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Maize *Adh-1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco

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The promoter region of a maize alcohol dehydrogenase gene (*Adh-1*) was linked to a reporter gene encoding chloramphenicol acetyl transferase (CAT) and transformed stably into tobacco cells using T-DNA vectors. No CAT enzyme activity could be detected in transgenic tobacco plants unless upstream promoter elements from the octopine synthase gene or the cauliflower mosaic virus 35S promoter were supplied in addition to the maize promoter region. CAT enzyme activity and transcription of the chimaeric gene were then readily detected after anaerobic induction. The first 247 bp upstream of the translation initiation codon of the maize *Adh-1* gene were sufficient to impose anaerobic regulation on the hybrid gene and S1 nuclease mapping confirmed mRNA initiation is from the normal maize *Adh-1* transcription start point.

Key words: alcohol dehydrogenase/anaerobiosis/maize/plant enhancers/T-DNA transformation

Introduction

When maize seedlings are flooded, there are major changes in the pattern of protein synthesis associated with the decrease in oxygen concentration. Synthesis of proteins present during aerobic conditions is repressed and a set of about twenty polypeptides (the anaerobic polypeptides) that are absent or at a very low concentration during aerobic growth, are preferentially synthesised (Sachs *et al.*, 1980). Several of these proteins, such as alcohol dehydrogenase (ADH), (Hagemen and Flescher, 1960) have now been identified as enzymes involved in anaerobic glycolysis (Laszlo and St. Lawrence, 1983; Kelley and Freeling, 1984a,b). Their synthesis is presumably an adaptive response that allows the plant to survive periods of flooding; this has been demonstrated experimentally in the case of alcohol dehydrogenase in maize seedlings (Schwartz, 1969). These 20 polypeptides comprise more than 70% of the total protein synthesised during anaerobic conditions (Sachs *et al.*, 1980) making the 'anaerobic response' a significant stress response, comparable to the heat-shock response in soybean and *Drosophila* (Schlessinger *et al.*, 1982).

During the first 24 hours of anaerobiosis the steady-state concentration of transcripts for a number of the anaerobic proteins increases (Gerlach *et al.*, 1982) and synthesis of RNA in nuclei isolated from anaerobically induced maize seedlings also increases, showing that gene expression is controlled primarily at the level of transcription for the two alcohol dehydrogenase genes, *Adh-1* and *Adh-2* (Larry Beach, personal communication).

To understand the molecular basis of this transcriptional regulation we have used DNA transformation experiments to determine whether there are promoter elments upstream of the coding region of the *Adh-1* gene of maize that regulate gene expression.

This upstream region of the gene has been linked to a reporter gene encoding the bacterial enzyme chloramphenicol acetyl transferase (CAT) and the hybrid gene has been introduced into *Nicotiana tabacum* (cv. Wisconsin 38) cells by stable transformation using *Agrobacterium tumefaciens* T-DNA vectors. CAT enzyme activity was monitored in these transgenic plants. We found this gene construct to have only extremely low expression in tobacco. However, activity was readily detected when sequences with enhancer-like properties derived form two constitutive genes, octopine synthase (*ocs*) and the 35S gene of cauliflower mosaic virus (CaMV), which are expressed in dicot plants, are placed upstream of the maize promoter region. Under these conditions, the first 247 bp of sequence upstream of the translation initiation codon of the maize *Adh-1* gene confers anaerobic regulation and accurate transcription initiation to the hybrid gene in transgenic tobacco.

Results

The construction of an Adh promoter expression system and transformation of tobacco

The promoter region of the maize *Adh-1* gene (Dennis *et al.*, 1984) containing the TATA box, transcription start site and most of the untranslated leader region was linked to the coding region of the bacterial gene, *cat*, encoding CAT (Bolivar, 1978), through a fusion in the 5′ untranslated region of both genes. Plant transcription termination and polyadenylation signals were provided at the 3′ end by a segment of the nopaline synthase gene (*nos*) (Depicker *et al.*, 1982; Bevan *et al.*, 1983) (Figure 1). The constructs were transformed into the nuclear genome of tobacco by

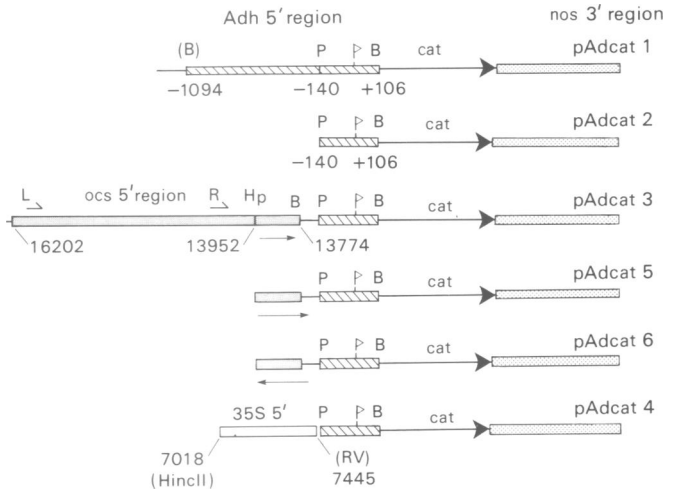

Fig. 1. Restriction maps of hybrid gene constructs. ▷ = transcription start; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; Hp, *Hpa*II; Rv, *Eco*RV; letters in brackets are sites filled in with Klenow fragment of *Pol*I; ocs = octopine synthase; nos = nopaline synthase. Nucleotide coordinates for *ocs* and 35S DNA are from Barker *et al.* (1983) and Franck *et al.* (1980). L and R indicate left and right T-DNA border repeats. The orientation of the *Bam*–*Hpa*II fragment of the ocs upstream region in pAdcat5 and pAdcat6 is indicated by the arrow.

