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Transcription of a zein gene introduced into sunflower using a Ti plasmid vector

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A maize genomic clone containing a zein gene (Z4) was inserted into the T-region of the T37 Ti plasmid. ***Agrobacterium tumefaciens*** **cells carrying this modified Ti plasmid were used to inoculate sunflower stemlets. Callus tissue active in nopaline synthesis was grown from a single transformed cell. DNA analysis of this tissue showed that the zein gene plus T-DNA was present in ~12 copies per diploid sunflower genome. A 1000 ± 100 base RNA homologous to a zein probe could be isolated from the engineered sunflower tissue and the 5′ end of this RNA was determined by S1 nuclease mapping. Two transcription start sites were detected. The positions of these transcription start sites and the ratio of the amounts of the two transcripts are identical for the Z4 gene in sunflower and in maize endosperm. Although the zein RNA isolated from the engineered sunflower tissue could be translated in a wheat germ system to yield an immunoprecipitable protein of the expected mol. wt., the presence of the zein protein in the sunflower tissue could not be demonstrated.**

Key words: zein /Ti plasmid/sunflower/transcription/promoter

Introduction

Crown gall tumorigenesis in dicotyledonous plants results from transfer, by a natural mechanism, of a specific section of the *Agrobacterium tumefaciens* tumor-inducing (Ti) plasmid to plant cell nuclei where it is integrated and stably maintained (for reviews, see Van Montagu and Schell, 1981; Ream and Gordon, 1982; Bevan and Chilton, 1982a; Caplan *et al.*, 1983). This transferred DNA, called T-DNA, is transcribed in the transformed plant cells (Willmitzer *et al.*, 1982, 1983; Bevan and Chilton, 1982b; Gelvin *et al.*, 1982), and the products of some of these T-DNA genes confer upon the plant cells the characteristics of uncontrolled growth and the synthesis of opines, compounds not found in normal plant cells. The genes encoding the enzymes responsible for opine synthesis are present in T-DNA, and the synthase genes for two opines, nopaline and octopine, have been identified and sequenced (Depicker *et al.*, 1982; Bevan *et al.*, 1983a; De Greve *et al.*, 1983).

The Ti plasmid has also been used as a vector to study the expression of foreign genes introduced into higher plant cells. The ability of foreign genes combined with various regulatory sequences to function in transformed plant cells has been studied by the use of site-specific insertion of genetic information into T-DNA (Matzke and Chilton, 1981; Leemans *et al.* 1981). Work so far has shown that the expression of foreign genes of non-plant origin in plant cells depends on the presence of adjacent plant regulatory signals. For example, a yeast alcohol dehydrogenase gene (Barton *et al.*, 1983) and a rabbit β-globin gene (Shaw *et al.*, 1983), both inserted into T-DNA with their own promoters, were not expressed in transformed plant cells. However, when the coding regions of various bacterial antibiotic resistance genes were fused to the nopaline synthase promoter (a promoter which allows constitutive expression of the nopaline synthase gene in crown gall tumors), expression of these genes was observed in the transformed plant cells (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983b; Fraley *et al.*, 1983).

The opine synthase genes and plant nuclear genes that have been sequenced (Messing *et al.*, 1983) share common structural features, such as a TATA box and a −80 sequence, in their 5′-flanking regions. The presence of these common elements suggests that some plant genes might be expressed from their own promoters after introduction into a different species. Such a case has been recently reported by Murai *et al.* (1983). They showed that a gene for the bean storage protein phaseolin was expressed in sunflower cells transformed with T-DNA, into which either a phaseolin genomic clone or a chimeric construction containing part of the phaseolin coding sequence joined to an octopine synthase promoter was inserted.

In this paper, we report the first evidence for transcription of a monocot gene in a dicot host plant. A genomic clone for the maize storage protein zein, introduced along with T-DNA into sunflower cells, is transcribed from its own promoter. The zein mRNA made in sunflower can be translated *in vitro* to yield an immunoprecipitable protein with the expected mol. wt. However, we have been unable to detect the presence of the zein protein in the engineered sunflower tissue.

Results

Construction of the modified Ti plasmid and isolation of engineered sunflower tissue

The zein genomic clone, λ (W22)Z4, was isolated and characterized by Lewis *et al.* (1981) and sequenced by Hu *et al.* (1982). The amino acid composition deduced from the nucleotide sequence indicates that it codes for a 27-kd zein polypeptide. This protein would have an apparent mol. wt. of 23 kd on an SDS gel (Park *et al.*, 1980).

