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Development of a heat shock inducible expression cassette for plants: Characterization of parameters for its use in transient expression assays

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Abstract

A heat-inducible expression cassette has been constructed to study the conditional expression of sense or antisense orientations of any sequence of interest in transgenic plants or plant tissues. The construct includes the promoter and all but 5 bases of the mRNA leader from the soybean *Gmhsp17.5-E* gene, the polylinker from pUC18 (modified to remove the ATG), and a fragment that contains the polyadenylation signal and site from the nopaline synthase gene. Analysis of transient expression of a construct containing the β-glucuronidase (GUS) coding sequence cloned in the cassette and introduced into *Nicotiana plumbaginifolia* protoplasts by electroporation shows that the promoter has high expression at heat shock temperatures. This construct is expressed at a roughly 80-fold higher level per unit time than a cauliflower mosaic virus 35S gene promoter-GUS construction. The heat shock promoter is regulated positively by supercoiling in this transient assay system. The level of expression of HS-GUS constructions with the polyadenylation sites from either the nopaline synthase gene or the *Gmhsp17.5-E* gene was similar. Constructs with a perfect fusion at the 5′ end had higher levels of expression than those with the corresponding nonperfect transcriptional fusion.

Introduction

Due to the difficulty of obtaining mutants in most plant species in important developmental or physiological processes, many investigators are limited to isolating developmentally regulated or inducible genes without regard to function with the hope to ultimately be able to identify their functions and the transcription factors that regulate their expression. A more in-depth understanding of the importance of the gene products, their developmental regulation or tissue specificity can be gained by expression of a gene coding sequence at times or in tissue types that are inappropriate or by overexpression of the sequence in transgenic plants. Further information can be gained by using antisense constructs to underexpress a sequence [27, 68]. Such research is gaining popularity, but the promoters most commonly used are either constitutive or light-inducible (see [16, 72] for recent reviews). A valuable tool for studying genes in any organism is one allowing conditional expression, either temporally or spatially, of a gene of interest. Ideal promoters for the conditional expression of genes in plants should fulfill several criteria: 1) a low level of expression under the uninduced condition, 2) a high level of expression under the in-

duced condition, 3) proper regulation in a number of heterologous systems, 4) induction that is readily reversible, 5) lack of tissue specificity, 6) an ability to interact with tissue-specific regulatory sequences to obtain tissue-specific expression, if desired, and 7) an inducing agent that does not perturb the normal physiology or development of the plant. To date, heat shock gene promoters provide the best candidates for satisfying these criteria.

High-level expression of a specific set of genes in response to a rapid or gradual rise in temperature from a temperature at which optimal growth and development occurs is a phenomenon common to nearly all organisms studied thus far (reviewed by [10, 39, 44]). The structural organization of these genes, termed the heat shock (HS) genes, and the promoter sequences that regulate their expression have been intensely studied. A 14 bp heat shock regulatory element consensus sequence (HSE) identified by Pelham [48] has recently been redefined by site-directed mutagenesis as either 3 or more repeats of a 5 bp sequence [1] or as repeats of a 10 bp sequence [75]. A protein factor, designated the HS factor or HS transcription factor (HSTF), that binds to this site has been identified in several organisms. In a careful analysis, Thomas and Elgin [66] showed that there is cooperativity in the binding of HSTFs to the multiple HSEs present in the *Drosophila hsp* 26 gene promoter. A factor in yeast that binds to this sequence has been identified, purified and its gene sequenced [62, 71]; Wu *et al.* [74] have purified the binding factor from *Drosophila*. In yeast, the HSTF binds to HSEs at both control and HS temperatures [61]. Its activity as a transcriptional activator possibly depends on its phosphorylation state [62] and the HSTF was shown to be absolutely required for growth at normal temperatures as well as at heat shock temperatures [62, 71]. In human and *Drosophila* systems, the factor appears to bind only after heat shock [33, 76]. Gilmour and Lis [23] have shown that at control temperatures, an RNA polymerase II molecule is bound to the *hsp* 70 gene in *Drosophila*; further work demonstrated that the polymerase stalls after synthesizing approximately 25 nucleotides of the transcript [53]. The binding of the HSTF in some manner re-engages the RNA polymerase to allow elongation of the transcript. Other protein factors may modulate the activity of heat shock promoters, including a TATA box binding protein, identified by Wu [73] that binds to the *hsp* 70 and *hsp* 82 gene promoters under both control and heat shock conditions. In addition, in two soybean heat shock genes, an AT-rich region serves to greatly increase the activity of these promoters [4, 13, 25].

We report the engineering of the promoter from the tightly regulated, highly expressed soybean 2019E heat shock gene [12] into a useful expression cassette. Parameters important to its use in a transient expression system and its eventual use in heterologous plant systems have been studied. The transient expression system allows rapid analysis of the expression of a sequence cloned into the cassette prior to its transformation into plants.

Materials and methods

General procedures

Fragments generated by restriction endonuclease digestions and used in the constructions described below were separated on agarose or acrylamide gels and purified as described in Maniatis *et al.* [41] or on low melting point agarose, melted and used directly as described by Struhl [64]. All vectors used for cloning were phosphatase-treated according to Barker *et al.* [3]. Ligations were done using T4 DNA ligase from New England Biolabs and, for sticky-end ligations, according to the manufacturers' recommendations; for blunt-end ligations the buffer of King and Blakesley [32] was used. For purification of fragments or plasmids from restriction digests, where necessary, the DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with chloroform/isoamyl alcohol (24 : 1). The DNA was precipitated twice with 2 volumes of ethanol after adjusting the sodium acetate concentration to 0.3 M. All transformations were done by diluting the ligation mix-

tures 5-fold with water and transforming competent cell prepared by the method of Hanahan [26].

Construction of the heat shock inducible cassette

The 1.55 kb fragment from pHin2019, a plasmid subclone of hsE2019 [12] containing the promoter upstream region, the region coding for the mRNA leader sequence and 72 bp of the open reading frame of the 2019E gene was subcloned into pUC19. To remove the protein coding sequence, the *Bam* HI fragment was purified by polyacrylamide gel electrophoresis, diluted to 0.2 μg/μl and resected with Bal31 (New England Biolabs) for 8 min at 25 °C as described in Maniatis *et al.* [41]. After repairing the ends with Klenow fragment, a *Hin*d III linker (New England Biolabs, No. 1038) was added [41] and the fragment cloned into pUC19. Fine structure restriction endonuclease mapping was used to establish the approximate location of the ends of the resultant clones. The *Hin*d III-*Eco* RI inserts from the clones with 3′ ends (relative to the gene sequence) just upstream but not including the protein coding sequence were subcloned into M13mp18 [69] for sequencing. One clone, designated pMA401, that contained the promoter region and all but 5 bp of the mRNA leader sequence was used for construction of the HS inducible expression cassette.

A 3.2 kb upstream promoter fragment of the 2019E gene, contained in the clone pMA402, was reconstructed by ligation of a *Bgl* II-*Cla* I fragment from pHin2019 (position − 3.2 kb to − 540 bp), a *Cla* I-*Hin*d III fragment from pMA401 (position − 539 to + 86 bp) and *Bgl* II-*Hin*d III digested pUC8J (obtained from W. Gurley), a derivative of pUC8 in which a *Bgl* II linker was added to the *Bam* HI site.

