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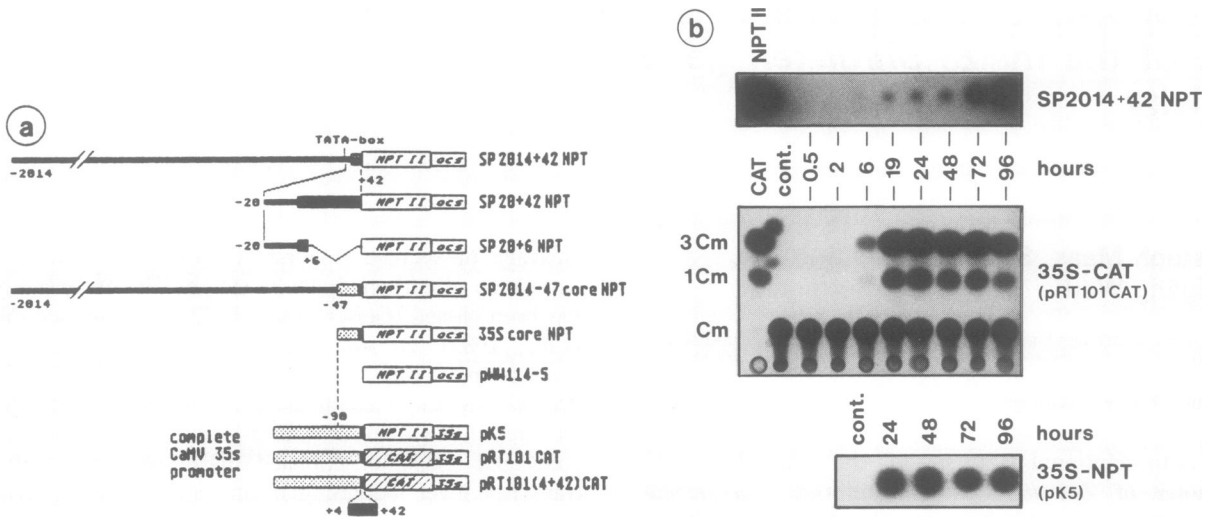

Fig. 1. (**a**) Schematic representation of chimeric gene constructions transfected into maize suspension protoplasts. The numbering of the sucrose synthase upstream fragments indicates the distance of the terminal nucleotides from the major transcription start site (+1) used in the maize endosperm (Werr *et al.*, 1985). The CaMV 35S core promoter fragment extends to the *Eco*RV restriction site at position −90. (**b**) Activity of the *Sh* promoter in comparison with the CaMV 35S promoter. Protoplasts of one large scale transfection were cultivated, isolated at different time points after transfection and monitored for expression of the chimeric SP2014+42 NPT and cotransfected 35S−CAT (pRT101CAT, Pröls *et al.*, 1988). The NPTII activity profile observed with the chimeric 35S−NPT gene (pK5) in a different transfection experiment is shown in comparison below. NPTII: protein extract from a tobacco plant stably transformed with a chimeric NPTII gene, CAT: chloramphenicol transacetylase enzyme Cm: chloramphenicol, 1Cm and 3Cm: acetylated reaction products.

