Virus Induction of Heat Shock Protein 70 Reflects a General Response to Protein Accumulation in the Plant Cytosol[1]

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Different cytoplasmically replicating RNA viruses were shown to induce a specific subset of heat-inducible heat shock protein 70 (*HSP70*) genes in Arabidopsis (*Arabidopsis thaliana*). To identify the inducing principle, a promoter::reporter system was developed for the facile analysis of differentially responding Arabidopsis *HSP70* genes, by infiltration into *Nicotiana benthamiana* leaves. Through transient expression of individual viral cistrons or through deletion analysis of a viral replicon, we were unable to identify a unique inducer of *HSP70*. However, there was a positive correlation between the translatability of the test construct and the differential induction of *HSP70*. Since these data implied a lack of specificity in the induction process, we also expressed a random series of cytosolically targeted Arabidopsis genes and showed that these also differentially induced *HSP70*. Through a comparison of different promoter::reporter constructs and through measurements of the steady-state levels of the individual proteins, it appeared that the *HSP70* response reflected the ability of the cytosol to sense individual properties of particular proteins when expressed at high levels. This phenomenon is reminiscent of the unfolded protein response observed when the induced accumulation of proteins in the endoplasmic reticulum also induces a specific suite of chaperones.

Organisms are continuously battered by internal and external stresses that perturb the balance of cellular control. In many cases, these lead to a destabilization of the cellular protein machinery through the incorrect folding and inactivity of expressed proteins. These effects are corrected through the recruitment of a suite of chaperone proteins designed to refold aberrant proteins or to target them for degradation. Hence, denatured or incorrectly folded proteins resulting from heat stress are countered by increased activity of multiple classes of chaperones, including heat shock protein 70 (HSP70).

HSP70 is one of the major classes of chaperone molecules and plays many roles in eukaryotic cells. In addition to its part in responses to abiotic stress, HSP70 also functions in response to pathogen attack. Hence, in plants, HSP70 and HSP90 are essential components of the hypersensitive response defense mechanism (Kanzaki et al., 2003), and HSP70 is induced by virus infection in both animal and plant cells (Aranda and Maule, 1998; Whitham et al., 2003).

HSP70 induction in response to plant virus infection is tightly controlled, spatially and temporally, such that recently infected cells (i.e. those showing high levels of virus replication and viral genome expression) accumulate *HSP70* mRNA and protein (Aranda et al., 1996; Escaler et al., 2000). This phenomenon applies to infections with a diverse range of viruses, including members from the Potyvirus, Tobravirus, Potexvirus, Geminivirus (Aranda et al., 1996; Escaler et al., 2000), and Cucumovirus (Havelda and Maule, 2000) genera. While the detailed mechanism of *HSP70* induction by plant viruses has not been investigated, for animal viruses specific viral proteins have been shown to be active (Aranda and Maule, 1998, and refs. therein). From studies of potyvirus induction of heat-inducible proteins (Aranda et al., 1996), however, it seems that the processes of heat and virus induction of HSPs are different.

Similarly, the purpose of HSP70 induction for plant viruses is uncertain. It might be expected to fulfill a requirement for rapid protein maturation and turnover during a short virus multiplication cycle. Alternatively, there is evidence that HSP70 may play a role in virus cell-to-cell movement. Hence, members of the Closteroviridae use a self-encoded HSP70 homolog as a virion component to assist the movement of the particle through plasmodesmata (Prokhnevsky et al.,

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2002). Furthermore, in a separate study (Aoki et al., 2002), host-encoded HSP70 was shown to traffic through plasmodesmata and, therefore, may be required for other viruses to cross the same cellular boundary.

In this work, we show that viruses do not encode a specific inducer of HSP70. Instead, it is the high-level production of virus-specific proteins in the cytosol associated with virus replication that induced a generic response to ectopic protein expression and accumulation, a process that involves a specific subset of chaperones. We also show that this response is not restricted to virus-encoded proteins and may reflect a process analogous to the unfolded protein response (UPR) in the endoplasmic reticulum.

RESULTS

Induction of Hsp70 in Arabidopsis

The *HSP70* gene family in Arabidopsis (*Arabidopsis thaliana*) comprises 14 members, of which five are predicted to be cytosolic (Sung et al., 2001). Since plant RNA viruses replicate in the cytoplasm, we studied these five members for their induction by a range of compatible viruses. In uninfected leaves, three of the five *HSP70*s (called heat shock cognate genes, *HSC70*; *AtHSC70-1*, *AtHSC70-2*, *AtHSC70-3*) were constitutively expressed at a very low level, although *AtHSC70-1*, *-2*, and *-3* and the remaining two genes, *AtHSP70* and *AtHSP70B*, were also induced by heat (Sung et al., 2001; Fig. 1A). Infection of Arabidopsis with *Turnip mosaic virus* (TuMV; genus Potyvirus) or *Turnip crinkle virus* (TCV; genus Carmovirus) induced four of the five genes in the systemically infected tissues, *AtHSP70B* remaining unaffected (Fig. 1A).

