ELSEVIER

Plant Science 103 (1994) 189–198

PLANT SCIENCE

Functional studies on a seed-specific promoter from a Brazil nut 2S gene

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Received 17 June 1994; revision received 17 August 1994; accepted 23 September 1994

Abstract

The 5′ upstream regulatory region of the Brazil nut 2S albumin gene was studied in order to identify sequences involved in gene expression. Restriction fragments from the regulatory region were fused to the reporter genes chloramphenicol acetyl transferase (CAT) and β-glucuronidase (GUS). The CAT constructs were used to transform tobacco leaf discs via *Agrobacterium* and the GUS-promoter fusions were introduced into bean embryos by particle bombardment. With both systems all promoter deletions conferred expression of the GUS and CAT genes. In the biolistically transformed bean embryos reporter gene activity dropped at position −460 base pairs upstream from the transcription initiation site. CAT activity in stably transformed tobacco plants was less markedly affected at this site but declined sharply after the deletions reached −210 bp upstream from the transcription initiation site. The temporal expression of the CAT gene controlled by the Brazil nut 2S promoter in developing, transgenic tobacco seeds parallels that of 2S albumin expression in Brazil nut. DNA retardation assays and footprint analysis using Brazil nut 2S promoter fragments and nuclear extracts from Brazil nut seeds allowed the identification of at least two sequence motifs, ACGT and CCAC, that might be involved in promoter activity.

Keywords: Brazil nut; 2S albumin; Promoter; Transient expression; Transgenic plants

1. Introduction

Genes encoding seed storage proteins are attractive as potential sources for sequences useful for the improvement of the quality of nutritionally important crops. In the seeds of many dicotyledons the dominant part of the storage proteins are water-soluble albumins that sediment with an S value of 2. Such 2S albumins are found in various phylogenetically rather distant species such as *Ricinus communis* [1], *Brassica napus* [2], *Arabidopsis thaliana* [3] and Brazil nut (*Bertholletia excelsa*) [4]. Whilst in *Ricinus*, *Brassica* and *Arabidopsis* either the cotyledons or the endosperm are storage organs, in Brazil nut the 2S albumins are synthesized exclusively in the embryonic axis, i.e. in the massive, swollen hypocotyl and the radicle [5]. The 2S storage albumins from

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SSDI 0168-9452(94)03996-M

Brazil nut are extremely rich in the essential sulphur amino acid, methionine [4,6,7]. This feature makes their genes promising candidates for introduction and expression in economically important but methionine-deficient crops.

The Brazil nut 2S albumin genes belong to a multigene family and at least one of them, BE2S1, is expressed exclusively in seeds at advanced stages of development [7,8]. The highly regulated temporal and spacial expression makes it an attractive model for studies on the mechanisms that control gene expression.

In many plant gene promoters *cis*-acting elements containing the palindromic ACGT G-box core have been described, e.g. in the promoters of the genes coding for the small subunit of ribulose biphosphate carboxilase (rbcS) [9–11], patatin genes [12] and the legumin B4 genes [13], among others. DNA-retardation assays point to the importance of the G-box motifs in the regulation of expression of many plant genes and recently Williams et al. [14] have demonstrated that the sequences flanking the G-box cores influence the specificity of binding of nuclear proteins to these *cis*-acting elements.

Interestingly, a comparison of the 2S promoter sequences from Brazil nut, *Arabidopsis* and *Brassica* [7] revealed the presence of four G-box core motifs in all three promoters. The first three are localized at nearly identical distances relative to the TATA-boxes. However, the spacing of the fourth, identical motif is less rigidly conserved. The conservation of these motifs in the different promoters suggests that they may be important for the transcriptional regulation of the 2S genes.

We therefore initiated functional studies of the Brazil nut 2S albumin promoter using GUS and CAT structural genes fused to intact and deleted 2S promoter sequence. The constructs containing the CAT structural sequences were stably introduced into tobacco plants via *Agrobacterium tumefaciens* and the GUS-constructs were assayed in bean embryos transformed using a particle gun.