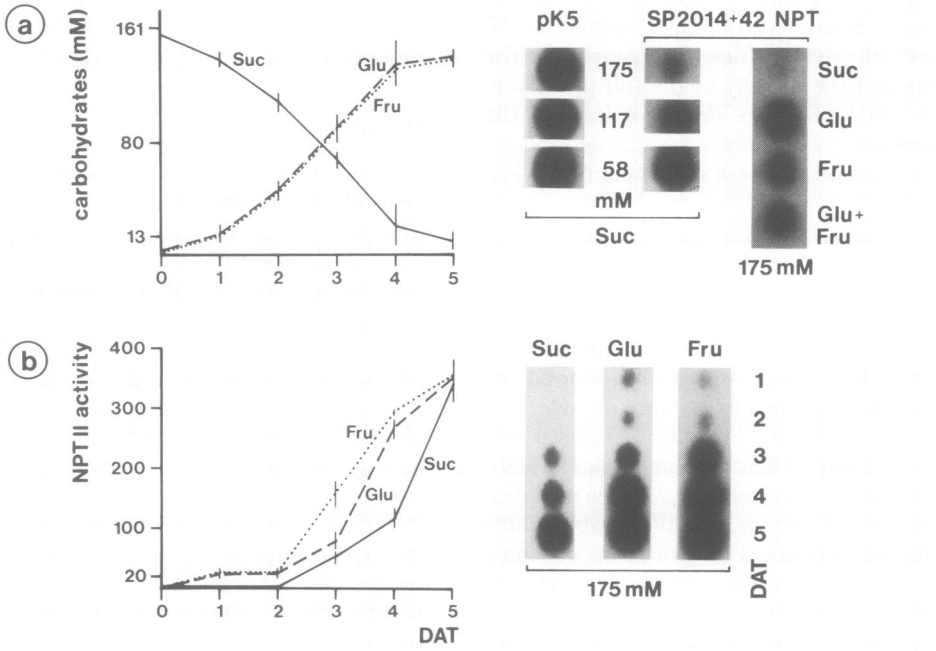

Fig. 2. (**a**) The *Sh* promoter is inhibited by high extracellular sucrose concentrations. The diagram indicates sucrose hydrolysis and accumulation of glucose and fructose in the protoplast medium. The higher differences observed between 2 and 4 days are due to faster hydrolysis of sucrose in different experimental series. Beside the diagram the NPTII activity is shown observed 2 days after transfection of protoplasts with either SP2014+42 NPT or pK5 (35S promoter) and cultivated in media with decreasing sucrose concentrations. Expression of the CaMV 35S promoter (pK5) was constant, while the activity of the *Sh* promoter increased at lower sucrose concentrations. The inhibition of the *Sh* promoter was not observed at 175 mM concentrations of glucose, fructose or glucose/fructose (each 175 mM). (**b**) Transient expression of SP2014+42 NPT in protoplasts cultivated on different carbohydrate sources. One large scale transfection experiment was split into three aliquots which were plated on media containing 175 mM sucrose, glucose or fructose and cultured for the time indicated beside the autoradiographs. Note that sucrose is hydrolysed during the cultivation period (see Figure 2a). The NPTII activity values in the diagram have been normalized on the level determined in sucrose-containing medium at day 1 after transfection. DAT: days after transfection.

The Sh promoter is inhibited by high extracellular sucrose levels

Analysis of the carbohydrate composition in the medium during protoplast cultivation indicated that the sucrose supplied as a carbohydrate source was completely hydrolysed within the 5 day cultivation period. As depicted in Figure 2a glucose and fructose accumulated to equimolar concentrations. Therefore transient expression of SP2014+42 NPT was compared after 2 days cultivation in medium supplemented with sucrose, glucose or fructose (each 175

Table I. Summary of transient expression experiments with various promoter NPTII constructions.

	5 days / 2 days	Glu + Fru / Suc
TATA-box SP2014+42 NPT (-2014, +42)	340 - 405	16 - 25
SP20+42 NPT (-20, +42)	240 - 320	8 - 20
SP20+6 NPT (-20, +6)	10 - 26	7 - 12
pRT101-5	1.1 - 1.7	1.0 - 1.3
SP2014-47 core NPT (-2014, -47)	15 - 30	6 - 7.5
35S core NPT	1.1 - 1.7	1.0 - 1.5

Transient expression of *Sh* promoter deletions was compared after cultivation in glucose or sucrose containing medium and at day 2 or 5 after transfection. The numbers are calculated from different experiments and give relative values normalized on the NPTII activity observed after 2 days cultivation on 175 mM sucrose media (=1).

mM, Figure 2a). Transcription from the *Sh* promoter as estimated by comparison of NPTII activities was ~20-fold higher in protoplasts cultivated in medium supplemented with monosaccharides rather than with sucrose. The transcriptional activities of the CaMV 35S (pK5) or *nos* promoters (pLGV1103) were unaffected by changes in the extracellular carbohydrate source (data not shown, see also 35S core NPT in Table I). Repression of *Sh* promoter could be released by lowering the molarity of sucrose in the cultivation medium from 175 mM to 57 mM which did not affect transcriptional activity of the CaMV 35S promoter (pK5 in Figure 2a).

However, the hydrolysis of sucrose in the cultivation medium cannot completely explain the activity profile of the *Sh* promoter. As shown in Figure 2b, activity profiles similar to those obtained using sucrose medium were observed if protoplasts transfected with SP2014+42 NPT were cultivated on media supplemented with either glucose or fructose (each 175 mM). A significant change in the concentration of both extracellular monosaccharides was not measurable during the cultivation period (data not shown, see also accumulation of monosaccharides in Figure 2a). Therefore the delayed increase in *Sh* promoter activity is not explained by changes in the extracellular carbohydrate concentrations.