Nopaline synthase 3′ polyadenylation site and signal fragment

A fragment containing the nopaline synthase gene from pTi37 (obtained from W. Hughes) was digested with Sau3AI and the resultant fragments cloned into pUC19 digested with *Bam* HI. Clones containing the fragment corresponding to the bases 666 to 418 from the nopaline synthase gene 3′ end [6] were identified by colony hybridization [24]. The Sau3AI fragment from one of these clones was purified by electrophoresis on a 5% polyacrylamide gel, the ends flushed with Klenow fragment and linkered with a 50 : 50 mixture of a *Bgl* II linker (New England Biolabs, No 1001) and an *Eco* RI linker (New England Biolabs, No 1018) as described above. The fragments were ligated into pUC8J digested with the same enzymes. Clones with the linkers in the desired orientation were identified by restriction endonuclease mapping followed by subcloning of the *Eco* RI-*Hin*d III inserts into M13 for sequence analysis using the chain terminations method of Sanger *et al.* [55]. One clone used for preparing the cassette described below was designated pMA404.

Modified polylinker

The ATG within the *Sph* I site of the pUC19 polylinker was removed by digestion of the plasmid with *Sph* I and treatment of the DNA with 2 units mung bean nuclease (New England Biolabs) per μg DNA at 30 °C for 30 min as specified by the manufacturer. The plasmid was recircularized by ligation using blunt-end ligation buffer [32] to make pMA403.

Construction of the heat shock inducible cassette

For construction of the heat shock inducible cassette, the *Bgl* II-*Hin*d III fragment from pMA402 (200 ng), the *Hin*d III-*Eco* RI fragment containing the polylinker from pMA403 (13 ng) and the *Eco* RI-*Bgl* II fragment from pMA404 (50 ng) were ligated together, the DNA from the resulting mixture purified by phenol : chloroform extractions and ethanol precipitations as described above, digested with *Bgl* II, purified on a low melting point agarose gel, and ligated into pUC8J

that had been digested with *Bgl* II. The desired construct (pMA405) was identified by restriction analysis. The cassette from pMA405 was transferred into pGG101, a pUC8 derivative in which the entire polylinker region was replaced with a *Bgl* II linker, thereby making pMA406.

Construction of HS promoter-GUS constructs

A *Hin*d III-*Sal* I fragment from pRAJ260 [29] containing the β-glucuronidase coding sequence was cloned into the corresponding sites in pMA406, creating pMA412. The *Bgl* II cassette was transferred into pMA105, a derivative of Bluescript KS$^+$ (Stratagene) in which a *Bgl* II linker was engineered into the *Eco* RI site. The 3′ end of the *nos* gene in the Bluescript derivative, pMA417, was exchanged with the 3′ end of the 2019E gene by digestion of pMA412 with *Sal* I, which removes the *nos* 3′ end, flushing the end with Klenow fragment and cloning a blunt-ended Sau3AI fragment from the 2019E gene that corresponds to bases 546 to 1026 [12]. In the derivative containing the 2019E gene 3′ end, termed pMA422, a *Bgl* II site was created at the 3′ end of the cassette allowing excision of the cassette by *Bgl* II.

Preparation of perfect 5′ and 3′ fusions

Perfect fusions, i.e., fusions containing all bases of either the 5′ or 3′ ends of the 2019E gene non-translated coding sequences fused directly to the translation initiation codon or termination codon of the GUS open reading frame (see Fig. 4), respectively, were created using the following oligonucleotides: 5′ fusion, CAGCTAAGAAAAAC-CAAAAGATGTTACGTCCTGTAG; 3′ fusion of GUS with the *nos* gene 3′ end, GGGAGG-CAAACAATAAAGAAGGAGTGCGTCGA-AGCAGATCGTTCAAACATTTGG; 3′ fusion of GUS with the 2019E gene 3′ end, GGAGG-CAAACAATGATCCATGTTATGGTTG. The oligonucleotides were synthesized on an Applied Biosytems 380 DNA Synthesizer and purified by HPLC by J. Wunderlich according to the manufacturers' directions. The dried detritylated oligonucleotides were dissolved in water and lyophilized to dryness several times; undissolved material was removed by centrifugation. The oligonucleotides were dissolved in 3 ml of 50 mM triethylammonium acetate and purified using C-18 Sep-Pak cartridges. After the cartridges were rinsed with 10 ml HPLC grade methanol, the oligonucleotides were added, the cartridges washed with 20 ml of HPLC grade water, and the oligonucleotides eluted with 3 ml of triethylammonium acetate/methanol (1 : 1). Fractions containing the oligonucleotides were dried, rinsed with 90% ethanol and redried.

The DNA template for making the 5′ fusion was prepared by single-stranded rescue of pMA421 in *Escherichia coli* strain CJ236 [36] using M13K07 as a helper phage [70]. For the 3′ fusions the *Sma* I-*Sal* I fragment from pMA417 was cloned into *Sma* I-*Sal* I digested M13mp19 and the *Ssp* I fragment from pMA422 was cloned into the *Sma* I site of M13mp18. Purification of the single-stranded M13 templates was done according to the method of Vieira and Messing [70]. The mutagenesis of the 5′ and 3′ ends were done as described by Kunkel *et al.* [36] with the exception that some of the annealings of the oligonucleotides to their templates were done at 37 °C for 20 min. For screening the colonies and plaques, oligonucleotides were end-labeled using the same procedure described by Kunkel *et al.* [36] except 100 μCi of [α-^{32}P]ATP was used in place of ATP. The probes were purified on NACS Prepac columns (BRL) using the ammonium acetate buffer procedure detailed by the manufacturer. After transfer to nitrocellulose by standard procedures [41], the colonies or plaques were screened by prehybridization 4–5 h in 6 × SSC, 5% non-fat milk followed by hybridization in the same solution with 0.1% SDS added. After hybridization overnight, the filters were washed three times for 10 min at room temperature in 6 × SSC, 0.5% non-fat milk, 0.1% SDS and 2 min at increasing temperatures until probe was hybridized to only those DNAs containing the desired mutation. Further characterization of the mutated con-

structs was done by restriction analysis and subcloning fragments into M13 or pUC118/119 and sequencing by the chain termination system of Sanger *et al.* [55]. The entire fragments from the *in vitro* mutated DNAs that were used for the constructions below were sequenced to confirm that the correct sequence was obtained during the *in vitro* DNA synthesis used to generate the mutations.

Constructions containing both the *nos* and 2019E gene 3′ end were made in all combinations of 5′, 3′ and both 5′ and 3′ perfect fusions by the ligation of 3 to 5 fragments each as detailed in Fig. 5 and its legend. The outcome of the ligations was biased toward those desired by phosphatase treating one of the two ends that were to be joined in a particular ligation. Those clones determined by colony hybridization [24] to contain at least two of the desired fragments were screened by fine structure restriction endonuclease mapping to identify the correct constructions.