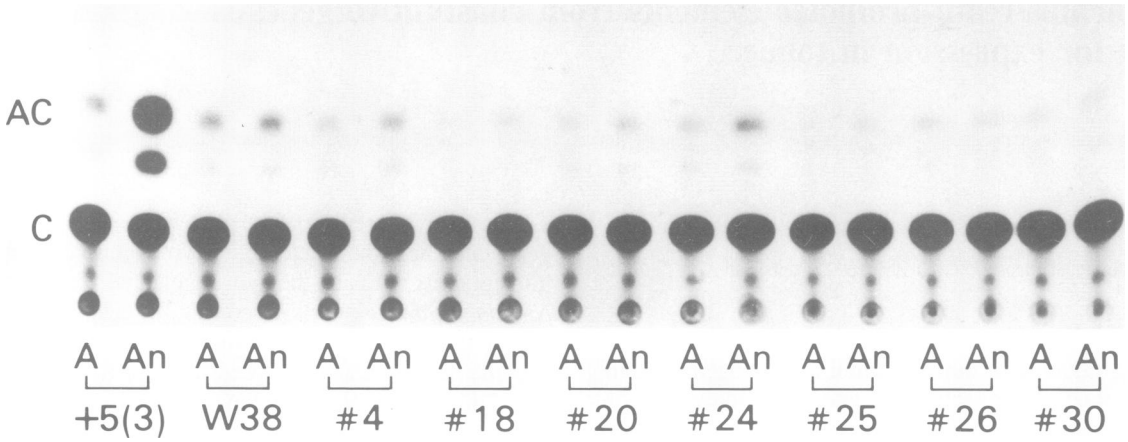

Fig. 2. CAT enzyme assays on leaf extracts of transgenic tobacco plants transformed with the hybrid gene construct pAdcat1 and of one plant [+5(3)] transformed with the construct pAdcat3. C, [^{14}C]chloramphenicol; AC, acetylated derivatives of [^{14}C]chloramphenicol; W38, assay on extracts of untransformed *N. tabaccum* cv. Wisconsin 38. A, reaction products of extracts from leaf tissue grown in air (aerobic). An, reaction products of extracts from leaf tissue incubated under argon for 40 h (anaerobic).

the T-DNA vectors of An *et al.* (1985) using kanamycin resistance as the selectable marker for stable transformation. Promoter activity was then assayed by CAT enzyme activity. Two plasmid constructs containing different lengths of the *Adh-1* promoter region were tested initially. The first (pAdcat1) contained maize DNA from bases −1094 to +106 (the transcription start point of the *Adh1-1S* gene has been determined by primer extension analysis to be 108-bp 5′ of the A residue of the ATG translation initiation codon, E.S.Dennis, unpublished data). The second (pAdcat2) contained a shorter sequence from −140 to +106, and each was linked to the *cat* reporter gene (Figure 1). If either of these two maize upstream segments contained *cis* acting regulatory signals capable of promoting anaerobic gene expression in tobacco then CAT enzyme activity should increase in the transgenic tobacco cells after a period of anaerobic induction. The most convenient tissue to assay for CAT enzyme activity was immature leaf tissue of transgenic plants growing in tissue culture. This tissue was responsive to anaerobic induction; analysis of ADH enzyme activity by starch gel electrophoresis (Schwartz and Endo, 1966) showed that at least one tobacco ADH isozyme, which was absent during aerobic growth, was induced by these anaerobic conditions. We tested 14 plants transformed with the pAdcat1 construct and although all contained the complete hybrid gene as determined by Southern hybridization (data not shown), the level of anaerobically induced CAT enzyme activity was at or near the background level for tobacco. Similar results were found for 29 plants transformed with the pAdcat2 construct (eight confirmed by Southern hybridization to contain the hybrid gene). Figure 2 shows the results of CAT enzyme assays on W38 tobacco with seven transgenic plants containing pAdcat1. In these seven transgenic plants, CAT enzyme activity was close to the background activity observed in tissues of untransformed W38 tobacco. In plants #4 and #24 there was a slightly elevated expression of CAT enzyme activity in the anaerobically induced tissue indicating that the hybrid gene may function at a low level. Further evidence for a low level of gene activity came from examining the transcription of the hybrid gene. Total RNA was isolated from plant #4 after 24 h anaerobic induction and was analysed by S1 mapping. A very low level of *cat* gene expression was detected (Figure 3a). We conclude that the hybrid gene does function in tobacco but the level of expres-