The increasing transcriptional activity is linked to cell wall biosynthesis

Microscopic analysis indicated that during the 5 day cultivation period protoplasts started regeneration of cell walls (see −DCB fluorescence photograph in Figure 3). The substrate for the synthesis of most cell wall polysaccharides including cellulose, is UDP-glucose, one of the products resulting from the cleavage of sucrose by the enzyme sucrose synthase, the product of the *Sh* gene. Therefore it was tempting to test whether inhibition of cell wall formation affects the activity of the *Sh* promoter as assayed by transient expression experiments. After one large scale transfection experiment with SP20+42 NPT, protoplasts were subdivided into two aliquots and cultivated for 5 days either in normal sucrose-containing media or in media supplemented with 2,6-dichlorobenzonitrile (DCB), a specific inhibitor of cellulose biosynthesis (Meyer and Herth, 1978). As shown in Figure 3 cell wall regeneration (+DCB fluorescence photograph in Figure 3) was inhibited by the herbicide and the late increase in *Sh* promoter activity was blocked. The control protoplasts cultivated in the absence of DCB showed normal cell wall regeneration (−DCB photograph in Figure 3) and increasing NPTII activity. The transient expression of the cotransfected pRT101CAT plasmid demonstrates that the transcription of the CaMV 35S promoter was not affected by the DCB treatment and that protoplasts were viable over the 5 day cultivation period in the presence of the cellulose biosynthesis inhibitor.

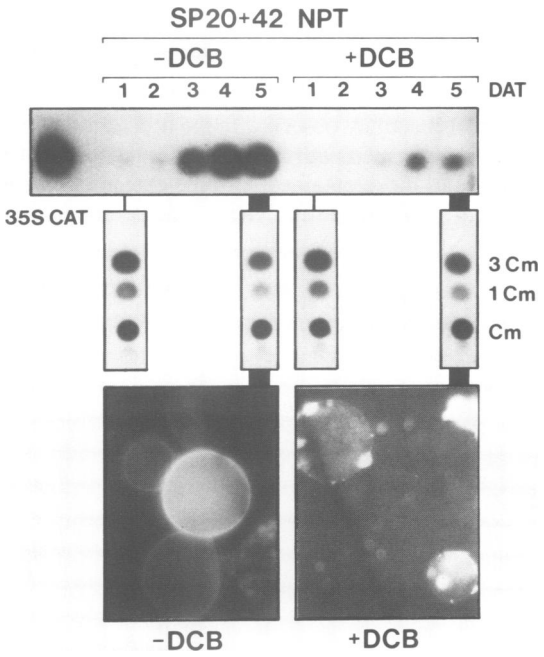

Fig. 3. Inhibition of cellulose biosynthesis blocks *Sh* promoter activity. Maize suspension protoplasts were cotransfected with SP20+42 NPT and pRT101CAT, divided in two aliquots and cultivated on sucrose medium (175 mM) for 5 days in the presence or absence of dichlorobenzonitrile (DCB), a specific inhibitor of cellulose biosynthesis. The delayed increase in NPTII activity of the *Sh* promoter is blocked by the herbicide. The activity of the cotransfected 35S CAT gene (pRT101CAT) indicates viability of the protoplasts in the presence of DCB up to 5 days after transfection (CAT: chloramphenicol transacetylase enzyme, Cm: chloramphenicol, 1Cm, 3Cm acetylated reaction products). The difference in cell wall formation dependent on the presence (+DCB) or absence (−DCB) of the herbicide is demonstrated by fluorescence staining of protoplasts 5 days after transfection (DAT). The entire surface is fluorescing in absence of DCB, while only fluorescing spots are detectable in presence of the cellulose biosynthesis inhibitor at higher exposure times. The chimeric SP20+42 NPT gene has been used in this experiment because it was performed subsequently to the deletional analysis of the sucrose synthase promoter.

