Theor Appl Genet (1990) 80:43–48

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# Molecular cloning and chromosomal location of genes encoding the "Early-methionine-labelled" (Em) polypeptide of *Triticum aestivum* L. var. Chinese Spring

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Received January 30, 1990; Accepted March 6, 1990
Communicated by J. W. Snape

**Summary.** The "Early-methionine-labelled" (Em) polypeptide is the most abundant cytosolic polypeptide found in mature wheat embryos. Using a near full-length cDNA clone as a hybridisation probe to detect genomic sequences by Southern blotting of electrophoretic separations of genomic DNA derived from *Triticum aestivum* L. var. Chinese Spring and a series of its aneuploid derivatives, we demonstrate that the Em polypeptide is the product of a small multigene family in which the copies are located on each of the long arms of the homoeologous group 1 chromosomes. Screening of a variety of genotypes additionally reveals a number of restriction fragment length polymorphisms associated with these loci. Screening of a library of genomic DNA cloned in the vector λEMBL 4 has resulted in the isolation of a genomic fragment containing two closely linked Em genes. These are separated by ca. 2.5 kb. Analysis of restriction enzyme digests of this clones fragment has identified it as originating from chromosome *1A*.

**Key words:** Wheat – Em genes – Group 1 chromosomes – RFLPs

## Introduction

The "Early methionine-labelled" (Em) polypeptide is the single most abundant cytosolic polypeptide present in mature (dry) wheat embryos (Grzelczak et al. 1982). Accumulation of this polypeptide normally occurs during the later, "maturation" stages of embryogenesis ("stage 3" according to the morphological criteria of Rogers and Quatrano 1983). Expression of the Em genes is positively modulated, at the transcriptional level, by the plant growth regulator abscisc acid (ABA) (Williamson et al. 1985; Marcotte et al. 1988). Expression of Em genes is also induced in response to osmotic stress, both in immature embryos and during subsequent germination, with ABA potentially acting as an intermediate in this response (Morris et al. 1990). In this property and in its biophysical characteristics (McCubbin et al. 1985), the Em polypeptide shares many features with polypeptides identified in a number of plant species, which accumulate both during late embryogesis and in response to water stress (Baker et al. 1988; Dure et al. 1989). These characteristics have led to the proposal that the Em polypeptide has an important function in the tolerance of seeds to the programmed desiccation inherent in their maturation, and in the ability of seedlings to withstand periods of drought (Grzelczak et al. 1982; McCubbin et al. 1985; Dure et al. 1989; Morris et al. 1990).

In view of the potential importance of Em gene expression in contributing to the survival of plants suffering adverse environmental conditions, and because of the response of these genes to a specific developmental cue (ABA), it is important to understand the molecular mechanisms by which Em gene expression is elicited and maintained. We have therefore determined the chromosomal location of the Em genes, within the wheat genome, and to facilitate the molecular analysis of Em gene expression we have isolated, by molecular cloning, a genomic fragment in which two Em genes are closely linked.

## Materials and methods

### *Plant material*

*Triticum aestivum* L. var. "Chinese Spring" and homoeologous group 1 nullisomic-tetrasomic and ditelosomic lines derived

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from it (Sears 1966) were obtained from the collection maintained at The Institute of Plant Science Research, Cambridge Laboratory.

*Preparation of high-molecular-weight DNA*

*DNA isolation for molecular cloning.* Etiolated wheat shoots (5d after germination) were harvested and frozen in liquid nitrogen. After homogenising in a coffee grinder, the fine powder was resuspended (1 g per 10 ml) in 50 m*M* TRIS-Cl, pH 8.0, 0.7 *M* NaCl, 10 m*M* $Na_2EDTA$, 2% (w/v) sodium dodecylsulphate, 10 m*M* dithiothreitol, 0.1 mg $ml^{-1}$ Proteinase K and incubated at 37 °C for 1 h. The slurry was gently extracted with a ⅔ vol. of phenol saturated with 10 m*M* TRIS-Cl, pH 8.0, 1 m*M* $Na_2EDTA$. A ⅓ vol. of chloroform was added prior to centrifugation (1,600 g, 5 min) to separate the phases. Extraction of the aqueous phase was repeated twice, followed by overnight digestion with 40 μg $ml^{-1}$ RNase A at room temperature. DNA was recovered by spooling on a glass rod, following addition of an equal volume of ethanol, and purified by CsCl density gradient centrifugation and subsequent dialysis against 10 m*M* TRIS-Cl, pH 8.0, 1 m*M* $Na_2EDTA$.

