PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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The expression of heat-shock genes in higher plants

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High-temperature stress or heat shock induces the vigorous synthesis of heat-shock proteins in many organisms including the higher plants. This response has been implicated in the acquisition of thermotolerance. The biological importance of a group of low-molecular-mass proteins in the response of plants is indicated by the conservation of the corresponding genes. The steady-state levels of mRNAs for these proteins shift from undetectable levels at normal temperature to about 20000 molecules per gene in the cell after heat shock. The analysis of 'run-off' transcripts from isolated soybean nuclei suggests a transcriptional control of gene expression. The DNA sequence analysis of soybean heat-shock genes revealed a conservation of promoter sequences and 5′-upstream elements. A comparison of the deduced amino acid sequences of polypeptides showed a conservation of structural features in heat-shock proteins between plants and animals. The implication of a common regulatory concept in the heat-shock response makes genes belonging to this family (15–18 kDa proteins) in soybean favourable candidates for investigating thermoregulation of transcription. We have exploited the natural gene transfer system of *Agrobacterium tumefaciens* to introduce a soybean heat-shock gene into the genomes of sunflower and tobacco. The gene is thermoinducibly transcribed and transcripts are faithfully initiated in transgenic plants. Experiments are in progress to define the regulatory sequences 5′-upstream from the gene. The expression of heat-shock genes in a heterologous genetic background also provides the basis for studying the function of the proteins and their possible role in thermoprotection.

0. Introduction

The heat-shock (hs) response is an instantaneous reprogramming of cellular activities (for a review see Nover *et al.* 1984). The synthesis and accumulation of heat-shock proteins (hsps) is frequently considered as a criterion for this response in living organisms. By definition, hsps are new proteins abundantly synthesized in response to hs. The specific biological functions of the different hsps are still unknown, although they are generally considered to confer thermal tolerance to potentially lethal heat treatment. Direct experimental evidence, though limited to mutant strains of *Escherichia coli*, yeast and *Dictyostelium*, suggests that the synthesis of certain hsps is essential for cell growth at elevated temperatures. There is only correlative evidence to suggest that hsps might play a part in the development of thermal tolerance in higher plants. In many organisms other stresses including ethanol, arsenite, anoxia and heavy metal ions will also evoke the synthesis of hsps. However, in higher plants, taking soybean as a model system, only arsenite elicits a response similar to that induced by hs (Lin *et al.* 1984). The pattern of hsps synthesized in plants differs from that in animals with respect to the abundance, complexity and size range of a group of low-molecular-mass (lmm) hsps (see, for example, Schöffl *et al.* 1984*a*; Key *et al.* 1983*a*, *b*, 1985*a*). This feature of the hs response in plants was originally discovered in soybean (Key *et al.* 1981; Schöffl & Key 1982) and most of our studies have focused on the lmm-hsps of this species. Although progress has been made in detecting the

hs phenomena in other plants, only soybean has been subsequently analysed at the RNA level (Schöffl & Key 1982) and hence to the gene level (Schöffl & Key 1983) to study the expression of these hsps in detail. The close parallels of the molecular characteristics of hsps and hs genes between soybean and other plants justify the emphasis given to soybean as a model system. In animal systems, most investigations have focused on the high-molecular-mass hsps, predominantly on the highly conserved hsp70, which is perhaps one of the most conserved proteins in Nature and its biological significance is probably the same in all organisms. In what follows we summarize briefly the current state of knowledge of the hs response in plants, with emphasis on the molecular analysis of genes for lmm-hsps in soybean. We shall also address the issues of gene transfer into heterologous plants by using different Ti plasmid vector systems and the analysis of transgenic plants.

1. Heat shock proteins and thermotolerance

Different plant species, including soybean (Barnett *et al.* 1980; Key *et al.* 1981), tomato (Scharf & Nover 1982; Nover & Scharf 1984), maize (Cooper & Ho 1983; Key *et al.* 1983*a*), tobacco (Barnett *et al.* 1980; Meyer & Chartier 1983; Kanabus *et al.* 1984), cotton (Key *et al.* 1983*a*; Burke *et al.* 1985), pea and millet (Key *et al.* 1983*a*) have been shown to undergo a transition in their protein synthesis upon an elevation of temperature of approximately 10 °C above their optimum growth temperature. When the temperature of 3-day-old etiolated soybean seedlings is increased from 28 to 40 °C the synthesis of different sets of hsps (molecular masses 15–18, 21–24, 27, 68–70, 84 and 92 kDa) predominates with only minimal synthesis of most of the proteins normally found at 28 °C (Key *et al.* 1981). The induction of hsp synthesis is very rapid with a maximum increase within the first 30–60 min and is continued for 6–8 h. After this time the pattern of normal protein synthesis returns. When seedlings are returned to 28 °C after an incubation at 40 °C (2–4 h), there is a rapid decline in hsp synthesis concomitant with a reappearance of normal proteins. The induction of hsp synthesis does not require an abrupt shift in the temperature; a gradual increase (simulating field conditions) is equally effective (Kimpel & Ley 1985*a*). The minimum temperature at which the hsps can be detected (37 °C) and the temperature at which full expression of the response is obtained (40 °C) are very similar in both types of experiment. There is a strong correlation between the synthesis and accumulation of hsps in soybean seedlings and the ability to survive short periods (2 h) of hs at otherwise lethal temperatures (e.g. 2 h, 45 °C) (Lin *et al.* 1984; Key *et al.* 1983*a*, *b*, 1985*b*). A short heat pulse (10 min, 45 °C) also triggers a transient synthesis of hsps to high levels provided that the seedlings are returned to 28 °C for 2–3 h (Lin *et al.* 1984). Interestingly, in these experiments, thermal tolerance develops during the recovery period, at which time hsps are being synthesized. Thermal tolerance can also be generated in seedlings by arsenite treatment (50 μM, 3 h) at normal temperature (Lin *et al.* 1984). Arsenite is the only chemical known to induce the full set of hsps in soybean (Key *et al.* 1985*a*, *b*). This strong correlation between the synthesis of hsps and thermal tolerance cannot entirely exclude the possibility that other factors, concomitant with the appearance of hsps, are responsible for the acquisition of thermal tolerance. Support for an involvement of alternative factors comes from a notable exception to the rule that all plant tissues synthesize the full spectrum of hsps. The pollen from *Tradescantia* does not synthesize hsps but seems to develop thermotolerance in pollen tube growth (Xiao & Mascarenhas 1985). However, there is so far no clear pattern

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

emerging from the studies of hsp synthesis in pollen. Lily pollen seems to synthesize hsps in the absence of proline (Zhang *et al.* 1984) and maize pollen synthesizes hsps that are atypical of other tissues of the plant (Cooper *et al.* 1984). It is possible that the development of thermal tolerance is the result of synergism between different factors (mechanisms) in the cell, with hsps playing a crucial role. The lack of suitable mutations in hs genes of higher eukaryotes renders it difficult to determine the function of hsps. The recently identified ATPase activity of hsp70 may be crucial for the functions of this conserved protein in multiple processes during hs and recovery (Pelham 1984; Lewis & Pelham 1985). The functional significance of other hsps is less well established but there is convincing evidence that the synthesis of a cluster of lmm-hsps seems to be essential for the development of thermotolerance in *Drosophila* (Berger & Woodward 1983) and *Dictyostelium* (Loomis & Wheeler 1982). In this light, attention needs to be given to the lmm-hsps in plants. All plants so far tested synthesize a number of highly abundant hsps with molecular masses ranging from 15 to 20 kDa (see table 1) and they show complex patterns on 2D O'Farell gels (Key *et al.* 1983*a*). These features make this group of proteins unique to plants, and their conservation of peptide structures is indicated by the sequence homologies between the corresponding genes (see §§2 and 3). The high-molecular-mass proteins (68–92 kDa) are less abundant in plants and they seem to be very similar as judged by their electrophoretic mobilities and immunological cross-reactions (Kimpel & Key 1985*b*). One of the unifying features of the 15–18 kDa hsps in soybean is their differential localization in the cell, which depends on the incubation temperature of the tissue. At temperatures above 40 °C these proteins co-purify with subcellular organelle fractions for nuclei, mitochondria and ribosomes (Lin *et al.* 1984). At low temperatures (30 °C) they dissociate from these fractions and become enriched in the post-ribosomal supernatant. Repeated cycles of hs and recovery relocate the previously formed proteins in the respective fractions (Key *et al.* 1982; Lin *et al.* 1984). The mechanism of this reversible aggregation and the biological functions of these processes are not yet understood. It is possible that the lmm-hsps self-aggregate to form large globular structures, known as hs granules in tomato cells (Nover *et al.* 1984) and soybean seedlings (Kimpel & Key 1985*b*). Stressing the analogy with *Drosophila*, lmm-hsps may become transient constituents of RNPs (Arrigo *et al.* 1985) and/or cellular matrices (Leicht *et al.* 1986). It should be noted that two other hsps from soybean (21 and 24 kDa) become specifically associated with mitochondria. Maize, pea and soybean synthesize abundant cytoplasmic polypeptides between 21 and 27 kDa, which are translocated into the chloroplast (Kloppstech *et al.* 1985; Vierling *et al.* 1986). The suggested processing of the protein precursor during transport would be consistent with their irreversible localization within the organelle. Another