The analysis of the GUS and CAT activities driven by the different 2S promoter fragments allowed us to tentatively identify promoter regions essential for optimal expression of the reporter genes. DNA retardation assays and DNA footprint analysis using promoter fragments demonstrated that the fragments contain sequences capable of binding nuclear proteins from seeds which are different from those nuclear proteins bound from leaf extracts.

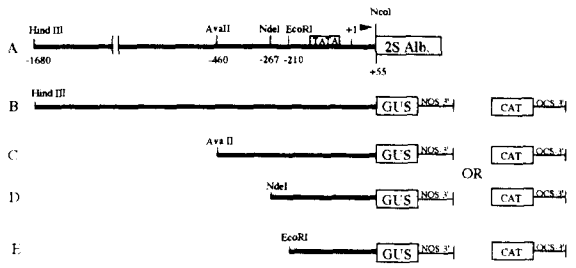


Fig. 1. Promoter fragments used in the transformation experiments. (A) Schematic representation of the promoter region of the *BE2S1* gene from Brazil nut. The position +1 corresponds to the initiation site of transcription and the position +55 corresponds to the initiation site of translation (*Nco*I site). (B–E). The 2S promoter fragments fused either to the GUS-NOS-3′ or the CAT-OCS-3′ coding regions.

2. Materials and methods

2.1. Promoter fragments and constructs

The constructions used in the transformation experiments are represented schematically in Fig. 1. The starting point for all constructions was a *Hin*dIII/*Pst*I 2S gene fragment spanning from 1680 bp upstream of the protein synthesis initiator ATG down to a unique *Pst*I-site within the 2S gene's coding region. As a first step the restriction fragments, indicated in Fig. 1, were subcloned into pUC19. The 2S coding region (*Nco*I/*Pst*I) was then replaced by a *Pst*I/*Nco*I fragment containing the CAT structural and the 3′OCS sequences from plasmid 35SCAT1 (E. Krebbers, Plant Genetic Systems, Gent, Belgium). After filling-in the staggered ends, the fragments were subcloned into the filled-in *Bgl*II site of the 1703A binary vector [15]. For the bombardment experiments, the promoter fragments were joined to a 2.2-kb *Eco*RI/*Nco*I GUS-coding fragment from plasmid pMJD67 [16].

2.2. Plant transformation

Plasmids containing CAT chimeric genes were

mobilized into *A. tumefaciens* strain C58C1 rif using the *E. coli* strain HB101 harbouring pRK 2013 [17]. Leaf discs of *Nicotiana tabacum* were transformed as described by Horsch et al. [18]. Transformed cells were selected on a shoot inducing medium containing 100 μg/ml kanamycin and young shoots were transferred to a root-inducing medium containing 100 μg/ml kanamycin and 200 μg/ml carbenicillin. Regenerated plants were grown in the greenhouse.

2.3. CAT assays

For each construct (Fig. 1), mature seeds and, in the case of the intact promoter, seeds at different developmental stages were collected from three transgenic plants, pooled and ground to a fine powder in liquid nitrogen. The decision to pool the seeds of three independent transformants was based on preliminary experiments where the three seed batches were analysed independently; no significant variation in CAT activity was found between the batches transformed with the same construct. 100 μg of powder was extracted with 500 μl of 0.25 M TRIS–HCl (pH 7.5) containing 2 mM phenyl methyl sulphonyl fluoride (PMSF) and vortexed for 20 min at 4°C. After centrifugation for 10 min in an Eppendorf microfuge the supernatant was incubated at 65°C for 10 min to reduce the background activity. The suspension was then centrifuged at 11 000 rev./min for 30 min at 4°C and the protein concentration in the supernatant was determined by the Bradford dye-binding method [19] (Bio Rad Laboratories). CAT activity was assayed as described by De Block et al. [20], using 5 μg of protein/assay.