Protoplast isolation from Nicotiana plumbaginifolia

Axenically grown haploid *Nicotiana plumbaginifolia* plants were maintained at 26 °C and a 8 h day length. Protoplasts were prepared from leaves approximately 3–6 cm long according to the method of Maliga [40] as modified by P. Evans (University of Georgia). Approximately 7–10 g of leaves were cut (2–3 leaves per plant) into small pieces (approximately 5 mm) and vacuum-infiltrated with W5G buffer (125 mM $CaCl_2$, 45 mM NaCl, 5 mM KCl, 100 mM glycine, pH 5.6), 3 times for 4 min each, and left for one hour. The W5G was removed and the tissue divided into roughly 1 g portions and placed into 100 mm diameter glass Petri dishes, and 20 ml of freshly prepared NM3E (for 100 ml: 1 g cellulysin, 0.5 g macerase, 100 ml M3: MS micronutrients [43], B5 vitamins [20], 75 μg/l $FeSO_4$ in solution with 37.5 μg/l Na_2EDTA, 0.075 μg/l KI, 14.6 mg/l glutamine, 400 mg/l NH_4NO_3, 293 mg/l $CaCl_2 \cdot 2H_2O$, 246 mg/l $MgSO_4 \cdot 7H_2O$, 68 mg/l KH_2PO_4, 250 mg/l xylose, 134 mg/l malic acid, 0.5 g/l inositol, 72 g/l glucose, pH 5.5, filter-sterilized) was added to each. The digestions were done at 26 °C overnight (usually 14 h) in the dark. After the digestion, the plates were swirled gently to release the protoplasts, filtered through a 105 μm nylon mesh and the suspension divided into 15 ml sterile plastic disposable centrifuge tubes. Approximately 1.5 ml of W5G was layered on top of the suspension and the protoplasts were floated by centrifugation at 800 ***g*** for 10–12 min. The protoplasts were carefully removed and washed once with W5G buffer and once with electroporation buffer (20 mg/l KH_2PO_4, 115 mg/l Na_2HPO_4, 7.5 g/l NaCl, 36.4 g/l mannitol, 4 mM $CaCl_2$, pH 7.2; the solution was prepared without $CaCl_2$, autoclaved, the $CaCl_2$ added and the solution filter-sterilized) by centrifugation as above. The yield was usually 12–14 × 10^6 protoplasts/g leaf tissue and the viability was 89–94% as assayed using the exclusion of bromophenol blue.

Electroporation of the protoplasts

The electroporation of the protoplasts was done essentially as described by Fromm *et al.* [18]. The protoplasts were resuspended at a concentration of 8 × 10^6 protoplasts/ml and were kept at room temperature until used. The cuvettes used for electroporation were placed on ice, 0.5 ml of protoplasts were added, 0.5 ml of electroporation buffer with filter-sterilized DNA was mixed with the protoplasts. The DNA was added in TE (10 mM Tris, 1 mM EDTA, pH 8.0) and the total amount of TE added to each sample within an experiment was the same. The samples were electroporated at 250 V, 750 μF, and 250 ms pulse length using the Promega Biotec X-Cell 450 electroporator which generates an exponentially decaying electric pulse. The samples were placed on ice for a minimum of 10 min, transferred to Petri dishes, incubated at room temperature for the same amount of time used for the 4 °C incubation, and then 8 ml of M3HH medium (M3 medium with 2 mg/l naphthaleneacetic acid and 1 mg/l N^6-benzyladenine) was added. All samples in an experiment that were to be compared

directly were done in the same 'set', that is, protoplasts and DNA were added to the cuvettes for all the samples in a set, the electroporation of the samples was done in succession within a set as was the chilling on ice, incubation at room temperature and addition of medium. Because of different numbers of samples within sets, the actual time interval of chilling and room temperature incubations after electroporation was different, varying from 8–12 min, but the interval for each sample within a set was the same and was carefully measured. After the media was added, the protoplasts were incubated at 22 °C for 22–24 h unless otherwise specified.

Preparation of DNA used for electroporation

All plasmids used were maintained in DH5α cells (BRL). The plasmid DNAs were prepared using the alkaline-lysis method [41] and purified twice on CsCl gradients. The plasmids were extracted once with freshly prepared phenol, once with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and once with chloroform : isoamyl alcohol. The DNA was precipitated twice from 0.3 M sodium acetate and once from 2 M ammonium acetate. The DNA was stored at − 70 °C and DNAs used in a particular experiment were freeze-thawed the same number of times. Linearization was done using a restriction enzyme site that flanks the cassette; the DNA was extracted and precipitated as described above.

Heat shock treatment of the protoplasts

For heat shock treatment of the protoplasts, the protoplasts were transferred into sterile flasks (about 1.8×10^6 protoplasts total) and incubated at the desired temperature for 2 h. All treatments were done in duplicate or triplicate. If more protoplasts were required for a heat shock treatment than would be obtained in one sample, 2 or more identical sets of samples were electroporated, the corresponding samples pooled and then samples for the heat shock treatments were aliquoted. The protoplasts were recovered by centrifugation at 800 ***g*** for 10–12 min. The supernatants were removed and the pellets frozen at − 70 °C until the assay for β-glucuronidase (GUS) activity was done.

β-Glucuronidase assays

The GUS assays were done using a fluorometric assay essentially as described by Jefferson [29]. The protoplasts were thawed into 0.5 ml of extraction buffer, sonicated for 10 s at 75 W using the microprobe of a Braun-sonic or Labline Ultratip Labsonic System Sonicator, and cooled on ice. The debris was removed by centrifugation for 15 min in an Eppendorf microfuge. The extract was diluted 1 : 10 into the reaction mixture. Using this amount of extract, less than 5% (usually much less) of the total fluorescence during a measurement was absorbed by the components of the extract. The reaction minus the extract was incubated at 37 °C for several minutes before the extract was added to initiate the reaction. A minimum of 4 time points were taken for all reactions (30 or 45 min time points) and the slope of the line determined by linear regression. The samples were kept in the dark at 4 °C until their fluorescence was measured. The fluorescence was measured on an Aminco SPF-5000 spectrofluorometer with the excitation and emission band pass set at 2 nm and 20 nm, respectively (at these band pass settings, photodecomposition resulted in a reduction of the measured fluorescence of less than 0.5% per min). The amount of extract used was chosen so that the activity was within the linear range of the assay (approximately the activity producing no more than 7.5 pmol methylumbelliferone per minute) and within the linear range of the spectrofluorometer (less than a total concentration of methylumbelliferone of approximately 1 μM). The activity was normalized to protein concentration (measured using the Biorad Protein Assay kit and bovine serum albumin as the standard).

