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Cis regulatory elements directing tuber-specific and sucrose-inducible expression of a chimeric class I patatin promoter/GUS-gene fusion

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Summary. The 5′-upstream region of the class I patatin gene B33 directs strong expression of the β-glucuronidase (GUS) reporter gene in potato tubers and in leaves treated with sucrose. *Cis*-acting elements affecting specificity and level of expression were identified by deletion analysis in transgenic potato plants. A putative tuber-specific element is located downstream from position −195. Nuclear proteins present in leaf and tuber extracts bind specifically to a conserved AT rich motif within this region. A DNA fragment between −183 and −143, including the binding site is, however, not able to enhance the expression of a truncated 35S promoter from cauliflower mosaic virus. Independent positive elements contributing to a 100-fold increase relative to the basic tuber-specific element are located between −228 and −195; −736 and −509, −930 and −736 and −1512 and −951. Sucrose inducibility is controlled by sequences downstream of position −228, indicating that the tuber-specific and sucrose-inducible elements are in close proximity.

Key words: Class I patatin – Promoter – Cis-elements – β-Glucuronidase

Introduction

The major glycoprotein of potato tubers is encoded by a gene family of 10–15 genes per haploid genome. The classification of these genes into two groups, according to structural features, is based on both RNA and expression studies, using potato plants transformed with chimeric genes consisting of patatin promoters fused to the β-glucuronidase reporter gene (Pikaard et al. 1987; Rocha-Sosa et al. 1989; Wenzler et al. 1989). Class I genes are strongly expressed in all cell types of the potato tuber except the periderm and are inducible in leaves by sucrose. Expression of class II genes is restricted to specific cell types in tubers and roots (Köster-Töpfer et al. 1989).

Sequence analysis of the class I promoter B33 (Rocha-Sosa et al. 1989) has revealed two structures that might be involved in its control i.e., a 208 bp direct duplication and a 37 bp AT rich motif occurring three times in the promoter region. The long direct repeats allow division of the 1.5 kb promoter fragment into four modules. In order to define *cis*-regulatory elements responsible for the specificity and the high levels of expression in potato tubers a deletion analysis of the 5′-upstream region was performed.

Here we report the results obtained with chimeric constructs consisting of different parts of the B33 promoter driving the β-glucuronidase gene (GUS) in transgenic potato plants after *Agrobacterium*-mediated gene transfer. Nuclear proteins were identified that bind to the conserved AT rich element within the region necessary for tuber-specific expression.

Materials and methods

Deletion constructs. A *Hpa*I restriction site was introduced into pP33P1 (Rocha-Sosa et al. 1989) by changing the sequence starting at position −740 from GGTAAA to GTTAAC via oligonucleotide-directed in vitro mutagenesis. The *Dra*I/*Xba*I fragment (−1512/−339), the *Dra*I/*Hpa*I fragment (−1512/−737) and the *Dra*-I/*Xho*II fragment (−1512/−509) were deleted in order to obtain constructs D3, D9 and D10, respectively. Deletion of the internal *Hpa*I/*Xba*I fragment was used to construct D1. Starting from D1, the *Dra*I/*Xba*I (−1512/−951) fragment was deleted to create D2. D7 and D8 were constructed by fusion of the *Dra*I/*Xba*I (−1512/−951) and *Hpa*I/*Xba*I fragment (−737/−339), respectively, to position −72 of the truncated 35S promoter from caulflower mosaic virus (CaMV). For this purpose the *Bam*HI/*Eco*RI fragment of pJO/44d (Ow et al. 1987) containing the luciferase coding region and *nos* terminator was replaced by the *Bam*HI/*Eco*RI fragment contain-

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ing the GUS gene and *nos* terminator from pBI101.1 (Jefferson et al. 1987). The 35S enhancer fragment extending from −373 to −32 was cloned as an *Eco*RI fragment into the *Eco*RI site of constructs D3, D5 and D6. All these constructs were subsequently transferred to pBin19 (Bevan 1984).