2.4. Quantification of GUS activity and histochemical visualization in bean embryos

The experimental details of the biolistic approach were as described in detail in Aragão et al. [16]. Twenty-five bombarded embryos of *Phaseolus vulgaris*, cv. Carioca were ground to a fine powder in liquid nitrogen and proteins were extracted in buffer (0.5 M NaCl, 50 mM Na_2HPO_4 (pH 7.2), 2 mM PMSF, 0.13 mg/ml leupeptine, 1% β-mercaptoethanol, 0.2% polyvinylpyrolidone). Protein concentration was determined using the colorimetric assay. The extracts were then prepared for fluorimetric assays using 4-methyl-umbelliferyl β-D-glucuronide.

In situ localization of GUS activity in embryos was carried out as described by Aragão et al. [16].

2.5. DNA retardation assays

Nuclear extracts from Brazil nut seeds close to maturity and from leaves were prepared as described before [21]. The promoter fragments shown in Fig. 8a were radiolabelled using the Klenow-fragment and either 32P-dATP or 32P-dCTP. Binding reactions were done in a final volume of 30 μl of binding buffer (10 mM TRIS–HCl (pH, 8.O); 50 mM NaCl; 7 mM β-mercaptoethanol; 10% glycerol) containing 4–8 μg of nuclear proteins, 2 μg poly (dl-dC) and 5000–10 000 counts/min of 32P-labelled probe.

After 30 min at room-temperature the reactions were loaded onto 5% polyacrylamide gels in 0.5 × TBE. The gels were run at 120 V and after completion of the run, fixed, dried and autoradiographed.

2.6. DNAase I footprint analysis

*Ava*II/*Nco*I fragments were used as a substrate for the DNAase protection experiments, after radiolabelling of the ends with [^{32}P]dATP. The fragments were purified on non-denaturing 6% polyacrylamide gels and after electroelution were resuspended in H_2O to approximately 10 000 counts/min/μl. The footprint reactions were carried out as described by Lichtsteiner et al. [22].

3. Results

3.1. Transient GUS expression in bean embryos and promoter strength

The chimeric gene constructs (Fig. 1B–E) were used to transform isolated bean embryos via particle bombardement. For each construct, 25 transformed embryos were pooled and assayed for GUS-activity. The mean values obtained from 5 independent series of experiments are shown in Fig. 2; all of the promoter fragments tested were able to drive GUS activity. After an initial drop when the *Hin*dIII/*Ava*II fragment was deleted, activity was found to stay at the same level with constructs C and D but with a further reduction with deletion E.

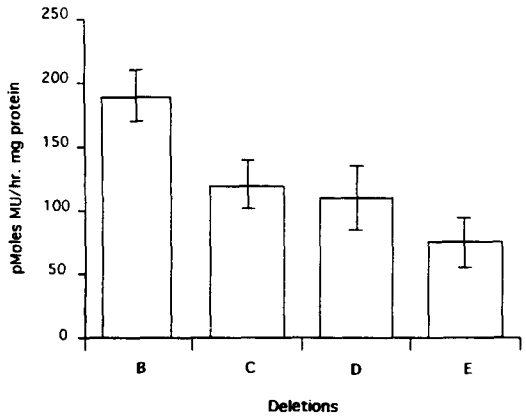


Fig. 2. Comparison of transient expression of BE2S1-GUS fusion constructs in bean embryos. (B–E) The constructs shown in Fig. 1. For each construct the mean value obtained from 5 independent experiments is shown. The bars delineate the range of the values.

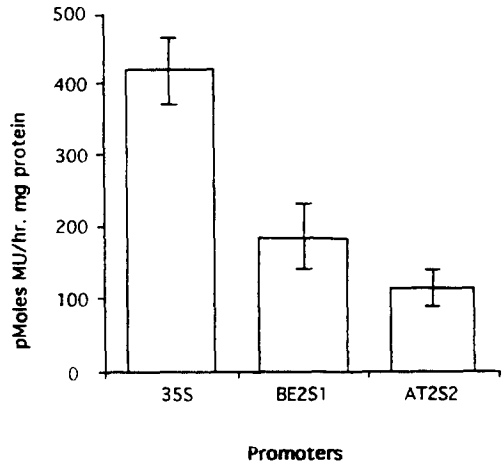


Fig. 4. Comparison of strength of 2S gene promoters from Brazil nut and *Arabidopsis* and of the CaMV 35S promoter. From left to right: 35S-, Brazil nut 2S- and *Arabidopsis* 2S-driven GUS activities. Assays as described in Materials and methods. The bars delineate the data range.