Results

Construction of the heat-inducible expression cassette

The heat-inducible expression vector was constructed as detailed in Fig. 1. The promoter chosen was from the soybean *Gmhsp17.5-E* gene (for simplicity this gene will be referred to as the 2019E gene, which is based on the designation of the corresponding cDNA), which in soybean has undetectable expression at normal growing temperatures but high expression at heat shock temperatures. The 2019E gene is among the most highly expressed heat shock genes in soybean [11]. The promoter fragment in the cassette has 3.2 kb of sequence upstream from the transcription start site and all but 5 bp (77 bp total) of the 5′ nontranslated leader sequence [12]. The 5′ terminal region of the leader sequence has been shown to be essential for translation of heat shock gene mRNAs at heat shock temperatures (reviewed by [45]). The cassette includes the polyadenylation signal and sites from the nopaline synthase gene (*nos*) contained on a 249 bp Sau3AI fragment previously characterized by Bevan [5] as containing all sequences essential for transcription termination. To facilitate cloning of DNA sequences into the cassette, a modified polylinker from pUC18 was placed between the promoter and 3′ end fragments of the cassette. Consistent with the Kozak model [34] of translation initiation in eukaryotes, ATG sequences upstream of the translation initiation codon can

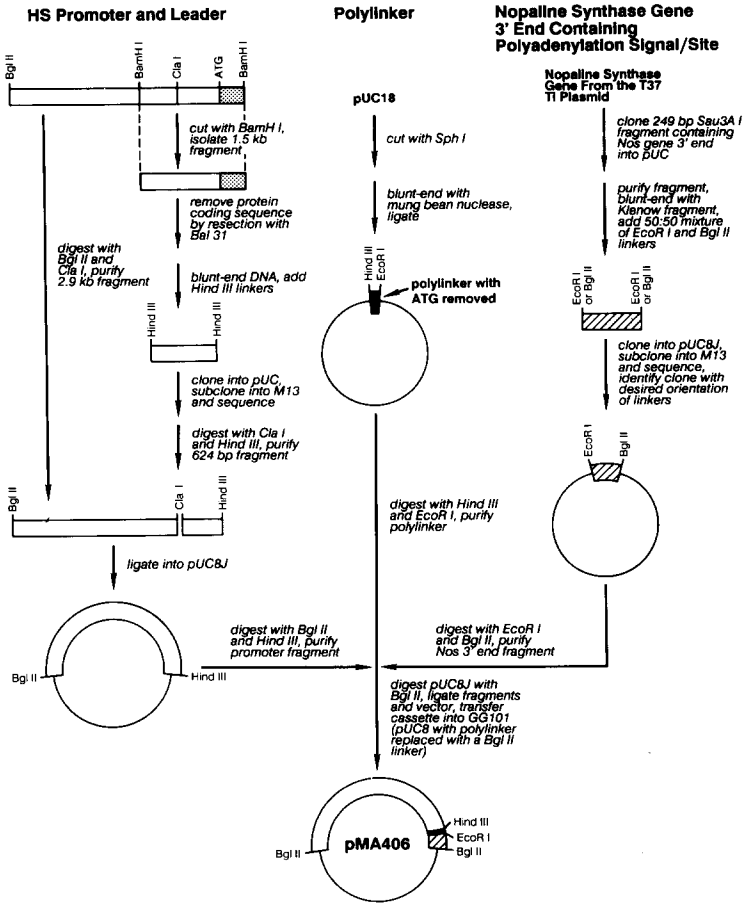

Fig. 1. Construction of heat inducible expression cassette.

severely reduce the level of translation of mRNAs in plants [5]. Accordingly, the polylinker used in the cassette was modified to remove the ATG (constituting the *Sph* I site) from the polylinker. The pUC derivative of the cassette (pMA406) has eight unique restriction endonuclease sites within the polylinker including sites for *Hin*d III, *Sal* I, *Acc* I, *Hin*c II, *Sma* I, *Xma* I, *Kpn* I, and *Sst* I.

Construction of HS-GUS chimeric genes and perfect translational fusions

One of the potential problems in constructing chimeric genes for introduction into plants is that transcriptional fusions, i.e., constructs where the fusion of the promoter and protein coding sequence occurs within the sequence encoding the mRNA leader sequence, may not expressed or are expressed at low levels [30]. To test whether sequences that are important to the transcription or translation of a chimeric HS-GUS gene have been removed from the 2019E portion of the construct or whether sequences that are inhibitory to the expression of the gene have been added by insertion of the GUS coding sequence, perfect fusions in which all of the 5′ or 3′ nontranslated sequences of the constructs are fused to the translation initiation or termination codons of the GUS gene, respectively, were made for all of the relevant constructions (see Fig. 5).

To test the importance of the 3′ end of the 2019E HS gene to the regulation of the 2019E gene expression during a 2 hour heat shock, HS-GUS chimeric genes containing either the *nos* (pMA417) or 2019E (pMA422) 3′ end sequence were prepared (Fig. 3). The Sau3AI fragment containing the 2019E gene 3′ end begins within the termination codon of the gene and extends 35 bp past the polyadenylation site that was mapped by Czarnecka *et al.* [12] and therefore should contain polyadenylation signals that precede the polyadenylation site and any conserved sequences that have been identified that lie 3′ to polyadenylation sites [51]. This fragment was exchanged with the *nos* gene 3′ end in pMA417 to create pMA422. Perfect fusions were made from the entire construct in Bluescript (5′ perfect fusion) or fragments of the constructs in M13 (3′ perfect fusions) using oligonucleotide-directed mutagenesis. Figure 4 shows the sequences that are deleted (underlined) or added (boxed) to create the perfect fusions. Chimeric HS-GUS-*nos* and HS-GUS-2019 genes containing perfect

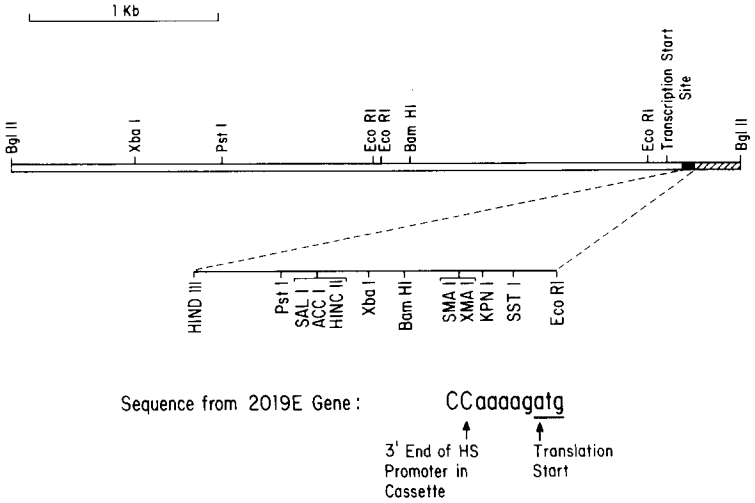

Fig. 2. Heat shock inducible expression cassette. The open box indicates the promoter from the 2019E heat shock gene; the solid box represents the polylinker region from pUC18 with the *Sph* I site removed; the hashed box denotes the 3′ end from the nopaline synthase gene (*nos*). Restriction enzyme sites in the polylinker written in upper case letters are unique in pMA406 (a pUC derivative). 2019E is the abbreviated designation of the *Gmhsp17.5-E* gene.

