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Stable Transformation of an Arabidopsis Cell Suspension Culture with Firefly Luciferase Providing a Cellular System for Analysis of Chaperone Activity in Vivo

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Using Agrobacterium, we developed a method to transform an Arabidopsis cell suspension culture. A stably transformed cell line expressing high levels of firefly luciferase (Luc) was used for in vivo studies of thermal denaturation and renaturation of the enzyme and the protective role of different chaperones. Luc activity was monitored under heat stress and recovery conditions in control, thermotolerant cells and cells expressing plant chaperones after transient cotransformation with plasmids encoding proteins of the heat shock protein Hsp90, Hsp70, or Hsp20 family. The effects of the expressed proteins were specific. The Hsp17.6 class I protein maintained Luc activity on a level comparable with that observed in thermotolerant cells and improved Luc renaturation. Although transient expression of Hsp90 did not protect Luc from thermal denaturation, it accelerated Luc renaturation during recovery. In contrast to the other chaperones tested, overexpression of Hsp70 alone had no effect on denaturation and renaturation of Luc but enhanced Luc renaturation if coexpressed with Hsp17.6.

INTRODUCTION

All organisms respond to supraoptimal temperatures with the synthesis of heat stress proteins (Hsps), which act as molecular chaperones to protect other proteins from damage by heat and to assist in normalization of cellular functions during recovery from stress (reviewed in Nover, 1991; Vierling, 1991; Parsell and Lindquist, 1993; Morimoto et al., 1994; Waters et al., 1996; Nover and Scharf, 1997). Proteins induced by heat stress can be assigned to 11 families conserved among bacteria, plants, and animals. Isoforms within an Hsp family have similar or identical biochemical functions but differ in their intracellular localization and in their regulation; that is, they can be constitutively expressed, heat stress induced, and/or developmentally regulated. Most of the Hsps represent proteins essential for proper folding, processing, and intracellular targeting of newly formed proteins. Frequently, members of different families cooperate as "chaperone machines," for example, the Hsp70/40 or the chaperonine complexes (Kimura et al., 1995; Buchner, 1996; Hartl, 1996; Rassow and Pfanner, 1996; Nover and Scharf, 1997).

During the last 10 years, our knowledge of the major constituents of these cellular chaperone machines has increased rapidly. However, the low molecular weight (lmw) Hsps belonging to the Hsp20 family have been neglected. These proteins represent the preponderant Hsps in plants with >20 different members. Isoforms are found in many cellular compartments, including plastids (Vierling et al., 1986, 1988; Chen et al., 1994) and mitochondria (Lenne, 1995) and in the lumen of the endoplasmatic reticulum (Helm et al., 1993). In the cytoplasm, two classes of lmw Hsps can be distinguished, class I and class II (reviewed in Caspers et al., 1995; Waters et al., 1996). Proteins of the Hsp20 family usually form oligomeric complexes of 200 to 800 kD, even when produced as recombinant proteins in *Escherichia coli* (Chen et al., 1994; Lee et al., 1995). Under heat stress conditions, they undergo changes resulting in a tight assembly of lmw Hsps into a detergent- and salt-resistant complex and subsequent formation of large cytosolic aggregates called heat stress granules (Nover et al., 1983, 1989).

The intriguing multiplicity and abundance of lmw Hsps in plants have led to speculations about their role as a protective matrix for other proteins (Kimpel and Key, 1985; Jakob and Buchner, 1994). Evidence to support this hypothesis has been published recently showing that Hsp18.1 maintains reporter proteins in a folding-competent state (Ehrnsperger et al., 1997; Lee et al., 1997). Several members of the Hsp20 family, for example, mammalian Hsp25 (Jakob et al., 1993), α-B-crystallin (Horwitz, 1992; Boyle and Takemoto, 1994; Nicholl and Quinlan, 1994), and plant Hsp18.1 and Hsp17.7 (Lee et al., 1995), exhibit in vitro chaperone activity. They protect citrate synthase or α-glucosidase against thermal aggregation in an ATP-independent fashion.

The data collected so far have led to speculations that other proteins and/or a certain cellular environment may be necessary for full chaperone activities of these proteins (Waters et

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al., 1996). To test this, we developed a system to determine chaperone activities in vivo by using firefly luciferase (Luc) as a reporter. This enzyme is reported to be a good indicator of different chaperone activities both in vivo and in vitro (Nguyen et al., 1989; Schröder et al., 1993). By an agrobacteria-mediated transformation, we generated an Arabidopsis cell line (At-luc1) expressing high levels of Luc. It was used for polyethylene glycol (PEG)–mediated transformation of protoplasts and transient expression of chaperones to analyze their effect on the kinetics of thermal denaturation and subsequent renaturation of Luc in vivo.

RESULTS

Stable Transformation of Arabidopsis Cell Culture by Agrobacterium

Following the method described by An (1985) for tobacco cells, we elaborated a protocol for direct transformation of Arabidopsis callus with Agrobacterium and for selection of a transgenic cell line expressing high levels of firefly Luc. For an optimal transformation and selection procedure, the following was essential for an efficient transformation. (1) The number of transformed calli increased with the cocultivation time of Arabidopsis cells with agrobacteria, reaching an optimum after 48 hr (Figure 1). Longer cocultivation led to reduced viability of the plant cells and thus to a delay of the growth of transformed microcalli. (2) As reported for tobacco cell cultures, transformation and selection required actively growing cultures. It was inefficient with stationary phase cultures. (3) For complete elimination of agrobacteria and selection of transformed calli, repeated cycles of dispersion, treatment with timenten, and growth on media containing 250 μg/mL timenten and 50 μg/mL kanamycin were required (see Methods). The efficiency of transformation and selection is best illustrated when comparing nontransformed and transformed calli after 3 weeks of cultivation on agar with and without kanamycin (Figure 2). At 50 μg/mL kanamycin, no growth was observed without prior cocultivation with agrobacteria, whereas up to 80 resistant microcalli were obtained from a single Petri dish after transformation.