The histochemical visualization of GUS-activity shown in Fig. 3A,B supports this result. The blue colour obtained with construct B was much more intense than that obtained with the deleted promoter fragments (only E is shown). Using the same constructs in a transient expression system, we evaluated the relative strength of the Brazil nut 2S promoter in comparison with the *Arabidopsis* 2S and the CaMV 35S promoters. The results are shown in Fig. 4; both 2S promoters work in the bean tissue and the Brazil nut promoter seems to be slightly more efficient than the analogous

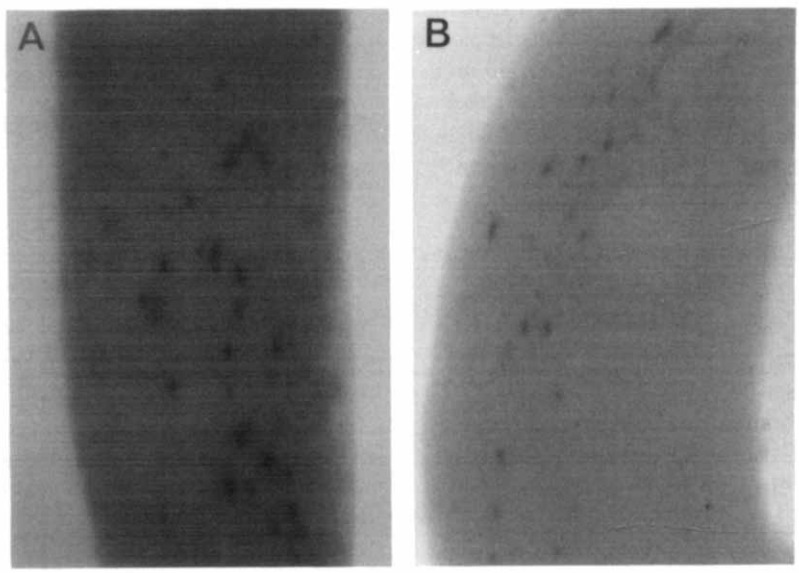


Fig. 3. Histochemical visualization of GUS activity in axis of mature bean embryo. (A) Full length promoter. (B) Construct 'E' as shown in Fig. 1.

Arabidopsis sequences. However, a 2–3-fold increase is obtained when the CaMV 35S promoter is used to drive the expression of the GUS-reporter gene.

3.2. CAT gene expression in stably transformed tobacco

For this series of experiments the different promoter fragments (Fig. 1) were fused to CAT-structural sequences. Tobacco leaf discs were then transformed and plants regenerated as described above in *Materials and methods*. CAT activity was determined in transgenic seeds that had been harvested 25 days after flowering when they are close to maturation. The results are shown in Fig. 5. With constructs C and D only a slight reduction of transcriptional activity can be observed. However, construct E results in only 20% of the activity obtained with the intact promoter.