*DNA isolation for Southern blotting.* DNA was extracted from samples of wheat leaves, essentially as described by Sharp et al. (1988).

*Southern blot analysis of wheat genomic DNA*

Wheat DNA (5 μg) was digested using 5 units of enzyme per microgram DNA. Digested DNA was separated by electrophoresis in 0.8% agarose gels, and transferred to Genescreen Plus (New England Nuclear) nylon membrane by alkaline transfer (Reed and Mann 1985). Prehybridisation, hybridisation and washing was performed essentially as described previously (Sharp et al. 1988) using a probe labelled with $^{32}P$ by the "oligo-labelling" method of Feinberg and Vogelstein (1983). Detection of Em-specific bands was achieved by using as a probe the Em cDNA sequences cloned in the plasmids p10–15 (Williamson et al. 1985). Hybridising sequences were detected by autoradiography using Kodak XAR-5 X-ray film.

*Construction of genomic libraries*

The protocols followed for the generation of genomic DNA libraries were those described by Maniatis et al. (1982). High-molecular-weight DNA was digested to completion with EcoRI and subjected to preparative agarose gel electrophoresis. DNA fragments of 15–20 kb, which were found to hybridise with the Em-specific cDNA probes in Southern blot experiments, were eluted electrophoretically from the appropriate segments of the gel.

Genomic DNA fragments were ligated with λEMBL 4 arms obtained by digestion with EcoRI and treated with alkaline phosphatase. The ligated DNA was packaged into phage heads and propagated on *E. coli* strain K803, and the resulting plaques were screened by hybridisation with the Em cDNA insert from p10–15. DNA isolated from phage derived from positively hybridising plaques was analysed by agarose gel electrophoresis and Southern blot hybridisation. Appropriate fragments were subcloned into the plasmid pUC 18 for further detailed analysis by restriction endonuclease digestion. In addition to Southern blot hybridisation using Em cDNA as a probe, a synthetic oligonucleotide, constructed using an Applied Biosystem model 381A oligonucleotide synthesiser, was prepared corresponding to the first 18 nucleotides of the Em protein-coding sequence (Litts et al. 1987). This was radioactively labelled using polynucleotide kinase and γ-[$^{32}P$]-ATP, for hybridisation with Southern blots of subcloned genomic fragments. This hybridisation was performed at 20 °C in 6 × SSC, 5 × Denhardt's solution, and the filter was washed at 45 °C with 6 × SSC (equivalent to $T_m$ −20 °C).

## Results and discussion

*Genomic organisation of Em genes*

Southern blot analysis was used to determine the organisation of Em genes within the wheat genome. Genomic DNA was isolated from euploid wheat (var. Chinese Spring) and digested to completion with HindIII, a restriction enzyme that has no recognition site within the Em cDNA sequence in plasmid p10–15 (Litts et al. 1987). Following agarose gel electrophoresis, blot transfer and hybridisation with the p10–15 sequence, ten strongly hybridising fragments were identified in the Chinese spring genome, with approximate sizes as indicated in Fig. 1.

Initial screening localised the Em-specific fragments to the homoeologous group 1 chromosomes. The chromosomal location of each fragment was then determined by the pattern of hybridisation with DNA isolated from lines that were nullisomic-tetrasomic for chromosomes of homoeologous group 1. Further intrachromosomal localisation of the Em genomic sequences was obtained by screening DNA from wheat lines in which particular pairs of chromosomes were ditelocentric. The results are shown in Fig. 1. Lines nullisomic for chromosome *1D* and tetrasomic for chromosome *1B* (Fig. 1: N1DT1B) lacked the 13.8-kb, 8.9-kb and 8.3-kb fragments, but showed more intense hybridisation (corresponding with the increased dosage) of the 14.8-kb, 11.2-kb and 4.4-kb fragments. Analysis of the ditelosomic lines demonstrated that the Em genomic sequences were derived from the long arms of the group 1 chromosomes. Thus, in DNA from the DT1BL line, there was no change in the pattern of hybridising fragments, whereas in DNA from the DT1BS line, the 14.8-kb, 11.2-kb and 4.4-kb chromosome *1B* fragments were missing. The assignment of the chromosome *1D*-specific fragments to the long arm of this chromosome was inferred from the observation that no fragments were deleted when DNA from the DT1DL line was analysed; the DT1DS line was not available for analysis. In addition to the ten strongly hybridising fragments observed, two additional, weakly hybridising fragments were observed with sizes of ca. 4.6 kb and 3.0 kb (marked "?" Fig. 1). These bands were apparent in DNA from all the group 1 nullisomic-tetrasomic lines, and so must derive from another homoeologous chromosome group. We have not determined the origin of these fragments.

