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Transcriptional control of plant storage protein genes

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SUMMARY

The accumulation of plant storage proteins is controlled primarily by the transcriptional activation of their genes. Two classes of storage proteins, the zygotic or seed-specific, and the somatic, such as tuber proteins, have been studied. Gene expression analysis in transgenic plants has defined small regions of the promoters of such genes that are able to confer the appropriate patterns of expression. Protein–DNA interactions, both *in vivo* and *in vitro*, have revealed proteins that bind to regions implicated in expression, and these may be transcription factors. Promoter deletion analysis has determined the role of some of these DNA-binding proteins, such as in determining tissue-specificity or levels of expression. A common theme linking the expression of both classes of storage proteins is the involvement of metabolite levels in directly controlling gene expression.

1. INTRODUCTION

Plants deposit and mobilize protein reserves during several different stages of development. The most well known example is the deposition of seed protein reserves in the endosperm and embryo, which are mobilized to provide nutrients during germination. The deposition of these zygotic storage proteins is under strict developmental and temporal control, with the genes encoding the various types of storage proteins being transcriptionally activated in endosperm tissue, embryo tissue, or both, at a particular stage of seed development. The regulation of genes expressed late in embryogenesis is relatively well characterized; the wheat Em gene contains an enhancer responsive to abscisic acid, and putative transcription factors have been cloned (Guiltinan *et al.* 1990). In addition, mutations such as *vp1* affect the transcription of several seed-specific sets of genes in maize, including genes regulated by abscisic acid such as the Em genes (Hattori *et al.* 1992). Genes expressed exclusively in the endosperm are also quite well characterized at the genetic and molecular level. A class of maize zein genes, all of which are expressed in the endosperm, are regulated by a B-Zip factor called *opaque 2*, which binds to a GCN4/jun motif found in the promoters of some classes of zein genes (Lohmer *et al.* 1991; Schmidt *et al.* 1992). This motif, together with other sequences conserved between different classes of genes also expressed in the endosperm such as wheat low molecular mass (LMM)-glutenin, is important for high level endosperm-specific gene expression (Hammond-Kosack *et al.* 1993; Colot *et al.* 1987). Less is known about the regulation of storage protein genes expressed in embryonic tissues however. *Cis* sequences conferring embryo-specific expression of the soybean conglycinin promoter have been identified (Chen *et al.* 1988), and the conglycinin and bean phaseolin promoters have also been shown to bind many nuclear activities, some of which are conserved between legume genes expressed in embryo tissues (Riggs *et al.* 1989; Chamberland *et al.* 1992). A quantitative element from the phaseolin promoter has been identified, but elements conferring tissue specificity remain elusive (Bustos *et al.* 1989).

Some of these zygotic storage proteins are also expressed during other stages of plant development. For example, some of the genes encoding Kunitz trypsin protein from soybean are also expressed in leaves, roots and stems, indicating a storage or proteinase inhibitor function in somatic tissues as well as the zygote (Jofuku & Goldberg 1989). There is another class of storage proteins expressed in somatic tissues, called the vegetative storage proteins (VSPs; Staswick 1990). The transcriptional activity of these genes is modulated rapidly in different organs by factors such as sucrose, amino acid levels, and jasmonic acid (Mason *et al.* 1992). It has been postulated that VSPs act as temporary N reserves during different stages of plant growth and in response to environmental factors. In trees, temporary protein reserves are laid down in the bark for use by the reactivated cambium after the winter (Coleman *et al.* 1991). In plants which use somatic tissues such as roots, bulbs and tubers as major storage organs, abundant proteins are deposited in these tissues for subsequent remobilization together with other storage reserves such as starch. In sweet potato, *Ipomoea batatas*, several abundant proteins such as β-amylase and sporamin (which has significant similarity to