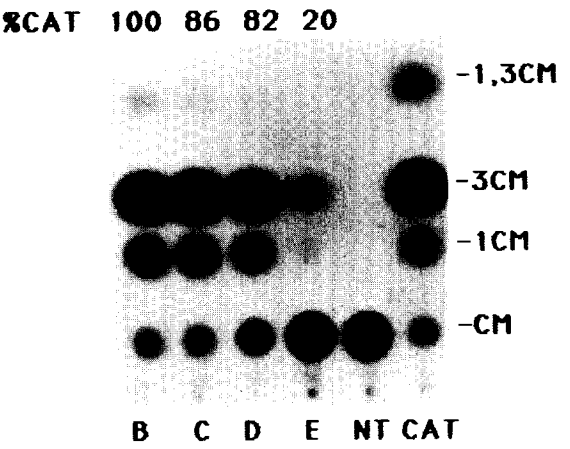


Fig. 5. CAT assays of seed protein extracts from transgenic tobacco plants transformed with constructs B–E (Fig. 1). For each assay, 5 μg of protein was used. NT, untransformed plant; ENZ, 5 units of chloramphenicol acetyl transferase (Boehringer); CM, chloramphenicol; 1-CM, 1-acetyl-chloramphenicol; 3-CM, 3-acetyl-chloramphe nicol; 1,3-CM, diacetyl chloramphenicol. Quantification was done by cutting out the acetylated chloramphenicol spots from the thin-layer foil and subsequent scintillation counting. The value obtained with the largest promoter fragment (B) was considered to represent 100% of activity.

3.3. Spatial and temporal regulation of CAT expression

In order to verify whether the spacial and temporal expression patterns conferred by the intact 2S promoter are maintained in the tobacco host, seeds from transgenic plants were harvested at 10, 15 and 25 days after flowering and their CAT activity tested. For each stage the seeds of three transgenic tobacco plants were pooled and the CAT activity determined. The results of this analysis are shown in Fig. 6. The nearly mature seed (25 days after flowering) show highest activity whilst only minimal CAT activity was observed in seeds harvested 10 days after flowering.

Leaves and stems of transgenic plants carrying the various constructs were tested for their ability to express CAT activity and determine whether one of the deletions might result in the relaxation of tissue specificity. The results for all constructs were identical to those shown in Fig. 7 for the intact promoter sequence: no CAT activity was detectable in either stems or leaves; experiments with the truncated promoters gave identical results (data not shown).

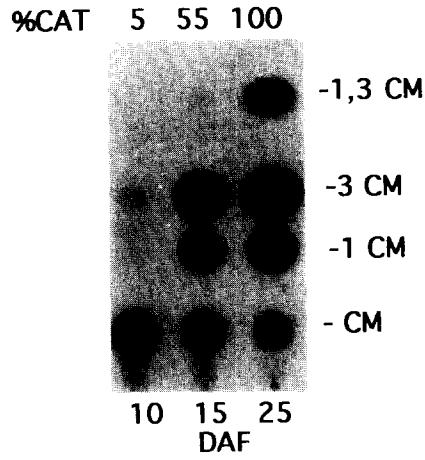


Fig. 6. CAT activity in transformed tobacco seeds at different developmental stages. Autoradiography showing the activity in extracts from seeds harvested at 10, 15 and 25 days after flowering (DAF). Quantification of the results shown on top was done as described in Fig. 5. 25 DAF seeds are considered to show 100% activity.

3.4. DNA-retardation assays and footprint analysis with Brazil nut 2S promoter fragments

In order to identify promoter elements that

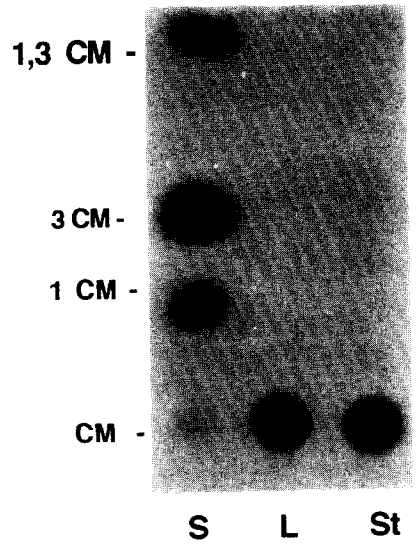


Fig. 7. CAT assay of protein extract from seeds (S), leaves (L) and stems (St) of transgenic tobacco transformed with construct B (Fig. 1). With the quantification method described for the previous experiments, no CAT activity can be shown either in leaves or in stems.

might be involved in the regulation of expression and specificity of the 2S gene through binding to nuclear proteins, a series of DNA retardation assays was performed with nuclear extracts from seeds and leaves.