Sequences near the transcription start are sufficient to mediate sugar type dependent and delayed promoter activity

An unexpected result was obtained during analysis of 5′ terminal promoter deletions. The longest deletion (SP20+42 NPT), removing the TATA-box of the *Sh* promoter, was still inhibited by high sucrose levels and its transcription increased

between 2 and 5 days after transfection (see Table I and Figure 4a). For this deletion, the maize sequences fused in front of the NPTII marker gene consist of only 20 bp of the *Sh* upstream region and 42 bp of untranslated exon 1. The final NPTII activity observed in the transient expression experiments performed with this promoter deletion reached similar levels to those obtained with the extended upstream regions, e.g. SP2014+42 NPT. The comparison of SP20+42 NPT to the promoterless NPTII cassette (pWW114-5 in Figure 4a) demonstrates that transcriptional initiation has to be mediated by the 62 bp *Sh* sequence element and not by sequences on the bacterial vector.

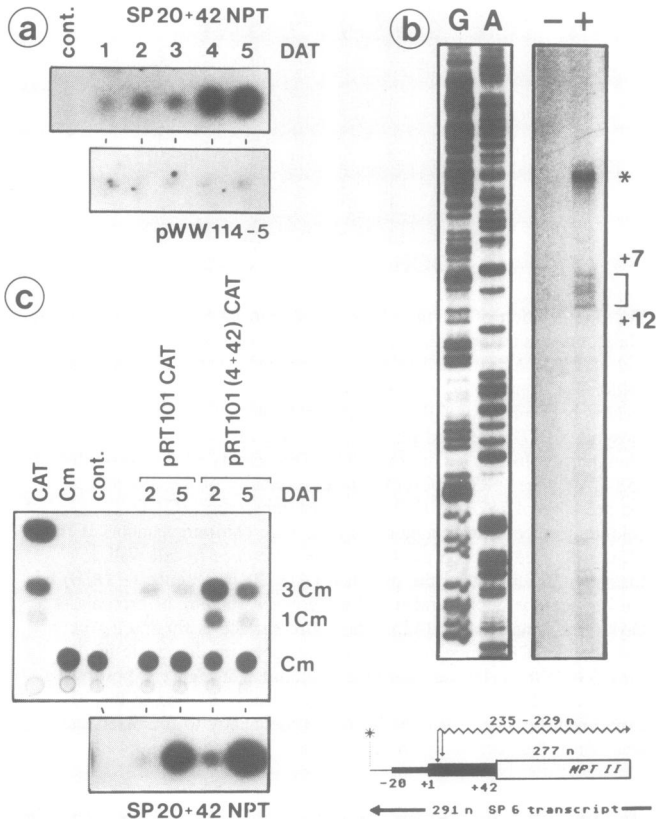

Fig. 4. (**a**) Time dependent NPTII activity profile of SP20+42 NPT in comparison with the promoterless pWW114-5 NPTII cassette. (**b**) RNA protection experiment performed with total cellular RNA isolated from protoplasts transfected with the SP20+42 NPT plasmid (+) or untransfected control protoplasts (−). The structure and the direction of the SP6 antisense transcript is shown below the autoradiograph, the length of the protected bands has been determined according to a DNA sequence (G, A lanes shown) run in parallel. The bracket on the margin indicates the initiation sites between position +7 and +12 relative to the main transcription start site used in the endosperm. The band marked (*) extends to the 3′ end of the SP6 transcript and can be explained by unterminated readthrough transcripts or SP20+42 NPT plasmid copies in the RNA preparation. (**c**) The exon 1 sequences in the *Sh* promoter NPTII fusions do not mediate a post-transcriptional response. The exon 1 +4 to +42 sequences were cloned into the transcription unit of pRT101CAT as indicated in Figure 1a. In the upper autoradiograph the CAT activities of pRT101CAT and pRT101(+4+42)CAT are compared. CAT activity decreased whether or not the maize leader sequences were present in the transcription unit. Below the corresponding CAT lanes, expression of cotransfected SP20+42 NPT shows the delayed increase in activity. Note that the insertion of the +4 to +42 sequences in the untranslated leader sequence positively affected expression of the CAT gene. Cm: chloramphenicol and acetylation products (1Cm, 3Cm), cont: protein extracts from protoplasts treated in absence of DNA.

This has been confirmed by RNase protection experiments (Figure 4b) which indicate transcripts initiating between position +7 and +12 relative to the main start site (+1) used in the endosperm (Werr *et al.*, 1985). The prominent longer band in the protection experiment coincides with the point of divergence between the SP6 transcript and the chimeric SP20+42 NPT construction. It may be explained, by some residual plasmid copies in the total RNA preparation, transcripts initiating in the pUC vector sequences or unterminated readthrough transcripts. As the very low background activity observed from the promoterless pWW114-5 NPTII cassette argues against a significant translation of the NPTII coding region from such invading transcripts, we have not further analysed the nature of this band.