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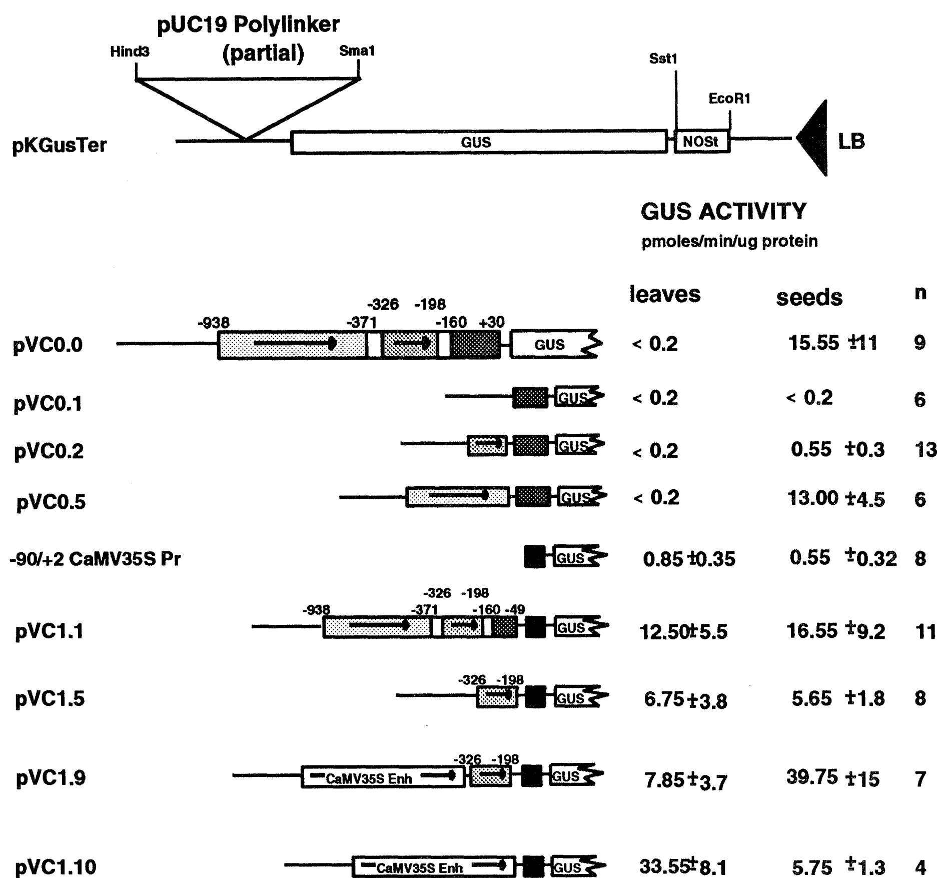

Figure 1. Structure of LMMglu–GUS fusions in the transformation vector Bin19. The VC0 series are derivatives of the LMMglu promoter TATA box region fused to different regions of the LMMglu promoter. The GUS activities resulting from expression of these fusions is shown in leaves and seeds harvested approximately 15 daa. The VC1 series are fusions of LMMglu promoter fragments to the −90 region of the CaMV 35*S* promoter. The 35*S* enhancer sequences are those found between −90 and −250 b.p.

Kunitz trypsin inhibitors) are deposited in the storage root (Nakamura *et al.* 1991). The steady-state levels of mRNA encoding both of these proteins increases in response to high level of exogenous sucrose. Similarly, sucrose levels determine, in part, the abundance of transcripts encoding two proteins found in potato tubers, patatin (Rocha-Sosa *et al.* 1989; Wenzler *et al.* 1989), and proteinase inhibitor II (Kim *et al.* 1991), which both account for most of the protein in developing tubers. Patatin is a lipolytic acyl hydrolase (Andrews *et al.* 1988), which may release fatty acids from membranes as part of a signalling response involving pathogen interactions (Parker 1987). In addition, the transcription of genes encoding granule-bound starch synthase (GBSS, Visser *et al.* 1991) and ADP-glucose pyrophosphorylase (AGPase, Muller-Rober *et al.* 1990), both enzymes of starch biosynthesis, was activated by high levels of sucrose in potato. In maize, sucrose-modulated gene expression causes differential activation and repression of genes encoding sucrose synthase isozymes (Koch *et al.* 1992). Finally, sucrose has been shown to repress the transcription of genes encoding enzymes of photosynthetic C fixation in maize (Sheen *et al.* 1990). These findings indicate an important role for levels of sucrose or its metabolites in regulating and integrating the activity of sets of genes in somatic storage tissues, and in contributing to, via effects on gene expression, the partitioning of carbohydrate in plants. The expression patterns of patatin and other somatic storage proteins could be determined by the high concentrations of sucrose found in sink tissues such as tubers (Humphreys 1988). Alternatively, factors specifying tuber expression may dictate the principle pattern of patatin expression, with separate mechanisms governing sucrose-inducibility in other organs. The control of zygotic storage protein gene expression by metabolites such as amino acids is presently being studied (Balconi *et al.* 1993), with an indication that N levels play a significant role in determining the overall expression levels of zein storage proteins via *opaque 2* in maize.