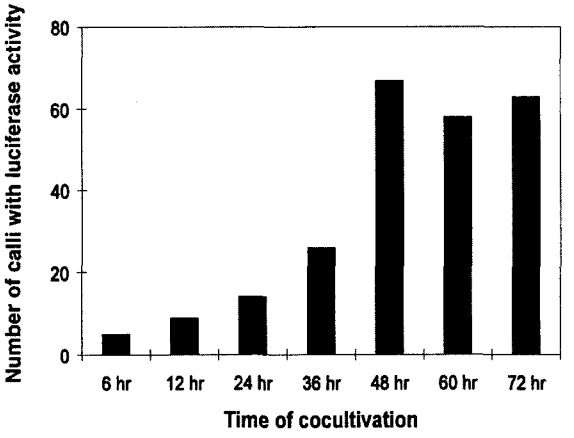

Figure 1. Influence of Cocultivation Time on Transformation Efficiency.

Different cocultivation times of Arabidopsis cells and Agrobacterium were tested. After coinfection, cells were plated on media containing 50 μg/mL kanamycin. After 3 weeks of growth, calli with Luc activity of at least 1000-fold above background level were selected. The number increased during and up to 48 hr of cocultivation and then slowly declined, probably as a consequence of the reduced viability of the cells.

Different kanamycin-resistant lines derived from a single Petri dish displayed a broad range of Luc activity levels (Table 1). The background activity of untransformed Arabidopsis calli was ~100 relative light units (rlu) per 10 sec per 10 mg of cells. Five of 53 microcalli isolated showed activities >500-fold above background level (Table 1). One cell line (At-luc1) with 94,000 rlu per 10 sec per mg fresh weight was selected and tested for the integration of the *luc* gene into the genome (Figure 3) and for long-term stability of the new genetic trait under selective and nonselective conditions. As expected for a stably transformed cell line, no significant difference in Luc activity was detectable even under nonselective conditions after 4 months of cultivation with weekly transfer to fresh medium (Figure 4A). A suspension culture generated from callus line At-luc1 provided the basis for an efficient protoplasting procedure (see Methods). If inoculated from a stationary phase culture, Luc activity was growth dependent (Figure 4B). The enzyme activity increased during the exponential growth phase and dropped to near background level in the stationary phase. If cells were transferred to fresh medium in their exponential growth phase, Luc activity remained at a high level. Therefore, growth phase cultures with subcultivation every 7 days were used for all of the following experiments.

Intracellular Localization of Luc

One drawback in using Luc as a reporter for chaperone activity is that the enzyme may be targeted to the peroxisomes (Gould et al., 1990). To examine the situation particular to our Arabidopsis cell culture, we isolated intact microbodies and separated them by sucrose gradient centrifugation. Fractions were tested for catalase, which in plants is expressed exclusively in microbodies, and Luc activity (Figure 5). Although >95% of the catalase activity was found in gradient fractions indicating separation of intact microbodies, >80% of total Luc activity was detected in the soluble cytoplasmic fraction on top of the gradient. This result is similar to the observation of other groups using Luc in mammalian systems (Schneider et al., 1996).

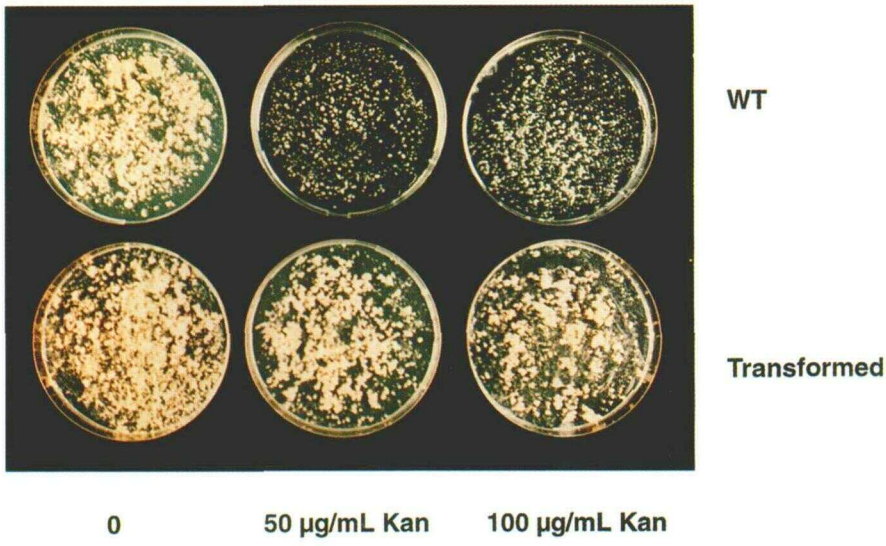

Figure 2. Transformation Efficiency at Various Kanamycin Concentrations.

Four-day-old Arabidopsis cells were cocultivated for 48 hr with Agrobacterium. After removal of bacteria, transformed cells were plated on agar containing the indicated concentrations of kanamycin (Kan) and were compared with nontransformed cell culture (WT). Wild-type cells did not grow in the presence of >25 µg/mL kanamycin.

