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Expression of DNA binding proteins in carrot somatic embryos that specifically interact with a *cis* regulatory element of the French bean phaseolin gene

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Abstract

Nuclear proteins from bean (*Phaseolus vulgarus*) embryos bind specifically to a 55 bp DNA sequence located upstream of the seed storage protein gene phaseolin. This sequence is capable of elevating gene expression in transgenic tobacco plants by as much as 150-fold when fused to a chimeric β-glucuronidase reporter gene. Results presented in this paper demonstrate that nuclear extracts from carrot embryos bind to a phaseolin DNA sequence that includes a phaseolin activator sequence. This specific DNA binding activity is modulated during somatic embryogenesis. Two separable protein species react specifically with the labeled phaseolin DNA fragment (58.0 and 51.7 kDa). These results suggest that the *cis*- and *trans*-acting elements controlling gene expression have been highly conserved during evolution.

Abbreviations: bp, base pairs; CAMV, cauliflower mosaic virus; GUS, β-glucuronidase; kDa, kilodalton; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

DNA binding proteins have been shown to associate specifically with promoter elements from many prokaryotic and eukaryotic genes and have been implicated in regulating transcription [17]. The existence of plant DNA binding proteins which interact with DNA upstream of a number of diverse plant genes has been demonstrated [5, 8, 10–11, 15–16, 27]. The *Phaseolus vulgarus* seed storage protein gene, phaseolin, has been shown to be expressed predominately in the seed tissues of bean, and is correctly expressed in transgenic tobacco plants [20]. A protein factor from bean cotyledon nuclear extracts binds specifically to a 55 bp sequence from the 5′ region of this gene [1, 6]. Fusion of this 55 bp DNA sequence 5′ of a truncated CaMV promoter and GUS reporter gene resulted in elevated expression of the GUS gene (in seeds, roots and in leaves) when transferred to tobacco plants. Thus, a functional correlation has been made between the protein binding site, and gene activation.

We have investigated the species specificity and

developmental regulation of DNA binding proteins which interact with a phaseolin transcriptional activator fragment using a carrot *in vitro* somatic embryogenesis system. Biochemical quantities of somatic embryo stages, including preglobular, proembryogenic masses, can be isolated from embryogenic carrot cultures [9, 28]. For this reason, carrot somatic embryogenesis has been used as a model for studying the developmental expression profiles of proteins and mRNAs specific to or modulated during embryogenesis [2–4, 12, 22, 24, 25, 28]. In this report, we demonstrate that nuclear protein factors from carrot somatic embryos bind specifically to the same DNA fragment as do immature bean cotyledon nuclear extracts, and that the developmental regulation of this activity is correlated with somatic embryogenesis. We also show that two separable protein species bind to a phaseolin *cis* regulatory element.

Materials and methods

DNA binding protein analysis. Protein extraction and quantitation, DNA manipulations, DNA binding assays, gel retardation analysis and autoradiography were described previously [1, 6, 7, 16].

Carrot tissue culture. Conditions for initiation of carrot cultures from sterilized seed grown hypocotyl sections and for the induction of carrot somatic embryogenesis in liquid cultures have been described [26].

SDS-PAGE. Proteins were separated by electrophoresis by the method of Laemmli [13] and visualized by silver staining by the method of Oakley *et al.* [19].

Nuclear protein gel blots. Nuclear proteins separated by SDS-PAGE were reacted with labelled DNA fragments by the methods of Miskimins *et al.* [18] with modifications as indicated in the legend to Fig. 4.

Results and discussion

Extraction of crude nuclear extracts from staged carrot somatic embryos

Carrot callus cultures were initiated from seed-grown hypocotyl sections on agar solidified media. The resulting calli were introduced into suspension culture after 12 weeks. After 2 additional weeks, the suspensions were transferred to media lacking 2,4-D to initiate somatic embryogenesis. Samples were examined by microscopy to determine the course of embryogenesis. Globular, torpedo and plantlet stages were predominant at 5, 14 and 25 days after transfer, respectively. Samples were obtained from: seed-grown hypocotyls, agar-grown calli at 6 and 12 weeks after induction, cells in suspension grown in the presence of 2,4-D, somatic embryos of globular, torpedo and plantlet stages and leaves of seed-grown plants (H, 6, 12, S, G, T, P and L respectively). Torpedo-staged extracts were previously shown to be enriched for nuclear proteins by western analysis with monoclonal antibodies specific to a rare nucleolar protein, B-36, present in both plant and animal cells [7]. Crude nuclear extracts prepared from these samples were quantitated and examined on silver-stained SDS-PAGE gels (Fig. 1). While overall the profiles of separated proteins are very similar, some marked differences can be observed. For example, two bands with apparent molecular weights of 69.0 and 42.5 kDa are predominant only in the samples representing suspension cultures and globular and torpedo somatic embryo stages (Fig. 1, lanes S, G and T). Several different proteins are abundant in the hypocotyl extracts as well as for the plantlet extracts (Fig. 1, lane H, 63.2 and 41.9 kDa, lane P, 41.3 kDa).