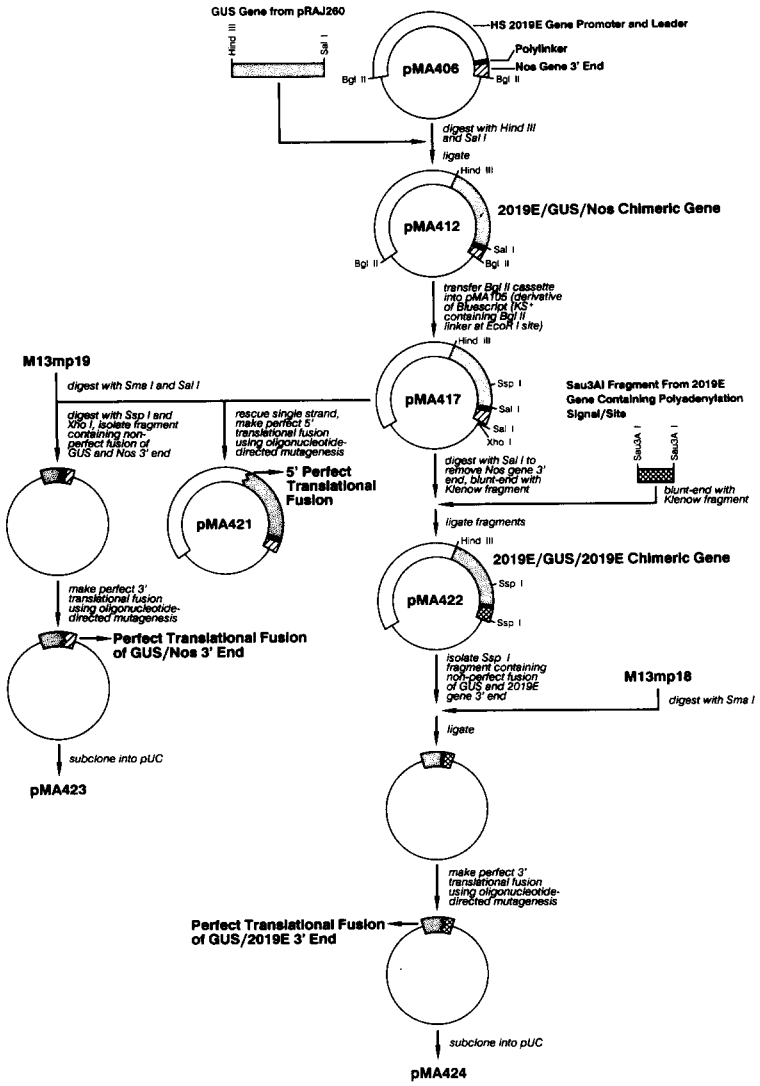

Fig. 3. Construction of heat shock promoter-β-glucuronidase (GUS) chimeric genes and perfect translational fusions. 2019E is the abbreviated designation of the *Gmhsp17.5-E* gene.

fusions at the 5′, 3′ or both the 5′ and 3′ ends were constructed from plasmids indicated in Fig. 5. The use of small fragments containing the perfect fusions (fragments C, F and J in Fig. 5) minimized the amount of sequencing required to verify the accuracy of the *in vitro* DNA synthesis.

Transient expression system

A moderate level of expression of the 2019E heat shock gene at control temperatures was observed in protoplasts from *N. plumbaginafolia* shoot cultures, *Arabidopsis thaliana*, and carrot suspension cultures (A. Merlo, personal communication). The induction of a stress response in protoplasts has been described previously and has been attributed largely to a response to the presence of an osmoticum [15]. When minimized by changing conditions of protoplast preparation and electroporation, the expression of the 2019E gene at control temperatures in the *N. plumbaginafolia* system is about 10% of the maximally induced level.

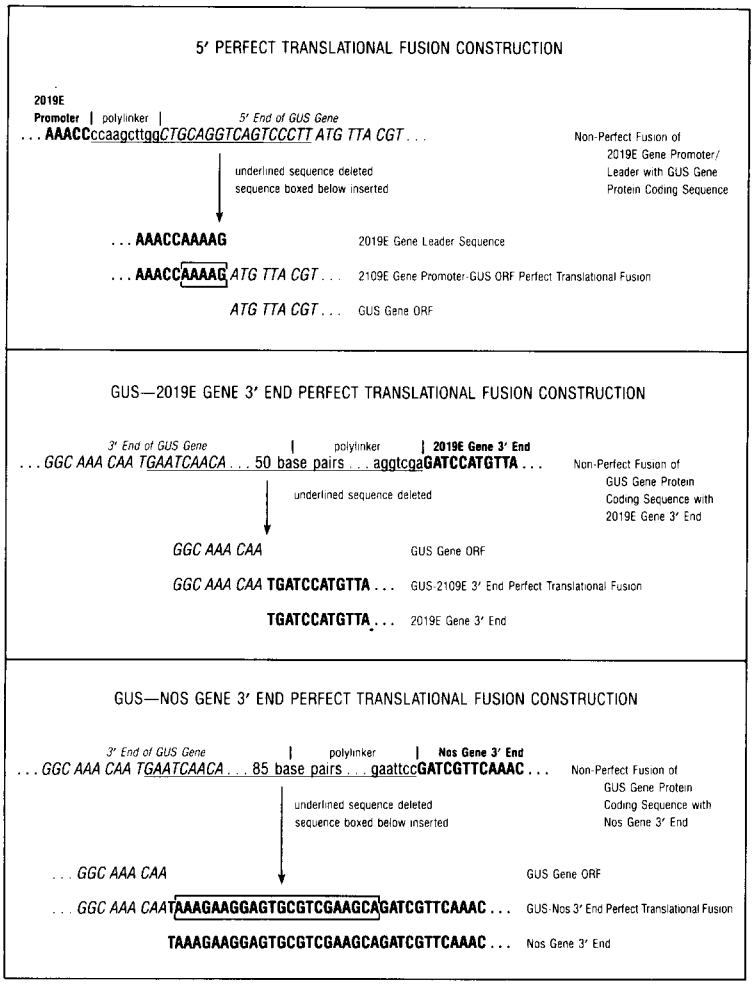

Fig. 4. Construction of perfect translational fusions by oligonucleotide-directed mutagenesis. 2019E is the abbreviated designation of the *Gmhsp17.5-E* gene.

A necessary concern when using a transient expression system is that the regulation of expression of introduced genes does not accurately parallel the regulation *in vivo* due to saturation of limiting amounts of transcriptional or other regulatory factors by multiple copies of the introduced gene. For example, Lauret and Baserga [37] have shown that a competing DNA expressing a protein product from apparently any higher eukaryotic gene essentially abolished expression of their test gene in Syrian hamster cells. To test whether factors necessary for the expression of heat shock genes are limiting in the *N. plumbaginifolia* system, 5 μg of a HS-GUS construct was mixed with increasing amounts of plasmids carrying either: 1) the heat shock inducible expression cassette without an insert, 2) the cassette containing the chloramphenicol acetyltransferase gene, or 3) the cassette containing the HSP70 gene from *A. thaliana*. All samples were brought to the same final DNA concentration, with Bluescript plasmid DNA added as necessary. None of the 'competing' DNAs affected the level of expression of the HS-GUS chimeric gene (data not shown), demonstrating that under the conditions of electroporation and the range of DNA concentrations (up to 30 μg) used in these studies, the factors necessary for expression of the