D11 was constructed by ligation of a *Dde*I/*Hpa*I (−800/−736) fragment, containing one copy of the conserved AT motif, to the −90 position of a truncated 35S promoter fused to the GUS coding region. For this purpose, the isolated fragment was treated with Klenow DNA polymerase in the presence of all four deoxynucleotides, and ligated to the *Sma*I site of plasmid pBinΔ35S (J. Sánchez-Serrano, personal communication). The various deletion constructs are schematically diagrammed in Fig. 1.

Agrobacterium tumefaciens and potato transformation were performed as described by Rocha-Sosa et al. (1989). Micropropagated shoot cultures were transplanted to 16 cm plastic pots containing P-type soil/sand (3:1) and cultivated in the greenhouse under long day conditions.

GUS assays and Northern blot analysis. GUS activity of protein extracts was determined as described by Jefferson (1987) using 4-methylumbelliferyl β-D-glucuronide as substrate. For histochemical analysis sections of freshly harvested tubers were cut with a razer blade and incubated in 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid), 50 mM $NaPO_4$ pH 7 for 16–20 h at 37° C. RNA was isolated according to Logemann et al. (1987). All other methods were performed according to Sambrook et al. (1989).

Sucrose induction of leaf discs. Leaves were floated on MS medium (Murashige and Skoog 1962) containing 7% sucrose under long day conditions (16 h light/8 h dark) for 2 days.

DNA binding studies. Preparation of nuclear extracts and gel shift assays were performed as described by Prat et al. (1989). A 144 bp *Dde*I fragment corresponding to −249/−105 was filled-in with Klenow polymerase and cloned into the *Sma*I site of pUC18. The inserted fragment flanked by polylinker sequences was excised as a 205 bp *Eco*RI-*Hin*dIII fragment and used for gel retardation analysis.

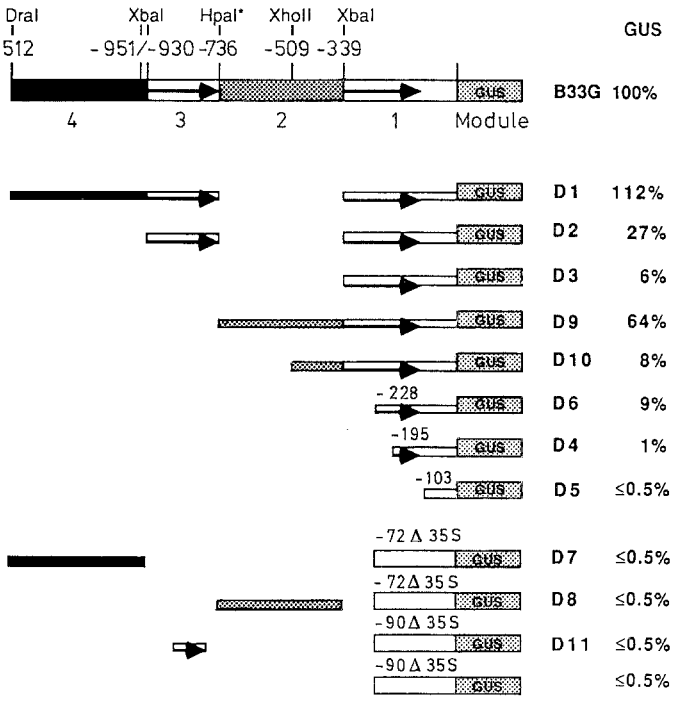


Fig. 1. Structure and level of expression of chimeric constructs in tubers. The module structure based on the 208 bp duplication and a partial restriction map of B33G is given in the upper part. Constructs D1–D11 contain different parts of the promoter fused to β-glucuronidase (GUS). In the case of D7, D8 and D11, parts of the B33 promoter were fused to the 35S promoter truncated at positions −72 (−72Δ35S) and −90 (−90Δ35S). The levels of GUS activity in mature tubers relative to the activity of the wild-type B33 promoters is given in the righthand column

