequence fragment is a combination of the restriction enzyme for the gene walker library and the sequencing primer. Eco = *Eco*RV; Stu/St = *Stu*I; ATG = the initiation codon; ARP = auxin repressed-like protein gene.

Sequence analysis of promoter regions

The sequences of amplified promoter regions were, in the first instance, screened for introns by comparison with the original cDNA sequence. Exon sequences were subjected to BLAST and TBLASTX searches to confirm their identity with the original source cDNA and to identify amino acid sequence motifs. Figure 3 shows the resulting translation of sequences downstream of the start codon, ATG, and the promoter sequence of the putative ARP gene. Amino acid sequence motifs typical of ARP were identified. Sequence analysis of the promoters showed that a TATA box (TATAA) was located 83-bp upstream of the ATG start codon (Figure 3). de Souza et al. (2009) also reported a cassava *Mec*1 promoter with the TATA box located 103-bp upstream of the ATG.

Promoter isolation and cloning

Primers were designed from the consensus sequence derived from the gene walking PCRs to amplify about 1.5 kb of the promoter sequence upstream of the ATG start codon (see Figure 3). Figure 4 shows the gel picture of the PCR-amplified promoter for the putative ARP gene. Restriction enzyme sites (*Bam*HI and *Nco*I) were incorporated into the primers to facilitate subsequent cloning in the plasmids.

The amplified promoter of the ARP gene was subsequently ligated into pGEM®-T Easy vector, and the recombinant plasmid was used to transform competent *E. coli* DH5α. Plasmids were isolated from bacterial cultures, restriction enzyme digested to size the insert (Figure 5) and sequenced to re-confirm the identity. The insert released after restriction enzyme digestion of the pGEM-T Easy recombinant plasmids was cloned into pCAMBIA 1305 vectors ready for transformation into cassava plants for tissue expression studies.

AGGACACTTTCGGCGGCAGGTTCGGCGGCCGAAAGTCCCTCCAGAGCCGAAAGTCAG
GCAGGTTCGGCGGCACCTTCGGCGGCCGAAACTCCCAGACAGAGACGAAACTCATGC
ATGTTCGGCGGCACCTTCGGCGGCCGAAACTGCCAGACAGAGACGAAAGTCTCCTTT
CGGGGGCAAGTTTCGGCAGCCGAAGGGCTGCCTCCCCAGCCATGTTCGGCGGCCGAA
AGTTCCTTCGGCTGCCGAACCTGGTTTCTGCCAAAGGGCAGAAACTTGGCTCCCAAT
GCACATTTCGCCTCCAAACTTATCAAACATGCATCAAACCTATTCTACAACACACAA
ACGCAAGCATACATGTTCCTAGGGGTCTCAAACCATCATAAACCCCATCTACAACAC
ATCAAGCATCCACATTGTTCAAGAACACACATTATACCCATAAACACAACCATAACC
TAAACATGCATTCTAACTCATAGATCTTGCATAAAACTTATTCAAAACATAAAACGA
GCTTAAGATCGGCTCTTACCTCTTGAAGATCGAGAGAGACGACCCAAAAACTCGGAG
TTGGGAGAGATTTGGTTCTTGAACCTCCAAGCTCCAAAACTTTGCTCAAAAGCTTAA
ATCTTCAAAACCAAGTTAAAACAAGTGAAAATCTTGAAAGATTTAGAGGAAGAACAT
CAAAAATGGGTGAGGGACGGCGGAGAGCTCACCTGGGCCGAAAATGGGGAAAAGCTC
GCCCGTTTTCGGCTAAGGGACCCTTTTATAGTGGCTGGCCAGACCACGTTCGGGGGC
CGAATGTGTCTCCGCATGCATGCCATGTTCGGCGGCCGAACCTGGACTTCCCTCACT
TATGCTTTCGGGGGCCTAAAGCACACCCGCAACGCATGCATGTTCGGCGGCCGAACT
TGAGGTTCGACGGCCGAACCTGAGTTTTCCTCCAATGCTATTTTCATGCAAAAACTC
ATTTTCTTTCATGCTTAAAACATAAAACACATTAAAATATTTCATAAAAACATGGTT
TTACCCTACTAGAGACTTCCGACATCCGAGATTCCACCGGATGGTAGGAATTTCGAT
ACCGGAGTCTAGCCGGGTATTACACTCATATAATAGAATTATCATCATTACACGAAA
TTTCTAGAAGAAAATATGCAATTCCAAAATCTTTACTGAAATTGAAAGAGTTTGCTG
TCATTTTAGAGACCATGCATAACCCAATTAGTAGGAAGCTTTTACGTTGCTCCCTTC
GATTTTTTTTTTCTGCAGATATTTTCTGCAGCCAACTTACCAGCCACTCAAACTAGG
ACTACCTAAGTAATATGTATTATTATGCTAAAATAACATCCTAGTACAAAATATTTC
ATATTTAAACATTGTTTAAAAATAAATATTTGCAATTCTTATCCTGTTTTATAAGAA
ATGAAATATAAATTCTCATAGATATTGGATTTGCCACGTGGAACACCCAGACTGGCT
ATGGGGCAATAAATGAATCTAAGAACTTGGTGGTCCGAAAGGTGACGCGTAAGATAG
CCGACAAAAATCTTGAGCCTCCAATGAAATGAGAGTTCTAGATATCGAGAGATAAGA
AGCTTTGTGAAATCCTGGACGTGGGATTTTCAGCAAGATAAGAAAATCTACACTTAA
CTCAT**TATAA**ATGCCCACCAAATCCACTTCTCTAATTCACCACTTGAATCAAACAAC
ATACAGAGGATTCAAACCCACACTCCTTTAAACA

```
1745 ATGgcagcctcaacaatggcccttcctcccttctcttgccggc
     M   A   A   S   T   M   A   L   S   S   P   S   L   A   G
1790 aaggcagtgaagctcacccccctctgccctgagctcatgggcaat
     K   A   V   K   L   T   P   S   A   P   E   L   M   G   N
1835 ggccgtttttcaatgaggaaaactgccagcaaggctgtttcctct
     G   R   F   S   M   R   K   T   A   S   K   A   V   S   S
1880 ggaagcccatggtacggtccagaccgtgttaagtacttgggtcca
     G   S   P   W   Y   G   P   D   R   V   K   Y   L   G   P
1925 ttctctggtgagcccccatcctacttgactggcgaattccc 1965
     F   S   G   E   P   P   S   Y   L   T   G   E   F
```