A post-transcriptional control mechanism mediated by the *Sh* 42 bp untranslated leader sequence of exon 1 present in the *Sh* promoter NPTII fusions seems unlikely for several reasons. Firstly, the insertion of the exon 1 sequences (+4 to +42) at a similar position in front of the CAT coding region of pRT101CAT did not change the time profile of CAT expression (Figure 4c). CAT activity was high 2 days after transfection and decreased by day 5 while expression of the NPTII enzyme from a cotransfected SP20+42 NPT construct increased between day 2 and day 5. Secondly, most exon sequences are deleted from the chimeric SP20+6 NPT gene. The NPTII activity observed with SP20+6 NPT was considerably lower than obtained with SP20+42 NPT, however, transcription was still inhibited by sucrose and increased late after transfection (Table I).

A third argument against a control mediated by the *Sh* exon 1 is implied by the following promoter fusion experiments. In the SP2014-47core NPT chimeric gene the *Shrunken* transcription start including the exon sequences is replaced by the CaMV 35S core promoter (SP2014-47core NPT see Figure 1a). Transient expression of SP2014-47core NPT was 6- to 7.5-fold higher in the presence of glucose than in the presence of sucrose and increased up to 30-fold when NPTII activity was analysed after 5 days of cultivation. Expression of the core promoter (35S core NPT in Figure 1a) was unaffected by the carbohydrate source and cultivation period. From the promoter fusion experiments the presence of at least one additional control element of similar function to that near the transcription start site has to be postulated upstream of −47 in the *Sh* promoter. The result also indicates that this type of transcriptional control can be transferred to other normally unaffected plant/viral promoters.

Discussion

Is the sucrose synthase 1 promoter feedback controlled?

The activity of the *Sh* promoter in maize protoplasts as monitored by transient gene expression was repressed by high extracellular sucrose concentrations but not by comparable concentrations of glucose or fructose in the protoplast medium. In addition, *Sh* promoter activity increased sharply when the transfected protoplasts started resynthesis of cell walls after 2−3 days of cultivation. Through the analysis of promoter deletions, a 26 bp region (SP 20+6) has been identified which responds negatively to high sucrose concentrations and positively to cell wall

regeneration. Within this 26 bp sequence covering the transcription start site of the maize *Sh* gene either two sequence elements, one responding to sucrose and one to cell wall synthesis, must be closely linked or both responses are mediated via the same signal transduction chain.

Such a chain could start with UDP-glucose, a reaction product from the cleavage of sucrose by the enzyme sucrose synthase, encoded by the *Shrunken* gene. Perhaps the *Sh* promoter is repressed at high intracellular UDP-glucose concentrations and active at low UDP-glucose levels. If DCB inhibits the transfer of glycosyl residues from UDP-glucose to the growing cellulose chains UDP-glucose accumulates and the activity of the *Sh* promoter stays low. The UDP-glucose level would also be affected at high extracellular sucrose concentrations if the disaccharide enters the protoplasts and is cleaved intracellularily by the sucrose synthase enzyme. Transcription and translation of the endogenous *Sh* gene has been verified in Northern and Western blot experiments (data not shown). Therefore both responses could be mediated via the UDP-glucose level and the signal involved could act at a single regulatory element.

If transcription of the *Sh* promoter is dependent on low intracellular UDP-glucose levels the maize endosperm cells where the *Sh* gene is highly transcribed (Heinlein and Starlinger, 1989; Chen and Chourey, 1989) may be like protoplasts regenerating cell walls. Except, that in the endosperm cells UDP-glucose is a possible substrate for the synthesis of starch (Tsai, 1974) rather than the biosynthesis of cellulose. The low UDP-glucose level at the high rates of starch synthesis in the endosperm cells therefore would induce *Sh* promoter activity. The translation of the *Sh* mRNA would consequently result in an increased level of the sucrose synthase enzyme supplying more UDP-glucose by the cleavage of sucrose.