Thermal Denaturation of Luc Expressed in Arabidopsis

Using cell line At-luc1, we investigated the thermal denaturation of Luc protein in vivo. Approximately 5000 protoplasts were dispersed in 25-µL aliquots in a microtiter plate. After lysis and injection of luciferin, they produced ~120,000 rlu in a 60-sec registration period (100%). For recording the thermal denaturation, protoplasts were incubated for the indicated time in the luminometer at 40, 41, and 42°C, respectively, before injection of the substrate (Figure 6). The Luc half-life was <3 min at 42°C, 5 min at 41°C, and 8 min at 40°C. For comparison, we measured Luc activity by adding luciferin under isoosmotic conditions without detergent and without ATP. In both experiments, the half-life declined with increasing temperatures (data not shown). Because we detected only 10% of total Luc activity by in situ measurement and it was not known whether this 10% was representative of the denaturation status for all Luc proteins, we monitored the overall Luc activity in all experiments by lysing the protoplasts.

In the following experiments, we used 41°C as denaturation temperature to study the effect of Hsps on thermal denaturation and renaturation. The most convenient way to demonstrate the protective effect of chaperones on the thermal stability of proteins is a heat preconditioning, which induces the synthesis of endogenous Hsps. At-luc1 protoplasts were made thermotolerant by a short heat shock pulse (15 min at 38°C) 3 hr before thermal Luc inactivation at 41°C (Figure 7A). Compared with the control protoplasts without preconditioning, the decrease in Luc activity was considerably slower (a half-life of 11 or 12 min). Lysis of protoplasts before Luc inactivation at 41°C eliminated this effect (Figure 7A). The kinetics of inactivation were identical for both types of lysates as well as for commercially obtained firefly Luc tested under the same conditions (Figure 7A).

Table 1. Selection of Cell Lines Expressing Luc Activity[a]

Relative Light Units/mg Fresh Weight	Number of Calli Obtained
>50,000[b]	5
>5,000	18
>1,000	15
<1,000	26

[a] Calli from a single transformation experiment growing on 50 mg/mL kanamycin were tested for Luc activity.

[b] Based on their relative activity, calli were divided into four groups ranging from very high Luc activity in group 1 to almost background in group 4.

Thermal Denaturation of Luc in the Presence of Different Chaperones

A major advantage of Arabidopsis cells with a stably integrated Luc reporter is the possibility of generating protoplasts for a PEG-mediated transient transformation with chaperone-encoding plasmids. For this purpose, 20 µg of

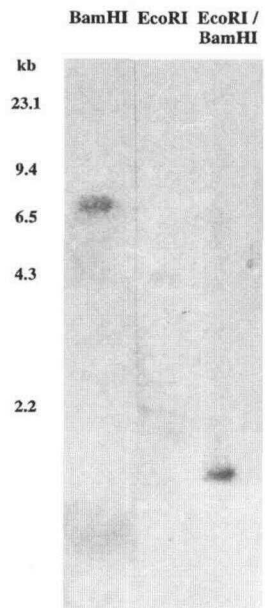

Figure 3. DNA Gel Blot Analysis of the *luc* Gene in Arabidopsis Cell Line At-luc1.

Genomic DNA from transformed cell line At-luc1 was digested with BamHI (first lane) and EcoRI (second lane) and double digested with EcoRI/BamHI (third lane), releasing the *luc* insert. The membrane was probed with a ^{32}P-labeled insert coding for *luc*. Molecular weight markers are shown at left in kilobases.

plasmid DNA carrying the gene of interest and 20 μg of carrier DNA (empty vector) were added to ~200,000 protoplasts in the presence of PEG (see Methods). After transformation, cells were incubated in the dark for 16 hr. For comparison, control cells and cells that had been allowed to acquire thermotolerance were always mock transformed with 40 μg of vector DNA.

For transformation, plasmids encoding Arabidopsis Hsp17.6 class I, petunia Hsp70, and Brassica Hsp90 were used. Expression levels were monitored by protein gel blotting and compared with those found in control and heat shock–preinduced cells (Figure 8). Although Hsp17.6 and Hsp90 were barely detectable or absent in control cells, they were expressed in preinduced or transformed protoplasts. The data were not conclusive for Hsp70 because its level in control protoplasts was relatively high.

When Luc thermal inactivation was tested in these protoplasts (Figure 7B), no effect was found with cells transformed with the Hsp90 and Hsp70 expression vectors, whereas Hsp17.6 had a clear protective effect. Hsp17.6 class I doubled the half-life at 41°C from ~4 min to 8 to 9 min, which is close to the value in preinduced protoplasts (Figure 7A). In addition, residual Luc activity observed after 20 min was at background level in lysed control and lysed thermotolerant cells but ~30% in thermotolerant cells and cells transformed with a lmw Hsp. In control cells, only 12 to 15% could be detected. Expression of both Hsp70 and Hsp17.6, which can accelerate renaturation during recovery (see below), did not significantly increase thermoprotection under heat stress (data not shown).

Special consideration should be taken in the analysis of the results obtained in the in vivo Luc inactivation assay. Because a significant difference was found in the time course between in vitro and in vivo inactivation (Figure 7A), small amounts of Luc liberated by the eventual decay of protoplasts may affect the analysis. For this reason, a similar experiment was performed to measure the activity in situ without any ATP and detergent but in the presence of lu-

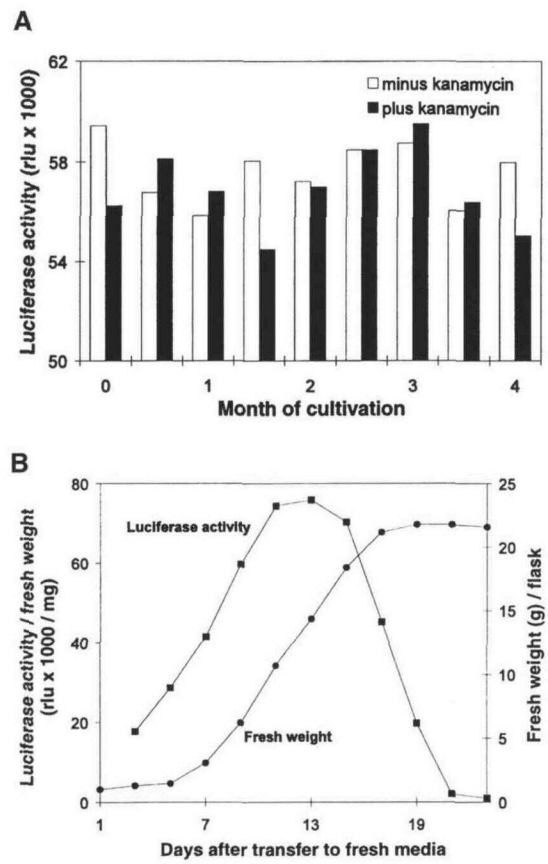

Figure 4. Properties of the Transformed Cell Line.