Figure 3. DNA sequence translation/analysis of auxin repressed-like protein gene promoter region. Typical ARP protein amino acid sequence motifs are shown. The initiation codon (ATG) and TATAA sequence typical of a eukaryotic promoter are shown in bold and underlined.
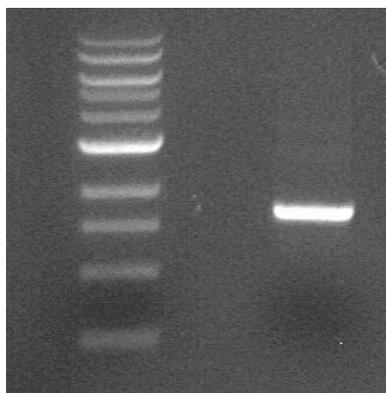
Figure 4. PCR amplification of cassava auxin repressed-like protein gene promoter. The primers were designed to amplify just the promoter (ATG inclusive). The marker shown is New England Biolabs 1-kb ladder (size details in Figure 1). About 1.5-kb size promoter was isolated.
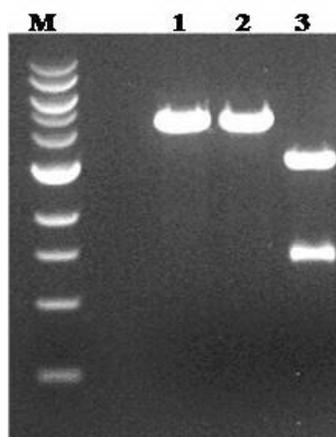
Figure 5. Restriction enzyme digestion of recombinant pGEM-T Easy plasmids with the cassava auxin repressed-like protein gene (ARP) promoter inserts. M = 1-kb ladder (sizes as shown in Figure 1); *lane 1* = ARP/*Bam*HI; *lane 2* = ARP/*Nco*I; *lane 3* = ARP/*Bam*HI and *Nco*I.

Analysis of gene promoter sequences employing the PLACE database identified important promoter motifs

Using the PLACE program (Higo et al., 1999), many regulatory elements known in other plants were identified in the ARP promoter sequence, such as light-responsive elements, ABA-responsive element, stress-responsive element, jasmonate- and elicitor-responsive elements, ACGT motif related to root expression and root-specific elements, among others (the motifs identified in the ARP promoter are listed in Table 1). Some of these regulatory elements have also been found in the cassava *Mec*1 promoter (de Souza et al., 2009) and in the *C54* promoter sequence (Zhang et al., 2003).