Thus, the Em cDNA probe used here identifies a set of homoeoloci which are designated *XEm-1A, XEm-1B*

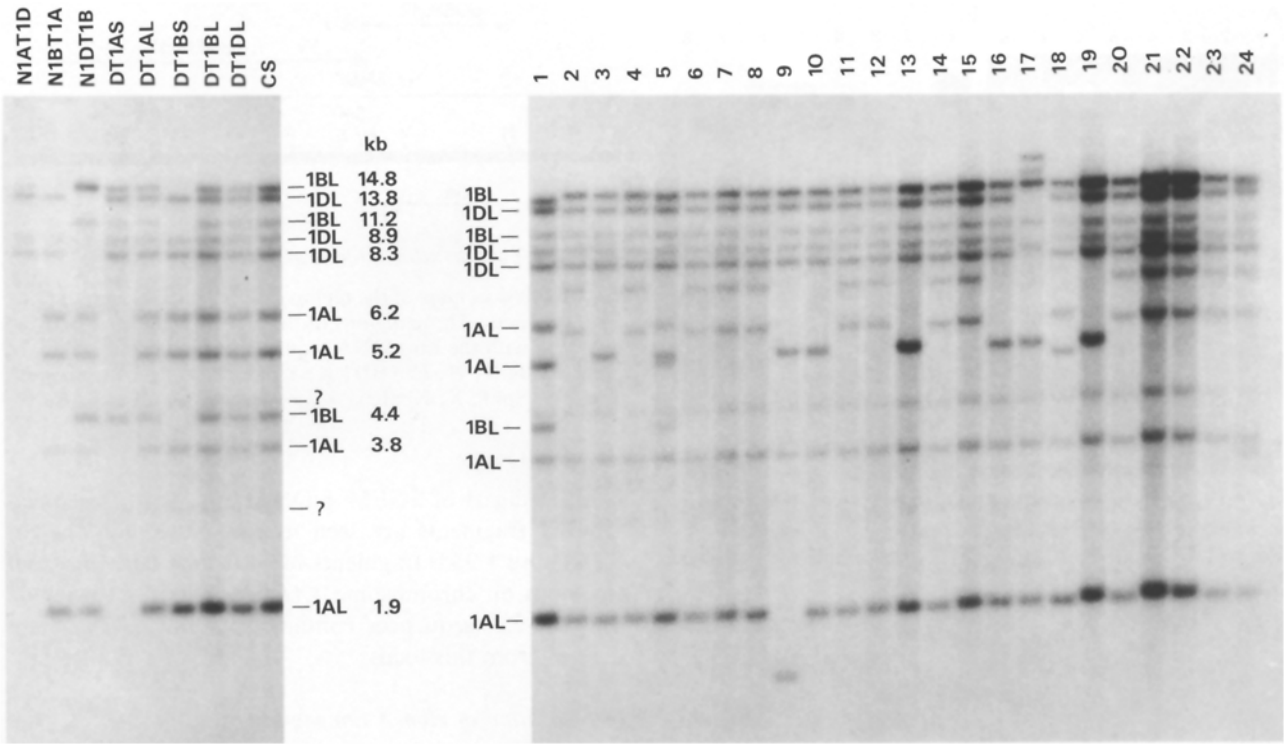


**Fig. 1** **Fig. 2**

**Figs. 1 and 2.** **1** Hybridisation pattern of Em cDNA sequences with HindIII-digested DNA of "Chinese Spring" and its aneuploids. The lines are indicated above the tracks. (CS = Chinese Spring; N1AT1D = nullisomic *1A*-tetrasomic *1D* etc,; DT1AS = ditelosomic for *1AS* etc.). The deduced chromosomal locations and estimated fragment lengths are indicated on the *right*. **2** RFLPs detected in HindIII digests of DNA from various wheat genotypes. The chromosomal location of the Chinese Spring fragments is indicated on the *left*. *Track 1*: Chinese Spring; *2*: Glennson; *3*: Maris Ranger; *4*: Sicco; *5*: Chinese Spring × Maris Sportsman $F_1$; *6*: Bersee; *7*: VPM1; *8*: Maris Huntsman; *9*: Sava; *10*: Capelle-Desprez; *11*: Manella; *12*: Bezostaya; *13*:Timgalen; *14*: Hobbit; *15*: Dwarf A; *16*: Mara; *17*: "Synthetic" (amphiploid from *Triticum dicoccum* AABB × *Aegilops squarrosa* DD; McFadden and Sears 1946); *18*: Jitpur; *19*: Minister Dwarf; *20*: Cheyenne; *21*: Poros; *22*: Avalon; *23*: Moulin; *24*: Sunstar

and *XEm-1D*, in accordance with the system for the nomenclature of wheat loci (Hart and Gale 1988). We have screened 24 wheat genotypes for restriction fragment length polymorphisms (RFLPs) at these loci as detected in HindIII digests (Fig. 2).

By comparing the pattern of hybridising fragments with that of Chinese Spring, an indication of the location of variation can be obtained. Almost all the varieties studied do not have the 14.8-kb and 4.4-kb *1BL* fragments of Chinese Spring, but possess a fragment larger