(A) The transformed cell line was grown under selective (50 μg/mL kanamycin) and nonselective conditions, respectively, and monitored for at least 4 months. Every week, 1 g of callus material was transferred to 120 mL of fresh media. In 2-week intervals, aliquots were analyzed for Luc activity. The At-luc1 cell culture maintained its high Luc activity even under nonselective conditions.

(B) Growth characteristics and Luc activity of the At-luc1 suspension culture: 1 g of a stationary phase culture was passed to 120 mL of fresh media. Growth rate and Luc activity (relative light units per milligram fresh weight) were analyzed.

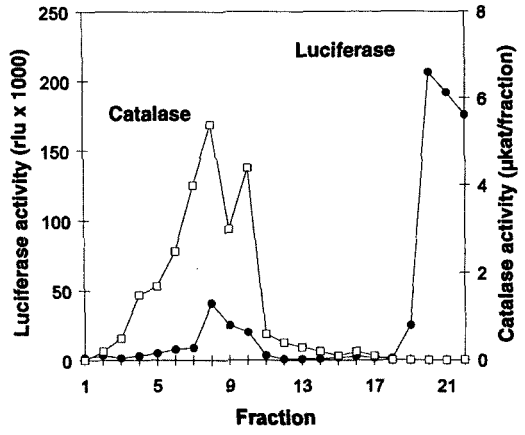

Figure 5. Subcellular Distribution of Luc and Catalase in Transformed Arabidopsis Cells.

Protoplasts prepared from 5 g of fresh weight of At-Luc1 were used to analyze the subcellular distribution of Luc. Cellular compartments were separated by sucrose gradient centrifugation and monitored for Luc and catalase activity.

ciferin. The apparent enzyme activity measured in situ was <10% of the activity measured in the protoplast lysate. A careful comparison of the time courses of Luc inactivation at 41°C in vivo yielded nearly the same results, irrespective of the mode of measurement, that is, in situ by the addition of luciferin to intact cells or in vitro by lysis before the addition of luciferin and ATP.

Reactivation of Luc Activity in Vivo

In the second set of experiments, we investigated whether the in vivo system is suitable for monitoring the reactivation of heat-inactivated Luc. Protoplasts were incubated for 20 min at 41°C, and Luc activity was measured before and after thermal inactivation. The residual Luc activity was 15 to 30%, depending on the cells tested (Figure 9). Immediately after heat treatment, reactivation of enzyme activity was monitored for 100 min. During this time, Luc activity of mock-transformed control protoplasts without preinduction showed only a minor increase in activity, but in preinduced cells, Luc activity was nearly completely restored (Figure 9). New synthesis did not contribute to the recovery, because similar results were obtained in thermotolerant cells treated with 10 µg/mL cycloheximide (Figure 9).

The best potential for reactivation of Luc activity was observed in cells expressing the Hsp90 protein. Enzyme activity was almost completely regained after 100 min of recovery at 27°C (Figure 10A). In addition, we observed no significant effect on the recovery effect of Hsp90 in the presence of 10 µg/mL cycloheximide (data not shown). Hsp17.6 improved the recovery of Luc up to 60% of the initial activity. Because after heat treatment there was a residual activity of 30%, the resulting increase of Luc activity was only twofold. Interestingly, this effect was enhanced to >80% if additional Hsp70 was present, whereas Hsp70 alone did not significantly contribute to Luc reactivation (Figure 10B).

DISCUSSION

The standard Arabidopsis transformation protocols (Meyerowitz, 1987; Bowman et al., 1988), starting with roots or leaves cocultivated with agrobacteria, require laborious procedures to regenerate plants as the basis for backcrossing to create homozygotes. The procedure could be accelerated by the recently developed techniques of vacuum infiltration of agrobacteria into the inflorescence of Arabidopsis with the goal of transforming generative cells directly (Bechtold et al., 1993). If the homozygous state is not required because cellular functions of a given gene product are to be analyzed, transformation of an Arabidopsis cell culture would be a rapid and convenient alternative. Following a technique described by An (1985) for tobacco suspension culture, we developed an efficient transformation and selection system for Arabidopsis. As a result, we created a stably transformed cell suspension culture (At-luc1) overexpressing firefly luciferase, which was used as a reporter for monitoring the influence of Hsps on protein denaturation and renaturation in vivo. This suspension culture could be used readily for protoplasting, PEG-mediated transformation, and transient expression of Hsps. Hence, a cellular system is available to test chaperones

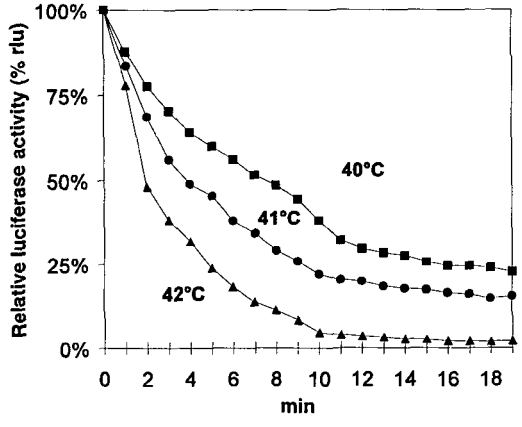

Figure 6. Luc Inactivation at Different Temperatures in Vivo.