TABLE 1. LOW-MOLECULAR-MASS HSPS IN PLANTS

species	molecular mass/kDa	hs temperature °C	reference
soybean	15–18	40–42	Key *et al.* (1981)
pea	17–20	37, 5–40	Key *et al.* (1983*a*)
maize	16–18	40	Cooper & Ho (1983)
millet	15–18	42–47	Key *et al.* (1983*a*)
cotton	21	41–46	Key *et al.* (1983*a*)
tomato	15–18	38–40	Nover & Scharf (1984)
tobacco	15–18	38–42	Kanabus *et al.* (1984)
Vicia faba	20	37, 5–40	F. Schöffl & E. Tacke (unpublished)
Tradescantia	16	41	Xiao & Mascarenhas (1985)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

set of proteins (27 kDa) is related to the hs response but its synthesis is also induced by other chemical or physical stresses in soybean. These proteins remain always in the 'soluble' fractions of the cytoplasm (Key *et al.* 1985*a*). The function of these proteins is not known. Their cellular levels, increasing 3–10-fold above normal control levels by any physical or chemical stress may reflect the level of common stress endured by soybean cells. No homologous counterparts have been yet identified in other plants.

2. mRNAs, transcriptional control and gene families

The reprogramming of protein synthesis during hs has been repeatedly investigated in many plant systems. The major characteristics of the hs response in soybean are (i) a rapid dissociation of polysomes followed by a reassociation of new polysomes (ii) the vigorous synthesis of hsps and (iii) the reduced capacity of cells to synthesize most normal proteins, even though most of their mRNAs were preserved during hs (Key *et al.* 1981). Only a few studies have addressed the question of how the reprogramming of gene expression is manifested at the RNA level. Two approaches have been used in the soybean system by Schöffl & Key (1982): (1) analysis of the overall changes in complexity and abundance distribution of poly(A)$^+$ RNA between hs (40 °C) and control tissue (28 °C) by kinetic hybridizations between RNAs and the corresponding cDNAs and (2) characterization of individual hsp-mRNA by using cDNA cloning, Northern blot analysis and hybrid-select translation. The overall analysis of RNA populations revealed the induction of a new kinetic class of highly abundant hs-specific poly(A)$^+$ RNA. The low complexity (1.8×10^4 nucleotides) in this kinetic component has the capacity for only 13–20 average-sized mRNAs. The proportion of these RNAs in total cellular poly(A)$^+$ RNA is increased to approximately 20% in 2 h, corresponding to 20000 molecules per sequence in the cell. Surprisingly, only the mRNAs for small hsps (15–24 kDa) and for the general stress proteins (27 kDa) seem to be present in this fraction. Hence mRNAs for the high-molecular-mass hsps have a lower abundance (presumably by a factor of 10) in the cell. The induction of a new abundance class for hs-specific mRNAs contrasts with the concomitant reduction in overall complexity of poly(A)$^+$ RNA. This reduction may be due to a general decline of steady state levels of non-hs RNAs. This hypothesis would be consistent with a significant preservation of many highly abundant mRNAs for normal proteins during hs (Key *et al.* 1981; Vierling & Key 1985). An alternative hypothesis proposes an hs-specific decline of selected mRNAs with no or little effect on other RNAs. This model is supported by significant decreases of auxin-regulated RNAs in soybean (Key *et al.* 1985*b*; Schöffl *et al.* 1986), α-amylase mRNA in barley (Belanger *et al.* 1986) and elicitor-induced sequences in parsley (Hahlbrock *et al.* 1985), whereas actin mRNAs in soybean do not decline (Key *et al.* 1985*b*). The mechanisms controlling the differential steady-state level of mRNAs during hs are not entirely understood. Transcriptional regulation seems to be involved in the decline of rRNA and auxin-responsive RNAs in soybean (Schöffl *et al.* 1986).

The construction of a cDNA library and the isolation of hs-specific clones (Schöffl & Key 1982) made it possible to analyse steady-state levels of mRNAs and transcriptional control of hs genes. A number of cDNA clones have been classified by their patterns of cross-hybridizations and by their proteins translated *in vitro* from hybrid-select mRNAs (Schöffl & Key 1982, 1983; Schöffl *et al.* 1984*a*; Czarnecka *et al.* 1984; Key *et al.* 1985*a*). At least four multigene families encoding 15–18 kDa hsps have been identified so far (table 2). The class

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

I family is represented by six individual cDNA clones, each of which corresponds to one out of 13 different lmm-hsps (15–18 kDa). One 18 kDa protein, previously thought to be unique (class II, described by Schöffl & Key (1982, 1983) and Schöffl *et al.* (1984*a*)) also meets the characteristics of a class I hsp as shown by double labelling of polypeptides and DNA sequencing (Key *et al.* 1985*a*). Another multigene family (class VI) seems to be closely related to class I by sequence homologies of genes (Key *et al.* 1985*a*). It is not known whether there is an overlap between the proteins of the two families or whether they are fully complementary to each other. There is probably an overlap in sequence homology with hsps of the two other small gene families (Key *et al.* 1985*a*) encoding three or four polypeptides between 21 and 24 kDa and four or five of 27 kDa (see table 2).

TABLE 2. MULTIGENE FAMILIES FOR HSPS IN SOYBEAN

family[1]	cDNA clones[2]	number of proteins[3]	size/kDa
class I	pFS1920	13	15–18
	pFS2059	—	—
	pFS1968	—	—
	pFS2005	—	—
	pFS2019	—	—
	pFS2026	—	—
	pCE53	—	—
class IV	pFS2033	3–4	21–24
class VI	pEC75	8–9	15–16
class III	pEC54	4–5	27
	pEC55	—	—

[1] See previous publications by Schöffl & Key (1982) and Schöffl *et al.* (1984*a*).
[2] Clones with prefix FS were identified by Schöffl & Key (1982); EC clones by Czarnecka *et al.* (1984).
[3] Polypeptides identified in 2D gels after hybrid selection and *in vitro* translation of mRNAs.

Sequence homologies between hsps are not confined to the multigene families in soybean. Cross-hybridizations between soybean cDNA probes and hs-mRNAs from other plants underscore the biological importance of the lmm hsps. Only the probes for the 15–18 kDa hsps in soybean hybridize significantly with pea, sunflower, millet, corn (Key *et al.* 1983*a*), parsley, *Vicia faba* and tobacco (Schöffl, unpublished). The homologies of genes among dicotyledonous plants seem to be much greater than the homologies between monocotyledons and soybean. The sizes of the cross-hybridizing hs-mRNAs (800–900 nucleotides) in plants suggest a correlation with only the lmm-hsps (see table 1). DNA probes for the 21–27 kDa hsps from soybean fail to detect homologous counterparts in other plant species. Compared with the 15–18 kDa hsps, these proteins also show a characteristically different localization in the cells of soybean (see §1). Despite the different roles suggested for these distinct groups of proteins, their synthesis is coordinately regulated. Transcriptional control has been implicated for the changes in steady-state concentrations of hs-mRNAs in analogy to the hs response in *Drosophila*, where transcriptional activation of hs genes correlates with dramatic changes in the puffing patterns of polytene chromosomes (Ritossa 1962). DNA sequence analysis of soybean hs genes (see §3) revealed additional support for a transcriptional mechanism in plants. Run-off transcription in isolated nuclei has been used to test this hypothesis (Schöffl *et al.* 1986). Only nuclei heat-stressed before their isolation synthesize the mRNAs of lmm-hsps. Hs genes from the class I family seem to be transcribed equally at high rates whereas other hs genes are

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

also heat-inducibly transcribed but apparently at lower rates of synthesis. A negative influence on the transcription of non-hs genes (Schöffl *et al.* 1986) contrasts with the concomitant activation of hs genes. The rapid induction of transcription is consistent with the appearance of detectable levels of hs-mRNA within minutes (Schöffl & Key 1982). Transcription of hs genes continues for more than 1 h in soybean seedlings as shown by the analysis of run-off transcripts by using cDNA probes (figure 1). The time course suggests a maximum increase in the rate of transcription within 15 min of the temperature shift from 28 to 41 °C. The rates of transcription seem to differ for different hs genes (lanes 3–8) but definite conclusions on individual rates cannot be drawn yet, because the apparent rates are influenced by several parameters. However, all the hs genes so far examined seem to be transcriptionally regulated. The synthesis of ribosomal RNA is, on the other hand, already induced at 28 °C (0 min) and continues to be synthesized during hs (lane 2). The strand-specificity of transcription is exemplified for one gene by using single-stranded DNA probes (Schöffl *et al.* 1986). Run-off transcripts hybridize only with the putative sense strand of gene hs6871 (lanes 10 and 13), but