The strategy for inserting this zein gene into the T-DNA of pTiT37 was similar to that used for the insertion of other genes into T-DNA (Matzke and Chilton, 1981; Barton *et al.*, 1983) as shown in Figure 1. In this construction, a 3.1-kb *Eco*RI-*Hind*III fragment containing the protein coding sequence of the Z4 gene was inserted, along with a kanamycin resistance marker (Km^R) and part of the pBR325, into the unique *Hpa*I site of the pTiT37 T-DNA *Bam*HI fragment 14a

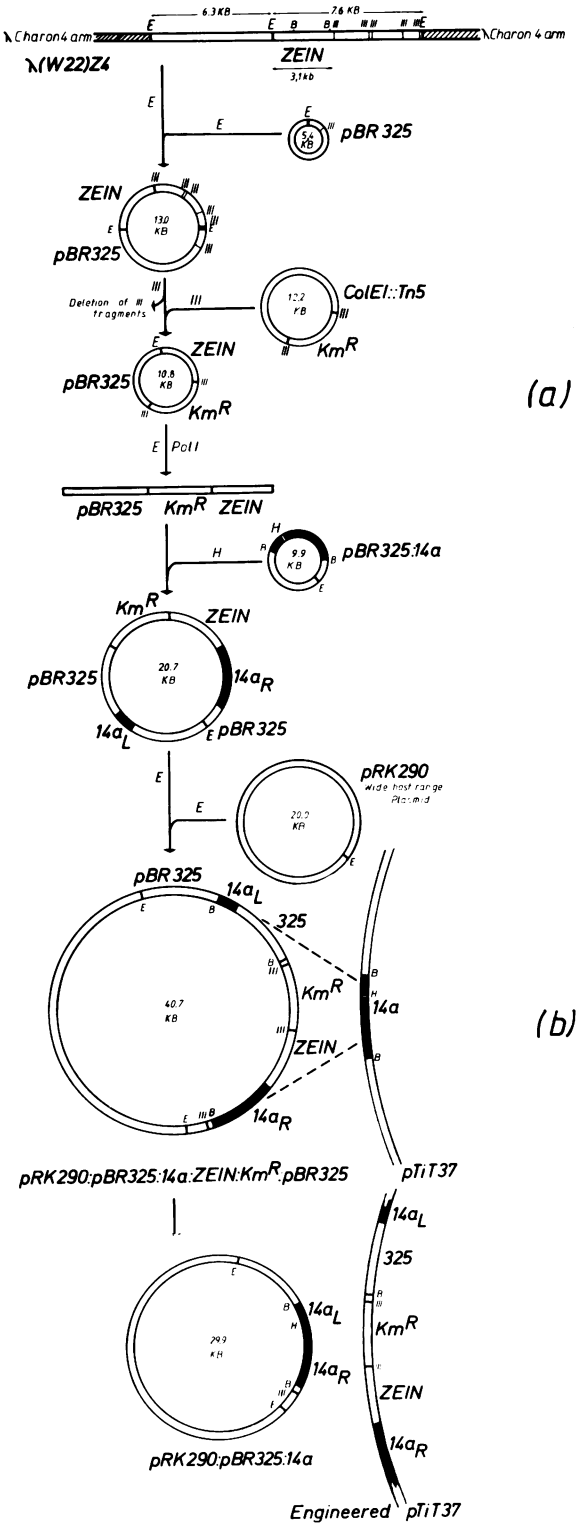

Fig. 1. (**a**) Construction of a wide-host range vector containing a 3.1-kb *Eco*RI-*Hind*III fragment of maize DNA with the zein gene and a kanamycin resistance marker (Km^R) inserted into the pTiT37 T-DNA *Bam*HI fragment 14a (Yadav *et al.*, 1980). (**b**) Double homologous recombination in *Agrobacterium* between the wild-type *Bam*HI fragment 14a of the resident Ti plasmid, T37, and the engineered *Bam*HI fragment (containing the zein gene) in the wide-host range vector. Agrobacteria containing the engineered Ti plasmid were selected by plating transformants on medium containing gentamycin and kanamycin after the introduction of R751pMG2, a plasmid which is incompatible with pRK290 and which encodes gentamycin resistance.

(Yadav *et al.*, 1980) in pBR325. The pBR325:14a:zein:Km^R: pBR325 was then linearized with *Eco*RI and ligated into the *Eco*RI site of the wide-host range vector, pRK290. *A. tumefaciens* strain A208 containing a wild-type T37 Ti plasmid were transformed with the pRK290:pBR325:14a:zein:Km^R: pBR325. We used plasmid incompatibility to select for double recombination between the engineered 14a fragment in the pRK290 construction and its wild-type counterpart in the Ti plasmid. After introduction, by conjugation, of R751pMG2, a plasmid which is incompatible with pRK290 and which carries a gentamycin resistance gene, we selected for bacteria resistant to kanamycin and gentamycin.

The structure of the engineered Ti plasmid was confirmed by Southern blot analysis (data not shown) and is shown in Figure 2B along with *Bam*HI and *Eco*RI maps of the wild-type pTiT37 (Yadav *et al.*, 1980; Barton *et al.*, 1983). As can be seen, the newly inserted DNA, including the zein gene, is present in a single large *Eco*RI fragment.