Results

Deletion constructs

Using the β-glucuronidase gene as a reporter it has been shown that a 1.5 kb promoter fragment of the class I patatin gene B33 provides all the information necessary for both tuber-specific and sucrose-induced expression in leaves of transgenic potato plants (Rocha-Sosa et al. 1989). Based on the presence of a 209 bp direct repeat, the 5′ upstream region of B33 was divided into 4 modules extending from positions −1512 to −951 (no. 4), −930 to −736 (no. 3), −736 to −339 (no. 2) and −339 to +18 (no. 1) (Fig. 1). A *Hpa*I restriction site at −739 was introduced by in vitro mutagenesis in order to separate modules 2 and 3. Module 2 was further divided into the regions from −736 to −509 and from −509 to −339. Progressive deletions from the 5′ end were used to subdivide module 1.

Modules 2, 3 and 4 were linked in different combinations to module 1 to examine the role in tissue specificity, inducibility and quantitative regulation of expression. All constructs were fused to the GUS reporter gene and transferred to pBin19. Potato plants were transformed via *Agrobacterium*-mediated gene transfer. Transgenic plants were shown by Southern analysis to contain intact copies of the chimeric genes (data not shown).

Five to 12 independent transformants grown under greenhouse conditions were analysed for each construct. GUS activity was monitored in tubers, tuberized stolons, leaves and stems of the different plants (Table 1). All constructs show identical tissue specificity, i.e. expression in tubers and tuberized stolons, but differ in their levels of expression by about two orders of magnitude. The results obtained by analysing expression at the protein level were confirmed by staining hand-cut sections of tubers with X-gluc (Fig. 2). The analysis on the GUS activity level was further substantiated by Northern blots using a radioactively labelled GUS probe (Fig. 3).

Table 1. β-Glucuronidase (GUS) expression in different tissues of transgenic potato plants transformed with chimeric patatin/GUS constructs

Construct	Tuber	Stolon	Leaf	Stem
B33G	717[a] (10)[b]	408(4)	<5(10)	8(10)
D1	805 (13)	201(11)	<5(12)	24(11)
D9	459 (5)	54(5)	11(5)	< 5(5)
D10	55 (8)	n.d.	<5(8)	7(8)
D2	197 (12)	24(7)	<5(10)	< 5(8)
D3	41 (9)	18(7)	<5(8)	6(6)
D6	67 (10)	19(5)	<5(8)	< 5(7)
D4	7 (6)	<5(4)	<5(6)	< 5(7)
D5	<5 (7)	12(7)	<5(7)	< 5(7)
D7	<5 (10)	n.d.	<5(10)	< 5(5)
D8	<5 (7)	n.d.	<5(7)	n.d.
D11	≤5 (12)	18(11)	17(11)	19(11)
−90Δ35S	≤5 (5)	10(5)	11(5)	<5(5)

[a] Average of GUS activities are given as pmol 4-methylumbelliferone released/μg protein per h
[b] Numbers of independent transgenic plants tested
n.d., not determined

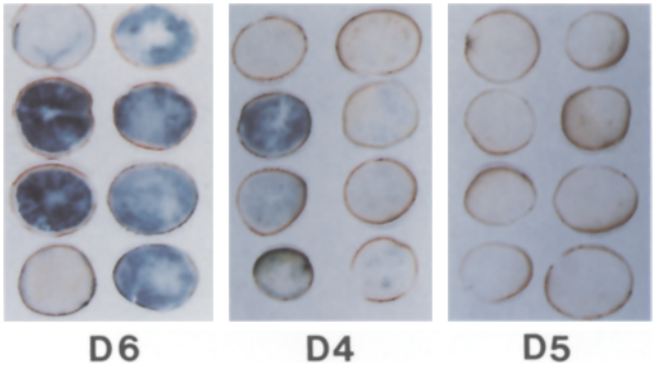


Fig. 2. Histochemical analysis of β-glucuronidase activity. Cross-sections of mature tubers from transgenic potato plants tansformed with constructs D6 (−228/+18), D4 (−195/+18) and D5 (−103/+18) were stained for GUS activity using X-gluc as a substrate. For each construct results obtained for tubers from eight independent transformants are shown