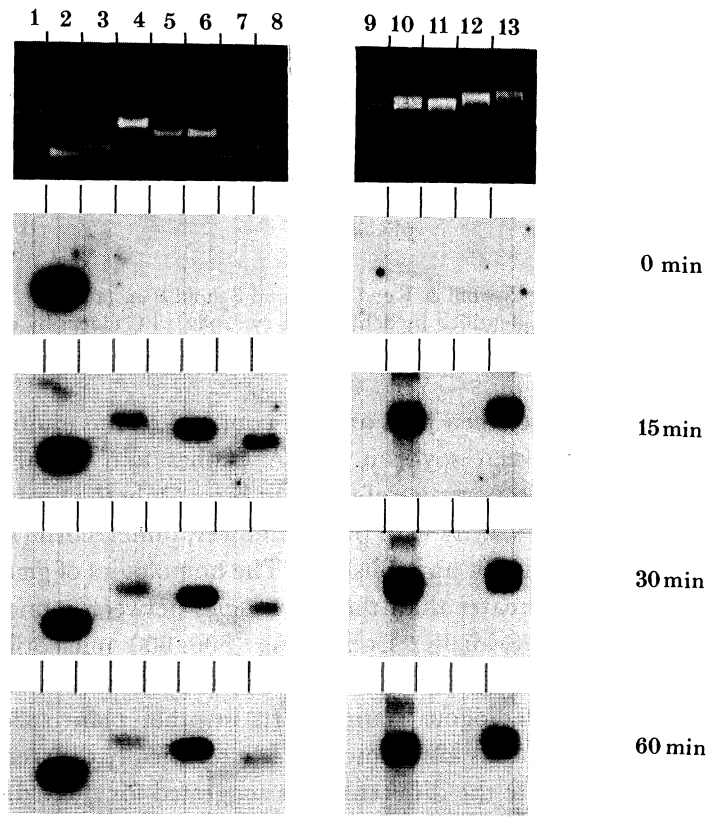

FIGURE 1. Identification of hs-specific mRNAs by run-off transcription in isolated soybean nuclei. Soybean nuclei isolated from seedlings previously subjected to hs (42 °C) for the indicated time periods. Run-off transcription *in vitro* was carried out in the presence of [^{32}P]UTP, specific transcripts were identified by Southern blot hybridization according to Schöffl *et al.* (1986). The DNA fragments used as gene probes are shown in the upper panel (ethidium bromide stain). Lane 1, pBR322-*AluI* digest (size marker); lane 2, clone 2014 (rDNA probe for 28S-rRNA); lanes 3–8, cDNAs for hsps from different gene families (see table 2): (3) clone 2026; (4) clone 2033, (5) clone 55, (6) clone 2059, (7) clone 75, and (8) clone 1968. Lanes 9–13, single-strained DNA probes for (+) sense-strand and (−) anti-sense strand of hs6871 DNA cloned into (10) mp9-I-8(+), (11) mp9-I-13(−), (12) mp9-III-4(−), and (13) mp9-III-7(+) according to Schöffl *et al.* (1986). Lanes 3, 5 and 7 hybridize also with hs-specific 'run-off' RNA as seen after prolonged exposure of the autoradiographs.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

not with the complementary DNA strand (lanes 11 and 12). Despite the strong evidence for transcriptional regulation of hs genes, we cannot entirely exclude a post-transcriptional nuclear processing operating on nascent RNA in control tissues. Turn-over rates of hs-mRNAs in the cytoplasm may also change with temperature. In *Drosophila*, hs-mRNAs were stable at 37 °C but rapidly destabilized to a half-life of about 2 h at 25 °C (DiDomenico *et al.* 1982). The apparent half-life of soybean hs-mRNA is about 1 h when seedlings are returned to 28 °C after 4 h hs (Schöffl & Key 1982). Assuming that decay is insignificant during hs, one can estimate an average efficiency of transcription of approximately 150 transcripts per minute per gene to cause the accumulation of 20000 transcripts per gene in the cell over a period of 2 h at 40 °C (Schöffl & Key 1982). Initial rates are possibly much higher, eventually decreasing after about 15 min as suggested by run-off transcription assays (figure 1). Such a profile of RNA synthesis may reflect the operation of self-regulatory mechanisms in hs response that have been attributed to the inhibitory functions of hsp70 in *Drosophila* (DiDomenico *et al.* 1982) and the homologous *dna*K protein in *E. coli* (Tilly *et al.* 1983). There must also be a selective mechanism for preferential translation of hs-mRNAs relative to the population of normal RNAs during hs and, conversely, a preference of normal RNAs after prolonged heat treatment (Kimpel & Key 1985*b*). The molecular basis for translational regulation is not yet known. However, translational preferences of hsp70 and hsp26 in *Drosophila* are known to require sequences in the 5′-untranslated leader of mRNA (McGarry & Lindquist 1985; Klemenz *et al.* 1985; Hultmark *et al.* 1986).

3. Heat shock genes and promoter structure

Several hs genes have been isolated from soybean genomic libraries by hybridization to the cDNA probes pFS1968 (Schöffl & Key 1983), pFS2019 (Czarnecka *et al.* 1985) and pFS2005 (Nagao *et al.* 1985). The analysis of genomic DNA fragments suggests a certain clustering of hs genes on the chromosome (Schöffl & Key 1983; Schöffl *et al.* 1984*b*). However, it is not known if all class I-genes map to one chromosomal location and whether they are interspersed by other hs genes. Chromosomal walking has not yet been undertaken to study the organization of putative gene clusters. A total of five complete genes (different members of the class I multigene family) and a number of cDNA clones have been sequenced in different laboratories.

The transcriptional units of hs genes were analysed by R-loop (Schöffl & Key 1983) and S1 nuclease mapping (Schöffl *et al.* 1984*b*; Czarnecka *et al.* 1985; Nagao *et al.* 1985). The protein coding sequences within transcribed regions were identified by large open reading frames beginning with ATG downstream from the 5′-initiation sites of transcripts. Intervening sequences have not yet been detected in any of the soybean hs genes. Other characteristics shared by these genes are a 5′-non-translated 'leader' sequence of approximately 100 nucleotides and multiple 3′-ends extending far beyond the deduced translational termination codons. It is not yet clear if some of the hs genes also have multiple 5′-initiation sites of transcripts or if this is an artefact of cross-hybridization (Key *et al.* 1985*a*). A comparison of the 5′-flanking regions encompassing the putative hs promoter revealed, not unexpectedly, significant homologies. Figure 2 sketches selected features of the presumptive hs promoters of five genes from soybean. The distinguishing components of the promoter structures resemble closely the regulatory elements present in the promoters of hs genes in *Drosophila* and *Xenopus* (Bienz 1985), *Caenorhabditis* (Russnack & Candido 1985), human (Wu *et al.* 1986) and maize (Rochester *et al.* 1986). The almost invariable TATA-like motif maps 19–24 bases upstream