Rapid Analysis of *AtHSP70* Induction in *Nicotiana benthamiana*

To study the mechanism of *HSP70* transcriptional induction and identify the viral inducers, it was necessary to have a rapid, reproducible, and quantitative assay. To assist in this analysis, we constructed promoter::reporter fusions using approximately 2-kb genomic fragments from upstream of *AtHSP70* and *AtHSP70B* fused to the β-glucuronidase (*GUS*) gene in Agrobacterium binary vectors (Fig. 1B, pHSP70::GUS, pHSP70B::GUS). While Agrobacterium-mediated transient expression provides a rapid assay, we found that this was not highly reproducible when Agrobacterium cultures were infiltrated into Arabidopsis leaves. As an alternative, we infiltrated the promoter::reporter constructs into expanded leaves of *N. benthamiana*. To confirm heat inducibility, the Agrobacterium cultures were infiltrated and the leaves were analyzed for GUS activity after 3 d following a heat treatment at 42°C. Neither of these constructs showed constitutive expression, but both showed clear GUS

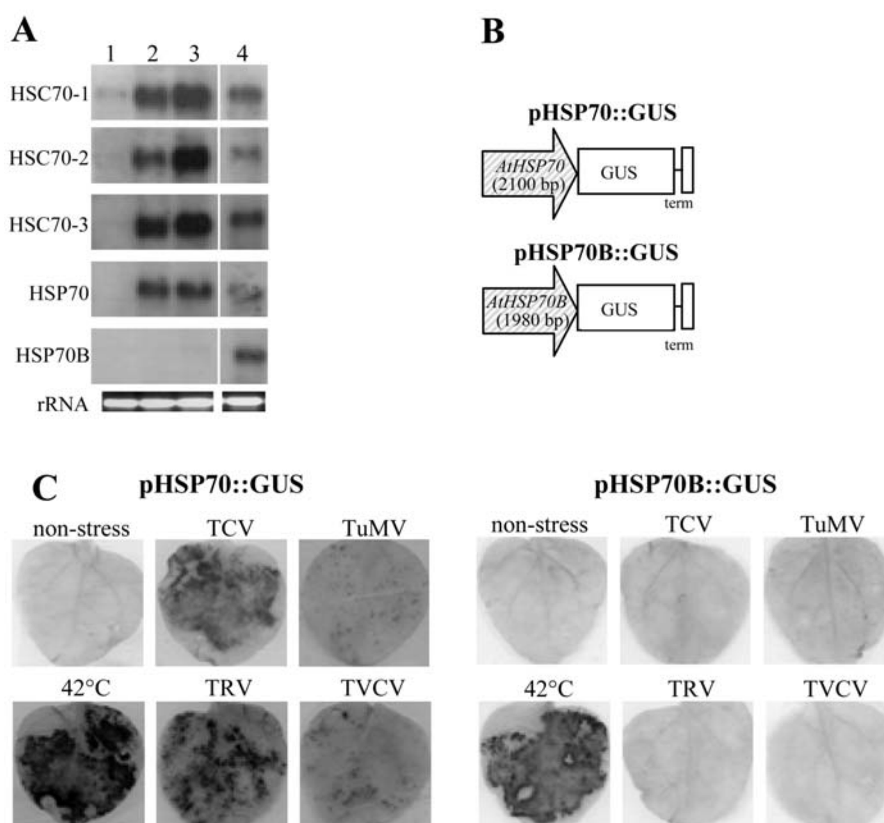

Figure 1. Induction of Arabidopsis *HSP70* genes by heat stress or virus infection. A, Total RNA preparations from Arabidopsis leaf tissues, untreated (lane 1), systemically infected with TuMV (lane 2) or TCV (lane 3), or subjected to heat stress at 42°C (lane 4), were analyzed on RNA blots using probes specific for each of the five cytosol-targeted *HSP70* genes (*HSC70-1*, *HSC70-2*, *HSC70-3*, *HSP70*, and *HSP70B*). B, Promoter::reporter fusion Agrobacterium binary constructs were made for *AtHSP70* and *AtHSP70B* utilizing 2.1 kb and 1.98 kb, respectively, of the upstream genomic DNA fused to GUS and the CaMV 19S terminator (term). C, Agrobacterium carrying the binary construct pHSP70 or pHSP70B was mixed with a second Agrobacterium culture carrying either an empty binary vector (non-stress and 42°C) or a binary vector including complete cDNAs for TuMV, TCV, TRV, or TVCV and coinfiltrated into expanded leaves of *N. benthamiana*. After 4 d, the leaves were stained for GUS activity. Data shown in A and C are representative of at least five independent experiments.