than 14.8 kb. This fragment probably represents a single fragment of 14.8 + 4.4 = 19.2 kb, in which two regions of homology with the cDNA sequence are brought together due to the loss of an interstitital HindIII site. The variety "Sunstar" (track 24) appears to have a different *1BL* polymorphism, with the loss of the 4.4-kb fragment and the gain of a fragment slightly shorter than the 3.8-kb *1AL* fragment. At the *1AL* locus, the 3.8-kb fragment was present in all varieties studied, the 1.9-kb fragment decreased in size in "Sava" (track 9) and the 6.2- and 5.2-kb fragments of Chinese Spring were replaced either by a large doublet (e.g. track 2) or by a single fragment (e.g. track 3) in all varieties except Jitpur. Only in the "Synthetic" line was there variation at the *1DL* locus, the polymorphism originating from the D genome contributed by *Aegilops squarrosa* (McFadden and Sears 1946).

### *Molecular cloning of Em genomic sequences*

Digestion of Chinse Spring DNA with the restriction enzyme EcoRI, followed by subsequent Southern blot analysis, revealed that the majority of genomic Em-specific fragments were between ca. 15 kb and 20 kb in length (data not shown; Litts et al. 1987). Since fragments of this size are convenient for cloning into the λ replacement vectors λEMBL 3 and λEMBL 4, such fragments were isolated by preparative agarose gel electrophoresis of an EcoRI digest of genomic DNA. Following elution of fragments from the gel, ligation with λEMBL 4 arms, packaging into phage heads and propagation, an unamplified library of ca. $6.10^5$ plaques was obtained. This was screened by hybridisation with the cDNA sequence from the plasmid p10–15, and a single hybridising plaque was identified.