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

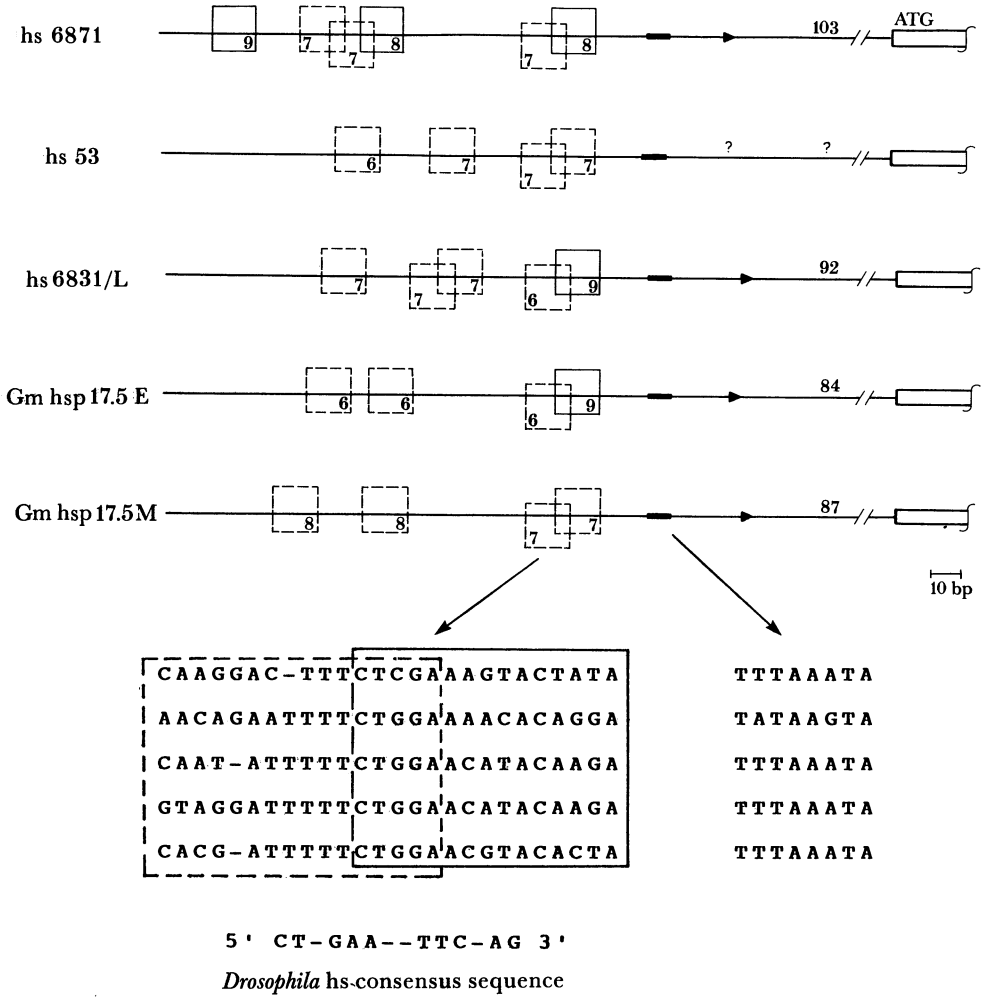

FIGURE 2. Conserved promoter elements in the 5′-flanking region of soybean hs genes. The analysis is based on DNA sequences for hs6871 (Schöffl *et al.* 1984*b*), hs53 and hs6831 (F. Schöffl, E. Raschke & C. Zühlke unpublished), Gmhsp17.5E (Czarnecka *et al.* 1985), and Gmhsp17.5M, L (Nagao *et al.* 1985). The boxes mark the position of short hyphenated dyad symmetries with the indicated homologies to the ten-base hs consensus core sequence. Closed bars, TATA box sequences; open bars, protein coding regions; arrowheads, mRNA start.

from the transcriptional start sites. Short imperfect dyad symmetries map 17–20 bases upstream from the TATA box sequences and additional elements are present at various positions in the 5′-flanking regions. The symmetrical core of the 14 base dyad consists of 10 residues 5′CT–GAA––TTC–AG3′ known as the *Drosophila* hs consensus sequence or heat shock element (HSE), for which 80% homology is required to induce transcription of chimeric genes by hs in COS cells and *Xenopus* oocytes (Pelham & Bienz 1982). Recent studies by Dudler & Travers (1984) revealed the importance of additional upstream sequences in the transcriptional regulation in *Drosophila*. HSEs with less than 80% homology to the consensus core sequence are present within this region. Footprinting analysis by Topol *et al.* (1985) provided evidence that, while the cooperative binding of the hs transcription factor (HSTF) to the two proximal HSEs is essential for efficient transcription, the additional binding to the upstream sites has an enhancing effect. The positioning of HSEs-like sequences in the 5′-upstream regions of soybean hs genes closely resembles the structure of the *Drosophila* hs promoter. Though several

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

other sequence motifs with homologies to potentially regulatory sequences in eucaryotes, as for example the SV40 enhancer (Laimins *et al.* 1982), glucocorticoid- and metallothionein-response elements (Karin *et al.* 1984), and Z-DNA sites (Wang *et al.* 1979) have been identified in soybean hs-genes (Czarnecka *et al.* 1985; Nagao *et al.* 1985), their significance in plant gene expression is unknown. These sites may possibly reflect the conservation of DNA–protein interactions but particular interactions may not necessarily cause the same type of regulatory function in every organism.

The DNA sequence analysis of the potential protein coding regions revealed an expected high homology between the five genes. The deduced amino acid sequences show a conservation of approximately 90% (for details see Schöffl *et al.* 1984*b*; Nagao *et al.* 1985). The degree of homology at the DNA level is similar. A comparison of the hydropathy profiles of the five proteins (figure 3) demonstrates impressively the conservation of hydrophobic and hydrophilic

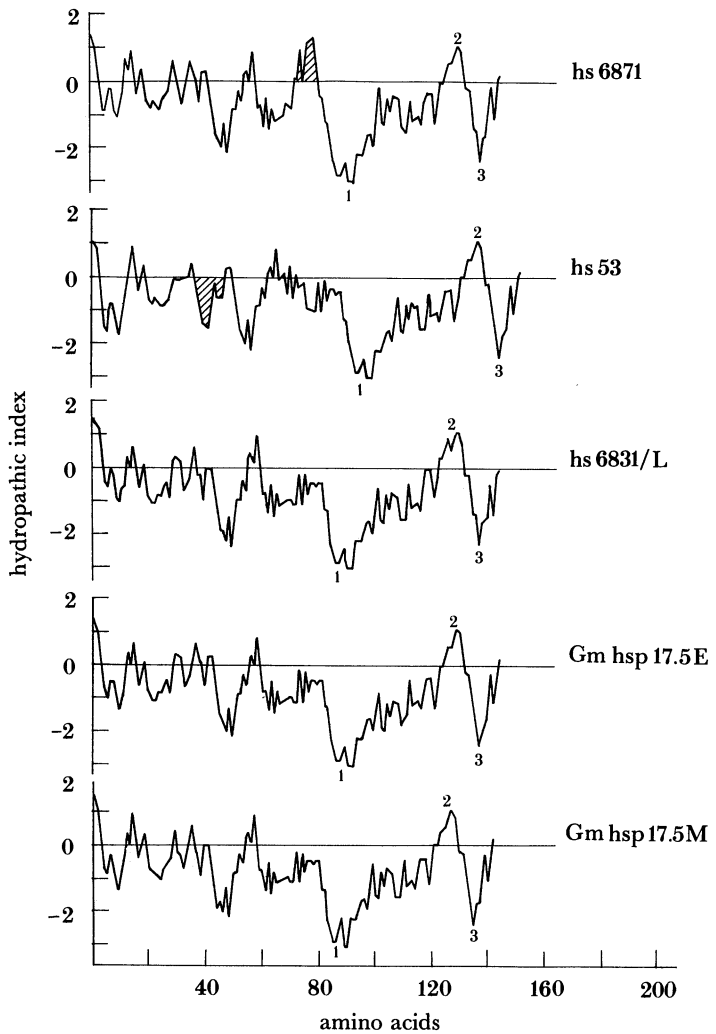

FIGURE 3. Hydropathic profiles of hsps. Deduced amino acid sequences (for origin of DNA sequence data see legend to figure 2) were progressively plotted by using a computer-aided approach (Kyte & Doolittle 1982) with a running average taken over nine amino acids. Sections above the centre lines correspond to hydrophobic regions, sections below the centre line to hydrophilic regions. The motifs in domains 1–3 are conserved in lmm-hsps of plants and animals. Hatched areas indicate differences between soybean hsps.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

characteristics throughout the polypeptide chains. Significant differences in profiles are confined to only two small regions within the hsps of hs6871 and hs53 (figure 3). The three characteristic domains in the profiles of soybean hsps (marked 1–3) are possibly the most important structural features and perhaps of functional significance. This type of analysis has been used to correlate structural features determined by crystallography with hydrophobicity and hydrophilicity along the amino acid sequences of proteins (Kyte & Doolittle 1982). A comparison of hydropathy plots of hsps from soybean and small hsps from *Drosophila*, *Xenopus* and *Caenorhabditis* revealed a remarkable resemblance of structural domains (Schöffl *et al.* 1984*b*; Nagao *et al.* 1985).

The most striking features are a hydrophobic domain (see figure 3, peak 2) including the highly conserved pattern of amino acids Asp/Asn-Gly-Val-Leu-Thr and a large hydrophilic domain (figure 3, peak 1). The hydrophilic domain is probably α-helical in the small hsps of *Drosophila* according to secondary structure predictions (Southgate *et al.* 1983). The third region is a small hydrophilic domain (figure 3, peak 3) located near the carboxyl terminus in all soybean and most *Drosophila* hsps. The resemblance of these structural features of hsps and the mammalian lens α-crystallins suggest common functional properties. This could be protein aggregation or other types of protein:protein interaction that seem to occur in soybean in a temperature-dependent fashion.