activity when leaves were heat stressed (Fig. 1C), confirming the basic expression characteristics observed in Arabidopsis. To assess the differential inducibility of pHSP70::GUS and pHSP70B::GUS by virus infection, each construct was coinfiltrated into *N. benthamiana* leaves with constructs carrying full-length viral cDNAs of TuMV, TCV, *Tobacco rattle virus* (TRV; genus Tobravirus), or *Turnip vein clearing virus* (TVCV; genus Tobamovirus), cloned after the cauliflower mosaic virus (CaMV) 35S promoter. All of the viruses are virulent on *N. benthamiana* and showed no symptoms over the duration of the experiment (3 d). Coinfiltration of the reporter constructs with an empty vector showed no GUS activity in either case. All four viruses showed induction of pHSP70::GUS but no induction of pHSP70B::GUS (Fig. 1C), confirming again the observations made following systemic infection of Arabidopsis. The strength of induction for the four viruses was consistently in the order TCV, TRV, TVCV, and TuMV, with only TCV giving induction close to that seen following heat shock.

Analysis of the Virus Inducer of *HSP70*

Two strategies were employed to identify the virus inducer of pHSP70::GUS: coexpression of individual viral cistrons or the subtraction of functional cistrons from the viral replicon. Experiments concentrated on TCV since, organizationally, it is among the simplest of the four viruses (Carrington et al., 1989; Fig. 2A) and because it gave the highest levels of induction in comparison with the other viruses. Constructs expressing each of the TCV cistrons after the CaMV 35S promoter (Fig. 2A) were coinfiltrated with pHSP70::GUS or pHSP70B::GUS. All of the constructs showed specific induction of pHSP70::GUS (Fig. 2B) but not pHSP70B::GUS (data not shown). To confirm that this was related to the expression of the viral proteins rather than the presence of the viral RNA or to the transcriptional activity of the CaMV 35S promoter, 35S::p38stop, which has two consecutive stop codons placed after the ATG, was tested. This showed a level of GUS activity similar to that from the empty vector control. Similarly, a series of mutated TCV genomes (Fig. 2A) were coinfiltrated with the reporter constructs. TCVΔ88, TCVΔ9, TCVΔ8, and TCVΔ38 have point deletions leading to frame-shift mutations. TCVΔ8, TCVΔ9, and TCVΔ38 are competent for viral replication, and other viral products were expected to accumulate. By contrast, TCVΔ88 and another mutant, TCVΔ88/28, were not competent for virus replication (Hacker et al., 1992). However, TCVΔ88 could theoretically produce a p28/p88 nonfunctional peptide of 400 amino acids from the expressed RNA, whereas, as a consequence of the two inserted stop codons, the TCVΔ88/28 mutant was prevented from protein expression. The predicted infectivity of all the mutants was confirmed by RNA-blot analysis after their infection of *N. benthamiana* (data not shown). After coinfiltration, every viral construct with protein-synthesizing capacity showed significant GUS induction with pHSP70::GUS (Fig. 2B) but not with pHSP70B::GUS (data not shown). Moreover, induction occurred whether viral protein expression was associated with virus replication (TCVΔ8, TCVΔ9, and TCVΔ38) or whether it came directly from the 35S-driven mRNA, as in the case of 35S::TCVΔ88 (Fig. 2B). By contrast, TCVΔ88/28 gave no significant induction.

In two additional series of experiments, we tested the hypothesis that viral induction of pHSP70::GUS represented a broad response to the expression of

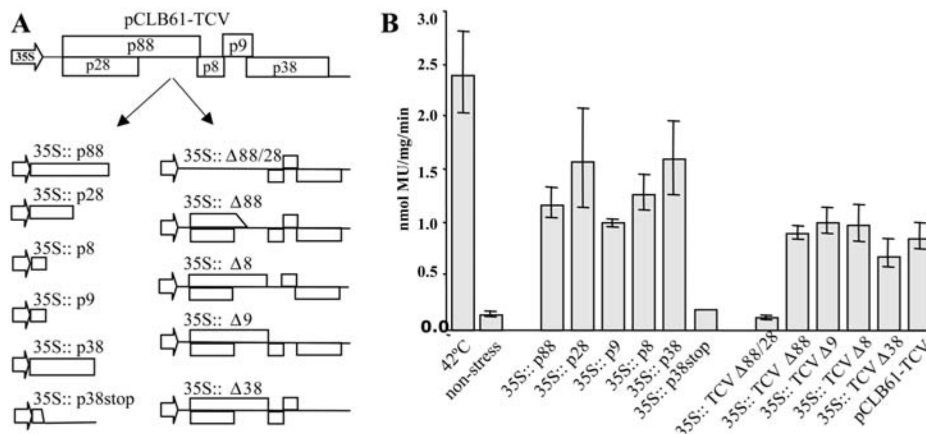

Figure 2. Induction of pHSP70 by the expression of individual TCV proteins or by TCV mutants. A, The genome of TCV was dissected into its component cistrons (p88, p28, p8, p9, p38) and each cistron cloned after the CaMV 35S promoter as a binary construct. A construct, p38stop, was the same as p38 except that two stop codons were inserted immediately after the p38 ATG. In parallel, virus mutants Δ88/28, Δ88, Δ8, Δ9, and Δ38 were similarly constructed. B, Agrobacterium culture carrying the binary TCV constructs or the empty vector (non-stress and 42°C) was mixed with a culture carrying pHSP70::GUS and infiltrated into expanded leaves of *N. benthamiana*. Quantitative GUS activity was measured fluorimetrically after 3 d. Assays were carried out in triplicate on individual extracts of infiltrated leaves from three plants. Bars show ±SE of the mean value.