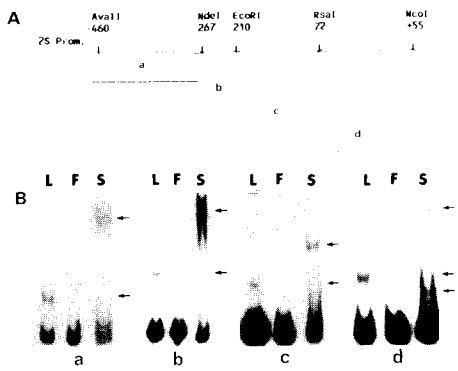


Fig. 8. DNA retardation assay. (A) Promoter fragments used in the assays. (B) Autoradiographs of the binding reaction: L, leaf extracts; F, free probe; S, seed extracts. The four fragments were analysed on different gels. Arrows point to complexes formed with the nuclear extracts.

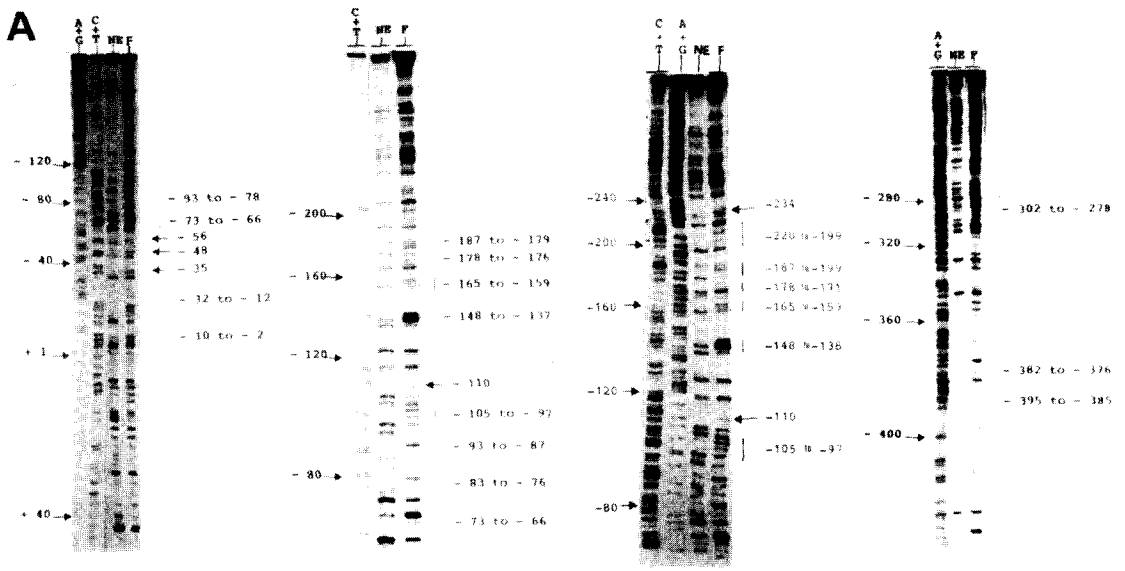
The fragments spanning the first 460 nucleotides upstream from the transcription initiation site (Fig. 8A) were used as probes since we have previously shown that the G-box motifs are found in this region. The results are shown in Fig. 8: the migration of all fragments was retarded by both types of extracts. However, the complexes formed with extracts from leaves and from seeds clearly differ. Nuclear extracts from leaf form only one specific, well-defined complex with all fragments. With extracts from seeds, however, the *Ava*II/*Nde*I fragment forms one complex, while in the case of the *Nde*I/*Eco*RI, *Eco*RI/*Rsa*I and *Rsa*I/*Nco*I probes two complexes are present (arrows). With these three probes, one of the complexes migrates identically to the one formed with leaf extracts; the others are of higher molecular weight.