4. Expression of soybean hs genes in transgenic plants

The conserved design of hs promoters (see §3) suggests the evolution of a universal mechanism for transcriptional regulation of hs genes. The heterologous expression of genes linked to the *Drosophila* hsp70 promoter in mammalian cells (Burke & Ish-Horowicz 1982; Corces *et al.* 1981; Mirault *et al.* 1982; Pelham & Bienz 1982) and *Xenopus* oocytes (Pelham & Bienz 1982) provided evidence for a ubiquitous hs-induced control system recognizing conserved promoter elements. Two strategies have been pursued to test the evolutionary conservation of hs genes and their activation in plants: (i) the construction of a chimeric gene containing a reporter sequence (neomycin phosphotransferase coding region) under the control of the hsp70 promoter from *Drosophila* (Spena *et al.* 1985) and (ii) the use of plant hs genes under the control of their natural promoter (Schöffl & Baumann 1985; Gurley *et al.* 1986; Rochester *et al.* 1986).

The natural gene transfer system of *Agrobacterium tumefaciens* (pTi) has been exploited to introduce the different genes into the genomes of sunflower (Schöffl & Baumann 1985; Gurley *et al.* 1986), tobacco (Spena *et al.* 1985; G. Baumann, M. Bevan & F. Schöffl, unpublished data) and petunia (Rochester *et al.* 1986). The synthesis of thermoinduced transcripts suggests the functional integrity of hs promoters across phylogenetic barriers. The levels of specific RNAs varied considerably for the different genes and expression systems; however, there is not yet sufficient information available to relate quantitative differences with differences in promoter structure and upstream sequences or with position effects, copy numbers, etc. The use of the sunflower tumour system, although averaging for possible position effects, shows a large discrepancy in steady-state levels between two hs-induced RNAs from closely related soybean hs genes, hs6871 (Schöffl & Baumann 1985) and Gmhsp 17.5-E (Gurley *et al.* 1986). This variation in apparent transcriptional efficiency may be due to the different hs conditions applied, peculiarities of the vector design, and intricacies of the tumour system. The use of

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

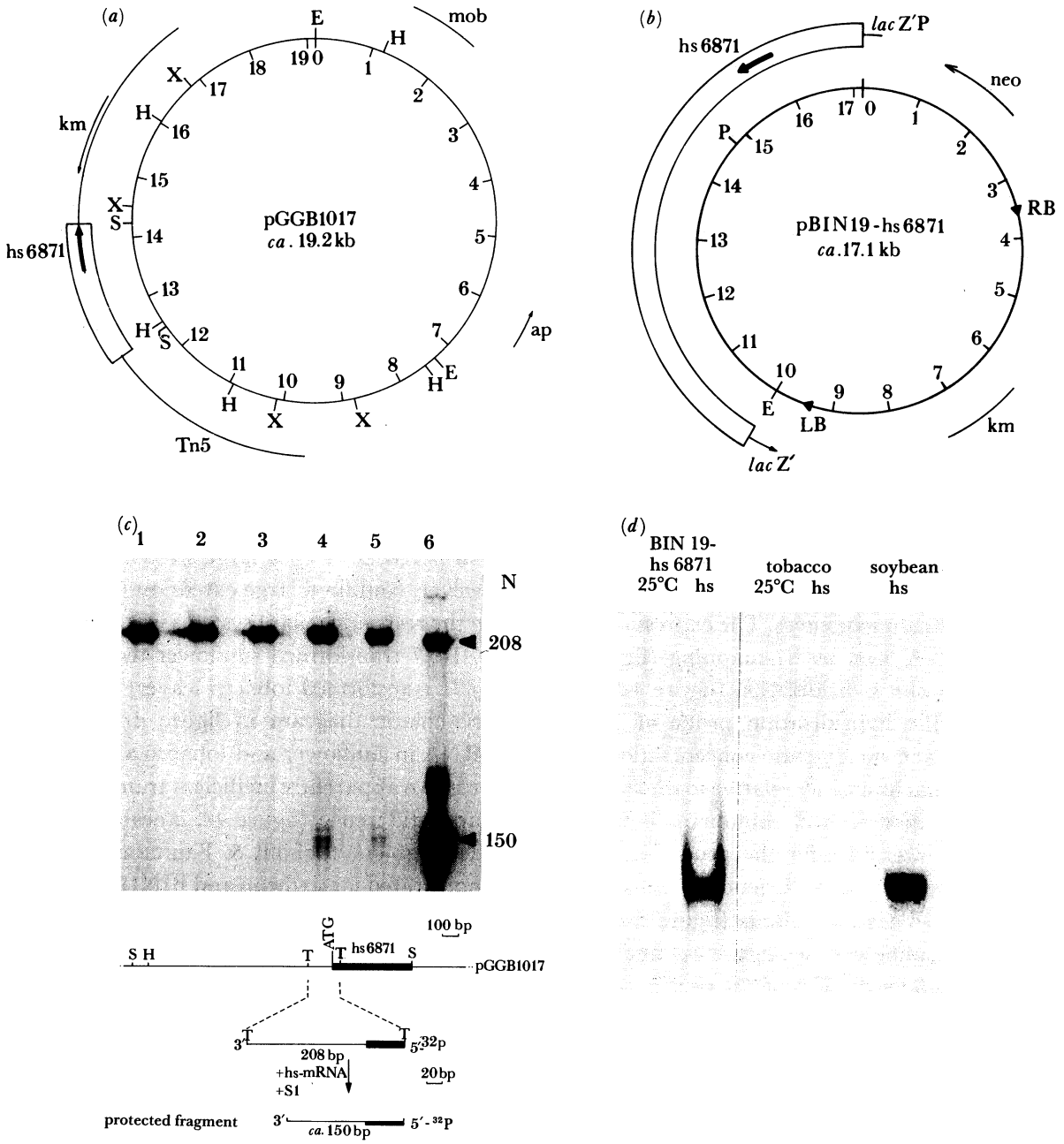

FIGURE 4. Transformation of plants with a soybean hs gene by using T-DNA derived vector systems. Schematic diagrams of vectors (*a*) pGGB1017 (intermediate) and (*b*) BIN19-hs6871 (binary) used for tumour induction in sunflower (Schöffl & Baumann 1985) or transformation of tobacco plants respectively. Transcription of hs6871 in sunflower tumour tissue was assayed by S1 nuclease mapping (*c*) or by Northern blot hybridization of RNA from transgenic tobacco plants (*d*) by using the gene-specific 208bp DNA fragment (see *c*). The schematic diagram (*c*) outlines the principle of the S1-mapping. The poly(A)$^+$ RNAs tested by this assay were from (1) sunflower hypocotyl and (2) control tumour tissue, both after 40 °C heat shock; pGGB1017 transformed sunflower tumours (3) cultivated at 25 °C and (4) after 40 °C hs; soybean hypocotyl (5) grown at 25 °C and (6) after 40 °C hs according to Schöffl & Baumann (1985). Thirty micrograms of total cellular RNA per lane was used for Northern blot hybridizations (*d*). Restriction endonuclease sites are marked for *Eco*RI (E), *Hin*dIII, (H), *Xho*I (X), *Sau*3A (S) and *Taq*I (T). LB. Left border sequence of T-DNA; RB, right border sequence; km, kanamycin resistance; ap, ampicillin resistance; neo, neomycin phosphotransferase; *lacZ'*: poly-linker in *lac*Z gene; P, *lac*Z-promoter.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

primary tumour systems to study regulation of gene expression is unsatisfactory for two reasons: the 'undifferentiated' status of callus tissue and the inseparable mixture of transformed and non-transformed cells. A general deregulation of gene expression is indicated not only by tissue proliferation but also by other examples such as 'overexpression' of Gmhsp 17.5-E and the constitutive transcription of developmentally regulated genes transformed into sunflower (Murai *et al.* 1983; Matzke *et al.* 1984).

In view of the ambiguities of gene analysis in primary tumours we have begun to investigate the expression of soybean hs genes in transgenic tobacco plants. The binary vector BIN19 (Bevan 1984) was used to introduce hs6871 into tobacco cells by leaf disk transformation (Horsch *et al.* 1985). Transgenic plants have been regenerated that inherit the transformed marker to the F_1 generation. Schematic diagrams of the vectors used for tumour induction in sunflower (pGGB1017) and transformation of tobacco (pBIN19-hs6871) are depicted respectively in figure 4*a*, *b*. The relevant characteristics in the design of the vector BIN19 are the replacement of tumorigenic genes by a selectable marker (neomycin phosphotransferase II) flanked by border sequences. The intermediate vector pGGB1017 contains, on the other hand, two stretches of DNA internal to the DNA region that can be used for homogenotization with tumorigenic pTiC58 (Schöffl & Baumann 1985). The integration of hs6871 into the multiple cloning site of BIN19 differs from the organization in pGGB1017 by a large stretch of DNA 3′-downstream from hs6871. Both constructions, however, contain a large extension (*ca.* 1 kb) of 5′-upstream sequences. The expression of hs6871 in the respective plant systems was analysed at the RNA level by S1-mapping (figure 4*c*: pGGB1017 transformed sunflower tissue) and Northern blot hybridization (figure 4*d*: BIN19–hs6871 transformed tobacco leaves) by using a 5′-specific hybridization probe of hs6871 (see schematic diagram in figure 4*c*). Large differences in steady-state concentrations of hs6871 RNA in sunflower and tobacco are shown by the signal intensities relative to the soybean standards. An apparently inefficient transcription of hs6871 in sunflower tumours is indicated by a large difference (figure 4*c*, lanes 4 and 6) in signal intensities for the protected 150-nucleotide fragment (Schöffl & Baumann 1985), whereas equally high efficiency of transcription is demonstrated for soybean and BIN19–hs6871 transformed tobacco plants (figure 4*d*). The mRNAs initiate at the same sites on hs6871 in soybean, sunflower (see figure 4*c*) and tobacco (data not shown).