virus proteins. For the first experiment, we selected the *Tobacco mosaic virus* (TMV; genus Tobamovirus) movement protein (MP), the P1 protein from *Pea seed-borne mosaic virus* (PSbMV; genus Potyvirus), the 6-kD protein (6K1) fused to the cylindrical inclusion (CI) protein from PSbMV (PSbMV-6K1CI), and protein p19 from *Cymbidium ringspot virus* (CymRSV; genus Nepovirus). After coinfiltration with pHSP70::GUS, all of these genes induced GUS activity (Fig. 3). In the second experiment, we compared the replicating TRV RNA1 and nonreplicating TRV RNA2 for induction of pHSP70::GUS. TRV is a bipartite RNA virus with the replication functions located on RNA1 and the MP and capsid protein genes located on RNA2 (MacFarlane, 1999). RNA1 can replicate in the absence of RNA2 and produces three proteins (MacFarlane, 1999). However, RNA2 requires RNA1-encoded functions for replication. In this experiment and in the absence of replication, RNA2 could produce the MP by translation of the CaMV 35S-driven mRNA. RNA1 and RNA2 cDNAs were cloned after the CaMV 35S promoter in a binary vector and coinfiltrated with pHSP70::GUS, as in the other experiments. Separately, both RNA1 and 2 showed significant increases in GUS activity. Interestingly, a combination of the RNAs (RNA1+2) showed an additive effect (Fig. 3).

HSP70 Is Induced by Plant Genes

Since virus protein expression broadly activated pHSP70::GUS but not pHSP70B::GUS, it was logical

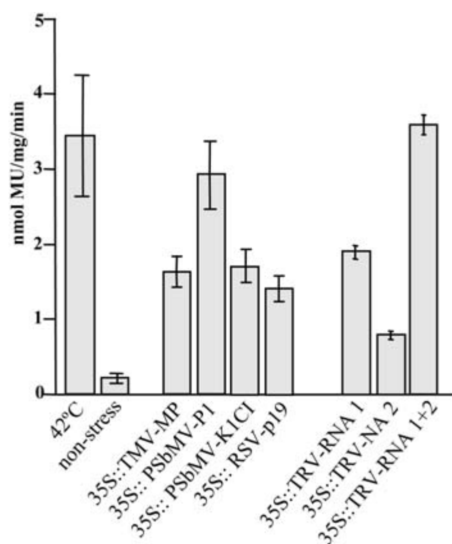

Figure 3. Induction of pHSP70 following expression of other plant viral gene products. Constructs for the expression of TMV-MP, PSbMV-6K1CI, PSBMV-P1, and CymRSV-p19 were coinfiltrated into *N. benthamiana* leaves with pHSP70::GUS or with the empty vector (non-stress and 42°C) as controls. All of these proteins showed significant induction of pHSP70::GUS as increased GUS activity. Similarly, leaves were coinfiltrated with pHSP70::GUS and constructs expressing TRV RNA1, RNA2, or RNAs 1 and 2 (RNA1+2). All assays were carried out on three replicates of infiltrated leaves from each of two plants. Bars represent ±SE.

to ask whether plant proteins would be perceived differently. To test this, we selected five random full-length Arabidopsis cDNA clones constructed as translational fusions to the fluorescent reporter enhanced green fluorescent protein (eGFP). The fusion proteins had all been shown to be targeted to the cytosol after expression using the 35S promoter (Koroleva et al., 2005). Separate coinfiltration of pHSP70::GUS with the five constructs expressing fusion proteins again showed strong GUS activity in *N. benthamiana* leaves (Fig. 4A) but no induction of pHSP70B::GUS. For the five proteins, the level of pHSP70::GUS induction was similar and at a level as high as that seen with the viral proteins in separate experiments. One protein, At1g74560, reproducibly induced pHSP70::GUS at a slightly lower level. In the absence of antibodies for these randomly selected proteins, the eGFP fusion proteins also allowed us to estimate the relative concentrations of the expressed proteins as GFP fluorescence. Although there was up to 4-fold difference in the level of protein accumulation, there was no correlation with induced GUS activity (Fig. 4B). Data collected using GFP-specific antibodies to detect the fusion proteins led to identical conclusions (data not shown).