Protoplasts of At-luc1 were incubated at the indicated temperatures in a luminometer. Every minute, an aliquot of the protoplasts was lysed, luciferin was injected, and the light emission was measured for 60 sec. The resulting relative light units were compared with the relative light units measured before denaturation, which was set as 100% (see Methods).

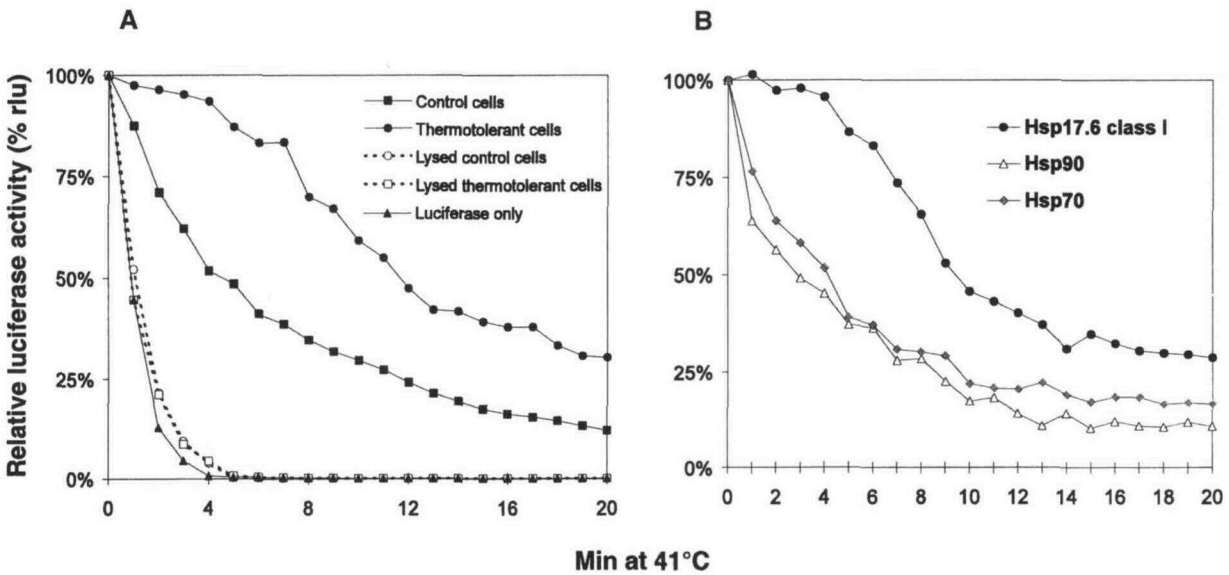

Figure 7. Luc Inactivation at 41°C in Vitro and in Vivo.

(A) Control protoplasts and protoplasts that were allowed to acquire thermotolerance by using a 15-min heat shock at 38°C plus a 3-hr recovery were incubated at 41°C. Luc activity was measured every minute and compared with the activity at room temperature (0), which was set as 100%. Luc activity in both cell lines was monitored over 20 min either in intact cells (closed squares and circles) or after lysis before incubation at 41°C (open squares and circles) and compared with the corresponding activity of purified Luc (closed triangles).

(B) Sixteen hours before thermal denaturation, protoplasts were transiently transformed with plasmids encoding the indicated chaperones (see Methods). Luc denaturation was evaluated as described in **(A)**. Expression of the chaperones was verified by protein gel blotting (see Figure 8).

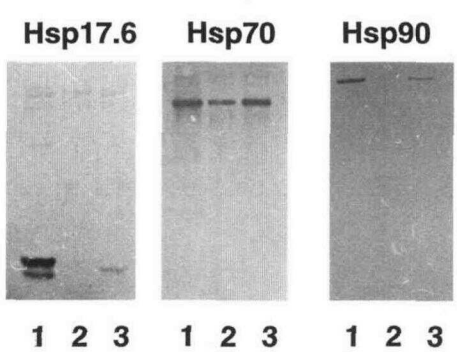

Figure 8. Chaperone Level in Transiently Cotransformed Protoplasts.

Total protein extracts were separated by SDS–gel electrophoresis, blotted, and incubated with the indicated antiserum detecting Hsp70, Hsp90, and Hsp17.6 class I. Lane 1 contains mock-transformed thermotolerant protoplasts; lane 2, mock-transformed control protoplasts; lane 3, protoplasts transformed with plasmids coding for Hsp90, Hsp70, and Hsp17.6 class I, respectively.

with regard to their influence on firefly Luc activity under heat stress and recovery conditions.

We used Luc as reporter protein, because it is rapidly and reversibly inactivated at elevated temperatures. Soon after its introduction (de Wet et al., 1987), it proved to be a valuable protein for studying thermal inactivation of proteins and the protective effect of Hsps (e.g., Nguyen et al., 1989; Schröder et al., 1993; Frydmann and Hartl, 1996). Our studies with transgenic Arabidopsis confirmed the suitability of Luc for this type of study with plant cells as well. We could show that under high temperatures, intracellular activity of the reporter enzyme was inactivated but to a much lesser degree than occurred in vitro. Remarkably, the highest protection of Luc was obtained in preinduced protoplasts. These cells were briefly heat stressed and allowed to recover before the experiment. In these cells, the whole set of endogenous Hsps is present, providing optimal conditions for protection during heat inactivation and recovery. Both types of cells served as a negative and positive control in our experiments.