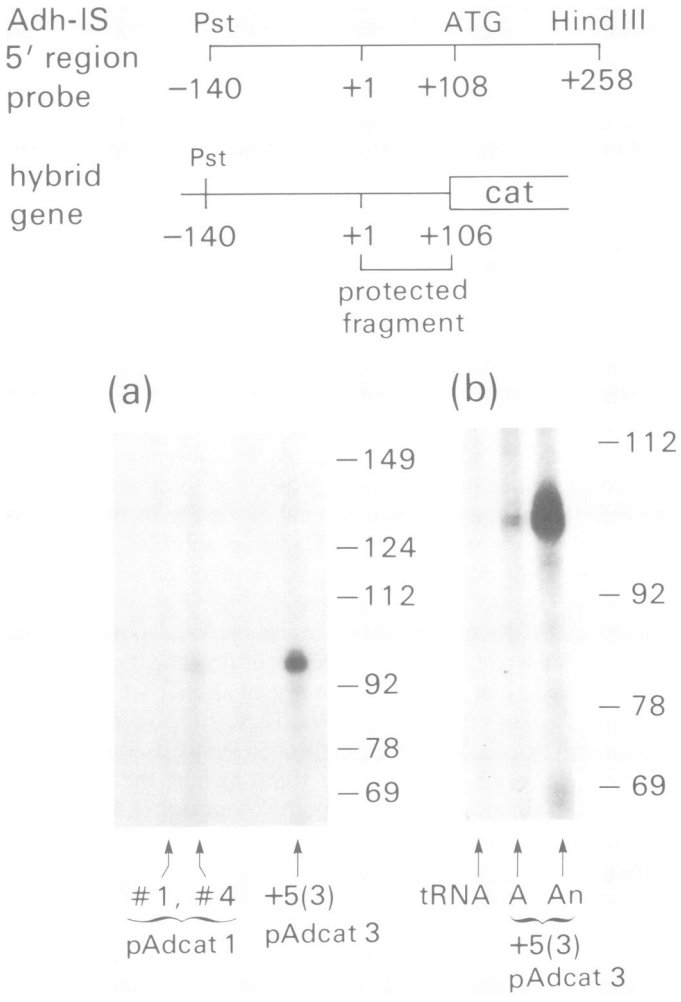

Fig. 3. S1 analysis of total RNA isolated from transgenic plants using a uniformly labelled probe from the *Adh1-1S* region. **(a)** RNA isolated from anaerobically induced transgenic plants. 100 μg of RNA from plants #1 and #4 transformed with pAdcat1 (see Figure 2 for CAT assays) and 25 μg of RNA from plant +5(3) transformed with pAdcat3 were assayed. **(b)** Anaerobic induction of RNA. 25 μg of RNA isolated from aerobic (A) and anaerobic (An) tissue of plant +5(3) were loaded. The control lane was loaded with tRNA.

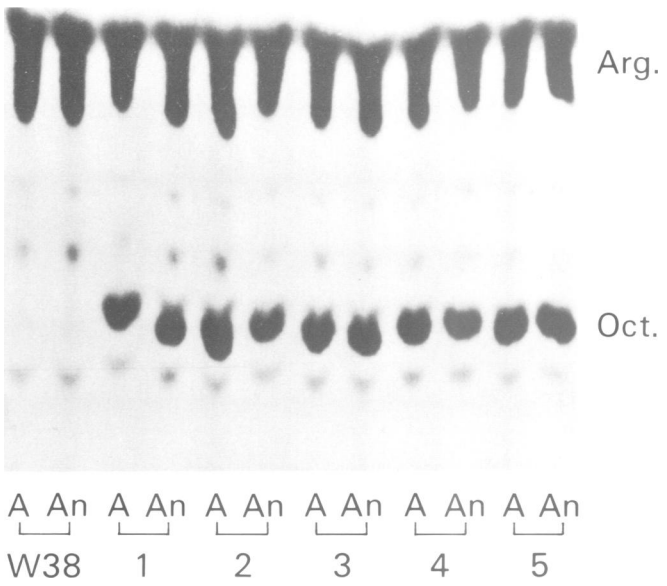

Fig. 4. Octopine synthase assays on five clones of the transgenic tobacco plant RGV-1 (Otten *et al.*, 1981; DeGreve *et al.*, 1982a) A, enzyme activity in aerobic leaf tissue. An, enzyme activity in anaerobic leaf tissue. W38, negative control using W38 tobacco. Oct = octopine, Arg = arginine.

sion is so weak that CAT enzyme activity is often below the level of detection. Similar results have been observed when a hybrid *cat* gene containing the maize *Adh-1* promoter was assayed in carrot tumour cells (M.Fromm, J.Callis and V.Walbot, personal communication).

Activation of the maize Adh1 promoter by an upstream element from the octopine synthase gene promoter

Since the S1 mapping data (Figure 3a) suggested that at least pAdcat1 functioned in tobacco although with very low efficiency, we attempted to increase the level of CAT enzyme expression by adding upstream promoter elements of constitutive plant genes to the *Adh-1* promoter region. The sequences required by plant gene promoters for maximum expression have been investigated in only a few cases. Preliminary studies of two promoters that function constitutively in tobacco cells, the octopine synthase promoter and the 35S promoter of CaMV have identified regions upstream of the TATA box that are important in regulating the level of gene expression (Koncz *et al.*, 1984; Odell *et al.*, 1985). The octopine synthase gene functions in tumours, leaves, roots, stems and seeds of tobacco (Otten *et al.*, 1981) and so it shows no tissue specificity. Moreover it does not respond to anaerobic induction. We have examined the expression of this gene in a transgenic plant, RGV1 (Otten *et al.*, 1981; DeGreve *et al.*, 1982a) and observed no difference in octopine synthase activity between aerobically or anaerobically treated leaf tissue (Figure 4). Therefore this gene was used as a source of a constitutive upstream promoter element. The region of this gene upstream of the *Bam*HI site (−112, de Greve *et al.*, 1982b) has been shown to be absolutely necessary for its expression (Koncz *et al.*, 1984). We tested whether this region would influence the expression of the *Adh-1* promoter when placed in approximately the same position relative to the transcription start site as it occurs in the octopine synthase gene. A 2.4-kb upstream segment of the *ocs* gene was inserted 5′ of the *Pst*I site (−140) of the maize *Adh-1* promoter as outlined in Figure 1. This consruct (pAdcat3) was inserted into the genome of tobacco and transgenic plants were tested for CAT enzyme activity. CAT enzyme activity was induced by anaerobiosis in at least six out of the nine plants tested (Figure 5). There was some variation in the level of CAT enzyme activity between individual transgenic plants. Nevertheless the relative level of CAT activity between the plants remained the same in repeated assays. Either copy number of the inserted gene or position of integration, as suggested by Jones *et al.* (1985), may account for this variation between plants.