After inoculating sunflower stemlets with the engineered agrobacteria and cloning of single cells from the resultant outgrowth (Binns *et al.*, 1982), a cloned sunflower callus tissue active in nopaline synthesis, was obtained. This tissue grew best on medium supplemented with kinetin. Southern blot analysis of the engineered sunflower tissue DNA verified that the entire pTiT37 T-DNA containing the zein gene was present in ~12 copies per diploid sunflower genome (Figure 3, lane 2; compare with T-DNA *Eco*RI map drawn in Figure 2B). As seen in lane 3 of this figure, the zein gene was found, as expected, to be present in a large *Eco*RI fragment. The structure of this integrated T-DNA in sunflower is very similar to what has been observed for pTiT37 tumors induced on tobacco (Yadav *et al.*, 1980; Lemmers *et al.* 1980; Barton *et al.*, 1983).

Expression of the Z4 gene in the engineered sunflower tissue

The expression of the zein gene was investigated by analyzing both RNA and protein of the engineered sunflower tissue. Figure 4 shows the results of a Northern blot experiment using the zein gene and other parts of the construction as probes. A strong signal was obtained with a nopaline synthase probe in the lane containing poly(A)-RNA from the engineered sunflower tissue (lane 2) but not with sunflower poly(A)-RNA from untransformed sunflower leaves (lane 3) or *Escherichia coli* rRNA (lane 1). This band, which migrated slightly slower than a 1.5-kb *E. coli* rRNA marker, corresponds to the size of the nopaline synthase mRNA in tobacco tumors (~1600 bases, Bevan *et al.*, 1983b). When the poly(A)-RNA preparations from the engineered sunflower tissue and normal sunflower were hybridized to a zein gene probe, an RNA molecule of 1000 ± 100 bases was detected only in the former (Figure 4, lane 5). The protein coding region of the Z4 gene is 804 bp (there are no introns; Hu *et al.*, 1982). Assuming that the length of the 5′- and 3′-untranslated regions and the poly(A) sequence add ~200 bp more to the mRNA, the size of the zein RNA we observe in the engineered sunflower tissue is within the range expected for an authentic Z4 transcript.

When the Z4 gene was used as a probe on total RNA from maize endosperm, a large, diffuse band of hybridization was visible (data not shown). The most prominent area of hybridization was slightly smaller than the size of the Z4 transcript in the sunflower tissue. A fainter area of hybridization migrated approximately with the sunflower Z4

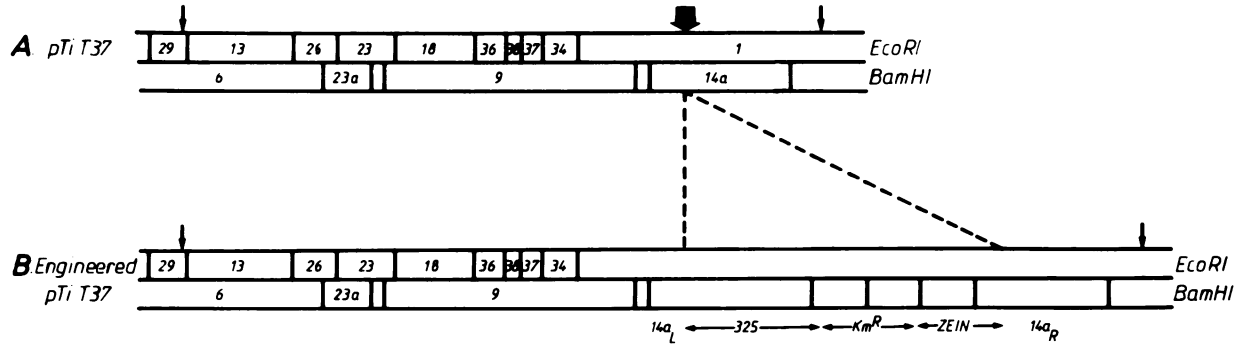

Fig. 2. (**A**) *Bam*HI and *Eco*RI restriction maps of the T-DNA region of the T37 Ti plasmid (Yadav *et al.*, 1980; Barton *et al.*, 1983). Small arrows denote the approximate location of the T-DNA borders (Yadav *et al.*, 1980; Lemmers *et al.*, 1980; Zambryski *et al.*, 1980); the large arrow shows the site of insertion of pBR325:Km^R:zein into the *Hpa*I site of *Bam*HI fragment 14a. (**B**) The structure of the engineered Ti plasmid. This structure was confirmed by Southern blotting (data not shown).

transcript. The smaller predominant transcripts are presumably transcribed from genes of the A30 subfamily which encode primarily 19-kd zein proteins (Messing *et al.*, 1983). Z4, also a member of this subfamily, encodes a 23-kd zein because of a duplication of 32 amino acids relative to the A30 sequence. Because of the poor resolution of the Northern blotting technique and the problems encountered when working with a multigene family it was not possible to tell if the size of the Z4 transcript is the same in sunflower and in maize endosperm or if the Z4 gene is in fact transcribed in maize endosperm. We could conclude, however, that the subfamily represented by the Z4 gene is transcribed in maize endosperm.