2. LOW MOLECULAR MASS GLUTENIN

Glutenins are prolamin proteins which are expressed specifically in the endosperm of developing cereal grains. Fusions of promoter regions to reporter genes and transformation of tobacco have roughly defined *cis* sequences necessary for endosperm-specific expression (Colot *et al.* 1987). The activities of the VC0 series of glutenin–GUS fusions shown in figure 1 demonstrate that sequences between −938 and −371 b.p. and −326 and −198 b.p. can act alone and in concert to direct seed specific expression.

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(*a*) sequence comparison of the 5'region around the endosperm box of cereal storage protein genes

Taglu1d1	Triticum aestivum	LMM glutenin 1D1	TAAAAGTGATACTATCTTGATAAGTGTGTGACATGTAAAGTTAATAAGGTGAGTCATATA
Taglut	wheat	LMM glutenin	CAAAAGTGATACTATCCTGATAAGTGCGTGACATGTAAAGTTAATAAGGTGAGTCATATG
Tagliabd	wheat	alpha/beta gliadin	TAAAAGTGATACTATCTTGATAAGTGTGTGAGTTGTAAAGTGAACAAGATGAGTCATGCG
Taglgb	wheat	gamma gliadin	CAAAGGGAACACAATCCATATAATTGTTTGACATGTAAAGTTAATAAGGTGAGTCATATA
Taagcnn35	wheat	alpha gliadin pseudogene	AAAATAGGCAATCTAGATTAGTGTTTGAGCTGTAAAGTGAATAAGATGAGTCATGCA
Tagliag2	wheat	alpha/beta gliadin	AAAATAGGCAATCTAGATTAGTGTTTGAGCTGTAAAGTGAATAAGATGAGTCATGCA
Tagliag3	wheat	alpha/beta gliadin	AAACTAGGCAATCTAGATTAGTGTTTGAGTTGTAAAGTGAACAAGATGAGTCATGCG
Taagcnn16	wheat	alpha gliadin pseudogene	CAAATAGGCAATCTAGATTACTGTTTGATCTGTAAAGTAAATAAGATGAGTCATGCG
Tagliaa	wheat	alpha gliadin	AAAATAGGCAATCTAGATTAGTGTTTGAGCTGTAAAGTGAATAAGATGAGTCATGCA
Tagliabb	wheat	alpha/beta gliadin	AAAATAGGCAATCTAGATTAGTGTTTGAGCTATAAAGTGAATAAGATGAGTCATGCG
Taggln	wheat	gamma gliadin	CAAAAGGAATGCAATCTAGATAATTGTTTGACTTGTAAAGTCGATAAGATGAGTCAGTGC
Taagcnn17	wheat	alpha gliadin pseudogene	ACAAATAGGCAACTAGATTACTGTTTGATCTATAAAGTAAATAAGATAAGTCATGCG
Tuglna	Triticum urartu	alpha/beta-type gliadin	AAAATAGGCAATCTAGATTAGTGTTTGAGCTATAAAGTGAATAAGATGAGTCATGCG
Tuglnb	Triticum urartu	alpha/beta gliadin pseudogene	AAAATAGGCAATCTAGATTAGTGTTTGAGCTATAAAGTGAATAAGATGAGTCATGCG
Hvg1hord	barley	gamma-1 hordein	TAGAAGGATTGGAAGATAGATACTTATGTGAGATGTAAAGTGAATAAGATGAGTCAGCAC
Hvb1horg	barley	B1 hordein	CAGAAGTGATACTAGCTTGATAAGTGCGTGACATGTAAAGTGAATAAGGTGAGTCATGTA
Hvghrdsp	barley	gamma hordein	TAGAAGGATTGGAAGATAGATACTTATGTGAGATGTAAAGTGAATAAGATGAGTCAGCAC
Scsec1b	rye (S. cereale)	Sec1 omega secalin gene	AGAAGGCAAAATCAAGATCAAGGTGTGTAGTGTAAAGTGAAAAAAATGAGTCATCAG
consensus sequence			CAATCTAGAT[A/T]AGTGT[T/G]TGANNT[G/A]TAAAGTNAATAAG[A/G]TGAGTCAT

(*b*) DMS hypomethylated sites in the 5'region of LMMG-1D1 in wheat endosperm at 18 DAA

Figure 2. Sequence comparison of the endosperm box region from several cereal storage protein genes expressed in the endosperm. For comparison, the positions of G residues involved in protein–DNA interactions in the endosperm is shown below, as identified by *in vivo* footprinting methods.