One plant, numbers +5(3) was studied in more detail. Measurement of the amount of [^{14}C]chloramphenicol that was acetylated indicated that anaerobiosis induced CAT enzyme activity 10 to 20 fold above background in the apical leaves of this plant and in shoots that developed on leaf pieces placed in tissue culture. S1 mapping (Figure 3b) showed a large increase in the concentration of mRNA transcribed under anaerobic conditions from the hybrid *cat* gene in this transformed plant. The increased transcription paralleled the increase in CAT enzyme activity. Furthermore the protected fragment of ~105 bp is in close agreement with the length expected if transcription of the hybrid gene is initiated at the normal site of the *Adh-1* gene. The effect of the *ocs* gene segment on gene expression can be appreciated by comparing the level of inducible CAT enzyme activity in plant +5(3) and the CAT activity in plants transformed with the construct pAdcat1 (Figure 2) or by comparing the level of *cat* gene transcription detected in plant +5(3) and plant #4 (Figure 3a).

Thus, in the heterologous *Nicotiana* system, expression from the maize *Adh-1* promoter was significantly increased by an upstream sequence from the constitutive *ocs* gene. It then became clear that the first 247 bp of the maize *Adh-1* promoter contain sufficient information both for anaerobic induction and for acurate initiation of transcription. The *ocs* segment itself does not supply any regulatory signal for anaerobic expression. As stated earlier, the *ocs* gene is expressed constitutively in all tissues of tobacco and its expression is not induced by anaerobic conditions. Furthermore, the *ocs* upstream region can be replaced in the promoter fusion construct by a similar upstream region from another constitutive promoter, the 35S promoter of CaMV.

A 309-bp fragment (−395 to −86, Odell *et al.*, 1985) from 5′ of the TATA box region of the 35S promoter was placed upstream and adjacent to the *Pst*I site of the pAdcat2 fusion to give pAdcat4 (Figure 1) and the construct was transferred into the genome of tobacco. Out of 23 transformants tested, 10 were induced by anaerobiosis to synthesise the CAT enzyme while the remainder showed background activity. Examples of several transgenic plants are shown in Figure 6. S1 analysis of RNA from one of these transformants showed that the concentration of mRNA from the chimaeric gene increased markedly during anaerobiosis and was accurately initiated from the maize transcription start site (data not shown). This upstream region of the 35S promoter was shown by Odell *et al.* (1985) to influence the level of expression of the 35S promoter. We have shown that it can also increase the expression from the maize *Adh-1* promoter in transgenic tobacco and that its effect is comparable to the *ocs* promoter upstream region.

Enhancer-like properties of the upstream region of the ocs gene

The behaviour of the upstream sequences of the *ocs* and 35S promoters is reminiscent of enhancers characterised in animal cells and their viruses. We have therefore investigated whether the *ocs* upstream segment has the orientation-independent property displayed by animal gene enhancers.

Previous work (Koncz *et al.*, 1984) demonstrated that the DNA segment further upstream of a *Hpa*II site (−292) in the *ocs* gene promoter region was not required for expression of this gene. Therefore, to study the *ocs* enhancer-like activity we chose the 176 bp fragment extending from the *Hpa*II site (−292) to the

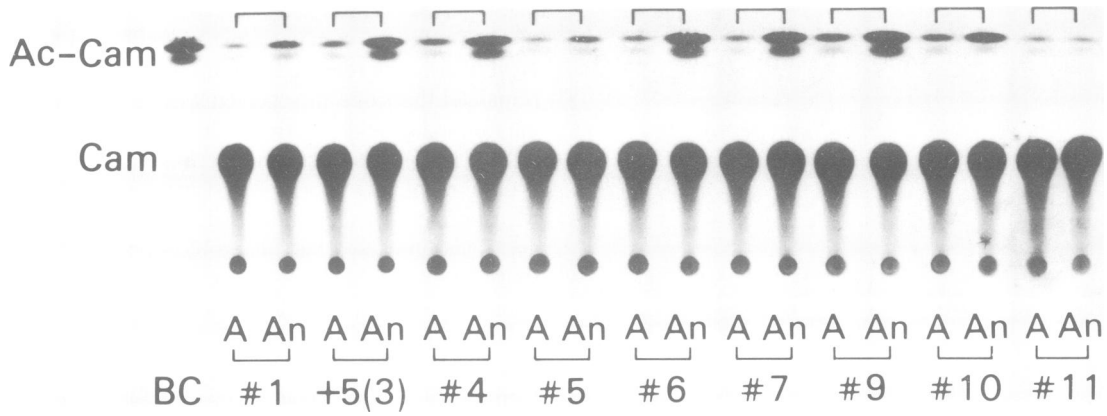

Fig. 5. CAT enzyme assays on leaf extracts of nine transgenic tobacco plants transformed with the hybrid gene construct pAdcat3. CAM, [^{14}C]chloramphenicol; Ac-Cam, acetylated derivative of [^{14}C]chloramphenicol; BC, control reaction using purified bacterial CAT. A, reaction products of extract of leaf tissue grown in air (aerobic). An, reaction products of extract of leaf tissue incubated for 40 h under argon (anaerobic).