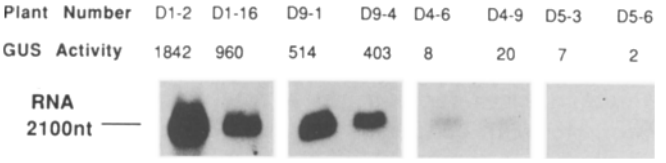


Fig. 3. Comparison of GUS activities [pmol 4-methylumbelliferone(4-MU) released/mg protein per min] and amounts of GUS mRNA. Total RNA (50 μg) isolated from tubers of transgenic potato plants transformed with constructs D1, D9, D4 and D5 was loaded in each lane fractionated by gel electrophoresis and blotted. Blots were hybridized to radioactively labelled GUS DNA. The blots for D1 and D9 were exposed for 16 h, for D3 and D5 for 72 h

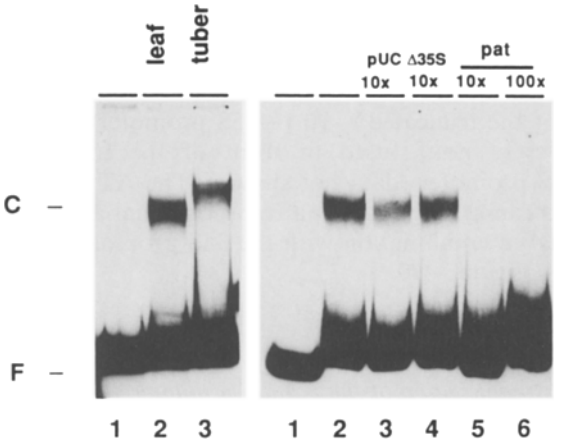


Fig. 4. Binding of nuclear proteins to a 145 bp *Dde*I fragments containing the AT rich element. A *Dde*I fragment containing the region from positions −249 to −105 was used as a DNA probe. The end-labeled DNA fragment was incubated with 20 μg leaf nuclear extract (lane 2, left panel), 20 μg tuber nuclear extract (lane 3, left panel and lanes 2–6, right panel) or without protein (lane 1, left and right panels) in the presence of 4 μg poly dI.dC as unspecific competitor DNA. The specific unlabeled competitor fragments and the degressive molar excess compared to the labeled fragment in the binding reaction are indicated at the top of lanes 3–6 (right panel). pUC (pUC18) linearized by *Eco*RI digestion; Δ35S, −90 to +8 fragment of the 35S promoter; pat, homologous fragment; F, free DNA; C, DNA bound to factor(s)

Identification of a putative tuber-specific element

Module 1 (D3; −339/+18) is sufficient to confer tuber-specific expression though at a level about 20-fold lower than that of the complete wild-type promoter. Progressive 5′ end deletions of D3 generated constructs D6 (−228/+18), D4 (−195/+18) and D5 (−103/+18). Both D6 and D4 still show tuber-specific expression, whereas no activity is detectable for the shortest construct, D5. The region between −195 and −103 thus seems to be necessary for gene expression. A tuber-specific element should therefore be located downstream of position −195.

DNA binding proteins

The deletion analysis indicates that sequences between −195 and −103 might contain elements necessary for the tuber-specific expression of the gene. This fragment includes one of three copies of a 37 bp AT rich conserved element in the promoter region. Nuclear factors binding to this region were identified by gel retardation assays. A *Dde*I restriction fragment containing the region between −249 and −105 binds nuclear proteins from extracts isolated from both leaves and tubers (Fig. 4). This binding cannot be competed with either pUC18 or a fragment from the 35S promoter (−90 to +8). Complexes with slightly different electrophoretic mobility are detected in extracts prepared from leaves or tubers, arguing for the presence of different factors in the two tissues. Similar protein complexes are found with fragments *Dde*I/*Hpa*I (−851 to −737) and *Sau*3AI/*Cla*I (−503 to −401) all containing a copy of the AT rich conserved element (data not shown) thus suggesting that the AT rich motif is the target for the binding of this protein or proteins.