A special problem using Luc in vivo may arise from the fact that it contains a peroxisomal target sequence (Gould et al., 1990), which may preclude its use as reporter for cytosolic chaperones. Our investigations demonstrate that this is not relevant for our transgenic cell line. More than 85% of the protein was cytosolic, probably due to the underdevel-

oped state of the peroxisomal system in dark-grown Arabidopsis cell cultures.

The main goal, however, was to establish a reporter system to investigate the chaperone capacity of plant Hsps in vivo. Most details of protein folding and chaperone function were elaborated using in vitro systems with a limited number of test proteins as substrates. For further progress, it is essential to develop in vivo systems to supplement these in vitro studies, because it became evident that other components are important for full activity of certain chaperone proteins, as outlined by Hendrik and Hartl (1993) or more recently described for Hsp70 (Höhfeld et al., 1995) or Hsp90 (Bose et al., 1996). Sometimes an organized link is necessary to establish effective folding (Freeman and Morimoto, 1996; Frydmann and Hartl, 1996). The benefit of accompanying in vivo studies already has been demonstrated for Hsp90 in mammalian cells (Nguyen et al., 1989; Pinto et al., 1991; Schneider et al., 1996) and DnaK/DnaJ/GrpE in *E. coli* (Schröder et al., 1993). The Luc-expressing Arabidopsis cell line described in this study can provide such a system for plants. The efficiency of transient expression in this system provides a basis for rapid testing of chaperone varieties and combinations as well.

Our experiments overexpressing three different types of chaperone proteins (Hsp90, Hsp70, and Hsp17.6) indicate the different functions of these proteins during heat stress response. When thermal Luc denaturation at 41°C and subsequent regeneration of activity at 27°C were used as an indicator of chaperone activity, Hsp17.6 had an effect on both processes. Although the effect of Luc reactivation after a given heat stress was only moderate, cells transformed with Imw Hsp prevented Luc inactivation as effective as thermotolerant cells, expressing the whole set of endogenous Hsps.

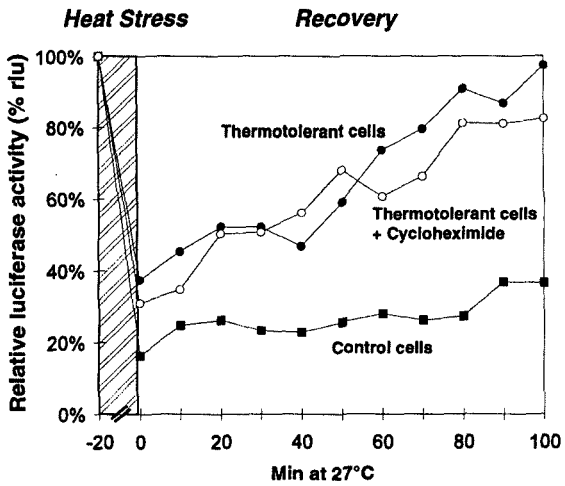

Figure 9. Recovery of Luc Activity after Thermal Inactivation at 41°C.

Control (closed squares) and thermotolerant (closed circles) protoplasts of At-luc1 were incubated for 20 min at 41°C before cells were allowed to recover for 100 min at 27°C. An aliquot of 5000 protoplasts was removed immediately after denaturation and then analyzed at 10-min intervals for Luc activity in vivo. The resulting relative light units were compared with the relative light units before denaturation. To test the effect of cycloheximide on Luc reactivation in thermotolerant cells, 10 μg/mL was applied to thermotolerant protoplasts immediately after heat stress (open circles).

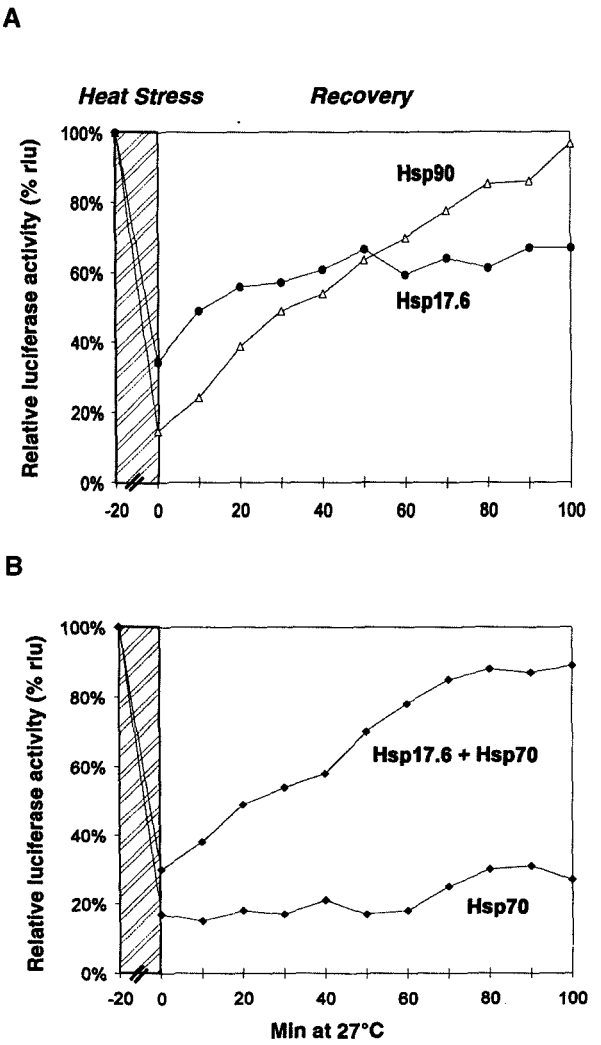

Figure 10. Recovery after Thermal Inactivation in the Presence of Cotransformed Hsps.