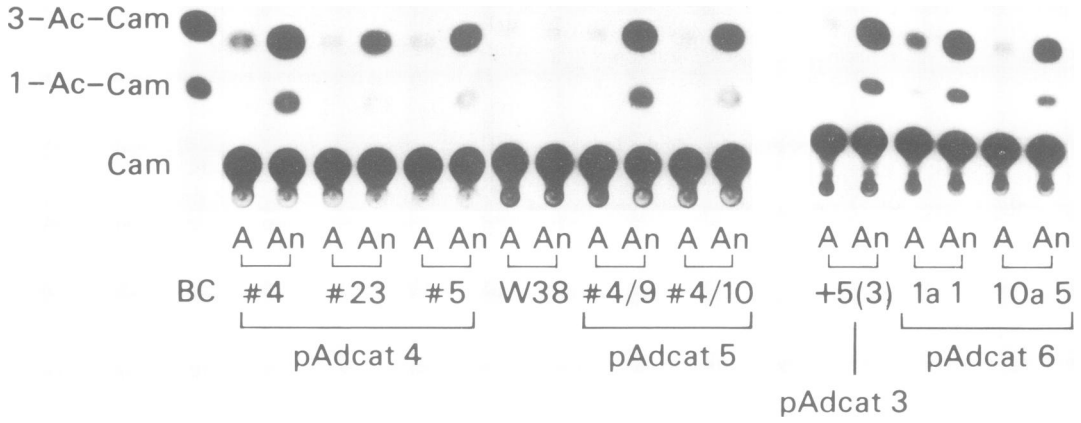

Fig. 6. CAT enzyme assays on leaf tissue extracts of transgenic plants transformed with hybrid gene constructs pAdcat4, pAdcat5 and pAdcat6. Cam, [^{14}C]chloramphenicol; 1-Ac-Cam and 3-Ac-Cam, acetylated derivatives of [^{14}C]chloramphenicol. BC, bacterial CAT. W38, assay on extracts of untransformed *N. tabacum* cv. Wisconsin 38. A, reaction products of extracts from plant material grown in air (aerobic). An, reaction products of extracts from plant material incubated under argon for 40 h (anaerobic).

*Bam*HI site (−116) rather than the larger 2.4-kb fragment used above in the construct pAdcat3. This 176-bp fragment was cloned in both orientations next to the *Pst*I site (−140) of the *Adh-1* promoter/*cat* gene fusion to give pAdcat5 and pAdcat6 (Figure 1). Anaerobically induced CAT enzyme activity was independent of the orientation of the *ocs* fragment. Ten out of 21 plants transformed with pAdcat5 and 11 out of 17 plants transformed with pAdcat6 expressed inducible CAT enzyme activity. CAT assays on several of these transgenic plants are shown in Figure 6. S1 mapping of mRNA from one of the pAdcat5 transformants and from one pAdcat6 transformant showed that transcription was initiated from the same site as in maize (data not shown).

These data show this 176 bp fragment of the upstream promoter region of the *ocs* gene has at least three of the properties of a eukaryotic enhancer element; it is essential for high-level expression of the *ocs* promoter (Koncz *et al.*, 1984), it enhances the expression of a heterologous promoter (*Adh-1*) and its function is independent of orientation. Further experiments are in progress to determine whether the element works at greater distances from the promoter, whether it functions when placed 3′ of a gene and to identify sequences within the fragment responsible for the enhancer-like activity.

Discussion

We have presented evidence that all the signals of the maize *Adh-1* gene required for anaerobic induction and accurate initiation of transcription in transgenic tobacco are within a 247-bp segment immediately upstream of the translation initiation codon. This fragment has also been identified as an anaerobic promoter by transient expression analysis of an *Adh-1*/*cat* gene fusion in maize protoplasts (Howard *et al.*, 1987; E.A.Howard and J.C.Walker, unpublished results). The results indicate that the information for transcriptional regulation of the maize *Adh-1* gene is contained within the promoter and not in the structural region or 3′ end of the gene.

In transgenic tobacco, this promoter segment alone functioned very weakly and its activity was detected only at the RNA level. However, by simply inserting DNA segments of constitutive promoters upstream of the maize promoter, high level and regulated expression of the reporter gene was observed. Analysis of the upstream segment of the octopine synthase gene showed that it had some characteristics of an enhancer element, namely the ability to increase the expression from a heterologous promoter independent of the segment's orientation. Although this element and the one from the CaMV 35S promoter which we

have studied in less detail, are from constitutive genes which are not regulated by environmental induction, their action in increasing expression of the hybrid gene did not override the anaerobic regulatory signal in the maize promoter. Rather, the enhancer-like element and the maize gene's regulatory signal were dependent on each other and acted co-operatively; the enhancer-like element was required to detect high level expression from the maize promoter and anaerobic induction was required to detect the enhancer-like effect of the *ocs* gene and CaMV 35S gene segments on the expression of the hybrid gene.

Having established a system where we are able to observe anaerobic induction of the maize *Adh-1* promoter in transgenic tobacco, we have begun to delineate further the *cis* acting regulatory signal sequences involved using deletion analysis. Similarly, we have begun deletion analysis of the 'enhancer' regions of the *ocs* and CaMV 35S promoters to identify the limits of the essential segments.