Histochemical analysis has shown that this expression occured exclusively in the endosperm of transgenic tobacco seed. Hybrid promoters containing the TATA box and as-1 element of the CaMV 35*S* promoter (Katagiri *et al.* 1989) and the −326 to −198 b.p. region (the VC1 series of constructs, figure 1) did not show any seed specific expression, and histochemical analysis of developing seeds showed expression in the endosperm, radicle tip and vascular tissue. This indicated that sequences other than the endosperm box region were required for high level endosperm-specific expression. A clue as to possible interactions between promoter regions is provided by the observation that the −326 to −198 b.p. endosperm box-containing region is able to alter the expression patterns of the 35*S* promoter such that it became highly active in seeds, and repressed in leaves, as shown by construct VC1.9. One interpretation for this is that the −323 to −198 b.p. region interacts with other regions of the LMW glutenin promoter which carry out an analogous function to those of the enhancer sequences in the B domain of the 35*S* promoter. These sequences are most likely in the −371 to −938 b.p. region of the glutenin promoter. Analysis of the −323 to −198 region has revealed a DNA motif which is highly conserved between several genes expressed in the endosperm of wheat, rye and barley. Within this motif, called the endosperm box, two subdomains with substantial homology to binding sites for known transcription factors can be defined (figure 2). Therefore one would predict the binding of at least two proteins to the endosperm box region, and that these proteins could interact with others to direct high levels of endosperm-specific expression. *In vivo* and *in vitro* analysis of protein–DNA interactions with the endosperm box region have characterized two of these binding activities (figures 3 and 4). The specificity of *in vitro* binding of the two activities for their cognate elements is the same as their *in vivo* specificity, indicating that the activities identified by *in vitro* and *in vivo* footprinting are functionally similar. ESBF-1 binds to the endosperm motif within the endosperm box about 12 days after anthesis (daa) when the glutenin gene is expressing at a relatively low level, while ESBF-2 binds to the GCN4-like motif from 15–18 daa, and at this time LMM glutenin transcription is maximal. It is conceivable that ESBF-2 is involved in directing high levels of expression, while ESBF-1 may determine the tissue-specificity of gene expression. What is the nature of these binding activities? It is likely that ESBF-2 is composed of a pair of b-ZIP proteins, due to the high degree of similarity of its binding site to sites known to bind b-ZIP proteins such as GCN4 and opaque 2. Experiments are in progress to determine which b-ZIP proteins are involved in activating transcription from the endosperm box motif. One of these may be similar to opaque 2 from maize, a positive regulator of zein gene expression in maize endosperm (Lohmer *et al.* 1991). The ESBF-1 binding site is being used to screen cDNA expression libraries in order to isolate binding proteins which may be candidates for ESBF-1.

3. PATATIN

As mentioned above, the tissue specificity of patatin expression could be determined principally by the accumulation patterns of sucrose in developing potato plants, as it is known that sink tubers in which patatin transcription occurs accumulate large amounts of