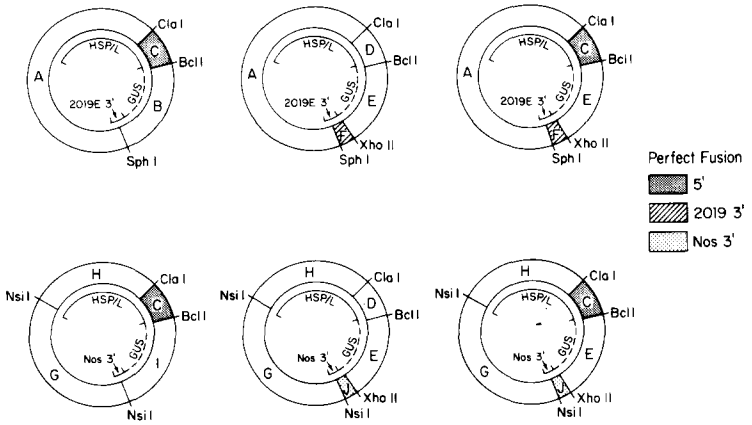

Fig. 5. Reconstruction of heat shock promoter-GUS chimeric genes containing perfect translational fusions at the 5′, 3′ or both 5′ and 3′ ends. The inner arc indicates the parts and extents of the chimeric genes: HSP/L: heat shock promoter and leader; GUS: GUS gene open reading frame; 2019E 3′: 3′ end from 2019E gene; *nos* 3′: 3′ end from nopaline synthase gene. The fragments used were derived from the following plasmids: A, pMA422; B, pMA422; C, pMA421; D, pMA422; E, pMA422; F, pMA424; G, pMA417; H, pMA406; I, pMA417; J, pMA423. 2019E is the abbreviated designation of the *Gmhsp17.5-E* gene.

HS-GUS chimeric gene at both the transcriptional and translational levels are not limiting.

Temperature and DNA concentration dependence of expression of the HS-GUS chimeric genes

The temperature dependence of expression of a HS-GUS-*nos* construct (pMA417) is shown in Fig. 6. The expression of the chimeric heat shock gene increases approximately 10-fold between 29 °C and 40 °C with a shallow slope of the curve between these temperatures; half-maximum expression occurs at 35 °C. Above 40 °C, expression drops dramatically.

The relationship of the expression of GUS enzyme activity versus HS-GUS DNA concentration (up to 30 μg) is not linear in the *N. plumbaginafolia* transient expression system (Fig. 7).

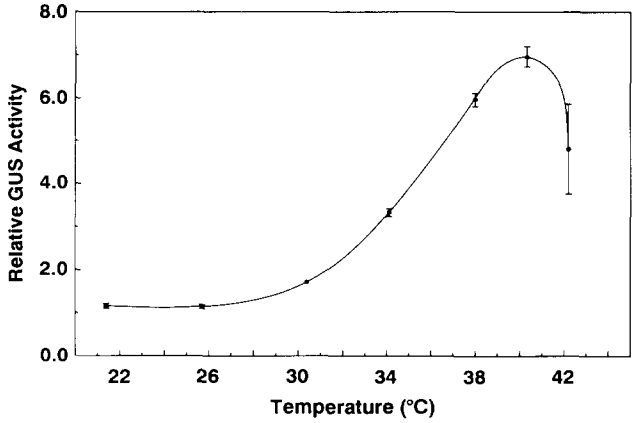

Fig. 6. Temperature-dependent transient expression of heat shock promoter-GUS chimeric genes in *Nicotiana plumbaginifolia* protoplasts. Supercoiled pMA417 was transferred to protoplasts by electroporation (10 μg DNA/10^6 protoplasts). After 22 h at 22 °C in the dark, protoplasts from several separate electroporations were pooled, divided into equal aliquots and three samples were incubated at each of the indicated temperatures for 2 h. The protoplasts were collected and GUS was extracted by the method of Jefferson [29].

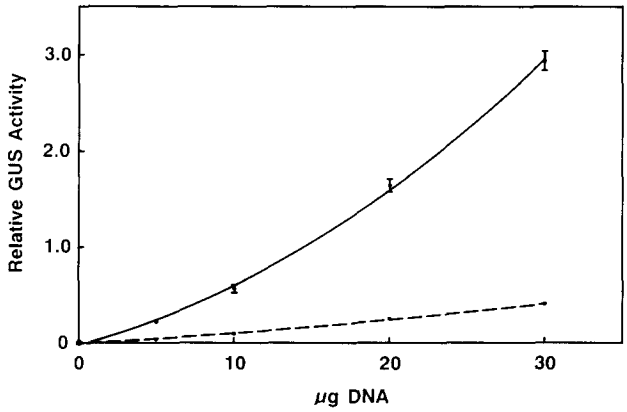

Fig. 7. DNA concentration dependence of expression of HS-GUS and HS-35S constructs. The amount of DNA indicated of the HS-GUS and 35S-GUS constructs was electroporated into protoplasts and the resultant GUS activity was measured.

This is true using pBI221 [29], a cauliflower mosaic virus (CaMV) 35S-GUS construct (assayed 24 h after electroporation) or the HS-GUS constructions and is independent of whether the DNA is linear or supercoiled. The DNA dependences of expression of the HS-GUS or 35S-GUS DNAs are similar but not identical in terms of proportional increases of expression per unit DNA added (Fig. 7). The upward slope of the curve in Fig. 7 is consistent with previously reported carrier effects showing increased transient expression with added nonspecific DNA, but this phenomenon is usually studied at much higher DNA concentrations [14]. It is presently unclear if the nonlinearity of expression with increasing DNA concentrations in the range of 5 to 30 μg DNA/ml is a peculiarity of the *N. plumbaginifolia* transient expression system.

Comparison of the HS-GUS and 35S-GUS constructions

The CaMV 35S gene promoter is considered to be one of the most highly expressed promoters in plants. Comparison of the expression of the supercoiled forms of plasmids containing GUS derivatives of the 2019E and 35S gene promoter constructions demonstrates that significantly higher levels of expression can be achieved using the HS-GUS constructs (Fig. 8). The accumulated GUS activity expressed from the 35S-GUS construct 24 h after electroporation is roughly one-tenth the activity produced during 2 h of heat shock in cells expressing the HS-GUS gene. On an hourly basis, assuming linearity of the increase of GUS activity between 18 and 24 h (data not shown) after electroporation of the 35S-GUS construct and comparing results using the supercoiled form of the plasmids carrying both constructs (Fig. 8), the HS-GUS construct is expressed at levels 80-fold higher than the 35S-GUS construct.

The HS-GUS constructs contained on supercoiled plasmids are expressed at a level 5-fold higher than when the same constructs are present on linearized plasmids (Fig. 8). By comparison there is no significant difference between the linear and supercoiled forms of the 35S-GUS construct. The results of these studies contrast with those reported for several plant and animal systems using electroporation to obtain stably transformed transgenic tissues or regenerated plants in which higher number of transformed cells are produced using linear DNA [49, 58].