A similar feedback transcriptional control has been described for the yeast *SUC2* gene which encodes a secreted form of the invertase enzyme catalysing sucrose hydrolysis. Transcription of the *SUC2* gene is repressed by glucose, a product of enzymatic sucrose hydrolysis (Sarokin and Carlson, 1984). The experimental data in yeast provides evidence that regulation of the *SUC2* gene involves signal transduction with cyclic AMP as second messenger. Cyclic AMP was initially shown to mediate *Escherichia coli* catabolite repression (review Magasanik and Neidhardt, 1987) and subsequently to be involved in signal transmission of a variety of eukaryotic genes (review: Roesler *et al.*, 1988). Flanking the transcription start of the *Sh* gene are six repetitive TCCC motifs (see Figure 5) exhibiting sequence homologies to AP2 binding sites mediating the cAMP response in the rat prolactin (CCCCTCCC, Cooke and Baxter, 1982) or tyrosine aminotransferase gene (TCCCTCCC, Becker *et al.*, 1987). However, until now cAMP has not been shown to be functional at the transcriptional level in plants. These fascinating problems of signal transduction mechanisms in plants warrant further investigations.

The Sh transcription start is an autonomous control element

Apart from temporal regulation, the SP20+42 *Sh* element initiates transcription efficiently in the absence of further upstream sequences. The lymphocyte specific *initiator* element at the transcription start of the terminal deoxynucleotidyltransferase gene is also sufficient for transcriptional initiation and control (Smale and Baltimore, 1989), while additional upstream elements are required for high transcriptional activity of the *initiator*. In contrast, high activity of the *Sh* transcription start does not appear to be dependent on upstream elements since sequences extending up to position −2014 did not significantly alter or increase the expression of the NPTII marker gene. This may be due, in part, to the redundancy of regulatory elements, as indicated by promoter fusion experiments (see SP2014-47 NPT in Table I). *Sh* promoter specific regulation was conferred on the unresponsive CaMV 35S core promoter by fusion with the SP2014-47 sequences. Therefore at least one additional control element exhibiting a similar function to that of the transcription start region must be localized upstream of position −47. As other small *Sh* promoter elements or individual binding sites for nuclear proteins (Springer *et al.*, 1990) enhance transcriptional activity if inserted in front of the CaMV core promoter (C.Maas and W.Werr, unpublished), the type of regulation exhibited near the transcription start of the *Sh* promoter seems to be dominant over the binding of positively acting transcription factors.

We have not directly compared the accuracy of transcription initiation from sites used in SP20+42 NPT to the CAP sites used when additional upstream sequences are present, because our main interest is to identify the target site for signal transduction. However, internal initiation of the SP20+42 *Sh* promoter element was detectable in RNase protection experiments as expected (Figure 4b). The CAP sites are scattered between position +7 and +12 in comparison with the main transcription start site used in maize endosperm (Werr *et al.*, 1985). The shifted cap sites may be explained by the absence of the natural TATA-box (position −32 to −24) which has been shown to be involved in positioning of the initiation complex in animal promoters (for example: Schatt *et al.*, 1990). A three nucleotide displacement of *Sh* CAP sites was also detected in maize roots after anaerobic induction of the *Sh* gene (Ortiz *et al.*, 1988) which indicates that initiation may vary *in vivo*.

As a first attempt to dissect the functions inside of the SP20+42 element, we removed most of the exon 1 sequences. Transient expression experiments performed with SP20+6 NPT demonstrated that the 26 bp *Sh* element reacts in a similar way to the SP20+42 element, negatively to high sucrose levels or positively to cell wall regeneration. The total NPTII activity, however, was considerably reduced in comparison with SP20+42 NPT. A positive effect on gene expression due to the presence of *Sh* exon 1 sequences was also observed with pRT101(+4+42)CAT (see Figure 4c) in which the exon was inserted at a similar position in front of the CAT coding region. Presently we cannot distinguish whether the exon sequences act at the transcriptional or post-

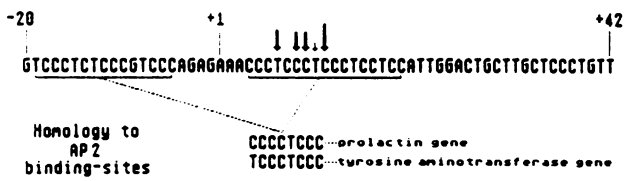

Fig. 5. Sequence of the sucrose synthase 1 transcription start (−20 to +42). The position of the initiation sites in SP 20+42 (see Figure 4b) are indicated by arrows. Sequence homologies to AP2 recognition sequences are marked.

transcriptional level. More important is the finding that the *Sh* untranslated leader sequences in front of the CAT coding region do not alter the transcription profile of the CaMV 35S promoter in the maize protoplasts. Therefore we feel quite sure that the 26 bp region surrounding the *Sh* transcription start (SP20+6) contains the transcriptional control element. In future experiments saturation mutagenesis of this 26 bp region may allow identification of the protein binding site and subsequently the signal transducing nuclear protein. This will hopefully be a first step to understanding one of the signal transducing chains controlling the transcription of genes involved in carbohydrate metabolism.