(A) Protoplasts transformed 16 hr before the experiment with the indicated chaperone expression plasmids encoding for Hsp90 and Hsp17.6 class I, respectively. Recovery of Luc activity was monitored as described in Figure 9.

(B) Recovery of Luc activity after a given heat shock in the presence of transiently transformed Hsp70 or Hsp70 coexpressed with Hsp17.6 class I.

In contrast, Ehrnsperger et al. (1997) could show clearly that in vitro under heat stress, the citrate synthase reporter was protected by Imw Hsps from aggregation but not from inactivation. This discrepancy supports their idea that Imw Hsps alone are not able to protect target proteins from being inactivated but that they provide an unspecific surface, which keeps the proteins in a folding-competent state while refolding to the native or active form is mediated by other cellular chaperones.

The apparent negative result obtained with the Hsp70 expression plasmids could be explained because a high level of endogenous proteins is already present in control cells, resulting in a moderate increase after transformation. The endogenous Hsp70 isoforms may be important for the relatively slow thermal inactivation of Luc in vivo as compared with the in vitro situation. Interestingly, a clear effect could be found if Hsp70 and a Imw Hsp act synergistically during renaturation: Hsp70 can enhance the ability of overexpressed small Hsps to aid Luc renaturation. This observation again supports the idea that both proteins act together during the proper refolding process, as suggested already by Ehrnsperger et al. (1997) or Lee et al. (1997).

The same interaction may be important during Luc inactivation in the presence of Hsp90 but with a significant difference. Although Imw Hsps have a strong effect under heat stress, preventing proteins from being inactivated and supporting the idea that these proteins act as a line of first defense (Buchner, 1996), proteins associated with Hsp90 were not protected from being inactivated but were kept in a folding-competent state. These proteins can refold to their native state only under recovery conditions. Other in vivo experiments focusing on Hsp90 (Schneider et al., 1996) have led to a model showing that Hsp90 together with Hsp70 is part of a multiprotein chaperone complex that determines whether an associated target protein is refolded or sentenced to be degraded. This model is summarized in an article recently published by Johnson and Craig (1997), which reviews data showing that partially unfolded proteins first interact with Hsp70 and a protein called Hip (Hsp70 interacting protein) (Höhfeld et al., 1995) before they assemble with Hsp90 and other components of this chaperone complex. Because this model is different from the one including Imw Hsps, it could explain why Imw Hsps are able to prevent Luc inactivation under stress whereas Hsp90 cannot.

METHODS

Plant Cell Culture

The *Arabidopsis thaliana* cell culture was obtained from D. Scheel (Institute for Plant Biochemistry, Halle, Germany). Cells were maintained in 125 mL of liquid growth medium (4.6 g/L Murashige and Skoog salts with vitamins [Duchefa, Haarlem, The Netherlands], 30 g/L sucrose, and 2 mg/L 2,4-dichlorphenoxyacetic acid) at 25°C by gentle agitation (130 rpm) in the dark. An aliquot of 4 to 5 g was transferred to fresh medium every week.

Vector Construction

For transformation of bacteria and plant cells, we used the firefly luciferase (*luc*) gene from *Photinus pyralis* (de Wet et al., 1987) fused to a pBIN19 binary vector derivative (Bevan, 1984). From the binary vector pBI121 (Clontech, Palo Alto, CA), the β-glucuronidase (*gus*) reporter gene was replaced by the *luc* gene from the pBluc plasmid obtained from H. Bujard (Zentrum für Molekularbiologie, Heidelberg, Germany). The resulting plasmid, pBIN19–luc, containing the *luc* gene under control of the 35S cauliflower mosaic virus promoter (Franck et al., 1980) and nopaline synthase terminator (*nos*), was used for transformation of *Agrobacterium tumefaciens* LBA4404.

For transient cotransformation, the pRT vector system was used (Töpfer et al., 1988). cDNAs encoding Arabidopsis Hsp17.6 class I, Petunia Hsp70, and Brassica Hsp90 were inserted into the pRT101 vector between the cauliflower mosaic virus 35S promoter and the *nos* terminator. *hsp17.6* class I was obtained from E. Vierling (University of Arizona, Tucson). *hsp70* from Petunia was obtained from J. Winter (Chiron Research Laboratory, Albany, CA), and the *hsp90* gene was a gift from P. Krishna (Department of Biochemistry, University of Minnesota, St. Paul).

Stable Transformation of Arabidopsis Cell Culture

Agrobacteria carrying the pBIN plasmid were grown in YEB medium (0.5% [w/v]) beef extract, 0.5% [w/v] peptone, 0.1% [w/v] yeast extract, 0.5% [w/v] sucrose, and 10 mM $MgSO_4$, pH 7.2) supplemented with 250 mg/L streptomycin (Duchefa) and 50 mg/L kanamycin (Duchefa) at 28°C to an OD_{600} of 1.5. Bacteria were collected by centrifugation (10 min at 4000 rpm) and resuspended in the same amount of cell culture medium. Four days after transfer to fresh medium, Arabidopsis cells (3 g fresh weight per 10 mL of medium) were incubated with 5 mL of an agrobacteria suspension in a Petri dish at 25°C in the dark with gentle agitation (130 rpm). After 48 hr, the cells were loaded onto a nylon net and washed with excess of cell culture medium to remove most of the bacteria. Remaining cells were resuspended in 40 mL of culture media, vortexed vigorously for 20 sec, collected by a short centrifugation (1 min at 600*g*), and resuspended in fresh cell culture media. This procedure was repeated three times using 250 mg/L timenten (ticarcillin plus clavulanic acid, 15:1; Smith-Kline Beecham AG, Thörishaus, Switzerland) in the last solution. This antibiotic combination was highly effective in removing the remaining bacteria but had no effect on plant cell growth. Cells were plated on plant cell growth medium with 0.6% Gelrite (Merck), 250 mg/L timenten, and the indicated concentrations of kanamycin (see Results). The dishes were stored at 25°C in the dark until calli formation was observed, usually after 2 or 3 weeks.