Specific binding of nuclear extracts from torpedo-staged embryos to the 402 BH phaseolin 5′ fragment

The 402 bp *Bgl* II to *Hha* I DNA fragment (402 BH) from the 5′ region of the phaseolin gene 177.4 [21] was isolated and end-labeled as pre-

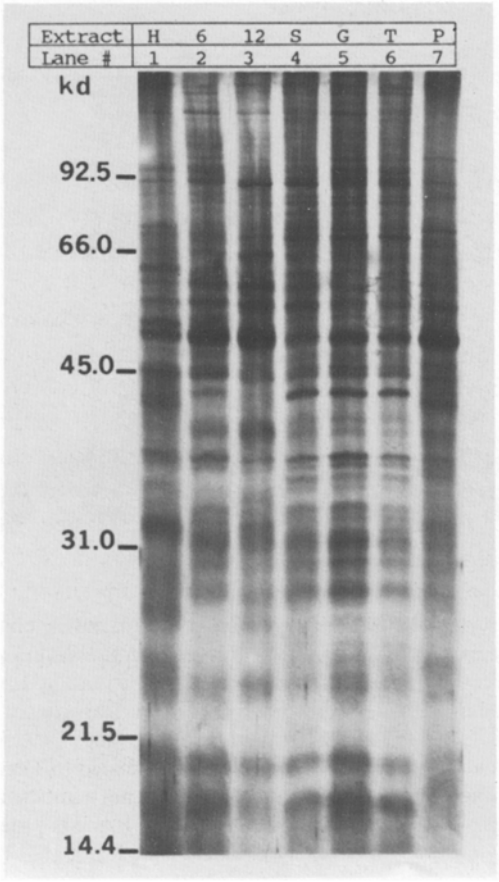

Fig. 1. Nuclear extracts from carrot tissues and embryogenic carrot cultures. Nuclear extracts were quantitated, separated by SDS-PAGE, silver-stained as described in Materials and methods. The extracts were made from: seed-grown hypocotyls, calli at 6 and 12 weeks after induction, suspension cultures, globular and torpedo somatic embryo stages and plantlets germinating from these cultures (H, 6, 12, S, G, T, P, respectively). The molecular weights (in kilodaltons) of standards run on an adjacent lane are indicated on the left.

viously described [1, 6]. This fragment, which is located between −783 to −381 5′ of the major CAP site of the phaseolin gene, was shown to bind nuclear proteins from developing bean cotyledons. The labeled 402 BH fragment was incubated with either the nuclear extract from torpedo-staged embryos (5 μg protein/lane in buffer D) or with buffer D (no protein), in the presence of 2 μg of non-specific competitor DNA (poly-dIdC-poly-dIdC). The mixtures were separated on a non-denaturing polyacrylamide gel. Autoradiography revealed that while the free probe ran as a single species (Fig. 2, lane 1) incubation with the carrot nuclear extract resulted in two bands of reduced mobility (Fig. 2, lane 2). Competition with unlabeled plasmids was used to assess the specificity of the binding activity. While pUC19 did not compete for the binding (Fig. 2, lane 3), the plasmid p402BH competed efficiently for the binding activity (Fig. 2, lane 4). Plasmid p402BH consists of pUC19 containing the same