Materials and methods

Construction of chimeric genes

All sucrose synthase promoter chimeric gene constructions were cloned in the same orientation into the polylinker region of pUC9 or 19. Cloning of the SP2014+42 NPT and the vectorless pWW114-5 NPTII cassette has been described in Werr and Lörz (1986). The small SP20+42 or SP20+6 promoter fragments have been inserted into the *Sma*I site of pWW114-5. The 35S core NPT gene was constructed by replacing an *Eco*RI–*Sph*I fragment from pWW114-5 by a fragment from the vector pPCV821-neo kindly provided by C.Koncz (Max-Planck-Institut für Züchtungsforschung, Köln). Fusion of the SP 2014-47 upstream sequences in front of the core promoter was achieved by using the compatibility of a *Sty*I restriction site (position −47) in the maize sequences and a *Xba*I site in front of the CaMV core promoter. The plasmid pRT101(+4+42)CAT was constructed by inserting a synthetic oligonucleotide into the *Sma*I restriction site in the untranslated leader sequence of pRT101CAT (Pröls *et al.*, 1988).

Transient expression analysis

Protoplasts were isolated from a maize suspension cell line, transfected with a 0.1 M $MgCl_2$, 0.45 M mannitol, 20 mM HEPES, 25% PEG solution and cultivated according to Maas and Werr (1989). To normalize individual transfection experiments 50 μg sucrose synthase promoter plasmids were mixed with 50 μg pRT101CAT and 100 μg of sonicated calf-thymus DNA and incubated with 2×10^6 protoplasts in 300 μl. Each transfection experiment was carried out in triplicate, protoplasts were incubated in Petri dishes at 28°C in the medium and for the time indicated in the figures. The three corresponding dishes were combined, protoplasts pelleted by centrifugation, divided into two aliquots and cells lysed by sonication prior to CAT and NPTII activity assays.

The estimation of CAT activity and protein concentration followed the protocol described in Maas and Werr (1989), both values were used to normalize differences in transfection efficiency. NPTII activity was assayed according to Reiss *et al.* (1984), protease activity was blocked by the addition of BSA (50 μg) and PMSF (1 mM) to the extracts. Autoradiographs were either scanned densitometrically or the radioactive spots were cut out and analysed in a scintillation counter.

RNA preparation and RNase protection analysis

Total RNA was prepared from protoplasts essentially as described by Logemann *et al.* (1987). For preparation of the antisense SP6 transcript an *Eco*RI–*Pvu*II fragment from the SP20+42 NPT chimeric gene was cloned into the pSP 64 vector (*Sma*I–*Eco*I, Melton *et al.*, 1984). This fragment contains the 20 bp upstream region plus pUC polylinker sequences and the 5′ end of the bacterial NPTII coding sequence. SP6 transcripts were prepared by using a SP6 transcription kit (Amersham, Code RPN 2006). Prior to the preparation of total RNA, protoplasts were incubated with DNase I according to Werr and Lörz (1986) to remove extracellular plasmid copies. After RNA isolation 20 μg of total RNA was treated with DNase I (RNase free; Boehringer Mannheim No. 776785) and precipitated in the presence of [^{32}P]UTP-labelled RNA probe ($1-2 \times 10^5$ c.p.m.). RNase protection analysis was performed essentially as described by Zinn *et al.* (1983). Hybridisation was performed at 45°C. Protected RNA fragments were analysed on denaturing polyacrylamide gels.

Carbohydrate determination and analysis of cell wall regeneration

Extracellular carbohydrate concentrations were determined by test kits available from Boehringer Mannheim GmbH (No. 139106). Cell wall regeneration was confirmed by fluorescence analysis with Tinopal CBS-X [CBS: disodium 4,4′-bis(2-sulfostyryl)biphenyl; Ciba-Geigy Corp.] as described by Huang *et al.* (1986). Cellulose biosynthesis was inhibited by adding the herbicide DCB (2,6-dichlorobenzonitrile, Aldrich Chem., D5,755-8) to the media N6-B 500 at 2 mg/l according to Meyer and Herth (1978).

Acknowledgements

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