Besides the zein gene, our construction also contains pBR325 DNA and a kanamycin resistance gene (see Figure 2B). However, with a pBR325:Km^R probe, no significant hybridization to any RNA preparation can be seen on the Northern blot (Figure 4, lanes 7, 8 and 9). Faint bands (between 1.9 and 3.9 kb and >3.9 kb) of hybridization are visible in lane 8. These are possibly due to transcripts originating at the promoter of the cytokinin autonomy gene. This is a T-DNA gene which is interrupted by the insertion of the zein: Km^R:pBR325. The promoter and the 5′ end of this gene are in *Bam*HI fragment 14a$_L$ (see Figure 2B) (Willmitzer *et al.* 1983). The large transcripts homologous to the 325:Km^R probe are thus probably read-through transcripts from this promoter. Since these faint bands are considerably longer than the zein trancript, we conclude that the signal we obtain with the zein probe is specific for an RNA transcribed from the zein gene and not due to read-through transcription from pBR325 or Km^R sequences.

We then attempted to determine the transcription start for the Z4 gene in sunflower and to compare it with the 5′ end of transcripts homologous to this gene in maize endosperm using S1 nuclease mapping. Poly(A)-RNA was isolated from engineered sunflower tissue as well as from normal sunflower leaves, and total RNA was obtained from maize endosperm. These were each hybridized to the 1.1-kb *Eco*RI-*Bam*HI fragment which contains the coding region for the N-terminal amino acids of the first fifth of the Z4 coding sequence (Lewis *et al.*, 1981) plus the 950 bp that are upstream from the ATG start codon (Hu *et al.*, 1982). This DNA was end-labeled at the 5′ end at a *Bam*HI site 170 bp downstream from the first nucleotide of the ATG start codon. After digestion with S1 nuclease, two protected fragments were present when poly(A)-RNA from the engineered sunflower tissue (Figure 5, lane 2) or total RNA from maize endosperm (lane 3) was hybridized to the labeled Z4 DNA. No bands were observed in the control with poly(A)-RNA from normal sunflower (Figure 5, lane 4). The lengths of the protected fragments correspond to transcription start sites which are 57 and 45 nucleotides upstream from the first nucleotide of the ATG start codon of Z4. These would correspond to positions which are 31 and 43 nucleotides, respectively, downstream from the first T of a possible TATA box of the Z4 gene (Hu *et al.*, 1982). There is another potential TATA box in the Z4 sequence which is only 15 nucleotides upstream from the start site of the shorter transcript.

We also tried to isolate the translation product of the zein RNA from the engineered sunflower tissue. To test for the presence of zein, we took advantage of the fact that this protein is soluble in 70% ethanol. Engineered sunflower tissue was repeatedly extracted first with acetone, then with 0.5 M NaCl and finally with 70% ethanol. The final extract was lyophilized and then separated by polyacrylamide gel electrophoresis (Laemmli, 1970). Gels were either stained with Coomassie blue or used for Western blots (Towbin *et al.*, 1979) and analyzed with polyclonal zein antiserum. Even loading material extracted from as much as 10 g of tissue into a single well did not give convincing evidence for the presence of the zein protein.

One possible explanation for this negative result would be that, for some unknown reason, the zein RNA is not translated in sunflower cells. To test this hypothesis, hybrid-selected poly(A)-RNA isolated from the engineered sunflower tissue was translated in a wheat germ system. The translation products were precipitated with polyclonal zein antiserum and then fractionated by polyacrylamide gel electrophoresis (Figure 6).

Translation of the Z4-selected poly(A)-RNA from the engineered sunflower tissue resulted in the synthesis of two immunoprecipitable proteins (Figure 6, lane 2) with an apparent mol. wt. of 24 000 for the main and 32 000 for the minor component. The Z4 gene encodes a mature zein protein with an apparent mol. wt. of 23 000 (its absolute mol. wt. is 27 000; Hu *et al.*, 1982) and the *in vitro* translation products of zein mRNAs are known to be somewhat larger than the mature zein proteins due to the presence of a signal peptide at the N terminus (Park *et al.*, 1980). Therefore, the 24-kd protein we observe is the size expected for an unprocessed Z4-encoded protein. The identity of the 32 000 mol. wt. protein is unknown; however, in a control experiment it was shown that a similar protein was also present in