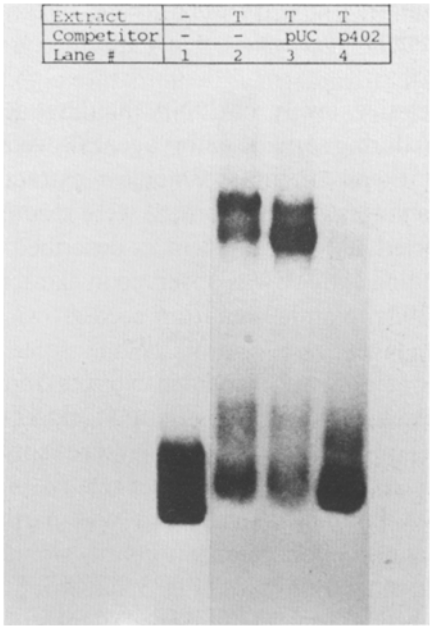

Fig. 2. Carrot nuclear extracts interact specifically with the 402 BH DNA fragment from the 5′ region of the phaseolin gene. DNA was isolated and end labeled as previously described. Binding reactions (20 μl) contained 2000 cpm of DNA, 2 μg of (poly-dIdC-poly-dIdC), 1 μl of binding buffer (200 mM Tris, 19 mM NaEDTA, 7.5 mM DTT, pH 7.5), either 5 μl of protein (1 μg/μl) in buffer D or 5 μl of buffer D alone. Competition experiments were performed by the addition of 1 μg of supercoiled plasmids (pUC19, unspecific; p402BH, specific). Reactions were incubated for 10 min at room temperature, 1 μl of loading dye was added and the products were separated by electrophoresis on a 5% polyacrylamide gel containing 0.25× TBE pH 7.5 buffer [16]. Gels were dried and autoradiographed as previously described. Lane 1, 402 BH fragment alone, lane 2, 402 BH fragment with carrot torpedo nuclear extract (T), lane 3, same as 2 with the addition of 1 μg unlabeled pUC19, lane 4, same as lane 2, with the addition of 1 μg unlabeled p402BH.

402 BH fragment used as the labeled probe. Even with five-fold excess of control plantlet- or seed-grown leaf extracts, no binding was observed (data not shown). These results differ from those observed with extracts prepared from bean hypocotyls prepared under different conditions [1]. The carrot globular nuclear extract also binds a 55 bp synthetic oligonucleotide (data not shown) which contains the phaseolin 5′ protein binding domain located within the 402 BH fragment [1].

Modulation of DNA binding activity during carrot somatic embryogenesis

The relative levels of DNA binding activities present during somatic embryogenesis were determined. Equal amounts of nuclear extracts from various stages of development were reacted with the labeled 402 BH fragment as described above. No binding activity was observed in extracts from hypocotyls, plantlets or from seed-grown leaves (Fig. 3, lanes 2, 8 and 9). While some DNA binding activity was evident in extracts from 6 and 12 week calli (Fig. 3, lanes 3 and 4), extracts from suspension-cultured cells, and cultures containing somatic embryos showed higher relative amounts of DNA binding activity; this was particularly true for cultures containing primarily globular and torpedo-staged embryos (Fig. 3, lanes 5, 6 and 7). The observation of low-level DNA binding activity in calli and suspension cultures is consistent with the hypothesis that 2,4-D induces cell proliferation [23] and gene expression programs characteristic of somatic embryogenesis in the absence of morphologically discernible embryos [4, 28]. Further somatic embryo development is inhibited by the presence of 2,4-D [9]. Our observation of increased levels of DNA binding proteins in globular and torpedo-staged embryos is correlated with the progression of embryogenesis upon removal of 2,4-D. Thus the expression of nuclear proteins that bind the phaseolin 5′ DNA binding site begins at an early stage of carrot somatic embryogenesis, and increases to a maximum at the globular and torpedo stages.

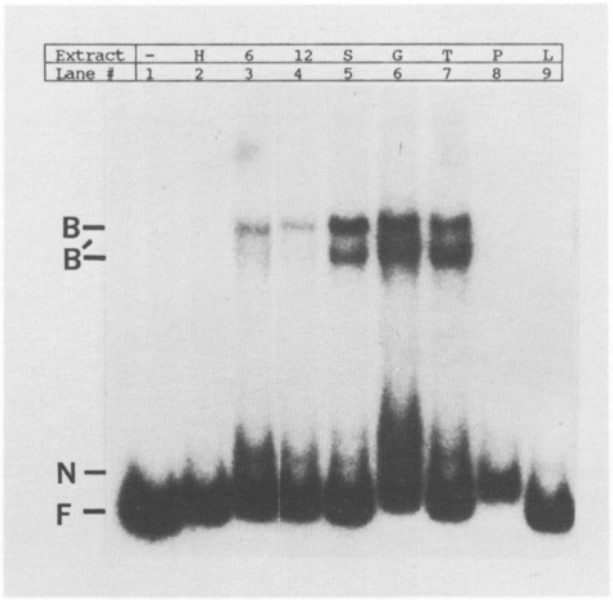

Fig. 3. Developmental regulation of carrot DNA binding proteins. Binding assay was performed as described in Fig. 2. Sources of nuclear extracts are as described in Fig. 1, with the addition of seed-grown carrot leaf nuclear extract (L). Lane 1, labeled 402 BH fragment alone, lanes 2–9, p402 fragment with the various extracts as indicated (6.3 μg). F, free fragment; N, non-specific band (does not compete with either pUC19 or p402BH, see also Fig. 2); B and B′, specifically bound DNA-protein complexes.