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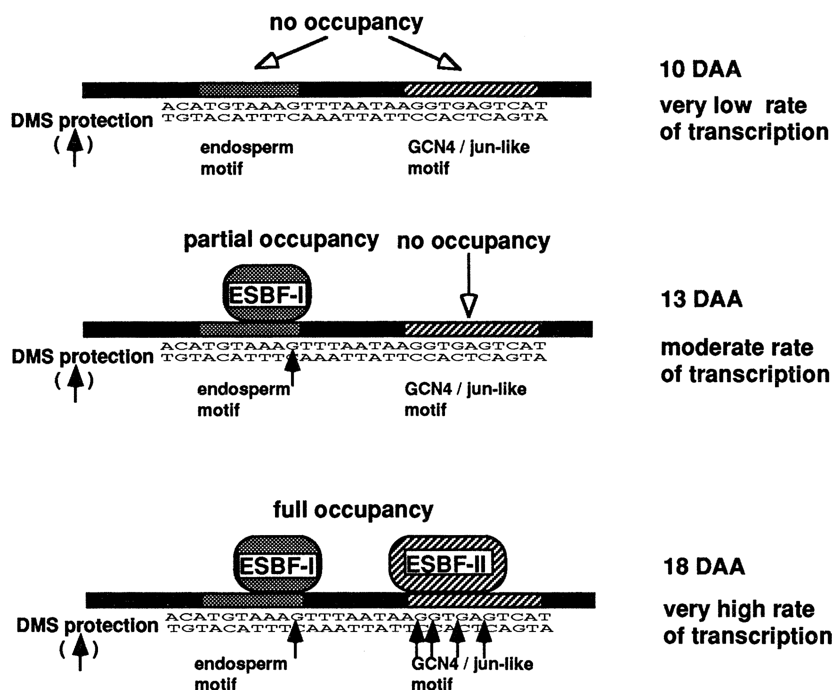

Figure 3. A model of protein–DNA interactions on the endosperm box. The binding activities ESBF-I and -II occupy sites, as identified by *in vivo* footprinting, at different times after anthesis. This occupation of sites has been correlated with different rates of expression of the LMMglu gene.

sucrose. Alternatively, patatin transcription could be governed by developmental signals in tubers, and by separate mechanisms in response to sucrose in other tissues. Promoter analysis experiments were carried out to test this hypotheses. The promoters of two patatin genes have been assessed for their ability to direct tuber-specific and sucrose-inducible gene pression in transgenic potato (Jefferson *et al.* 1990;] *et al.* 1990). Both studies have demonstrated a criti role for sequences located between −250 ε −150 b.p. as being important in confering both tu specificity and metabolite responsiveness. This reg

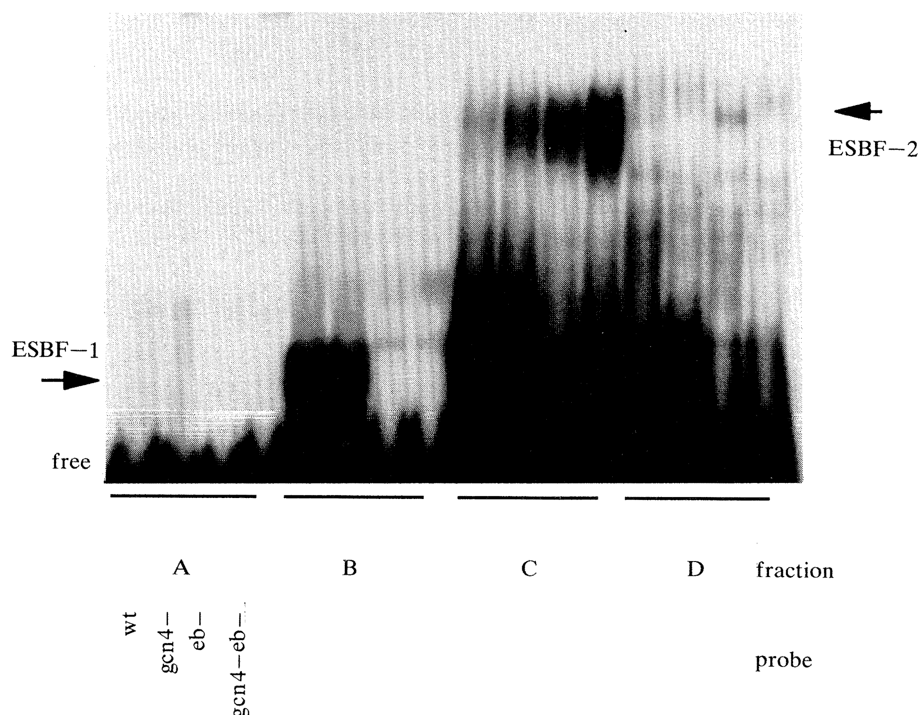

Figure 4. *In vitro* DNA–protein interactions on the endosperm box, as identified by EMSA. Binding activities of two mobilities with specific binding to each of the two motifs identified by *in vivo* footprinting are evident. Mutations altered in the G residues identified by footprinting were introduced into the endosperm box and used as probes with nuclear protein extracts from developing wheat endosperm. These data confirm the identification of two different DNA binding activities identified by *in vivo* footprinting.