The low levels of hs6871-specific transcripts in sunflower tumours may be attributed to tissue-specific effects and the low proportion of transformed cells in primary tumours. It is not known whether the 3′-flanking sequences of hs6871, lacking in pGGB1017, affect the transcription or mRNA stability (Schöffl & Baumann 1985). The genomic DNA fragment encompassing this gene in BIN19–hs6871 seems to contain all the sequences required for efficient expression in transgenic tobacco plants. Experiments are in progress to define the regulatory sequences 5′-upstream from this gene by a series of *Bal* 31-generated deletions.

Concluding remarks

Much progress has been made in the characterization of *hs* phenomena in a large array of organisms. Several features of the hs response are conserved, with plants being distinguished by the complexity and abundance of the lmm-hsps (15–20 kDa). The precise biological function of these proteins is not yet known, but several lines of evidence link them with the acquisition of thermal tolerance. The genes encoding these proteins in soybean are grouped into several multigene families. The deduced amino acid sequences of proteins of the largest

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

and best characterized family are about 90% homologous in soybean. A high degree of conservation of these genes is indicated by cross-hybridization between soybean DNA probes and hs-specific mRNAs from different plant species. The hydrophobic and hydrophilic characteristics of the polypeptides suggests a conservation of structural features in lmm-hsps between plants and animals. These structural features may be related functionally with the potential of these proteins to form large aggregates and to interact with other cellular components.

The transcriptional regulation of gene expression correlates with a conserved design of hs promoters in animals (*Drosophila*, *Xenopus*) and plants (soybean, maize). The evolutionary implications of this conservation lead to a model of a universal mechanism for the activation of hs genes (for review see Bienz 1985). The following steps are important in this model: (i) activation of inactive transcription factors (HSTF); (ii) stable binding of HSTF to hs promoter elements (HSE); (iii) initiation of transcription by RNA polymerase II. The activation of HSTF may possibly be regulated by ubiquitin (Munro & Pelham 1985).

We have demonstrated that a soybean hs gene hs6871 (17.3 kDa) is efficiently expressed and faithfully regulated in transgenic tobacco plants, and Rochester *et al.* (1986) have shown that a hsp70 gene from maize is properly expressed in transgenic petunia. However, it is not yet known whether the multiple 5′-HSE sequences are in fact the important binding sites for potential HSTFs in plants. Dissection of the hs promoters will be required to define the important sequences for *cis*-active regulation of transcription.

The highly conserved nature of lmm-hsps provides a good argument for functional activity of these proteins in a heterologous genetic background. This issue is crucial for future studies of biological effects (viability of cells, development of thermal tolerance) induced by constitutive expression of individual hsps (Pelham 1984) or by anti-sense RNA production (McGarry & Lindquist 1986).

Future analyses of hs genes in transgenic plants will certainly include members of other hsp families from soybean, especially those genes that trans-localize their corresponding hsps into chloroplasts (Vierling *et al.* 1986; Kloppstech *et al.* 1985) and mitochondria (Lin *et al.* 1984). These genes probably represent other sets of hsps that are important or perhaps even unique to the hs response in plants. Other favourable candidates would be the genes encoding the 27 kDa stress proteins in soybean. However, the lack of homology with the stress proteins of other plants may restrict their biological significance to soybean. The high homology (68%) between hsp70 from maize and *Drosophila* (Rochester *et al.* 1986) suggests common function for these proteins in animals and plants.