Induction Is Related to the Strength of the Ectopic Promoter

All of the constructs in the preceding assays were on binary vectors that also contained functional genes. Particularly, they carried the gene for resistance to the herbicides bialophos or kanamycin expressed from the Agrobacterium nopaline synthase promoter (NOS). The absence of pHSP70::GUS induction with the empty vector control implied that NOS-expressed genes escaped detection within the cytosol. To test this formally, one strongly inducing viral gene, 6K1CI from PSbMV (Fig. 3), and one Arabidopsis gene, At3g23600 (Fig. 4A), were cloned with NOS replacing the CaMV 35S promoter. Coinfiltration of these constructs with pHSP70::GUS showed no significant induction of GUS activity (Fig. 4C). The NOS promoter is weaker than the 35S promoter, as shown by the relative accumulation of the At3g23600 protein when detected on an immunoblot using anti-GFP antiserum (Fig. 4D).

DISCUSSION

HSP70 expression is induced in response to diverse biotic and abiotic stresses. We and others have reported the induction of *HSP70* by a wide range of viruses in diverse host plants, including pea (*Pisum sativum*), *N. benthamiana*, squash (*Cucurbita pepo*), tobacco (*Nicotiana tabacum*), and Arabidopsis (Aranda et al., 1996; Escaler et al., 2000; Havelda and Maule, 2000; Jockusch et al., 2001; Whitham et al., 2003). Based on the differential induction of heat-inducible genes

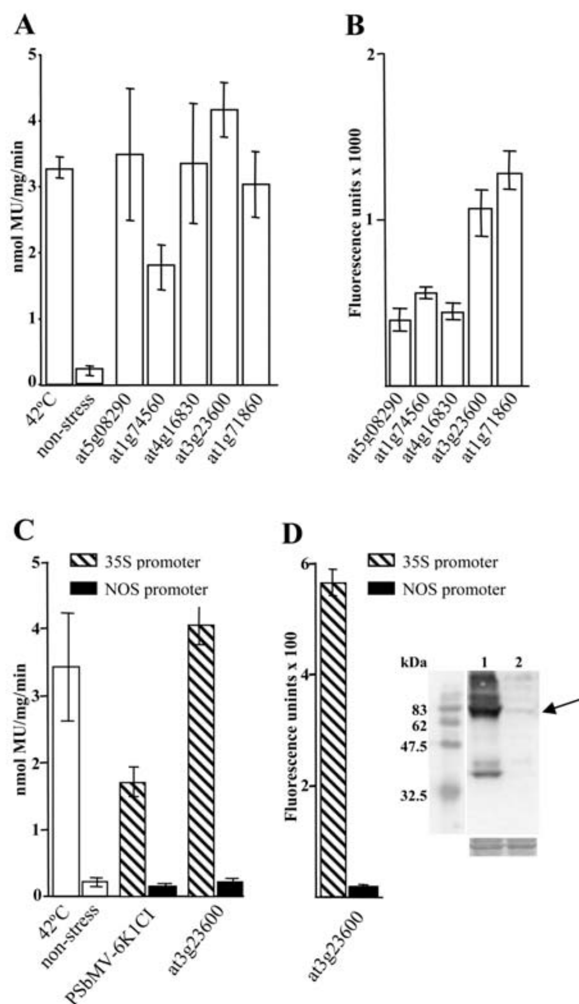

Figure 4. Induction of pHSP70 by the expression of nonviral proteins, relative to protein accumulation and promoter strength. A, Five full-length Arabidopsis cDNAs (At5g08290, At1g74560, At4g16830, At3g23600, At1g71860) fused to eGFP were tested for their abilities to induce pHSP70::GUS. The predicted functions of the selected genes are as follows: At5g08290, thioredoxin-like protein; At1g74560, nucleosome assembly protein inhibitor; At4g16830, related to cyclic nucleotide regulation of PAI-1 mRNA stability (Tillmann-Bogush et al., 1999); At3g23600, contains KOG3043 (predicted hydrolase related to dienelactone hydrolase); and At1g71860, similar to protein Tyr phosphatase 20. The expression constructs were coagroinfiltrated with pHSP70::GUS into leaves of *N. benthamiana*; empty vector (non-stress and 42°C) controls were analyzed in parallel. Quantitative GUS activity was measured fluorimetrically after 3 d. B, Tissues from the experiment in A were analyzed for the concentration of expressed proteins based on a fluorimetric assay for the fused eGFP. C, Two proteins, At3g23600 fused to eGFP and PSbMV 6K1CI, were expressed using the CaMV 35S or the NOS promoters, and the levels of pHSP70::GUS induction were compared after 3 d. D, The expression level from the CaMV 35S or NOS promoters was assessed for At3g23600 using a fluorimetric assay (left) or immunoblot assay (right) for eGFP to assess accumulation of the protein product. In the immunoblot assay, lanes 1 and 2 are from plants expressing At3g23600.eGFP from the CaMV 35S or NOS promoters, respectively. The predicted size of the fusion protein is indicated (arrow). The panel beneath confirms equal protein loadings as stained plant protein (large subunit of Rubisco). Bars represent ±SE.

by virus infection and heat shock, we also proposed that there may be specific induction mechanisms that would meet the particular needs of viral genome replication and expression and of viral pathogenesis (Aranda et al., 1999). Following this rationale, we aimed to identify the nature of the viral inducer using the model plant Arabidopsis. In line with the cytosolic location for the replication of most plant RNA viruses, we focused particularly on the family of cytosolic HSP70s. Of the five cytosolic *HSP70* genes, all but one were induced by a variety of different RNA viruses although all the genes were heat inducible, again indicating that the mechanism of transcriptional induction by viruses may be specific.