The upstream copy of the AT motif cannot activate a truncated 35S promoter

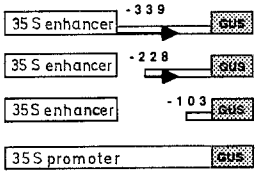
Fusion of promoter sequences to heterologous truncated promoters can be used to examine the function of *cis*-regulatory elements. As either the AT rich motif or sequences downstream from −143 might be responsible for the basic tuber-specific activity retained in D6 (−195/+18), the *Dde*I/*Hpa*I (−851/−737) fragment containing the upstream copy of the AT rich sequences was fused to the truncated −90Δ35S promoter (construct D11). Analysis of GUS activity shows no significant difference in the levels of expression of the plants transformed with this construct compared with control plants transformed with the truncated 35S construct (Table 1). Consistent with this, histochemical analsis of roots shows similar distributions of GUS activity in transgenic potato plants containing the GUS gene, either under control of the truncated −90Δ−35S promoter or with the AT rich motif fused in front of the truncated −90Δ35S promoter (data not shown). The AT rich region thus cannot function as a transcriptional activator when used in combination with the 35S promoter truncated at position −90.

Three different positive regulatory elements are present in the upstream region of the patatin promoter B33

Deletion of sequences from the 5′ end of the 1.5 kb promoter fragment of the class I patatin gene down to position −195 leads to a stepwise reduction in GUS expression to 1% of the original level. Three different positive regulatory elements that are responsible for the stepwise reduction were identified.

No significant influence on the level of expression is found when D3 (−339/+18) and D6 (−228/+18) are compared. However the tenfold difference between D6 (−228/+18) and D4 (−195/+18) suggests the presence of a positive element (A) between −228 and −195. One positive regulatory element is thus present in module 1. Construct D2 (−930/−736 + −339/+18) contains a duplication of the sequence from −143 to −345, including positive element (**A**). This construct has a fourfold higher activity than D3 (−339/+18), which carries only a single copy of the regulalory element (A). This is in accordance with the observation that D3 (−339/+18) alone is 5–6 times more active than D4 (−195/+18); here again, the duplication is associated with a 4- to 5-fold increase in activity, up to 27% relative to the wild type promoter. Module 2 (−736/−339) together with module 1 (−339/+18) in construct D9 leads to a tenfold increase of activity relative to D3 (−339/+18) alone. Relative to the wild type promoter this is more than 50% of the total activity.

Construct D10, containing a truncated copy of module 2, shows levels of expression similar to those seen with D3. This suggests that a positive element (B) present in module 2 lies between −736 and −509. Deletion of module 2 from the wild-type promoter has no significant influence on the activity as seen in D1. On the other hand, D1 can be considered as a fusion of module 4 (−1512/−951) to D2 (−930/−736 + −339/+18). The fusion leads to a further increase in GUS activity by a factor of 4, from 27% to 112%. Module 4 should thus contain a third independent positive element (C).



	GUS activity (pmol/µg/h)		
	tuber	leaf	stem
35S-D3 (3)	96	380	935
35S-D6 (4)	103	1897	1721
35S-D5 (9)	55	606	1022
35S-GUS(7)	531	550	nd

Fig. 5. Structure and expression of deletion constructs D3, D6 and D5 fused to the 35S enhancer (−373/−32). In the right panel the GUS activities (pmol 4-MU released/µg protein per h) in different organs of the transgenic plants are displayed. Numbers of inpendent transformants analysed are given in *brackets*

Modules 2 and 4 are unable to induce expression of a truncated heterologous promoter

Fusion of modules 2 (−736/−339) and 4 (−1512/−951) to position −72 of the truncated 35S promoter in constructs D8 and D7, respectively, results in no detectable GUS activity above background. The positive elements (B) and (C) are thus unable to confer tuber-specificity on the basic 35S promoter. Furthermore, they are not able to increase expression to detectable levels.