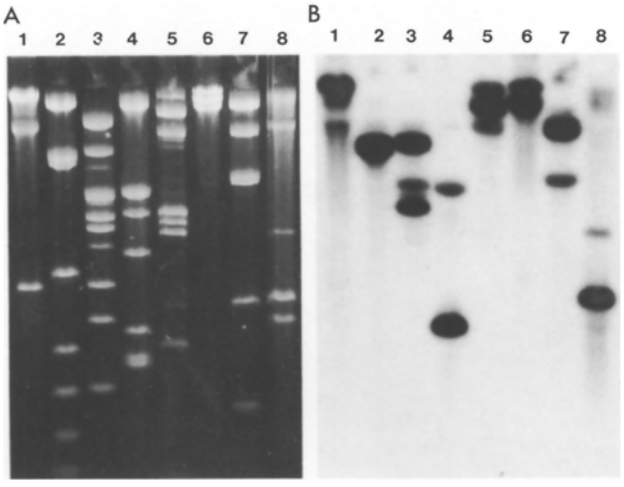


**Fig. 3 A and B.** Southern blot analysis of λGEM 1 DNA digested with a panel of restriction enzymes. **A** stained gel; **B** autoradiograph. *1*: BamHI; *2*: BglII; *3*: ClaI; *4*: HindIII; *5*: PstI; *6*: SalI; *7*: SmaI; *8*: XbaI

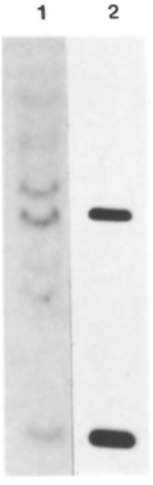


**Fig. 4.** Identification of cloned Em sequences in HindIII digests of genomic wheat DNA and λGEM 1 DNA. *Track 1*: genomic DNA; *track 2*: λGEM 1 DNA

Following plaque purification, DNA isolated from the positive phage (designated λGEM 1, for Genomic Em) was digested with restriction endonucleases and analysed by agarose gel electrophoresis and Southern hybridisation with Em cDNA probes. Southern hybridisation of λGEM 1 DNA revealed a number of hybridising fragments, depending on the enzyme used for digestion (Fig. 3); from these and other digestions (data not shown), the overall length of the cloned fragment was estimated to be 15 kb.

Two hybridising fragments were produced following digestion with HindIII, corresponding to sizes of ca. 5 kb and 2 kb, respectively. When a HindIII digest of Chinese Spring genomic DNA was compared directly with a

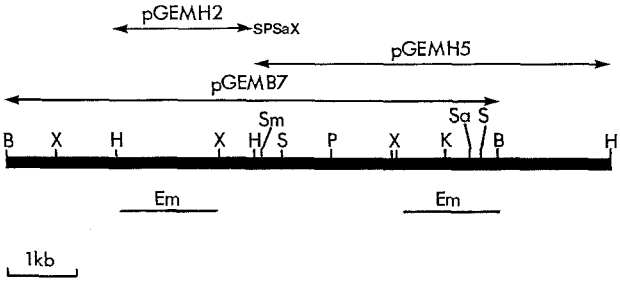


**Fig. 5.** Physical map of the region of λGEM 1 DNA containing EM sequences. Those regions of the cloned sequences found to hybridise with the Em cDNA sequence are indicated below the map. B: BglII; H: HindIII; K: KpnI; P: PstI; Sa: SalI; Sm: SmaI; S: SphI; X: XbaI

HindIII digest of λGEM 1 DNA (Fig. 4), these cloned HindIII fragments are seen to comigrate with the ca. 5.2 kb and 1.9 kb fragments identified as deriving from the locus on chromosome *1AL*. We therefore conclude that the Em sequences contained within λGEM 1 are derived from this locus.

*Organisation of cloned Em sequences*

To facilitate further analysis of the sequences cloned in λGEM 1, the two hybridising HindIII fragments and a single, 7-kb BglII fragment were subcloned in the plasmid pUC18 (the BglII fragment by ligation into the BamHI site of the vector). Digestion of each of these recombinant plasmids (designated pGEM H5, pGEM H2 and pGEM B7, respectively), with the enzymes known to cut at single sites within the multiple cloning site of the vector, enabled a map of these sites within the cloned sequence to be determined. This map is presented in Fig. 5. The extent of the pGEM H2, pGEM H5 and pGEM B7 subclones is indicated above the map, as is the disposition of these sequences relative to the restriction sites within the multiple cloning site.

The Em-encoding sequences within λGEM 1 were defined by Southern blot analysis of these digests. The results of this analysis indicated that there were two separate regions that hybridised with the Em cDNA. These regions of hybridisation are indicated in Fig. 5, and the evidence for the extent of their physical separation is presented in Fig. 6.