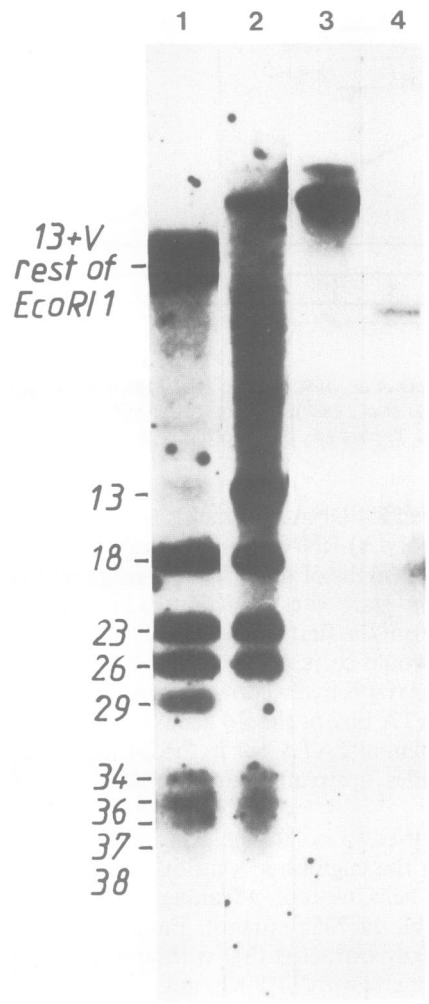

Fig. 3. Southern blot analysis of the T-DNA structure in the engineered sunflower tissue. **Lane 1:** *Eco*RI digest of a MINI-Ti (an *E. coli* plasmid containing the entire pTiT37 T-DNA; de Framond *et al.*, 1983) reconstruction probed with MINI-Ti. The equivalent of 12 copies of T-DNA per diploid sunflower genome was loaded on the gel. The bands of the MINI-Ti reconstruction are numbered on the left according to the *Eco*RI map of the pTiT37 shown in Figure 2A. **Lane 2:** *Eco*RI digest of engineered sunflower tissue DNA, same probe as in **lane 1**. Note the identity of the expected internal T-DNA fragments (see Figure 2B; compare also with the standard pTiT37 T-DNA *Eco*RI fragments in the MINI-Ti digest in **lane 1**) and the same hybridization intensity as in **lane 1**. This indicates that T-DNA is present in ~12 copies per diploid nucleus in the engineered sunflower tissue. **Lane 3:** same digest as in **lane 2**, probed with pBR325:Km^R:zein. As expected, the zein gene is present in the largest *Eco*RI fragment (see Figure 2B). **Lane 4:** control with DNA from normal sunflower. Same probe as in **lanes 1** and **2**. The weak hybridization may be due to some homology between T-DNA and normal plant DNA as also noted by others (Thomashow *et al.*, 1980; White *et al.*, 1983).

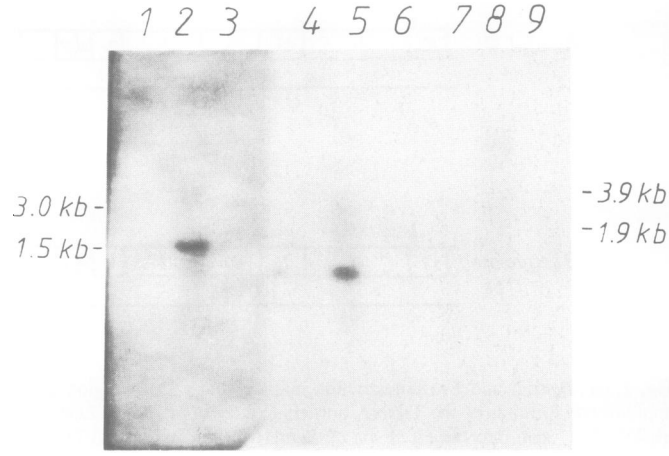

Fig. 4. Northern analysis of poly(A)-RNA isolated from normal sunflower leaves (**lanes 3, 6** and **9**), engineered sunflower tissue (**lanes 2, 5** and **8**) and *E. coli* rRNA (**lanes 1, 4** and **7**). 10 μg of RNA were run in each lane. The probes used were: **lanes 1, 2** and **3**: *Hind*III fragment 23, a fragment from pTiT37 containing the nopaline synthase gene (Hernalsteens *et al.*, 1980; Leemans *et al.*, 1981); **lanes 4, 5** and **6**: the 2-kb *Bam*HI fragment which contains the 3′ two-thirds of the zein coding sequence (Lewis *et al.*, 1981); **lanes 7, 8** and **9**: pBR325:Km^R. The probes were isolated by electroelution from agarose gels and then radioactively labeled to approximately the same specific activity. Equal numbers of counts from each probe were added to the hybridization reactions. The positions and sizes of rRNA markers from *E. coli* (left) and sunflower (right) are indicated.

the translation products of poly(A)-RNA from normal sunflower (Figure 6, lane 3), and we presume that this represents a sunflower protein which has some homology with zein.

When total RNA from maize endosperm was selected with the Z4 gene, translated *in vitro* and the products precipitated with polyclonal zein antiserum, two bands appeared (Figure 6, lane 1). Of these, one is the same size (24 000) as that seen when mRNA isolated from the engineered sunflower tissue was translated (Figure 6, lane 2) and is presumably the translation product of Z4 mRNA. A more predominant band was also present which had an apparent mol. wt. of 20 000. This band represents the 19-kd zein proteins plus their signal peptides which are the major proteins encoded by the A30 subfamily mRNAs (Messing *et al.*, 1983). Z4 is a member of the A30 subfamily, but because of a duplication of 32 amino acids relative to the A30 sequence, it encodes a 23-kd zein (Messing *et al.*, 1983). However, the Z4 DNA will also select out of endosperm total RNA those mRNAs transcribed from other genes in the A30 subfamily which encode mainly 19-kd zeins. Thus the pattern of the *in vitro* translation products which we find with the Z4-selected maize endosperm RNA corresponds to the results previously obtained by others (Park *et al.*, 1980; Lewis *et al.*, 1981).