Fusion of the 35S enhancer to the truncated patatin promoter leads to constitutive expression in all tissues

In order to study whether the lack of detectable activity in D5 (−103/+18) is due to the loss of a tuber-specific element or to the loss of some other positive element, D5 (−103/+18), D6 (−228/+18) and D3 (−339/+18) were fused to the 35S enhancer fragment (−373/−34). In all three cases strong activity was found in leaves, stems and tubers. The GUS activity of the fusion promoters was in the same range as that associated with the wild-type 35S promoter (Fig. 5).

A sucrose-responsive element is located within 228 bp of the patatin promoter

In order to identify the elements responsible for tuber-specific expression and sucrose induction in leaves, leaf discs of transformed plants were floated on medium containing 7% sucrose (Fig. 6). All deletion constructs down to position −228 retained inducibility, though the level of expression was only five times above background. If the tenfold drop of activity in tubers in D4 (−195/+18) relative to D6 (−228/+18) is reflected in leaf expression, activity should drop below the back-

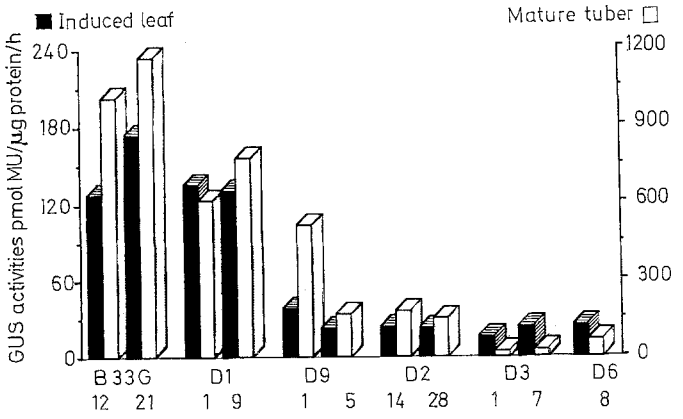


Fig. 6. Comparison of the GUS expression level of different deletion constructs in mature tubers and leaves after sucrose treatment. *Black bars* represent GUS activity in induced leaves corresponding to the left scale, whereas *white bars* represent GUS activity in mature tubers. Numbers of individual transformants are indicated on the X-axis

ground level and thus become undetectable. Tuber-specific expression and sucrose induction could not, therefore, be separated in this set of experiments.

Discussion

Previous work has demonstrated that a 1.5 kb promoter fragment of the class I patatin gene B33 is sufficient to direct high levels of tuber-specific GUS expression in transgenic potato plants upon fusion to the GUS reporter gene (Rocha-Sosa et al. 1989). Furthermore, the chimeric gene can be induced in leaves if plantlets are kept on media containing high levels of sucrose. These results suggest that the gene is under both developmental and metabolic control. In order to gain a better understanding of the regulation of the gene, the effects of different deletions within the promoter region were analzyed in transgenic potato plants.

The results described here suggest that sequences directing tuber-specific expression are located downstream from position −195. One copy of an AT rich motif present three times in the promoter is localized between −183 and −143. AT rich elements have been described as positive elements necessary for constitutive expression in yeast promoters (Struhl 1985). In plants, nuclear factors recognizing specific AT rich sequences have been described which bind to the promoter regions of several genes (Jofuku et al. 1987; Jensen et al. 1988; Bustos et al. 1989). AT rich elements have also been reported to act as activators in the leghemoglobin (Jensen et al. 1988), β-phaseolin (Bustos et al. 1989) and ribulose-1,5-bisphosphate carboxylase small subunit (Datta and Cashmore 1989) genes. Similar sequences are present in negative regulatory elements, as in the case of the phytohemagglutinin gene (Riggs et al. 1989) and the *cab-E* gene (Castresana et al. 1988). By contrast, in the case of th β-conglycinin gene this element has been shown not to have any significant influence on promoter activity (Allen et al. 1989). The AT rich elements found upstream of the β-phaseolin gene and of a class II patatin gene confer strong expression in transgenic tobacco when fused to the minimal 35S promoter (Bustos et al. 1989; S. Prat unpublished observation).