Digestion of pGEM H2 and pGEM H5 with HindIII released the cloned inserts, each of which hybridised with the cDNA probe. Digestion of pGEM B7 with HindIII released a nonhybridising 1.5-kb fragment, corresponding to the leftmost Bgl II-HindIII fragment in Fig. 5, a 1.9-kb HindIII fragment that hybridised with Em cDNA and that was identical with the fragment cloned in pGEM H2, and a ca. 6-kb fragment corresponding to the rightmost 3.2-kb HindIII-BglII fragment fused with the vector DNA (Fig. 6, tracks 1–3).

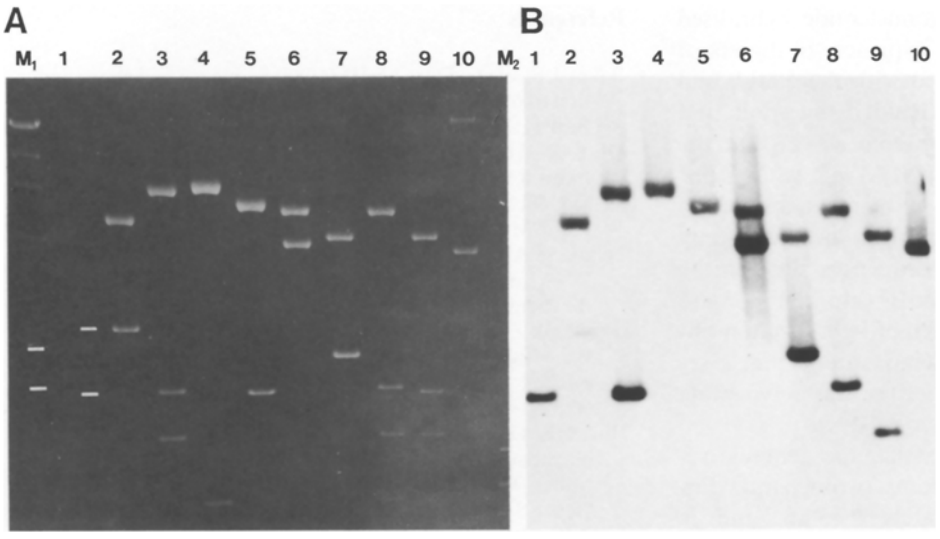


**Fig. 6 A and B.** Hybridisation patterns of Em sequences following digestion of plasmid subclones. *1*: pGEM H2 + HindIII; *2*: pGEM H5 + HindIII; *3*: pGEM B7 + HindIII; *4*: pGEM H5 + PstI; *5*: pGEM H5 + XbaI; *6*: pGEM B7 + PstI; *7*: pGEM B7 + XbaI; *8*: pGEM B7 + HindIII/PstI; *9*:pGEM B7 + HindIII/XbaI; *10*: pGEM H2 + XbaI. Tracks marked *M* contain restriction fragment size markers, respectively $M_1$: $\lambda$ DNA + HindIII: 23.1, 9.4, 6.5, and 4.4 kb 2.3, and 2.0 kb; $M_2$: $\phi$X 174 DNA + HaeIII 1353, 1096, and 872 bp. **A** Stained gel; **B** autoradiograph. The *bars in tracks* $M_1$ and *1* of **A** mark the positions of bands that were of insufficient staining intensity for photographic reproduction

Digestion of pGEM H5 with PstI, an enzyme that cleaves at a single site within the cloned sequence (Fig. 5), released a nonhybridising fragment of 1kb and a hybridising fragment of ca. 6 kb corresponding to the vector DNA fused with the remaining cloned sequence (Fig. 6, track 4). Digestion with XbaI, which has two closely adjacent cleavage sites within the insert (Fig. 5), released a nonhybridising fragment of 2 kb and a hybridising fragment of ca. 5.6 kb corresponding to a vector-insert fusion (Fig. 6, track 5). These results indicated that the the 1.9-kb HindIII fragment showing homology with Em cDNA, located at the left end of the map in Fig. 5, was separated from the region of Em homology within the 5-kb HindIII fragment located at the right end of the map.