It is not yet clear why the maize promoter was inefficient in tobacco cells in the absence of the *ocs* and 35S gene segments. Either the maize promoter was not recognised efficiently by the tobacco transcriptional mechanism or the mRNA from the hybrid gene construct was unstable and reporter gene expression was not observed until the level of transcription was increased. Nevertheless, the anaerobic signal must be sufficiently conserved between the distantly related plant species, maize and tobacco, to allow regulated expression of the *ocs/Adh-1/cat* and 35S/*Adh-1/cat* hybrid gene constructs in transgenic tobacco.

The entire maize *Adh-1* gene with its flanking region has also been transferred to tobacco and no expression of maize ADH enzyme activity or mRNA was observed (Llewellyn *et al.*, 1985) which is consistent with the results presented in this report and confirms that the promoter functions only weakly in tobacco. So far, expression of only three monocot genes in transgenic dicot plants has been reported; a maize zein gene in sunflower tumour cells (Matzke *et al.*, 1984), a wheat chlorophyll *a/b* binding protein gene in tobacco and petunia (Lamppa *et al.*, 1985) and a maize heat-shock gene in petunia (Rochester *et al.*, 1986), so in the absence of negative reports it is difficult to assess whether the lack of expression of the maize *Adh-1* promoter in tobacco is an exception or whether it represents a more general behaviour of monocot promoters in dicot plants. In order to investigate the possibility of differences between monocot and dicot promoters we have isolated an anaerobically induced *Adh* gene from the dicot species *Pisum sativum* to make direct comparison between the monocot and dicot promoters of a homologous gene in expression assays (Llewellyn *et al.*, submitted). If species-specific gene expression in plants is widespread, then this could pose problems for the use of interspecific gene transfer between distantly related plants as a tool for plant improvement. Our results provide an example of how this problem can be simply overcome by recombinant DNA techniques and transformation; by adding DNA segments analogous to constitutive enhancers in animal systems, it was possible to markedly increase the level of regulated expression of a plant promoter transferred to a distantly related host species. These enhancer-like sequences may have a more general application in increasing foreign gene expression in new host plants.

Materials and methods

Construction of maize Adh-1 expression vectors

The maize promoter region was isolated from a genomic clone of the *Adh1-1S* gene (Dennis *et al.*, 1984) as a *Bam*HI−*Hin*dIII fragment containing 1200 bp 5′ and 205 bp 3′ of the translation initiation codon and cloned into pBR322. The promoter fragment was resected from a unique *Sac*II site 11 bp 3′ of the ATG codon using the 3′−5′ exonuclease activity of T4 polymerase followed by S1 nuclease digestion and repair with the Klenow fragment of PolI. A synthetic *Hin*dIII linker (Biolabs) was added and the plasmids resulting from recircularisation were subcloned in M13 vectors and sequenced. One plasmid (pAd1) was chosen that was deleted to +106, 2 bp upstream of the A residue of the translation initiation codon. Plant polyadenylation signals were supplied by the 3′ end of the nopaline synthase gene on a 1.7 kb *Eco*RI−*Pst*I fragment of pLGV2382 (Herrera-Estrella *et al.*, 1983a) to give pAd2. The 5′ *Bam* site was filled in with Klenow fragment of PolI and the *Hin*dIII site changed to a *Bam*HI site using synthetic linkers. A *Bam*HI fragment from pNOSCAT4 containing the coding region of the bacterial *cat* gene was inserted downstream of the maize promoter. pNOSCAT4 contains the *Taq*I fragment of the *cat* gene of pBR325 (Bolivar, 1978) to which *Bam*HI linkers had been added (L.Herrara-Estrella, personal communication). The unique *Sal*I site was converted to a *Bgl*II site and the resulting plasmid pAdcat1 was sequenced across the *Adh1-1S* promoter/*cat* gene fusion. The *Pst*I fragment containing the hybrid *cat* gene was then subcloned into pUC8 to give pAdcat2. pAdcat1 was co-integrated with the binary vector plasmid pGA472 (An *et al.*, 1985) at the unique *Bgl*II site such that transcription of the *neo* gene used for selection of transformants and the *cat* gene were in the same direction. pAdcat2 was co-integrated at the *Hin*dIII site of pGA472 in both orientations.