To explore the induction process in more detail, we transferred the induction principle to the more experimentally tractable and reproducible host for transient expression, *N. benthamiana*. Using 2.1 kb and 1.98 kb of upstream genomic DNA for *HSP70* and *HSP70B*, respectively, for the construction of promoter::reporter fusions, we established that the transcriptional regulation by heat and by virus infection was preserved after infiltration into leaves of *N. benthamiana*. This was a specific response to virus infection since no induction of either construct was observed after infiltration of Agrobacterium carrying an empty binary expression vector.

Three experimental strategies were used to identify the viral inducer of *HSP70*: (1) the expression of individual viral cistrons of TCV; (2) the deletion of functional cistrons from the TCV replicon; and (3) the expression of miscellaneous cistrons from other RNA viruses. This approach had the potential to identify single inducers, combinatorial inducers, indirect inducers (e.g. indirect effect of RNA replication), or classes of inducers (e.g. viral MP class or silencing suppressor protein class). TCV has a relatively simple genome that includes all the essential factors for virus replication and pathogenesis. In addition, we tested four genes from other viruses. TMV-MP assists virus movement by modifying plasmodesmata (Waigmann et al., 1994). The precise function of PSbMV-P1 is not known, but P1 from the closely related potyvirus tobacco etch virus has been shown to be an ancillary factor to the potyvirus RNA interference suppressor HC-Pro (Kasschau and Carrington, 1998). Again, the precise function of PSbMV-6K1CI is not known, but the CI protein accumulates in recently infected cells as characteristic inclusions positioned over plasmodesmal openings (Roberts et al., 1998) and has therefore been proposed to have a role in virus movement. CymRSV-p19 is the suppressor of RNA interference for this virus (Havelda et al., 2003). We also used the bipartite nature of TRV to distinguish between viral protein expression and the properties of a viral replicon for a single virus.

The surprising outcome from all these experiments was that the only consistent correlation was that induction of *HSP70*, but not *HSP70B*, was linked to the translatability of the CaMV 35S-driven expression

cassette. From the appropriate controls, we excluded any roles for the CaMV 35S promoter or viral RNA sequence as effective inducers. The generic nature of this induction was confirmed when we tested the expression of five randomly selected cytosolically targeted Arabidopsis genes for *HSP70* induction. Since these genes were expressed as fusions to GFP, we could use the GFP as an assayable marker for protein accumulation. From this it was apparent that there was no correlation for the different genes between protein accumulation and the transcriptional activation of pHSP70::GUS. Nevertheless, for a particular gene, the amount of protein accumulating was related to the strength of the promoter. Hence, 35S::At3g23600 resulted in significantly more protein accumulation and was a much more effective inducer than NOS::At3g23600. A similar difference in transcriptional induction was observed for 35S::PSbMV-6K1CI and NOS::PSbMV-6K1CI.

Since the various genes expressed from the CaMV 35S promoter show very different levels of pHSP70::GUS induction and yet induction can be modulated by reducing the level of expression (i.e. from the NOS promoter), it appears that the plant cell perceives a characteristic of individual proteins that correlates with their specific total level of accumulation. The most likely characteristic is the proportion of each protein that achieves a misfolded state. This is supported by the important observation that TMV mutants with temperature-sensitive coat proteins are more able than wild-type TMV to induce HSP70 and that this correlates with protein aggregation in vivo (Jockusch et al., 2001).

HSP70 expression is autoregulated by HSP70 binding to heat shock factor (HSF), preventing the trimerization necessary for HSF binding to the heat shock element and the activation of *HSP* transcription (Morimoto, 1998). Heat causes protein denaturation and the diversion of HSP70 to protein repair, releasing HSF. The difference between pHSP70::GUS and pHSP70B::GUS shows that heat-induced protein damage is not the only induction mechanism but that *HSP70*, and probably the other virus-induced *HSC* genes, are linked to a mechanism that may be able to sense misfolded protein directly.

Such a phenomenon is very reminiscent of the much-studied UPR. In this case, inhibition of protein glycosylation, for example, using tunicamycin, results in the accumulation of incompletely folded proteins in the endoplasmic reticulum and the transcriptional induction of a suite of chaperones, such as the luminal binding protein BiP (Rutkowski and Kaufman, 2004). Initially characterized in yeast (*Saccharomyces cerevisiae*), the UPR has been demonstrated in Arabidopsis (Martínez and Chrispeels, 2003; Noh et al., 2003). Our data are consistent with the existence of a parallel mechanism for sensing misfolded proteins in the plant cytosol.