The major research activities in future will be directed to gaining insight into the mechanisms of transcriptional and translational control switches. Other important areas of research concern the physiological aspects of the hs response. The open questions are: What is the role of hsps and hs granules (Nover *et al.* 1984) and what are the mechanism(s) of aggregation–dissociation and selective localization? Is *de novo* synthesis of hsps required to generate short-term tolerance in field grown plants (Kimpel & Key 1985*a*)? Is there any relation between short-term and long-term adaptions to high-temperature stress (Berry & Björkman 1980; Wu & Wallner 1984)? Is the impaired fertilization of crop plants at high temperature due to the inability to synthesize hsps in pollen (Xiao & Mascarenhas 1985)? Answers to these questions will undoubtedly be important for future improvement of crop productivity. Genetic engineering and the generation of transgenic plants will certainly play a major role in conveying these answers.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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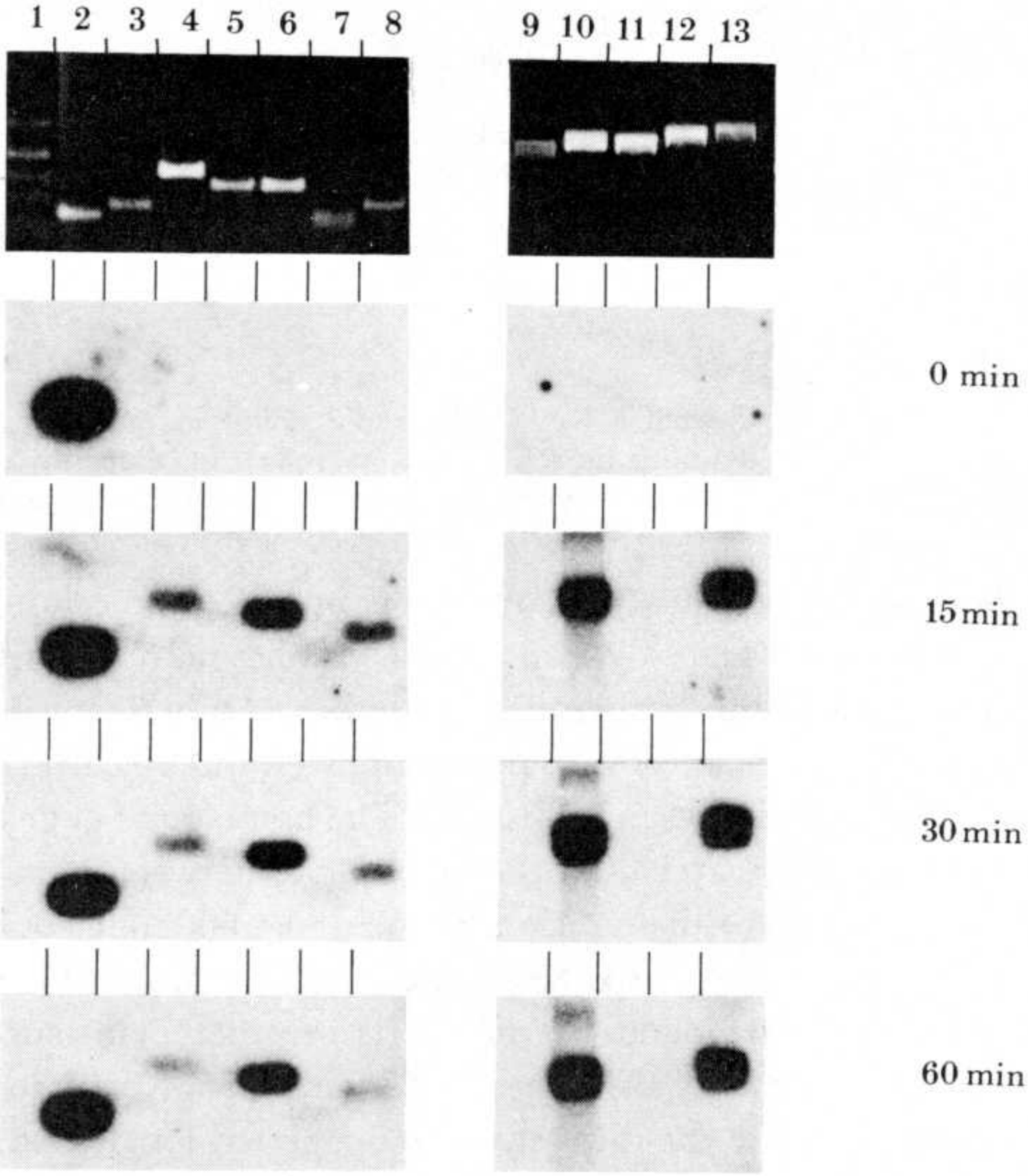
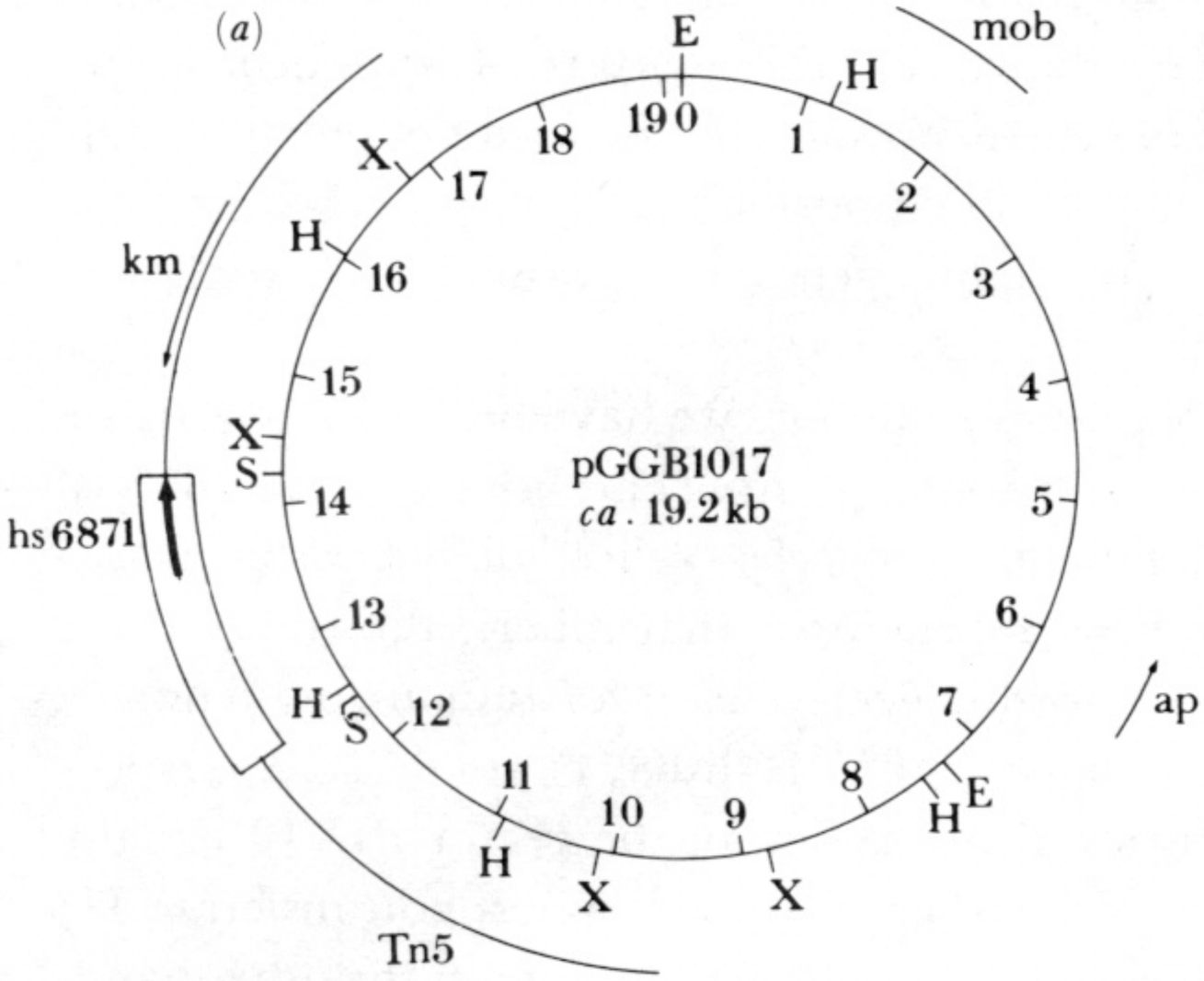
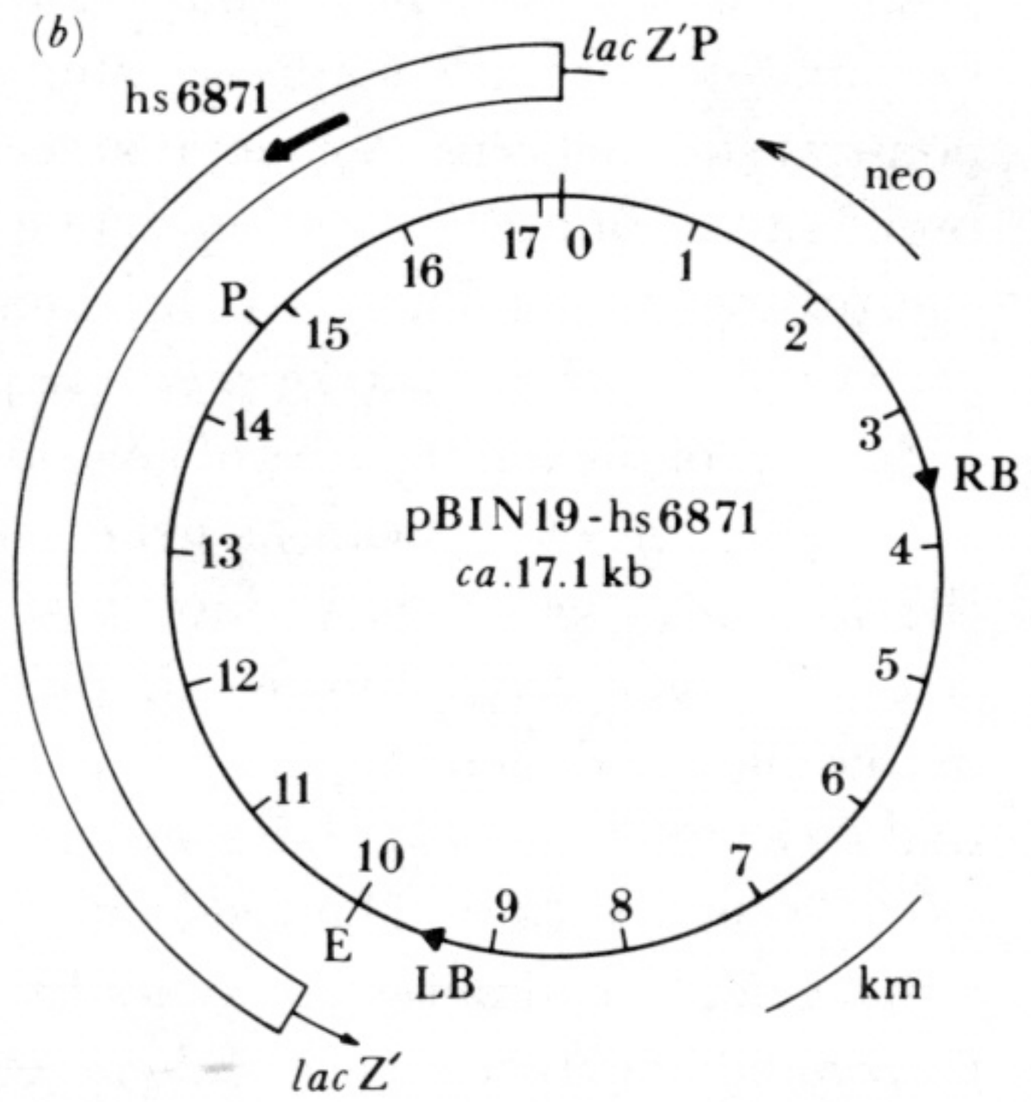
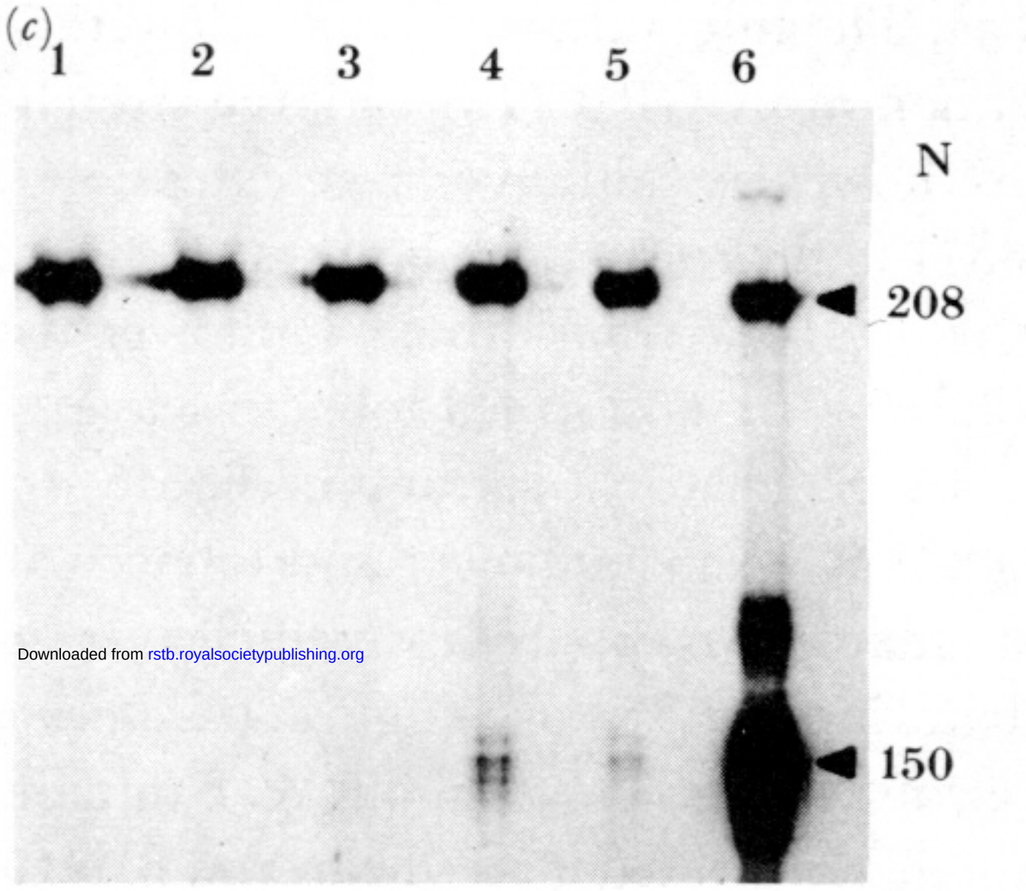