Table 1. Putative *cis* regulatory motifs identified in the cassava auxin repressed-like protein gene (ARP) promoter sequence by *in silico* analysis using the Plant *cis*-acting Regulatory DNA Elements (PLACE) database.

Name	Function/similar to	Relative position in the ARP promoter	Sequence	Identity (%)
EIN3	Ethylene-insensitive 3 binding site	1683	GGATTTGGTGGGCATTTATAATGAGTTA	76.9
GREGION	"G region" found in tobacco (N.t.) PRB	1598	CAAAGCTTCTTATCTCTCGATATC	73.7
RSE	"RSE (root-specific element)" found in bean	1467	GTTCCACGTGGCAAATCCAATATCTA	60.9
AGTA	"AGTA repeat" in pumpkin (C.s.) ascorbate oxidase	1260	AAAAAATCGAAGGAGCAACGTAAAAGC	69.2
SUREA	"SURE-a²", sugar-responsive element	635	AAAACCAAGTTAAAACAAGTGAAAA	68.0
IDE2	IDE2 (iron-deficiency-responsive element 2)	1177	CTGAAATTGAAAGAGTTTGCTGTCATT	53.8
JERE	"JERE" (jasmonate- and elicitor-responsive element)	1406	TTCTTATCCTGTTTTATAAGAAAT	60.9
BOXC"PSAS1	Box C" in pea asparagine synthetase	286	TCCCAATGCACATTTCGC	70.6
IDE1	IDE1 (iron-deficiency-responsive element 1)	345	CGCAAGCATACATGTTCC	70.6
GLUTE BOX1	"Box 1" of rice (O.s.) glutelin Gt3 gen	1536	TATCTTACGCGTCACCTTT	70.6
OCSENHAN	"OCS enhancer element" in octopine synthase	1231	TAGGAAGCTTTTACGT	78.6
WAR	"WAR (wounding activating region)" in *Brassica*	442	TTTATGGGTATAATGTGTGT	55.0
OCSGMGH24	"OCS element" found in soybean (G.m) GH2	325	AGGTTTGATGCATGTTTGATAAGT	55.0
REGION1	"region 1" ABRE-like sequence found in rice	829	CGGCGGCCGAACCTGG	68.8
AS1	"as-1 (activation sequence 1)" in CaMV 35S promoter	1512	TGGTGGTCCGAAAGG-TGACGCGTAAGAT	60.9
GLUTEBP2	"Glutelin BP-2"; binding with nuclear factor	612	TTGCTCAAAAGCT-TAAAT	77.8
BOX2	Box 2 of bean (P.v.) chs 15 promoter; SBF-1	1017	TTTTTATGAAATAT	76.9
LREBOXII	"BoxII", light-responsive element (LRE)	1465	TCCACGTGGC	100.0
BOX1	"Box I" found in the tobacco (N.t.) plastid	467	CATTCTAACTCATAGATCTTG	63.2
ABF	ABF (as-1-like box binding factor) binding site	1169	AAATCTTTACTGAAATTGA	83.3
ALF2	"ALF-2 (as-1-like sequence binding factor 2)	299	GGAGGCGAAATGTGCATT	61.1
LREBOX3	"LRE (light-responsive element) Box III	661	AAGATTTTCACTTGT	83.3
TEF1BOX	"tef1 box" found in *Arabidopsis*	477	TATGAGTTAGAATGCATGTTTAGG	57.9
23BPZM	23-bp sequence found in maize (Z.M.)	1630	AGCAAGATAAGAAAATCTACA	68.4
23BPUAS	"23-bp UAS (upstream activating sequence)	1203	TTTAGAGACCATGCATAACCCAA	55.0
AT1BOX	"AT-1 box (AT-rich element)" found in the promoter	1008	AATATTTTAATG	83.3
GLUTEBOX1	"Box 1" of rice (O.s.) glutelin Gt2 gene	116	CATGCATGAGTTTCGTCT	71.4
HSRE	"HSRE (HSR203-responsive element)" in tobacco	614	CAAAGTTTTGGA	83.3
TDBA12	TDBA12 binding site found in tobacco	55	TGACTTTCGGCT	90.0
ANAEROBIC CIS	20-bp anaerobic cis-regulatory sequence in maize	1254	CGAAGGGAGCAACGTAAAAG	65.0
BOXICHS	"Box 1 consensus sequence in the promoters	315	ATGCATCAAACCTATT	76.9
COREOS	CORE (coordinate regulatory element for antioxidant)	1363	AATATTTCATATTTAAACATTGTTTAAA	53.6
LEGUMINBOX	"legumin box" in legA 5' legumin gene	1486	TCCCCAGCCATGTTCGGCGGCCGAAAGTT	60.7
DR5GMGH3	"DR5", a highly active synthetic auxin-responsive element	163	ACTTTCGTCTC	90.0
ABRE	ABA-responsive element	1677	TCCACTTCTC	90.0
GAGA	"GAGA element" found in the promoter	540	AAGATCGAGAGAGACGAC	84.6

Table 1. Continued.