Discussion

The results presented here show that a zein gene, when incorporated with T-DNA into the sunflower genome, is transcribed from its own promoter. We estimate from the autoradiogram of the Northern blot that the zein RNA is nearly as abundant as the nopaline synthase RNA in the engineered sunflower tissue. Murai *et al.* (1983) have recently reported that a bean storage protein (phaseolin) gene is also transcribed from its own promoter in transformed sunflower cells. Sunflower and bean are both dicots; maize is a monocot. Our data are thus the first evidence showing that the promoter of a monocot gene can be functional in a dicot host plant.

The results of the S1 nuclease mapping experiment demonstrate that the zein subfamily represented by the Z4 gene is apparently transcribed in maize endosperm. As discussed previously, the Z4 gene belongs to the A30 subfamily (Messing *et al.*, 1983). The members of this subfamily share 90–100% DNA sequence homology (Messing *et al.*, 1983) also in the 5′-untranslated regions (Hu *et al.*, 1982; Pedersen *et al.*, 1982). Since RNAs transcribed from Z4 and

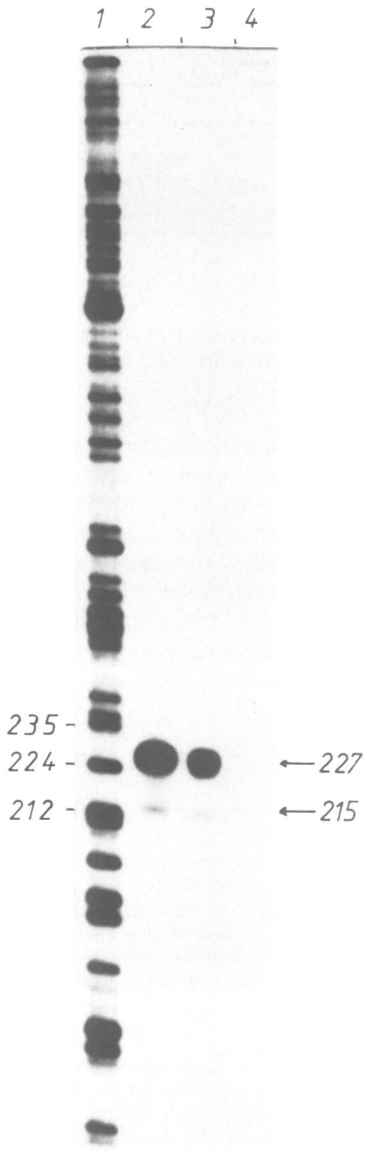

Fig. 5. S1 nuclease mapping of the transcription start of the Z4 gene in sunflower and maize. 50 μg of poly(A)-RNA from the engineered sunflower tissue (**lane 2**) or from normal sunflower leaves (**lane 4**) and 30 μg of total RNA isolated from maize W22 endosperm (**lane 3**) were hybridized for 12 h at 45°C to a denatured 1.1-kb *Eco*RI-*Bam*HI fragment containing the N-terminal fifth of the zein protein coding sequence and ~950 bp of 5′-flanking sequence. This DNA fragment had been 5′ end-labeled at the *Bam* site which is 170 bp downstream from the first nucleotide of the ATG start codon. Following the hybridization reaction, 600 units of S1 nuclease were added and the mixtures incubated for 90 min at 23°C. Fragments protected from S1 digestion were separated on a 6% polyacrylamide/7 M urea sequencing gel. Equal numbers of counts from the S1 reactions were loaded into the wells. **Lane 1** shows the C>T ladder of the part of the Z4 gene sequence which was used for sizing the protected fragments. The numbers on the left give the size, in nucleotides, of the indicated fragments in the sequencing ladder; the numbers on the right give the approximate size of the protected fragments. These numbers correspond to the distance, in nucleotides, of the 5′ end of the Z4 transcript from the labeled *Bam* site.

other A30 genes which are homologous to Z4 in their promoter regions might give similar results in an S1 nuclease mapping experiment, it is not possible to state with absolute certainty that the Z4 gene is actually transcribed in maize endosperm. Because of the high degree of homology among the members of the A30 subfamily, it must be emphasized

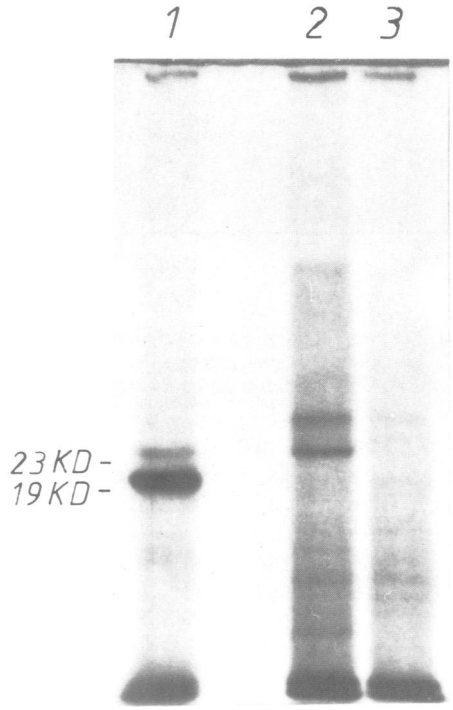

Fig. 6. Fluorogram of a polyacrylamide gel showing immunoprecipitated, *in vitro* translation products of Z4-selected RNA. The hybrid selection was performed with: **lane 1**, 30 μg of total RNA isolated from maize W22 endosperm; **lane 2**, 120 μg poly(A)-RNA isolated from the engineered sunflower tissue; **lane 3**, 210 μg of poly(A)-RNA isolated from normal sunflower leaves. The positions and sizes of a Coomassie blue stained zein standard run on the same gel are indicated by the bars on the left.