As reported for other AT rich elements, we show that a *Dde*I fragment extending from −245 to −105, including a copy of the AT rich motif, binds nuclear factors with different electrophoretic mobility in leaf and tuber extracts. This could reflect either proteolytic degradation in the leaf extract, cell-specific modification of the DNA binding protein, or the presence of distinct polypeptides in the two tissues. In chimeric promoters where the AT rich sequence is fused to a truncated −90 Δ35S promoter, no alteration in level or specificity of expression is detectable. This argues against a function of the AT rich sequence as a tuber-specific element, but suggests that sequences downstream of position −143 play a role in the organ specificity of the patatin B33 promoter. We cannot exclude the possibility that an interaction between the AT rich motif and other elements is necessary. In animal cells, AT rich sequences have been reported to bind the high-mobility-group (HMG) class of nuclear proteins (Solomon et al. 1986). Similarly, HMG-1-like proteins have been shown to interact with different AT motifs in soybean nodulin promoters (Jacobsen et al. 1990). Like HMG-related proteins, the AT rich motif binding factors detected here could have a general role in the regulation of transcription. These elements, in close proximity to the actual tissue specific element, might contribute to the global activity of the gene.

No tuber-specific activity was found for the fusions of module 2 (−736/−339), containing another copy of the AT rich motif, to the truncated 35S promoter. This further supports the idea that sequences downstream form position −143 govern promoter specificity. We could not detect binding of nuclear factors to this region in gel retardation assays (data not shown) though we cannot exclude the possibility that this failure was due to the in vitro conditions which we used.

The 35S enhancer has been used in several systems to define tissue specific elements in heterologous promoters (Stockhaus et al. 1989; Stougaard et al. 1987). In the case of patatin promoter fusions, the 35S enhancer confers constitutive expression in all tissues when fused to truncated patatin promoters D3 (−339/+18), D6 (−228/+18) and D5 (−103/−18). GUS activity in tubers is even reduced relative to the wild-type 35S promoter. Similar effects of the 35S enhancer on heterologous truncated promoters have been found for the proteinase inhibitor II gene (Keil et al. 1989) and the *rbcS-3A* gene (Kuhlemeier et al. 1989). Further experiments will be necessary in order to identify the tuber and sucrose responsive elements unequivocally.

Three different positive regulatory elements were identified, each one leading to a four to tenfold induction, although their combined influence is not simply additive. Deletion of element B has no deleterious effects on GUS expression in D1, though it can confer a tenfold increase in expression is fused to D3 (−339/+18). Duplication of element A seems to have a synergistic effect

on expression. The B33 patatin gene differs from other class I genes like PS20 (Wenzler et al. 1989) and B24 (W. Frommer, unpublished results) mainly by the presence of the 208 bp repeat. The fivefold higher expression of the B33 gene as compared to the B24 gene (W. Frommer, unpublished results) might thus reflect the presence of two type B elements. However, the positive elements identified in the B33 promoter seem to be different from general enhancers, as none of them is able to *cis*-activate truncated 35S promoters.

Apart from their tuber specificity, Class I patatin genes can be induced in potato leaves by metabolic signals (Rocha-Sosa et al. 1989; Wenzler et al. 1989). It would be interesting to see whether tuber specificity and inducibility by metabolic signals are conferred by the same *cis*-acting elements. The results obtained from the analysis of sucrose induction with the deletion constructs show that a sucrose-responsive element must be located within 228 bp of the patatin promoter and should therefore be positioned in the same region as the tuber element. Due to the low level of GUS expression in sucrose-induced leaves compared to tubers we were, however, unable to pinpoint this element within a smaller fragment. No differences in expression were found for the 35S enhancer/patatin fusion promoters under low and high sucrose conditions.

The three positive elements influencing expression in tubers also seem to be active in sucrose induction. The patatin promoter therefore does not contain a single strong enhancer element but rather possesses several positive elements that are unable to activate a heterologous truncated promoter.

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