This conclusion was confirmed by the results of hybridisation with fragments derived by digestion of the plasmid pGEM B7, shown in Fig. 6 (tracks 6–9). Digestion with PstI produced two fragments (ca. 5.2 and 4.2 kb), each of which hybridised with the probe. Digestion with XbaI released two nonhybridising fragments of ca. 0.6 and 2.3 kb, and two hybridising fragments of ca. 2.3 and 4.4 kb. Double-digestion with HindIII yielded the 1.9-kb HindIII hybridising fragment in the case of HindIII–PsI digestion, and a 1.5 kb HindIII–XbaI fragment (track 9) due to the presence of an XbaI site within the 1.9-kb HindIII fragment (track 10). No hybridisation occurred with the 400-bp fragment released by digestion of pGEM H2 with XbaI, thus limiting the extent of Em homology further to within the 1.5-kb sequence bounded by the HindIII and XbaI sites.

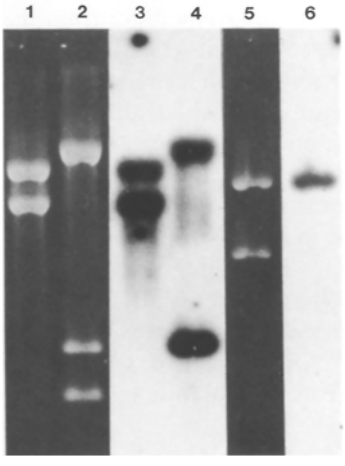


**Fig. 7.** Localization of N-terminal Em-coding sequences in plasmids pGEM B7 and pGEM H5 using oligonucleotides encoding the first six amino acids of the Em polypeptide. *1+3*: pGEM B7 + PstI; *2+4*: pGEM B7 + HindIII; *5+6*: pGEM H5 + KpnI. Tracks *1*, *3*and *5* show the stained fragments. Tracks *2*, *4* and *6* show the autoradiograph

These two regions of Em homology are separated by ca. 2.5 kb of nonhomologous DNA in $\lambda$GEM I. These could correspond either to two separate Em genes, or to coding regions of the same gene separated by a long intervening sequence. In order to distinguish between these possibilities, digests of the cloned sequences were hybridised with a synthetic oligonucleotide corresponding to the first six codons of the Em mRNA, derived from the published sequence of p10–15 (Litts et al. 1987).

Figure 7 shows that the oligonucleotide hybridised with each region of Em-specific sequence. In digests of pGEM B7, hybridisation occurred with the 5.8-kb and 1.9-kb fragments resulting from HindIII digestion, and with the two fragments resulting from cleavage at the single PstI site. Digestion of pGEM H5 with KpnI yielded a 4.7-kb vector-insert fusion fragment, and a 3-kb insert-specific fragment. Hybridisation occurred only with the former fragment. Since sequences between the BglII site and the rightmost HindIII site in Fig. 5 are absent from pGEM B7, the pattern of hybridisation obtained with the two subclones demonstrated that a sequence encoding the N-terminus of the Em polypeptide lies between the KpnI site and the BglII site.

We conclude from these data that the genomic sequence cloned in λGEM 1 contains two separate Em genes, separated by approximately 2–3 kb, and is derived from the long arm of chromosome *1A*. This conclusion is supported further by the results of sequence analysis (as yet incomplete), indicating that the Em-homologous regions of pGEM H2 and pGEM H5 encode separate transcripts (T. S. Futers, A. C. Cuming, unpublished results). From an inspection of the restriction sites surrounding these hybridising sequences, it is apparent that the Em genes isolated in this study differ from the Em gene described by Marcotte et al. (1988), which is characterised by a pair of KpnI sites that straddle the Em promoter region. This region has been identified by its ability to direct the transcription of a reporter gene (encoding the enzyme β-glucuronidase), in an ABA-dependent manner, following transfection of rice protoplasts. It will clearly be of interest to identify similar regulatory sequences associated with the Em coding sequences contained within λGEM 1 DNA. Additionally, sequence analysis of these cloned fragments will enable us to determine whether "consensus" ABA-responsive sequence elements of the type described by Marcotte et al. (1989) can be identified.

*Acknowledgements.* We are grateful to the Science and Engineering Research Council for its continued support of this work. We also thank Dr. T. Gallagher (University College, Dublin) and Dr. Cathie Martin (John Innes Institute) for the provision of λ vectors and their advice and encouragement in the early stages of this project.

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