pAdcat3, pAdcat5, pAdcat6. A 2.4-kb fragment of the T-DNA of pTiAch5 (De Vos *et al.*, 1981) extending from the *Eco*RI site (coordinate 16202, Barker *et al.*, 1983) to the *Bam*HI site (13774) was cloned into pUC8. This fragment contains the left border repeat of TR-DNA and the right border repeat of TL-DNA and the region directly upstream of the *ocs* promoter. The *Pst*I fragment of pAdcat1 containing the hybrid *cat* gene was inserted to give pAdcat3. To decrease the size of the T-DNA sequence and eliminate the border sequences we cloned the 176-bp sub-fragment from *Bam*HI (13774) to *Hpa*II (13952) into pBR322, digested with *Bam*HI and *Cla*I, which resulted in pOCS1. The *Bam*HI site is at −116 and the *Hpa*II at −292 with respect to the transcription initiation site (+1) of the octopine synthase (*ocs*) gene (de Greve *et al.*, 1982b). The small *Bam*HI−*Eco*RI fragment from pOCS1 was inserted into pUC8 and then the *Pst*I fragment of pAdcat1 inserted to give pAdcat5. pAdcat6 was constructed as follows: pOCS1 DNA was cut with *Pst*I and *Eco*RI then treated with Klenow fragment and ligated to *Pst*I linkers. After *Pst*I digestion and removal of excess *Pst*I linkers, the *Pst*I fragment of pAdcat1 was inserted. This fragment carried part of the *amp* gene and when inserted in the correct orientation, it complemented the deletion created in the *amp* gene of pOCS1 by the initial *Pst*I/*Eco*RI digestion. This placed the *ocs* 5′ region upstream of the *Adh-1* promoter but in an inverted orientation with respect to pAdcat5. A *Hin*dIII linker was inserted at the *Sal*I site of this plasmid to give pAdcat5. The plasmids pAdcat3, pAdcat5 and pAdcat6 were cointegrated at the *Hin*dIII site of pGA472. The orientation of pAdcat3 in pGA472 was chosen such that border repeats of the binary vector were not aligned in direct orientation with the TL and TR border repeats contributed by the 2.4 kb octopine T-DNA fragment. This was done to avoid the use of these border repeats and consequent elimination of the hybrid *cat* gene during transformation of tobacco. pAdcat5 and pAdcat6 were cloned into pGA472 in both orientations. The frequency of CAT enzyme positive transformants was independent of the orientation of co-integration (results not shown).

pAdcat4. A fragment of the 35S promoter of CaMV from the *Hin*cII site (coordinate 7018, Franck *et al.*, 1980) to the *Eco*RV site (7445) was cloned into the filled *Bam*HI and *Sal*I sites of pUC8 such that the *Eco*RV site was joined to the former *Sal*I site. The *Pst*I fragment of the pAdcat1 used in previous constructs was inserted to give pAdcat 6. The cleavage site of the *Eco*RV site used in this construct is at −90 with respect to the start of transcription (+1) of the 35S transcript (Odell *et al.*, 1985). pAdcat6 was co-integrated with pGA472 at the *Hin*dIII site.

The binary vector constructs were transferred from *Escherichia coli* to the avirulent *A. tumefaciens* strain LBA4404 Rif^r (Hoekema *et al.*, 1983) by triparental mating (Ditta *et al.*, 1980). Transconjugants containing the binary vector co-integrated with the *Adh-cat* constructs were selected on LB medium (Maniatis *et al.*, 1982) containing rifampicin (50 μg/ml) and tetracycline (5 μg/ml) then purified through a single colony on the same medium containing in addition, carbenicillin (5 μg/ml).

Transformation of tobacco leaf pieces

Leaves of *N. tabacum* cv. Wisconsin 38 growing in tissue culture on MS (Murishige and Skoog medium, Murishige and Skoog, 1962) were cut into pieces (1 cm^2) under liquid MS medium to prevent desiccation and added to a cell suspension of the transforming *Agrobacterium* strains. The bacteria were grown on YM slopes (Ellis *et al.*, 1979) for 24 h and then resuspended in 10 ml MS liquid medium. After 20 min the infected leaf pieces were transferred to MS agar and incubated for 24 h at 25°C. The leaf pieces were then washed in sterile water and transferred to MS9 shoot induction medium (MS medium, 0.5 mg/l indole acetic acid, 1.0 mg/l benzyl aminopurine) containing 100 μg/ml kanamycin sulphate and 500 μg/ml cefotaxime. When shoots were 1−2 cm high they were transferred to MS medium containing the same antibiotics. Plants that formed roots were

maintained under kanamycin selection and assayed for CAT activity. The plant material used in the assay was both young leaves of transgenic plants or shoot cultures initiated from leaf pieces of transgenic plants on medium containing kanamycin sulphate. The plant material was made anaerobic by incubation at 28°C on MS agar in an argon atmosphere for 40–48 h. Later, 18 h of anaerobiosis was found to be sufficient for induction.

CAT assay

The assay was performed as described by Herrera-Estrella *et al.* (1983b) except that the extraction buffer contained 0.1% ascorbic acid and 0.1% cysteine–HCl. Each μg of tissue was extracted with 1 μl of extraction buffer, and cleared by centrifugation. To 50 μl of supernatant, 0.2 μCi of [^{14}C]chloramphenicol (Amersham) was added then made to 1 mM acetyl CoA and incubated at 37°C for 30 min. The ethyl acetate extract of the reactions were concentrated by evaporation and then chromatographed on silica gel plates in chloroform–methanol (95:5). The gel plates were sprayed with fluor (0.4% PPO in 1-methyl-napthalene) and autoradiographed for 16 h at −80°C.

Octopine synthase assay

The assay was modified from Otten and Schilperoort (1978) by using 1 μCi of [U-^{14}C]arginine (Amersham). A single leaf was removed from each plant and cut along the midrib into two pieces. One half was incubated on MS agar in the dark for 36 h at 28°C (aerobic). The other half was incubated under the same conditions but under an atmosphere of argon (anaerobic). 2.5 μl of a 20 μl reaction was loaded for electrophoresis at pH 1.7 (1 M acetic acid, 0.75 M formic acid). Fluorography was performed as described above for the CAT assay.

Molecular analysis of transgenic plants

The preparation of genomic tobacco DNA and its analysis by Southern blot hybridization was as described in Dennis *et al.* (1984). Cytoplasmic RNA was isolated from aerobically grown shoot cultures or cultures incubated under an argon atmosphere for 24–48 h (Sachs *et al.*, 1980). S1 nuclease mapping was performed as previously described (Dennis *et al.*, 1984) using a single-stranded DNA probe (Hudson *et al.*, 1985). The probe extended from the *Pst*I site upstream of the maize *Adh1-1S* TATA box through to the *Hin*dIII site in the first intron (Dennis *et al.*, 1984).