The differential virus induction of pHSP70::GUS and pHSP70B::GUS suggests that the transient increase in HSP70 accumulation associated with active virus replication is part of a broader response triggered by the accumulation of viral structural and nonstructural proteins. So far, it is only for members of the Closteroviridae that a definitive role has been identified for an HSP70-like protein in virus infection. It seems certain, however, that HSP70 induction for wild-type viruses would not be irrelevant, as the induced HSP70 could contribute to viral protein folding and turnover and could play a part in other virus functions necessary for the development of disease, e.g. cell-to-cell trafficking.

MATERIALS AND METHODS

Plants, Bacteria, and Viruses

Arabidopsis (*Arabidopsis thaliana*) Columbia-0 and *Nicotiana benthamiana* plants were grown in pots in a growth cabinet at 20°C with a photoperiod of 8 h light/16 h dark.

All the intermediate DNA constructs presented in this work were used to transform *Escherichia coli* DH10B cells. Binary plasmids were transformed into *Agrobacterium tumefaciens* strain C58C1::pGV2260.

pBINTRA6 and pTV00 plasmids generating infections with TRV RNA1 and RNA2 (Ratcliff et al., 2001) were kindly provided by Prof. David Baulcombe (Sainsbury Laboratory, John Innes Centre, Norwich, UK). pGreen-TuMV, containing a full-length TuMV genome, has been described previously (Dunoyer et al., 2004). pBIN61-TVCV, generated by Dr. J.-W. Park (J.-W. Park, R.S. Nelson, and A.J. Maule, unpublished data), contained a TVCV full-length cDNA (Hii et al., 2002) cloned into the binary pBIN61 expression cassette. All TCV-derived constructions were based on the pCLB61-TCV plasmid, which contains a full-length genome of TCV strain M (Oh et al., 1995) cloned in the pBIN61 binary vector (Bendahmane et al., 2000) between the CaMV 35S promoter and the CaMV 19S terminator.

Construction of the Promoter::Reporter System

To create the constructs pHSP70::GUS and pHSP70B::GUS expressing the *GUS* reporter gene, 2.1 kb and 1.98 kb of genomic DNA upstream from the ATG codon of the Arabidopsis AtHSP70 and AtHSP70B genes (Sung et al., 2001), respectively, were amplified by PCR and cloned in place of the CaMV 35S promoter upstream of *GUS* in a pJIT166 vector (http://www.pgreen.ac.uk). Subsequently, the complete cassette was released by digestion with the appropriate restriction enzymes and cloned into the pGreen-0229 binary vector (Hellens et al., 2000).

Constructs Used for Transient Expression

TCV mutants were generated by digesting unique restriction sites present in each of the TCV open reading frames (ORFs) of pCLB61-TCV and blunt ending with T4 DNA polymerase (Promega, Madison, WI) according to manufacturer's instructions. Thus, pCLB61-TCV was digested with *Avr*II (nucleotide position 106), *Eco*RI (nucleotide position 2,369), *Aat*II (nucleotide position 2,597), or *Hin*dIII (nucleotide position 3,354) to obtain 35S::TCVΔ28, 35S::TCVΔ8, 35S::TCVΔ9, and 35S::TCVΔ38, respectively.

To obtain 35S::TCVΔ88/28, two stop codons after the ATG of the p88 ORF were introduced by PCR amplification using pCLB61-TCV as template and the antisense primer 5′-AAACTCGAGtta**CAT**CGTGAATAGAGAGAAG-3′ and sense primer 5′-AATCTCGAGtaaAGGTACTACCCTGAGGTTC-3′ (corresponding to nucleotide positions 116-134 and 61-45, respectively [ATG in bold, stop codons in lowercase, and an extra *Xho*I restriction site to facilitate cloning underlined]). The PCR product was digested with *Xho*I and religated.

To generate plasmids containing the individual TCV cistrons (e.g. 35S::p88, 35S::p28, 35S::p8, 35S::p9, and 35S::p38), cDNAs corresponding to each full-length TCV ORF were amplified by PCR from the pCLB61-TCV plasmid using specific sense and antisense primers tagged with restriction sites and cloned into the pBIN61 binary vector. To obtain 35S::p38stop, TCV p38 ORF was PCR amplified using the same antisense primer used to create

35S::p38 and a sense primer containing two consecutive stop codons after the ATG. The PCR product was cloned into the pBIN61 binary vector.

Other viral cistrons from TMV, PSbMV, and CymRSV also were cloned into binary vectors. To prepare 35S::TMV-MP, 35S::PSbMV-6K1CI, 35S::PSBMV-P1, and 35S::CymRSV-p19, the full-length coding sequences for these viral proteins were amplified by PCR using specific primers containing appropriate restriction sites and cloned either in pBinPlus (van Engelen et al., 1995) or pBIN19 binary vectors.