Comparison of 5′ and 3′ nonperfect and perfect fusion constructions

Figure 9 shows the relative transient expression of GUS activity using HS-GUS constructs which

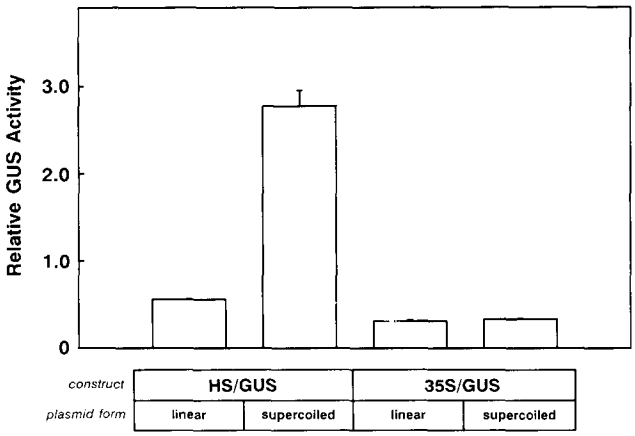

Fig. 8. Comparison of transient expression of a linear or supercoiled 35S-GUS construct (pBI121 [29]) with the expression of a linearized or supercoiled HS-GUS construct. Conditions of the electroporation and GUS activity assays are the same as detailed in the legend to Fig. 6 except 6.25 μg DNA/10^6 protoplasts were used. Protoplasts electroporated with the HS/GUS constructions were subjected to heat shock for 2 h. Protoplasts containing the 35S-GUS construct were harvested 24 h after electroporation. Determinations were done in duplicate.

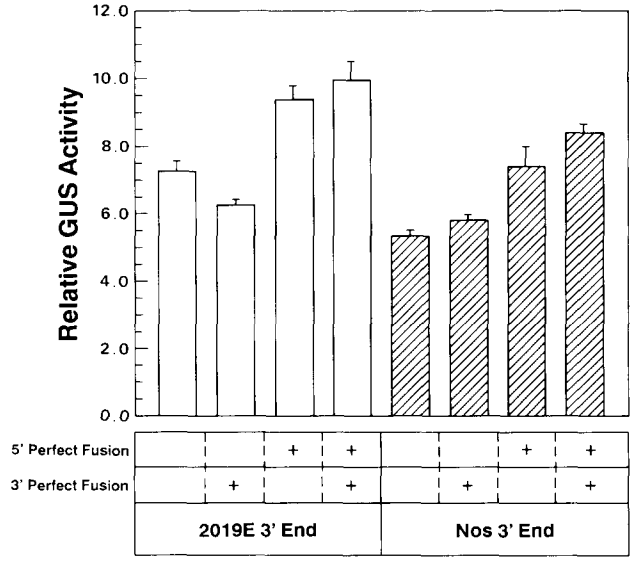

Fig. 9. Transient expression of heat shock-GUS chimeric gene constructions: Comparison of chimeric genes containing nonperfect translational fusions and perfect fusions and comparison of chimeric genes with the 3′ ends from the *Nos* and 2019E genes. Conditions of the electroporation and GUS activity assays are the same as detailed in the legend to Fig. 6 except 6.25 μg DNA/10^6 protoplasts was used and all plasmids were linearized with *Xho* I. All samples were subjected to heat shock for 2 h at 40 °C. Background activity has been subtracted from each sample. Determinations were done in duplicate. 2019E is the abbreviated designation of the *Gmhsp17.5-E* gene.

differ in having a nonperfect or perfect fusion of the 5′ or 3′ end or in having either the 2019E or *nos* gene 3′ end as described above. All DNAs used for electroporation in these experiments were linearized to avoid differences in expression due to possible different degrees of supercoiling of the plasmids. A comparison of the relevant sample pairs in Fig. 9 shows all HS-GUS-2019

and HS-GUS-*nos* constructions with a perfect 5′ fusion express approximately 40% higher GUS activity than the corresponding constructions with a nonperfect fusion. In contrast, there is only a small, if any, difference in the level of expression of a perfect fusion relative to a nonperfect fusion at the 3′ end in constructs with either the 2019E or *nos* gene 3′ end.

Discussion

A number of plant promoters have been isolated that are inducible to high levels of expression and could potentially be useful in inducible expression vectors; these include the anaerobically inducible alcohol dehydrogenase gene [17], light-inducible genes and genes induced by plant growth regulators [35]. Many of these systems suffer from an inability to achieve homogeneous distribution of the inducing agent in plants or a pattern of expression that is limited to specific tissues or organs. An alternate approach that seems promising is the use of prokaryotic- or lower eukaryotic-inducible and regulated promoters. For example, Gatz and Quail [22] have demonstrated that the prokaryotic Tn*10 tet* repressor-operator system functions correctly in plants.

The results presented here demonstrate that the soybean 2019E gene promoter can be expressed to high levels in *Nicotiana* species. The significance of several parameters were evaluated for the use of the promoter as part of an inducible cassette in transgenic plants. Moreover, the *N. plumbaginifolia* protoplast transient assay system is shown to be useful for quick analysis of constructs without using the labor- and time-intensive generation of transgenic plant tissue or plants. Importantly, this allows the systematic and thorough evaluation of constructs to identify the most ideal constructs for analysis in transgenic plants. The heat shock inducible cassette was designed to allow construction of a chimeric gene in a pUC derivative which can be used for transient expression experiments: subsequently, the cassette can be transferred easily and with high efficiency to a T-DNA vector by standard cloning procedures. Alternately, a gene of interest can also be cloned directly into pMA445, a derivative of the T-DNA vector pGA470 [2] containing the heat shock inducible expression cassette.

The advantage of using a heat shock promoter in an inducible cassette is that the heat shock response and the promoter elements that regulate the response are conserved not only between species but even between kingdoms, suggesting a high probability that a plant heat shock promoter will be properly regulated in most plant species. Moreover, with the possible exception of pollen [42], all plant tissues studied thus far undergo a heat shock response. The maize HSP70 [52] and *Drosophila* HSP70 [63] gene promoters have been shown to be heat inducible in heterologous plant species. Another soybean heat shock gene has been shown to be properly expressed in transgenic tobacco plants [4]. The 2019E gene promoter used here has been shown to be highly expressed in sunflower tumor tissue [25], maize protoplasts (J. Walker, personal communication), carrot protoplasts (A. Merlo, personal communication), *Arabidopsis thaliana* protoplasts (P. Strozycki, personal communication) and transgenic tobacco (W.M. Ainley *et al.*, manuscript in preparation). Despite the lack of detectable expression in soybean [11] there is expression at control temperatures in all protoplast systems tested thus far. In stably transformed plants, the expression of the 2019E gene promoter at control temperatures is 1–2% of the heat-induced level of expression (W.M. Ainley and J.L. Key, unpublished results).