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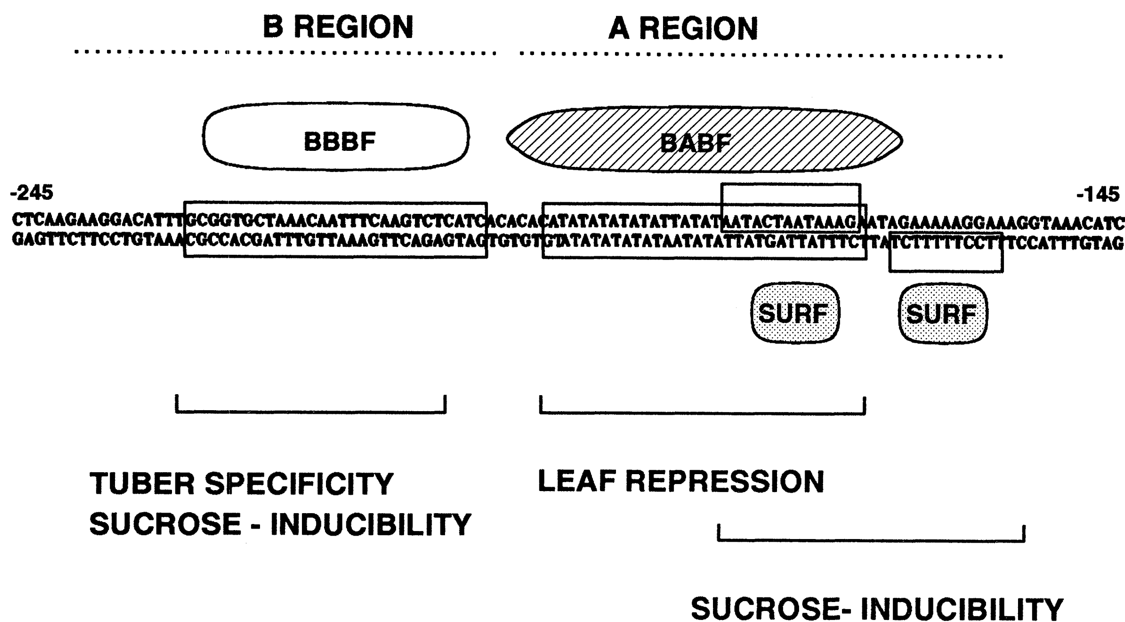

Figure 5. Model of protein–DNA interactions on the patatin promoter. The A+B repeat region, extending from −245 to −145 b.p. from the transcription start site, is shown, and regions specifically bound by the different activities are shown as boxed regions on the sequence.