that we are comparing the start sites of transcription of the Z4 gene in sunflower with the transcription start sites of members of the A30 subfamily (including, possibly, Z4) which are transcribed in maize endosperm.

The results of the S1 nuclease mapping experiment also show that two transcriptional start sites of the Z4 (and/or related) gene(s) are used *in vivo*, not only in maize endosperm but also in the engineered sunflower cells. Figure 7 indicates these start sites and their positions relative to the two possible TATA boxes in the Z4 sequence (Hu *et al.*, 1982). Within the limits of this analysis, the positions of the transcriptional start sites for the Z4 gene and the ratio of the amounts of the two transcripts are similar in sunflower and maize.

The only data available concerning the trancription start sites of other zein genes are those of Langridge and Feix (1983). They determined the transcription starts of a zein genomic clone, pML1 (listed as a member of the B49 subfamily; Messing *et al.*, 1983), in maize endosperm and in HeLa and *Xenopus* extract systems (Langridge and Feix, 1983). Transcription of pML1 was shown to occur from two widely spaced promoters, and the phenomenon of double starts at each promoter was also observed. One pML1 transcript started either 52 or 65 bp upstream from the first nucleotide of the ATG start codon; another began either 1038 or 1048 upstream from the ATG. The DNA sequences of Z4 and pML1 are only 60–70% homologous; thus, the transcription of the two genes cannot be strictly compared. However, it is interesting to note that transcription begins at similar distances from the ATG start codon for the Z4 gene and for the shorter transcript of pML1. In addition, both genes are transcribed from double starts although, in the case

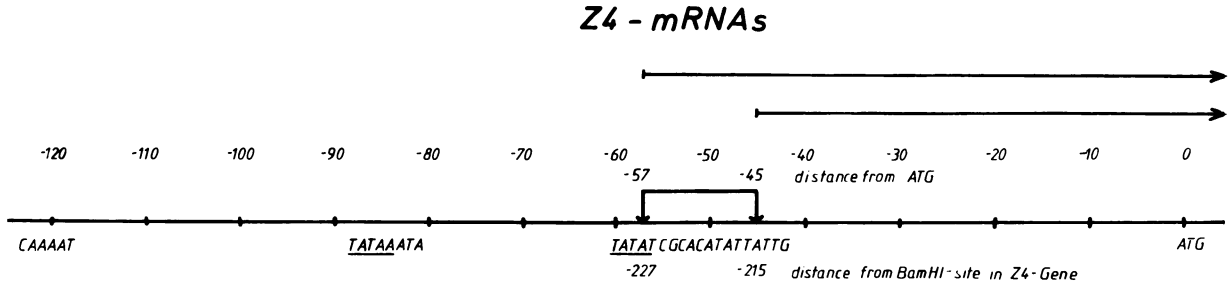

Fig. 7. Schematic representation of Z4 transcription start sites in sunflower and maize endosperm. The ATG start codon ('0') is shown at the far right. The two lines at the top indicate the start and direction of transcription. The distance of the transcription start sites from the ATG and the labeled *Bam*HI site are given. The two possible TATA boxes in the Z4 sequence (Hu *et al.*, 1982) are underlined.

of pML1, both start sites appear to be used with equal frequency (Langridge and Feix, 1983). We find no evidence for a second, longer transcript of Z4 in sunflower or maize. The function of these double starts is not known. It is possible that in this case they are due to the presence of two potential TATA boxes in the Z4 sequence (Hu *et al.*, 1982; see Figure 7). In any case, the signal for the double starts is recognized by both the maize and sunflower RNA polymerases. Since there are 50–100 genes in the zein family (Messing *et al.*, 1983), one cannot say at present whether the similarities found between Z4 (and/or other A30 genes) and pML1 transcription are typical for all zein genes.

No conclusions can be drawn at this time as to whether the zein transcript is translated in the engineered sunflower cells. Our extraction procedure for the zein protein did not give positive results. The zein RNA isolated from the sunflower tissue can be translated in a wheat germ system to yield a protein with a mol. wt. of ~24 000 and can thus be considered a functional mRNA. The 32-kd protein which is also visible in the translation products of Z4-selected poly(A)-RNA is obviously a sunflower protein with some homology with zein since this band is also visible in the translation products of normal sunflower poly(A)-RNA. This contention is also supported by the observation that a transcript somewhat larger than the zein transcript was visible after a long exposure of the Northern blot in lanes containing poly(A)-RNA from engineered and normal sunflower probed with the zein gene.