In order to characterize potential *cis*-acting elements in the promoter region more precisely, footprint analyses were performed using the *Ava*II/*Nco*I fragment. The results are shown in Fig. 9A. The protection pattern against DNAase I action corroborates the results obtained with the DNA-retardation assays: proteins from seed nuclei are able to protect multiple sites in the *Ava*II/*Nco*I promoter fragment. Two of the protected regions are located around the TATA box, seven in a 155 nucleotide region spanning from −65 to −220 and two more can be identified in the highly AT-rich region from −220 to −420. In Fig. 9B these results are summarized and, interestingly, most of the protected regions (underlined in the figure) contain, or are flanked by TGCA/ACGT and CCAC motifs. The latter motif is found in the CaMV 35S promoter [23] and also in the 'vicilin' consensus sequence [24].

4. Discussion

4.1. Specificity and strength of the Brazil nut 2S album in promoter

In this study we have initiated a functional analysis of a seed-specific promoter from a Brazil nut 2S albumin gene. This was accomplished by fusing different promoter fragments to the CAT and GUS reporter genes and introducing the constructs either biolistically into bean embryos or via *Agrobacterium tumefaciens* into tobacco.



B

*AvaII
ACCTCTCCAA CATTCTCTCT CTCTCCTATA ATGAATATAC

-420
ATCAATAAAA CTGATCAAAA ATGAAAAGAT ATTATTGTTG TAATATATAA TTATATTTAG

-360
AATTACTAAC AAACGATGTC CTAGAAAACT GGCTCCAATC AATTCTCAAT TTAATTCATA

-300 *NdeI
CACCATCGCT TAATTCATCG GACAACCATA TCATATGTTT AACGGTATTC CTAAAATTAT

-240 *EcoRI
AATAACTAAC TAAATCATAT ACTTTCTCCG AATTCCAGAC TAAAAAATCA ATGCTTCGAT

-180
CAGTTTGATA AGCAAACAGA CAACGTTTTT GGCAACGTCT GCATGAAATT CGACGTGGAA

-120 *RsaI
GCTCCGTTCC GCCACCTCAT CTCAGCCCAC TCCCGCCATC CTCCACGTAC TCCCACGTGC

-60
AAAGTTCCAA AAGCCCGAAA ATCCACCATA AATATAGAAA CCCCCACCAC CAAGCTCTCC

+1 *NcoI
ATCCACCACT GCTCTGTATC ACATATACCA ACCCACGCTT ATACCCAGAA TCACCATG

Fig. 9. Footprint analysis of the *AvaII*/*NcoI* promoter fragment using nuclear extracts from Brazil nut seeds. (A) Analysis, on 6% acrylamide, 8 M urea sequencing gels, of the *AvaII*/*NcoI* promoter fragment labelled at the *NcoI* site (first three panels) and at the *AvaII* terminal (right hand panel). 'C + T'/'A + G', pyrimidine and purine lanes, respectively; 'NE', fragment incubated with seed nuclear extracts and subsequently digested with DNAse I; 'F', DNAse-digested fragment unexposed to nuclear extract. The vertical bars at the right indicate the regions where DNA is protected from enzyme action, the numbers give the position in the promoter fragment. (B) Sequence of the promoter fragment used in the DNAase I protection experiments. The protected regions identified in Fig. 9A are underlined. ACGT/TGCA and CCAC motifs are boxed.

None of the deletions used in this study altered the seed-specificity conferred by the Brazil nut 2S promoter. The truncated promoter containing 210 nucleotides upstream of the transcription initiation site still retains the ability to drive the expression of the reporter gene in a seed-specific manner.

This suggests that the signals responsible for seed-specific expression might be located within this stretch of nucleotides.

Furthermore, the analysis of transgenic tobacco seeds at different stages of maturation showed that also the temporal expression pattern is conserved in the new biological environment. Similar results have been obtained with an analogous 2S promoter (AT2S1) from *Arabidopsis thaliana* in transgenic *Brassica* and tobacco. However, in contrast to our results, the *Arabidopsis* gene is transcribed during the early stages in the seed development of transgenic tobacco and its activity declines 20 days after fertilization [15]. In agreement with our findings, Guerche et al. [25] demonstrated an increase of activity for all four AT2S genes during *Arabidopsis* seed development.