The high levels of expression that can be achieved by the 2019E gene promoter throughout a range of elevated temperatures suggests that a temperature can be chosen for long term (e.g., several weeks) experiments in which only minimal effects, if any, on the normal physiology or development of the plants would be expected. In *N. plumbaginifolia* protoplasts, 50% of maximal expression occurs at 35 °C, 5 °C below the temperature at which maximal expression occurs, suggesting that even a mild heat shock treatment should achieve a reasonable level of expression in transgenic plants of any gene cloned in the cassette. The heat shock response is regulated

such that it shuts off after a period that depends on the temperature of the heat shock [10, 45]. For long-term expression studies, transgenic plants would have to be subjected to several cycles of heat shock and recovery; the actual length of the cycles and temperatures used would have to be determined empirically. Such a strategy has been suggested for using *Drosophila* heat shock gene promoters in expression vectors [47]. We have used this approach to show that morphologically normal heat shock induced shoots can be formed by daily heat shocks of leaf discs from transgenic plants containing the *ipt* gene (producing cytokinin, a plant hormone which can induce shoots) from *Agrobacterium tumefaciens* Ti plasmids [8] cloned into the heat inducible expression cassette (W.M. Ainley *et al.*, manuscript in preparation). This experiment, taken together with the high levels of expression of the 2019E gene promoter suggests that long term expression of heat shock promoter constructs in transgenic plants is feasible. In addition, if higher levels of expression of introduced DNA are required, the expression can be doubled by obtaining homozygous F2 plants.

The expression of a chimeric HS-GUS construct with a perfect 5′ fusion is expressed at roughly 40% higher levels than the corresponding construct with a nonperfect fusion. This contrasts with the results of Jones *et al.* [30] in which several chimeric gene constructs with transcriptional fusions are poorly expressed, if at all, while translational fusions were expressed at high levels. It is unclear if this problem is specific to their constructs or is a general problem. Because the *Sph* I site (containing an ATG) in the polylinker in the cassette has been removed, eight restriction sites are available for cloning of sequences. Reporter genes have been cloned into both the *Hin*d III and *Sal* I sites in the cassette and are expressed at high levels in protoplasts. We have not determined if inserts at the other sites are expressed at levels as high as the constructs studied here. Together, six inserts have been cloned into the cassette by nonperfect fusions and all have been expressed in transgenic plants (W.M. Ainley, R.T. Nagao and J.L. Key, unpublished data). Based on this, it seems likely that most sequences can be expressed when cloned directly into the cassette. However, to assure the highest levels of expression, perfect 5′ translational fusions for the chimeric gene constructs can be made.

Creation of a perfect 5′ fusion of the HS-GUS constructions adds 5 bases of the 2019E gene leader sequence, removes 27 bases of the GUS untranslated leader region and polylinker of the vector. A consequence of this sequence change is that an A residue occurs at the −3 position relative to the ATG initiation codon in the perfect fusion compared to a C residue at this position in the nonperfect fusion. Without further study there is no way to predict the consequences of most of the sequence alterations made in constructing the perfect fusions; however, based on the sequence for optimal translation initiation in eukaryotic systems [34] the difference at the important −3 position would predict the perfect fusion would be translated at a significantly higher level than the nonperfect fusion. The results of Taylor *et al.* [65] while not a complete analysis, suggest that maximal translation of plant mRNAs require the same sequence environment surrounding the ATG initiation codon as other eukaryotes. Specifically, one experiment showed that a C to A change at the −3 position (the same difference that occurs between the perfect and nonperfect fusions) increases expression of a chitinase gene two-fold. The results comparing the perfect and nonperfect 5′ fusion of the HS-GUS constructs are qualitatively but not quantitatively similar to this.

There is no significant difference in the level of expression of constructs containing the nonperfect and perfect fusions of the 3′ nontranslated regions from the *nos* or the 2019E genes in the transient expression systems, suggesting that the sequences required for transcription termination and polyadenylation do not lie close to the 3′ end of the protein coding region of the *nos* gene (this region is deleted in the nonperfect fusion) and that, at least to the extent of the changes made in the constructions shown in Fig. 4, the spacing of the 3′ nontranslated region relative to the open reading frame of these mRNAs is not critical.

Constructs containing the 3′ end of the *nos* gene are expressed at only slightly lower levels than those with the 2019E gene 3′ end during a 2 h heat shock at the temperature giving maximal expression of the heat shock promoter. This indicates that the 2019E gene 3′ end contains no sequences that are necessary for the regulation or enhancement of the expression of this heat shock gene under these conditions, providing only functional transcription termination and polyadenylation sites. The polyadenylation signals and sites in plant genes are presently poorly understood and no strongly conserved consensus sequence has been identified [31].

There are several reports that the 3′ nontranslated mRNA sequences are involved in mRNA stability, the 3′ end often making mRNA turnover responsive to developmental or environmental changes (for reviews see [50, 57]). While the presence of the 2019E gene 3′ end does not allow any apparent preferential expression of the 2019E gene mRNA during a 2 h heat shock, it may have a role in modulating the steady-state mRNA levels during lower temperature or long-term heat shock treatments, or during recovery at normal temperatures subsequent to a heat shock treatment. Simcox *et al.* [59] demonstrated that when part of the 3′ end of the *Drosophila* hsp70 gene mRNA is removed, the transcript no longer is rapidly degraded at control temperatures. The importance of the 2019E gene 3′ end under various heat shock regimes is currently being investigated.

The HS-GUS construct is expressed at levels 80-fold higher than the 35S-GUS construction in the transient expression assay (Fig. 8). The only valid comparison between two promoters is one which compares constructs having an identical transcription unit, differing only in the promoter regions 5′ to the transcription start site. While by this criteria the comparison here is not appropriate, due to the magnitude of the differences between the expression of the two promoter-GUS constructs, it seems quite likely that the heat shock promoter is a much stronger promoter than the 35S gene promoter.

An unexpected result from this work is that the 2019E gene promoter is expressed at a significantly higher level (5-fold) when in a supercoiled form relative to a relaxed form. While the importance of torsional stress of some genes has been well-documented in prokaryotes [38], it has only recently been shown using an *in vitro* transcription system to affect the expression of eukaryotic genes [28]. The recent demonstration that DNA complexed into nucleosome structures can be supercoiled without disruption of the nucleosome association supports the potential role for changes in the topological form of a eukaryotic gene sequence to regulate its expression [21]. Moreover, Tsutsui *et al.* [67] have identified binding sites in the nuclear matrix that are specific for supercoiled DNA. Whether such an association represents the same binding to the nuclear matrix that has been ascribed to an actively transcribed gene remains to be shown. Two plant heat shock genes that have been the most extensively studied, the 2019E gene [12, 13, 25] and the *hs6871* gene [4], both have an AT-rich region upstream from the start of transcription which contributes significantly to their expression. It is interesting to speculate that such regions may have a role in supercoil-dependent expression of these heat shock genes either as AT-rich regions which are characteristic of topoisomerase binding sites [54] or by representing regions that melt during heat shock thereby increasing the torsional stress in adjacent regions. The latter possibility would be a simple and energetically inexpensive mechanism for regulating the expression of heat shock genes that would be independent of transcription or translation.

While the heat shock inducible expression cassette is highly expressed at HS temperatures, even higher levels of expression might be possible. This might be accomplished by altering the sequence of the heat shock elements of the 2019E gene promoter so that they are identical to the HSE consensus sequence, using all or part of an mRNA leader sequence that allows extremely efficient translation such as the omega sequence from the tobacco mosaic virus RNA [19, 60], or addition of introns which has been demonstrated both in plants [9] and animals [7] to enhance expression.

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