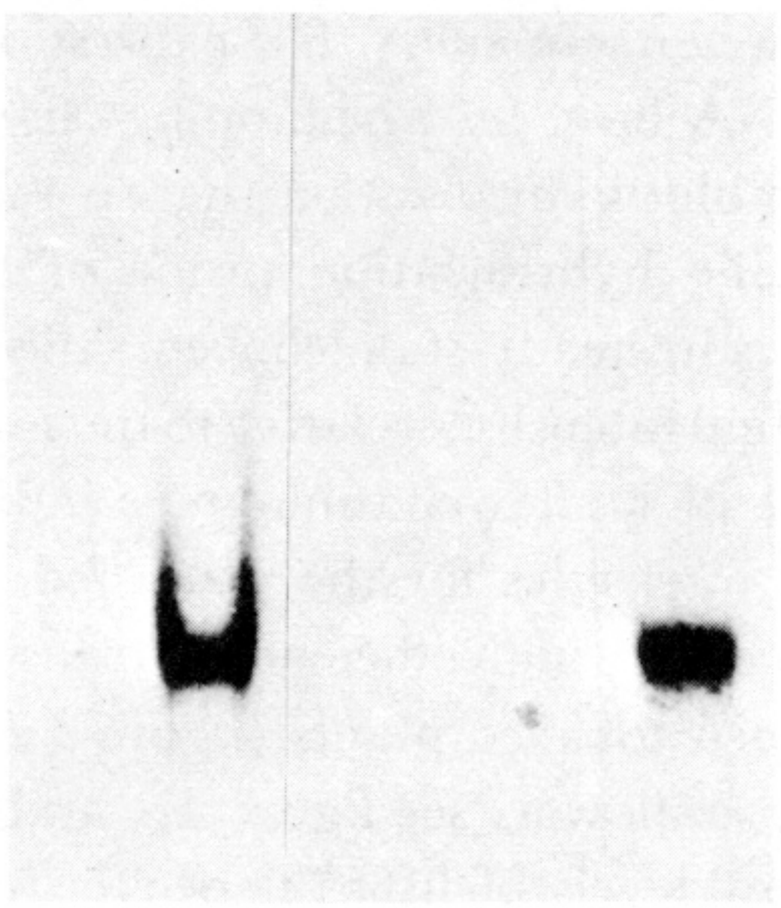
FIGURE 1. Identification of hs-specific mRNAs by run-off transcription in isolated soybean nuclei. Soybean nuclei isolated from seedlings previously subjected to hs (42 °C) for the indicated time periods. Run-off transcription *in vitro* was carried out in the presence of [^{32}P]UTP, specific transcripts were identified by Southern blot hybridization according to Schöffl *et al.* (1986). The DNA fragments used as gene probes are shown in the upper panel (ethidium bromide stain). Lane 1, pBR322-*AluI* digest (size marker); lane 2, clone 2014 (rDNA probe for 28S-rRNA); lanes 3–8, cDNAs for hsps from different gene families (see table 2): (3) clone 2026; (4) clone 2033, (5) clone 55, (6) clone 2059, (7) clone 75, and (8) clone 1968. Lanes 9–13, single-strained DNA probes for (+) sense-strand and (−) anti-sense strand of hs6871 DNA cloned into (10) mp9-I-8(+), (11) mp9-I-13(−), (12) mp9-III-4(−), and (13) mp9-III-7(+) according to Schöffl *et al.* (1986). Lanes 3, 5 and 7 hybridize also with hs-specific 'run-off' RNA as seen after prolonged exposure of the autoradiographs.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES









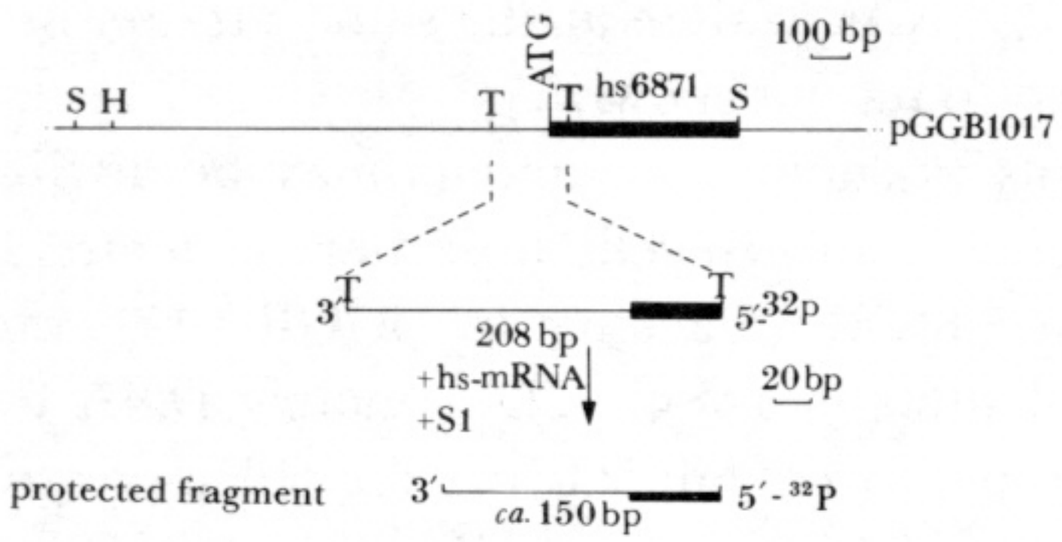


FIGURE 4. Transformation of plants with a soybean hs gene by using T-DNA derived vector systems. Schematic diagrams of vectors (*a*) pGGB1017 (intermediate) and (*b*) BIN19-hs6871 (binary) used for tumour induction in sunflower (Schöffl & Baumann 1985) or transformation of tobacco plants respectively. Transcription of hs6871 in sunflower tumour tissue was assayed by S1 nuclease mapping (*c*) or by Northern blot hybridization of RNA from transgenic tobacco plants (*d*) by using the gene-specific 208bp DNA fragment (see *c*). The schematic diagram (*c*) outlines the principle of the S1-mapping. The poly(A)$^+$ RNAs tested by this assay were from (1) sunflower hypocotyl and (2) control tumour tissue, both after 40 °C heat shock; pGGB1017 transformed sunflower tumours (3) cultivated at 25 °C and (4) after 40 °C hs; soybean hypocotyl (5) grown at 25 °C and (6) after 40 °C hs according to Schöffl & Baumann (1985). Thirty micrograms of total cellular RNA per lane was used for Northern blot hybridizations (*d*). Restriction endonuclease sites are marked for *Eco*RI (E), *Hin*dIII, (H), *Xho*I (X), *Sau*3A (S) and *Taq*I (T). LB. Left border sequence of T-DNA; RB, right border sequence; km, kanamycin resistance; ap, ampicillin resistance; neo, neomycin phosphotransferase; *lacZ'*: poly-linker in *lacZ* gene; P, *lacZ*-promoter.