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References

An,G., Watson,B.D., Stachel,S., Gordon,M.P. and Nester,E.W. (1985) *EMBO J.*, **4**, 277–284.

Barker,R.F., Idler,K.B., Thompson,D.V. and Kemp,J.D. (1983) *Plant Mol. Biol.*, **2**, 335–350.

Bevan,M.W., Barnes,W.M. and Chilton,M.-D. (1983) *Nucleic Acids Res.*, **11**, 369–385.

Bolivar,F. (1978) *Gene*, **4**, 121–136.

DeGreve,H., Leemans,J., Hernalsteens,J., Thia-Toong,L., DeBeukeleer,M., Willmitzer,L., Otten,L., Van Montagu,M. and Schell,J. (1982a) *Nature*, **300**, 752–755.

DeGreve,H., Dhaese,P., Seurinck,J., Lemmers,M., Van Montagu,M. and Schell,J. (1982b) *J. Mol. Appl. Genet.*, **1**, 561–574.

Dennis,E.S., Gerlach,W.L., Pryor,A.J., Bennetzen,J.L., Inglis,A., Llewellyn,D., Sachs,M., Ferl,R. and Peacock,W.J. (1984) *Nucleic Acids Res.*, **12**, 3983–4000.

Depicker,A., Stachel,S., Dhaese,P., Zambryski,P. and Goodman,H.M. (1982) *J. Mol. Appl. Genet.*, **1**, 561–574.

De Vos,G., DeBeuckeleer,M., Van Montagu,M. and Schell,J. (1981) *Plasmid*, **6**, 249–253.

Ditta,G., Stanfield,S., Corbin,D. and Helinski,D.R. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7347–7351.

Ellis,J.G., Kerr,A., Van Montagu,M. and Schell,J. (1979) *Physiol. Plant Pathol.*, **15**, 311–319.

Franck,A., Guilley,H., Jonard,G., Richards,K. and Hirth,L. (1980) *Cell*, **21**, 285–294.

Gerlach,W., Pryor,A., Dennis,E., Ferl,R., Sachs,M. and Peacock,W.J. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2981–2985.

Hageman,R. and Flescher,D. (1960) *Arch. Biochem. Biophys.*, **87**, 203–208.

Herrera-Estrella,L., De Block,M., Messens,E., Hernalsteens,J.P., Van Montagu,M. and Schell,J. (1983a) *EMBO J.*, **2**, 987–995.

Herrera-Estrella,L., Depicker,A., Van Montagu,M. and Schell,J. (1983b) *Nature*, **303**, 209–213.

Hoekema,A., Hirsch,P.R., Hooykaas,P.J. and Schilperoort,R.A. (1983) *Nature*, **303**, 179–181.

Howard,E.A, Walker,J.L. Dennis,E.S. and Peacock,W.J. (1987) *Planta*, in press.

Hudson,G.S., Farrell,P.J. and Barrell,B.G. (1985) *J. Virol.*, **53**, 528–535.

Jones,J.D., Dunsmuir,P. and Bedbrook,J. (1985) *EMBO J.*, **4**, 2411–2418.

Kelley,P.M. and Freeling,M. (1984a) *J. Biol. Chem.*, **259**, 673–677.

Kelley,P.M. and Freeling,M. (1984b) *J. Biol. Chem.*, **259**, 14180–14183.

Koncz,C., Kreuzaler,F., Kalman,Z. and Schell,J. (1984) *EMBO J.*, **3**, 1029–1037.

Lamppa,G., Nagy,F. and Chua,N.-H. (1985) *Nature*, **316**, 750–752.

Laszlo,A. and St. Lawrence,P. (1983) *Mol. Gen. Genet.*, **192**, 110–117.

Llewellyn,D., Dennis,E. and Peacock,W.J. (1985) In van Vloten-Doting,L. Groot,G.S. and Hall,T. (eds), *Molecular Form and Function of the Plant Genome*. Plenum Publishing Corporation, pp. 593–608.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning; A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Matzke,M.A., Susani,M., Bunns,A.N., Lewis,E.D., Rubenstein,I and Matzka,A.J.M. (1984) *EMBO J.*, **3**, 1525–1531.

Murishige,T. and Skoog,F. (1962) *Physiol. Plant.*, **15**, 473–497.

Odell,J., Nagy,F. and Chua,N.-H. (1985) *Nature*, **313**, 810–812.

Otten,L. and Schilperoort,R. (1978) *Biochim. Biophys. Acta*, **527**, 497–500.

Otten,L., DeGreve,H., Hernalsteens,J.-P., Van Montagu,M., Schieder,O., Straub,J. and Schell,J. (1981) *Mol. Gen. Genet.*, **183**, 209–213.

Rochester,D., Winer,J. and Shah,J. (1986) *EMBO J.*, **5**, 451–458.

Sachs,M., Freeling,M. and Okimoto,R. (1980) *Cell*, **20**, 761–767.

Schlessinger,M.J., Ashburner,M. and Tissieres,A. (eds) (1982) *Heat Shock from Bacteria to Man*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schwartz,D. (1969) *Amer. Nat.*, **103**, 479–481.

Schwartz,D. and Endo,T. (1966) *Genetics*, **83**, 125–136.

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