It is still possible that for some reason the zein mRNA is not translated in sunflower cells. If the zein protein is made in sunflower, our techniques may not be sensitive enough to detect it; alternatively, the protein could be made and then degraded. Murai *et al.*(1983) found degradation of phaseolin protein into two peptides in sunflower tissue engineered to contain the phaseolin gene. They also showed that authentic phaseolin was broken down into the same two peptides when incubated in a sunflower extract. When we added zein protein to a sunflower extract, however, no degradation was observed (data not shown).

The expression of the zein gene in maize, like that of phaseolin in bean, is normally highly regulated. Why then are both genes transcribed from their own promoter in undifferentiated sunflower tissue? In the case of zein, it is possible that the flanking sequences required for regulated transcription are not present in this construction. Other explanations can be envisioned, such as the lack of maize-specific regulatory molecules, or the insertion of the T-DNA containing the zein gene into a chromosomal location which is constitutively transcribed.

We do not know yet if it is possible to regenerate whole plants from the engineered sunflower tissue and we could therefore only test for zein gene expression in the undifferentiated callus. However, this Ti plasmid construction, with the insertion of genetic information into the *Hpa*I site of *Bam*HI fragment 14a, should allow the regeneration of whole tobacco plants from the transformed cells (Barton *et al.*, 1983). One could then test for tissue-specific expression in the regenerated plants. Such work is now underway with this and other zein constructions.

Materials and methods

Enzymes and chemicals

T4 DNA ligase and DNA polymerase (Klenow fragment) were purchased from New England Biolabs (Frankfurt, FRG). S1 nuclease was obtained from Bethesda Research Laboratories (Neu-Isenburg, FRG). Restriction enzymes were purchased from New England Biolabs, BRL or Amersham. All radiochemicals and a nick translation kit were obtained from the Radiochemical Centre (Amersham, UK). A 5′ end-labeling kit was purchased from New England Nuclear (Vienna, Austria). Mixed alkyltrimethylammonium bromide, oligo(dT)-cellulose and antibiotics were purchased from Sigma (Munich).

Bacterial strains and plasmids

The sources and descriptions of *A. tumefaciens* strain A208, ColE1::Tn5, *Bam*HI fragment 14a, pRK290 and R751pMG2 can be found in Barton *et al.* (1983). Bacterial growth media, plasmid isolation and procedures for conjugations and transformations are also described in Barton *et al.* (1983).

Inoculation of sunflower stemlets and cloning of sunflower tissue

Sunflower stemlets were inoculated with engineered agrobacteria and single cell cloning of the resultant outgrowth was performed as described in Binns *et al.* (1982). However, vancomycin instead of carbenicillin was used to free the transformed plant cells of the bacteria (the engineered agrobacteria are resistant to carbenicillin because of the presence of pBR325 sequences in the construction).

Plant DNA isolation and Southern hybridization

Plant DNA was isolated according to the procedure described in the Cold Spring Harbor Plant Molecular Biology Laboratory Manual (1982). Agarose gel electrophoresis and Southern blotting were performed as described by Thomashow *et al.* (1980). The 12 copy reconstruction mixture used for quantification of T-DNA was made with 10 μg of calf thymus DNA and the necessary amount of MINI-Ti DNA (de Framond *et al.*, 1983) based on a DNA content of 9.8 pg per diploid sunflower nucleus (Smith, 1977).

Plant RNA isolation and Northern blotting

Total RNA was isolated from engineered sunflower tissue and from normal sunflower leaves using the CTAB procedure described by Taylor and Powell (1982). Total RNA was isolated from W22 maize endosperm according to the procedure of Park *et al.* (1980). Poly(A)-RNA was selected by passage over oligo(dT)-cellulose. Formaldehyde agarose gel electrophoresis and Northern blotting was done according to Barinaga *et al.* (1981).

S1 nuclease mapping

This was done using the procedure of Maniatis *et al.* (1982).

Preparation of zein antiserum

Zein antiserum was prepared by injection of rabbits with 1–2 mg of purified zein protein (dissolved at a concentration of 1 mg/ml in 0.25% SDS and mix-

ed 1:1 with Freund's complete adjuvant). After 4 weeks, the injections were repeated except that Freund's incomplete adjuvant was used. Two weeks after the second injection, blood samples were taken and checked for zein antibodies. The rabbits were then bled by heart puncture and serum prepared.

Hybrid selection, in vitro translation and antibody precipitation

DNA-containing nitrocellulose disks [the DNA used was pBR325:zein (7.6 kb)] were prepared according to Cochet *et al.* (1979). *In vitro* translation was performed using the method of Roberts and Paterson (1973) with the modifications of Richter *et al.* (1980). Zein protein was precipitated from the *in vitro* translation reactions with zein antiserum. The precipitates were dissolved in sample buffer and run on 12.5% polyacrylamide gels in the presence of SDS (Laemmli, 1970). The gels were prepared for fluorography according to the procedure of Bonner and Laskey (1974).

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