With the methodology used in this study we could not detect any deviation from the spatial expression pattern observed in Brazil nut: the intact, as well as the truncated, promoters drive reporter gene activity exclusively in the seeds of the transgenic plants. Furthermore, in Brazil nut as well as in transgenic tobacco seeds, activity is highest towards the end of the maturation period. Results from the experiments with biolistic transformation of bean embryos were less specific; although all the constructs were expressed in the embryonic tissue, it is, at present, impossible to know whether the seed and developmental specificity has been maintained since it was not possible to regenerate intact plants. GUS reporter gene expression in the biolistically transformed material is analogous to the CAT expression in stably transformed tobacco, indicating that assays of this type might be useful as a rapid test of promoter strength. The performance of genetically engineered plants is highly dependent upon the strength of the promoter used to drive the expression of the gene of interest. Because of their nutritional value and seed-specificity the Brazil nut 2S gene and its promoter are of interest for the engineering of seed properties. Sequences coding for Brazil nut 2S albumins have been introduced and expressed successfully in various host plants such as *Brassica* [25] and tobacco [26], albeit not under the control of the homologous promoter. The evaluation of the strength of the Brazil nut 2S promoter in comparison with an analogous promoter from *Arabidopsis* and the 35S promoter from CaMV showed that the latter drives transient GUS-expression in bean embryos at least 50% more efficiently than the 2S promoters.

4.2. cis-acting regulatory domains

The deletions tested in the course of this work showed that the region between −267 and −210 nucleotides upstream from the transcription initiation site is essential for efficient gene expression but that seed-specificity is not lost when these sequences are deleted. It is very likely therefore that *cis*-acting sequences conferring seed-specificity are located downstream from position −210. The DNA retardation assays presented here support the notion that this region might be involved in the regulation of gene expression through transcription factors that bind to specific *cis*-acting sequences. Most of the complexes are formed with the *Eco*RI/*Rsa*I- and the *Rsa*I/*Nco*I-fragment. In addition the DNA retardation assays show clearly that nuclear extracts from seeds and leaves differ substantially with respect to their DNA-binding proteins. Our experiments have shown that nuclear proteins from Brazil nut seeds form one to several DNA–protein complexes while extracts from leaves give only one, possibly the same, well-defined complex with all probes. In the case of seed extracts the complexity of the DNA–protein interactions in the 2S promoter region is confirmed by the footprint analysis with seed extracts. The results are summarized in Fig. 9B where the regions protected from DNAase I action are underlined. The analysis of the sequences present in the protected regions shows that all contain, or are immediately flanked by, two core motifs (boxed in Fig. 9B). The first, the core motif of the G-box, ACGT, has been shown to be highly conserved in 2S albumin gene promoters from phylogenetically distant plants. The second motif, CCAC, is also found at the core of the medial and

distal region of the CaMV 35S RNA promoter [23] and in the 'vicilin' consensus sequence [24].

Recently it has been reported that tissue-specificity of legumin expression is determined by a core sequence 'CATGCATG', a motif that is present in the promoter region of many plant genes [27]. Part of this motif, namely the TGCA palindrome, forms the core of a *cis*-acting DNA sequence in the promoter of wheat histone genes [28]. It is also present in the CaMV 35S RNA promoter, the promoter of nopaline synthase and the promoter of the *Arabidopsis rbcS-1A* gene. Because of its wide distribution in a variety of genes that are all highly regulated, although dependent on quite different physiological and environmental factors, it seems that TGCA/ACGT represents a conserved core around which different flanking regions could define tissue and/or spatial expression patterns and responses to *trans*-acting elements.

Our results show that transcriptional regulation is most probably achieved through the combination of certain basic regulatory elements [29], two of which are, in the case of the 2S albumin promoter, the TGCA/ACGT and CCAC cores.

Acknowledgements

We thank Drs. M. Vincentz, M. Carneiro, E. de Almeida, L. Caldas and Nicholas Harris for critical reading of the manuscript. This work was supported by grants from CNPq/FINEP (Brazil).

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