Specific binding of labeled DNA to fractionated protein extracts

To estimate the molecular weights of the proteins responsible for the DNA binding activity, nuclear extracts were separated by SDS-PAGE, transferred to nitrocellulose and reacted with the nick-translated 402 BH fragment. Five separable species of protein were observed to react with the probe (Fig. 4, lane 1, 78.2, 66.6, 58.0, 51.2 and 26 kDa). To determine the specificity of these reactions, cold competitor DNAs were included (Fig. 4, lanes 2 and 3). The 78.2 kDa species did not compete with either pUC19 or with p402BH plasmid. The 66.6 kDa (a very faint band) and the 26 kDa species competed with both of the plasmids. Therefore, these three species can be considered to react non-specifically with the phaseolin protein binding sequence. Two species

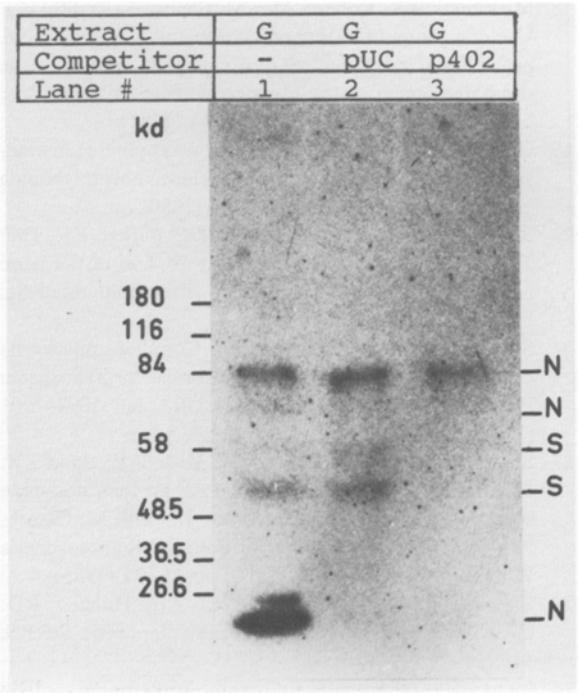

Fig. 4. Specific binding of phaseolin 402 BH fragment to carrot nuclear protein gel blots. Proteins were seperated by SDS-PAGE and transferred to nitrocellulose. 40 μg of extract from globular-stages embryos was run in each lane. Filters were incubated for one hour with gentle shaking in 5% non-fat milk, 10 mM Hepes pH 8.0. Isolated 402 BH DNA fragment was nick-translated according to Maniatis *et al.* [16] to specific activities of 2 × 108 cpm/μg. Probe was added to the filters at a final concentration of 106 cpm/ml in 2.5 ml of binding buffer (10 mM Hepes pH 8.0, 0.1 mM NaEDTA, 25 mM NaCl, 1 mM MgCl, 1 mM DTT and 0.25% non-fat milk) and incubated for 1 hour at room temperature with shaking. Filters were washed twice in binding buffer with 0.3 M NaCl for 1 hour each wash, air-dried, and autoradiography was performed as described. Cold competitior DNA (100 μg) was added 30 minutes prior to adding labeled probe; lane 2, pUC19 DNA, lane 3, p402BH DNA. The molecular weights (in kilodaltons) of standards run on an adjacent lane are indicated on the left. The positions of reacting species are indicated on the right with either S, for specific binding or N for non-specific binding.

(58.0 and 51.7 kDa) were competed only with the p402BH plasmid, demonstrating their binding specificity with the 5′ phaseolin DNA sequence. A 60 kDa protein was identified in nuclear extracts from soybean embryos, which interacts with a DNA sequence from the 5′ region of a seed-specific lectin gene using similar methods [11]. Given the seed-specific expression of the soybean lectin and bean phaseolin genes and the sequence similarity of the protein binding sites 5′ of the genes [1, 6], it is possible that the 58 kDa protein from carrot and the 60 kDa protein from soybean are evolutionary related.

Concluding remarks

We have demonstrated that carrot somatic embryos express DNA binding protein(s) which are capable of interacting specifically with a DNA sequence from the phaseolin seed storage protein gene. Since this DNA sequence has been shown to act as a transcriptional activator in transgenic tobacco plants, the carrot DNA binding proteins identified in this study may be involved in the regulation of gene expression during embryogenesis.

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