The eGFP variant was excised by *Xba*I digestion of the pEGFP plasmid (CLONTECH, Palo Alto, CA) and cloned into the pBIN19 binary vector to obtain 35S::eGFP. Clones containing eGFP fusions to At5g08290, At1g74560, At4g16830, At3g23600, and At1g71860 were obtained from Dr. J. Doonan (John Innes Centre, Norwich, UK; Koroleva et al., 2005). The genes were selected at random from a collection of cloned fusions to eGFP shown to have cytosolic destination after expression from the CaMV 35S promoter.

To clone the coding regions of PSbMV-6K1CI and At3g23600 into an expression cassette containing the NOS promoter, 35S::PSbMV-6K1CI or At3g23600 constructs were digested with suitable restriction enzymes to release the ORF, which was cloned into a pJIT vector carrying the NOS promoter (http://www.pgreen.ac.uk). Subsequently, these expression cassettes were released by digestion with appropriate restriction enzymes and cloned into the pGreen-0229 binary vector to create NOS::PSbMV-6K1CI and NOS::At3g23600.

Transient Expression by Agroinfiltration

Transformed *A. tumefaciens* was grown in Luria-Bertani broth plus appropriate antibiotics at 28°C for 48 h. After centrifugation, the pellets were resuspended in infiltration solution (10 mM MES, pH 5.5, 10 mM $MgCl_2$, 100 mM acetosyringone) to an optical density of 0.5 at 600 nm and incubated at room temperature for 2 h. Equal volumes of individual cultures were mixed prior to infiltration. For each Agrobacterium mixture, two expanded leaves on three independent 3-week-old *N. benthamiana* plants were pressure infiltrated using a syringe. After infiltration, plants were maintained under the same growth-cabinet conditions. For heat stress conditions, plants were transferred to 42°C for 90 min prior to GUS activity measurement.

GUS and GFP Assays

GUS activity was measured by incubating alcohol-cleared tissues with the colorimetric substrate 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (1 mM; Sigma, St Louis) in 100 mM sodium phosphate, pH 7.0, 0.1% (v/v) Triton X-100, 10 mM EDTA, pH 8, buffer, or, more regularly, fluorimetrically. GUS activity was measured individually in three plants infiltrated with each Agrobacterium mixture. For the fluorometric assay, protein extracts from infiltrated patches were prepared by grinding 0.2 mg of leaves in liquid nitrogen and resuspending the frozen powder in 2 volumes of ice-cold phosphate buffer (50 mM sodium phosphate, pH 7.0, 10 mM Triton X-100, 10 mM *N*-lauroylsarcosine, 1 mM 2-mercaptoethanol). Samples were centrifuged for 5 min at 4°C, and supernatants were recovered, frozen in liquid nitrogen, and stored at −70°C until use. GUS assays were carried out (Jefferson et al., 1987) using a buffer containing 4-methylumbelliferyl-β-glucuronide (Sigma). Fluorescence was measured quantitatively with a Wallac 1420 VICTOR multilabel counter fluorometer (Wallac Oy, Turku, Finland) fitted with an excitation filter of 355 nm and an emission filter of 460 nm. All measurements were repeated three times.

Relative GFP concentration in the protein extracts was measured fluorimetrically (Remans et al., 1999) by reference to a standard curve constructed using recombinant GFP (rGFP; CLONTECH). Briefly, 60 μg of total protein was dissolved to a final volume of 150 μL in phosphate buffer in an opaque 96-well plate. Fluorescence was quantified in the Wallac fluorometer fitted with an excitation filter of 405 nm and emission filter of 510 nm (eGFP) or with an excitation filter of 488 nm and emission filter of 510 nm (rGFP). For the standard curve, 0.05 to 1 μg of rGFP was mixed in a final volume of 50 μL containing 600 μg mL^{-1} of total protein from untreated *N. benthamiana* plants and brought to 150 μL in phosphate buffer.

Immunoblot Analyses

Equalized protein extracts (30 μg) were separated by 12.5% SDS-PAGE, blotted to nitrocellulose, and analyzed using a GFP monoclonal antibody (CLONTECH) following the manufacturer's instructions. The specific reactivity was visualized using alkaline phosphatase-conjugated goat anti-rabbit serum and nitroblue tetrazolium as a colorimetric substrate, following standard techniques.

Northern-Blot Analysis

Total RNA from noninfected and systemically virus-infected Arabidopsis leaves was extracted with Trizol reagent (Sigma) following the manufacturer's instructions. RNAs were electrophoresed on 1% (w/v) denaturing formaldehyde-agarose gels and transferred to nylon membranes (Roche, Mannheim, Germany). After immobilization by UV cross-linking, the blots were hybridized with specific digoxigenin-labeled RNA probes for each of the five cytosolic Arabidopsis HSP70 genes, as described previously (Pallas et al., 1998).

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