contains a highly conserved repeat motif consisting of a subset of two repeats, called A and B. The A+B motifs are repeated twice upstream of the proximal repeat, and progressive deletion of the repeats leads to steadily declining tuber activity and inducibility, providing further clues as to the importance of the A+B repeat motif. Recent experiments have studied the effects of small deletions in different regions of the A and B motif, and have also studied the function of this region in synthetic promoters (C. Grierson *et al.* unpublished results). In addition, *in vitro* protein–DNA interactions in this region were studied in order to correlate protein binding to any functional domains identified by deletion analysis. A dramatic decrease in both tuber and sucrose-induced activity was observed when 24 b.p. of the B repeat region was removed, such that the promoter expressed at low levels in both leaves and tubers. Nevertheless there was a residual sucrose-inducibility of about five- to tenfold. *In vitro* footprinting and EMSA (electrophoretic mobility shift assay) revealed that a nuclear protein of very low abundance bounds specifically to this region. This protein, called BBBF, was found in stems and tubers, and to a lesser extent in leaves and not at all in roots. Larger deletions that also remove the A/T rich region of the A+B repeat, as well as the B repeat, caused an increase in activity in leaves relative to tubers, and had little effect on residual sucrose-inducibility. Further internal deletions that removed the proximal region of the A repeat finally abolished this residual sucrose-inducibility, indicating the presence of sequences able to confer a small degree of sucrose-inducibility in addition to the activities of the B repeat region. Two classes of protein have been found to interact with the A repeat: the distal A/T-rich region bound an abundant activity, called BABF, which has several characteristics of the HMG (high mobility group) class of nucleoproteins (Jacobsen *et al*, 1990). But it was neither heat nor acid stable, and had a higher relative molecular mass than typical HMGs. Interestingly, leaf and tuber binding BABF formed complexes with the A+B repeat of different mobilities, which may reflect different forms fo BABF in leaves and tubers. The second class of binding activity, called SURF, bound specifically to a proximal region of the A repeat, called the SURE sequences, which have significant similarity to sequences in another promoter regulated by sucrose, namely sporamin from sweet potato. These binding activities may be similar to the SP8BF activity binding to similar sequences in the sporamin promoter (Ishiguro & Nakamura 1992). It is possible that these sequences, found twice in the A region of the A+B repeat, may confer the residual fivefold sucrose induction observed. Figure 5 summarizes our present understanding of protein–DNA interactions and function of the patatin proximal A+B repeats. The data indicate a central role for the B repeat and its cognate binding factor, BBBF, in conferring tuber specific expression on the minimal patatin promoter, and contributing to a large proportion of sucrose inducibility. The function of the distal A/T-rich region is more difficult to interpret as specific deletions and mutations have not been made that effect only that region. Preliminary indications are that it may be involved in repressing patatin activity in organs such as leaves. Finally, a residue of sucrose-inducibility is conferred by the proximal region of the A/T-rich repeat, and the SURE repeats are interesting candidates for the *cis* sequences responsible. Until the individual elements identified have been analysed by specific mutagenesis little more can be deduced about the function of the A+B repeat. Cloning DNA binding proteins with the appropriate binding specificity and the assessment of transcriptional activation or repression functions of these are the next steps necessary to increase our understanding of this

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particularly complex promoter. Experiments are now being carried out to define the role of these repeats in the absence of the adjacent *cis* sequences. In addition, the role of the B repeat and its cognate binding factor BBBF is being investigated. These approaches involve devising a transient assay system for tuber specificity and sucrose inducibility, and cloning binding activities using South-western screens of cDNA expression libraries.

4. CONCLUSIONS

The aim of these analyses has been to define the conditions and signals necessary for storage protein gene expression. We have studied two representatives of both classes of protein, the zygotic and somatic, to contrast the ways in which they are regulated and define common principles governing the regulation of both classes of genes. Some progress has been made in defining one of the groups of *cis* sequences in each gene studied which is responsible for key aspects of gene regulation, such as tissue specificity, and in assessing interactions between *cis* sequences resulting in the full spectrum of gene expression. This work provides a suitable basis for cloning DNA binding proteins which may be candidate transcription factors. The characterization of transcription factors will then provide a basis for understanding, at a mechanistic level, how developmental controls on gene expression can be integrated with controls exerted by metabolites. For example, as nearly all of the available N provided to wheat plants is sequestered in the grain, it is clear that stringent controls must be exerted on N partitioning, and one of the ways in which this could be achieved is by transcriptional regulation of genes encoding the N-sink products, i.e. storage proteins. The beginnings of how this may be achieved are evident in studies on the LMM glutenin promoter, where ESBF-1 may control tissue specificity and ESBF-2 responsiveness to N levels. It will be interesting to determine which metabolites may be involved in determining gene expression; in the case of zygotic storage proteins N is transported to developing seeds as ureides or asparagine, and seed-specific asparaginases and other deaminases release NH_3 which is then assimilated by the GOGAT cycle to form glutamine, the principle source of N for amino acid biosynthesis. Some of the molecules in this series of reactions, or the translocated N compound itself, may be involved in signalling gene activation. Similarly, the response of the patatin promoter to high exogenous concentrations of sucrose is most likely not due to sucrose levels per se defining conditions for activation, but rather one or several of the metabolites of sucrose. These may be metabolic intermediates leading to starch biosynthesis, as patatin expression is coordinated with starch biosynthesis both in tubers and other regions of the potato plant. Recent work using antisense down-regulation of genes encoding enzymes of starch and sugar metabolism to alter metabolite levels (Stitt, this symposium; Muller-Rober *et al.* 1992) has provided the necessary material to assess the effects of sugar levels and fluxes on patatin transcription.