Name	Function/similar to	Relative position in the ARP promoter	Sequence	Identity (%)
GAGA8	"GA octodinucleotide repeat"	540	AAGATCGAGAGAGACG	84.6
TCA1MOTIF	TCA-1 (tobacco nuclear protein 1) binding site	969	TCATTTTCTTT	90.0
TEFBOX	"tef-box" found in *Arabidopsis*	794	GGGGGCCGAATGTGTCT	69.2
C1GMAUX28	"C1"; DNase I protected sequence	1015	TAAAAACATGGTTTTA	68.8
SARE	Salicylic acid-responsive element found in CaMV	1613	TGGACGTGGGATTTTCAGCAA	52.6
WAR	"WAR (wounding activating region)" in *Brassica*	299	TCCAAACTTATCAAACATGC	83.3
JERE	"JERE" (jasmonate- and elicitor-responsive element)	1689	GAATTAGAGAAGTGGATTTGGTGG	47.6
ACGTROOT1	"ACGT motif" related to root expression	1465	TCCACGTGGC	90.0
SREN	Stress-responsive element (SRE) in tobacco	1295	TGGTAAGTTGGCT	69.2
TRANSTART	Plant consensus sequence for translation start	1385	TAAACAATGTTT	83.3
ACGTROOT1	"ACGT motif" related to root expression	1459	GCCACGTGGA	100.0
ABREA	ABA-responsive element (ABRE A)	1459	GCCACGTGGA	100.0
GLUTEBP2	"Glutelin BP-2"; binding with nuclear factor	1557	AGGCTCAAGATTTTTGTCG	66.7

ACKNOWLEDGMENTS

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

Beeching JR, Han Y, Gómez-Vásquez R, Day RC, et al. (1998). Wound and defense responses in cassava as related to post-harvest physiological deterioration. *Recent Adv. Phytochem.* 32: 231-248.

Cock JH (1985). Cassava: New Potential for a Neglected Crop. Westfield Press, Boulder.

de Souza CR, Aragao FJ, Moreira EC, Costa CN, et al. (2009). Isolation and characterization of the promoter sequence of a cassava gene coding for Pt2L4, a glutamic acid-rich protein differentially expressed in storage roots. *Genet. Mol. Res.* 8: 334-344.

Dellaporta SL, Wood J and Hicks JB (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep.* 1: 19-21.

El-Sharkawy MA (2004). Cassava biology and physiology. *Plant Mol. Biol.* 56: 481-501.

Higo K, Ugawa Y, Iwamoto M and Korenaga T (1999). Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* 27: 297-300.

Lu G and Moriyama EN (2004). Vector NTI, a balanced all-in-one sequence analysis suite. *Brief. Bioinform.* 5: 378-388.

Montagnac JA, Davis CR and Tanumihardjo SA (2009). Nutritional value of cassava for use as a staple food and recent advances for improvement. *Compr. Rev. Food Sci. Food Safety* 8: 181-194.

Puonti-Kaerlas J (1998). Cassava biotechnology. *Biotechnol. Genet. Eng. Rev.* 15: 329-364.

Restrepo S, Velez CM, Duque MC and Verdier V (2004). Genetic structure and population dynamics of *Xanthomonas axonopodis* pv. *manihotis* in Colombia from 1995 to 1999. *Appl. Environ. Microbiol.* 70: 255-261.

Sambrook J, Fritsch E and Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Thomson JA (2008). The role of biotechnology for agricultural sustainability in Africa. *Philos. Trans. R. C. Lond. B. Biol. Sci.* 363: 905-913.

Verdier V, Restrepo S, Mosquera G, Jorge V, et al. (2004). Recent progress in the characterization of molecular determinants in the *Xanthomonas axonopodis* pv. *manihotis*-cassava interaction. *Plant Mol. Biol.* 56: 573-584.

Zhang P, Bohl-Zenger S, Puonti-Kaerlas J, Potrykus I, et al. (2003). Two cassava promoters related to vascular expression and storage root formation. *Planta* 218: 192-203.