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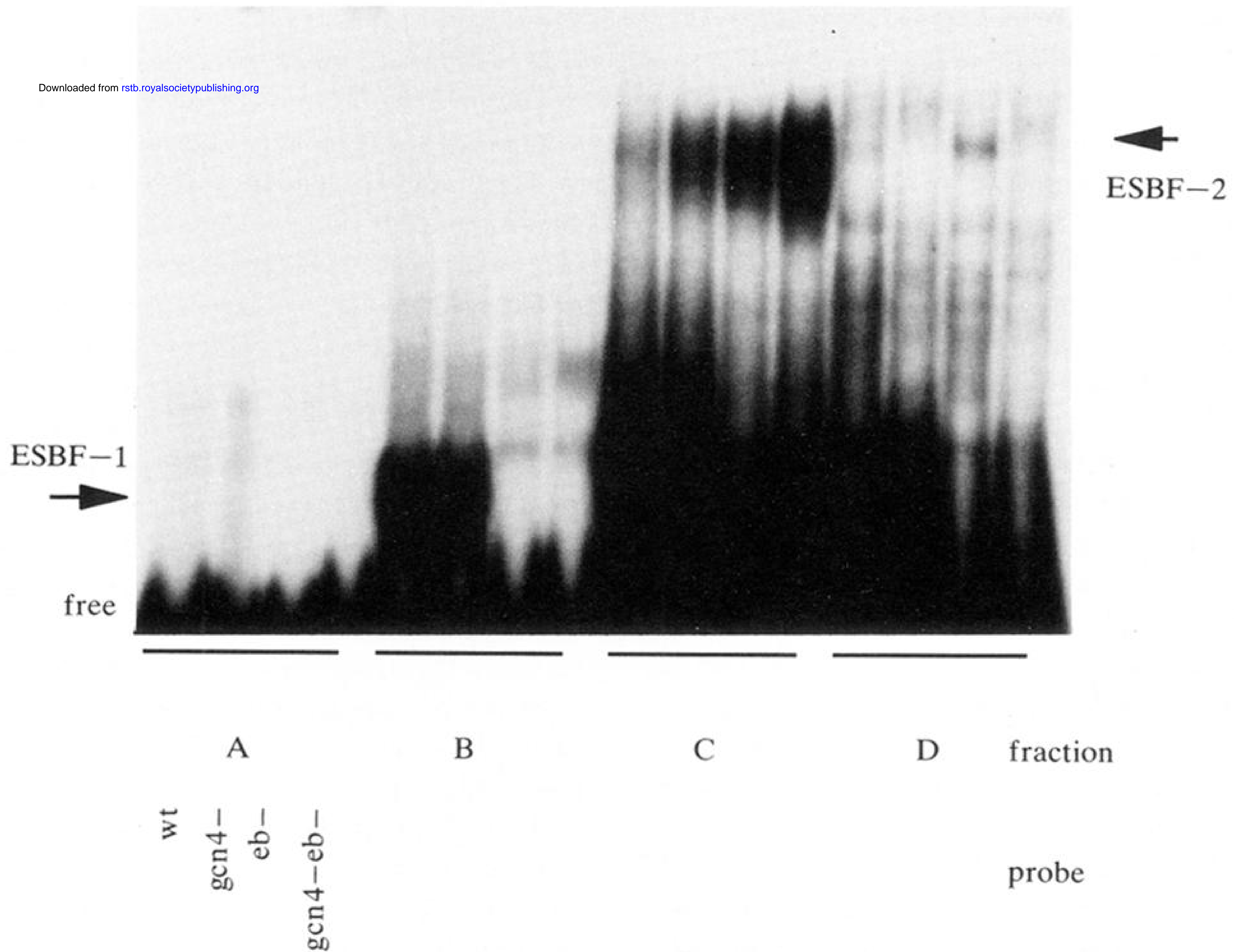

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igure 4. *In vitro* DNA–protein interactions on the endosperm box, as identified by EMSA. Binding activities of two ıobilities with specific binding to each of the two motifs identified by *in vivo* footprinting are evident. Mutations ıtered in the G residues identified by footprinting were introduced into the endosperm box and used as probes with ıuclear protein extracts from developing wheat endosperm. These data confirm the identification of two different ⁾NA binding activities identified by *in vivo* footprinting.