Propagation of Transformed Cells

Calli exhibiting high Luc activity were pressed through a steel sieve (125-μm pore size) to obtain very small cell aggregates and then further diluted in cell culture medium. Cells were plated in low density

on Petri dishes containing plant cell growth media with 0.6% Gelrite, 250 mg/L timenten, and 50 mg/L kanamycin. This was repeated two times. This procedure was designed to eliminate residual bacteria nesting within the calli and obtain clones from a single transformation event. A minimum of 1.5 g of a fast-growing callus was transferred to 25 mL of liquid growth medium containing 50 mg/L kanamycin. At that time, agrobacteria were usually no longer present in the culture; otherwise, timenten was added again. The suspension was kept in the dark at 25°C rotating at 130 rpm. Critical for propagation of cell calli in liquid medium is the optimal ratio of Arabidopsis cells to liquid media. Every 2 or 3 days, 10 to 20 mL of cell culture media was added to the cells. Depending on the growth rate, the amount of liquid was slowly increased up to 125 mL. The entire procedure, from the initial transformation to the stable suspension culture of At-luc1, took 3 or 4 months.

Preparing Protoplasts and Transient Transformation of Arabidopsis Suspension Culture

Protoplasts were obtained by incubating 1 g of cell culture for 4 hr under gentle agitation in 0.4 M mannitol, 7 mM $CaCl_2$, 3 mM Mes, pH 5.7, 0.2% (w/v) macerocyme, and 0.5% (w/v) cellulase. Cells were passed through a steel sieve (125-μm pore size) to disintegrate remaining cell aggregates. Protoplasts were collected by 10 min of centrifugation at 600*g*. Transient transformation was performed by a method based on Doelling and Pikaard (1993). Cells were washed twice in 10 mL of 0.4 M mannitol, 70 mM $CaCl_2$, and 5 mM Mes, pH 5.7, and resuspended in 2 mL of MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 5 mM Mes, pH 5.7). Protoplasts were placed on ice, and the density was adjusted to 10^6 cells per mL. Ten micrograms of plasmid DNA and 10 μg of carrier DNA were added to 100,000 protoplasts in the MaMg solution. For DNA uptake, 0.4 mL of 40% (w/v) polyethylene glycol (PEG) 6000, 0.4 M mannitol, and 0.1 M $Ca(NO_3)_2$ were added. After 20 min, the solution was diluted with 0.4 M mannitol, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, and 1.5 mM Mes, pH 5.7. Cells were collected by 10 min of centrifugation at 600*g* and resuspended in Murashige and Skoog cell culture medium supplemented with 0.4 M mannitol. Protoplasts were incubated under gentle agitation for 16 hr at 22°C in the dark.

Luc Assays

Assays were performed in a microlumat LB96P (EG & G Berthold, Wildbad, Germany). For selection of clones with high Luc activity, 50 mg fresh weight was transferred into each well of a microtiter plate and incubated in 0.1 mL of buffer containing 25 mM glycylglycine, 15 mM K-phosphate, pH 7.8, 15 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT, and 1 mM ATP. By automatic injection, 100 μL of reaction buffer with 1 mM luciferin was added. Light emission was measured immediately after injection for 10 to 30 sec as indicated (see Results).

For measuring the thermal inactivation of Luc in vivo, the incubation chamber of the luminometer was heated to 41°C. Equal amounts of protoplasts were distributed over 21 wells of a microtiter plate. The first well, representing time point 0, was measured immediately. The other wells were measured within 60-sec intervals, representing a total time of 20 min at 41°C for the last sample. For measuring Luc activity, protoplasts were lysed by addition of 100 mM Triton X-100 in reaction buffer containing 25 mM glycylglycine, 15 mM K-phosphate, pH 7.8, 15 mM $MgCl_2$, 4 mM EGTA, 1 mM DTT, 2 mM ATP, and 1 mM luciferin. This detergent concentration was sufficient to release the reporter enzyme from the cells but did not affect Luc activity (data not shown). After lysis, light emission was monitored for 60 sec.

For renaturation experiments, protoplasts were heated to 41°C for 20 min in a water bath. Immediately after heat shock, 20 aliquots were distributed to a microtiter plate and kept at 27°C. Luciferin in reaction buffer was added to 1 aliquot every 10 min, as described above, and Luc activity was recorded. For testing the effect of cycloheximide, the inhibitor was added immediately after heat shock. All figures presenting data of in vivo inactivation and renaturation show the average value of five independent experiments.

Isolation of Peroxisomes

To analyze the intracellular Luc distribution, protoplasts (5 g) were collected by centrifugation (10 min at 600*g*). Peroxisomes were isolated with modifications according to Feierabend and Engel (1986). Cells were resuspended in isolation buffer (50 mM Tricine–HCl, pH 7.5, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM $Na_2S_2O_4$, and 0.25 M mannitol). After four strokes with a glass potter, the tissue was immediately loaded onto a linear (28 to 60% in isolation buffer) sucrose gradient. After centrifugation (for 3 hr at 61,000*g*) at 4°C, 22 fractions of 500 μL each were collected. Catalase activity was measured by adding an aliquot to 10 mM H_2O_2 in a buffer containing 100 mM K-phosphate buffer, pH 7, and monitoring the decrease of H_2O_2 absorption at OD_{240}.

Protein Gel Blot Analysis

To monitor the expression of plasmid-encoded Hsps in transiently transformed protoplasts, ~5000 protoplasts were boiled in SDS sample buffer, and proteins were separated on a 15% SDS–acrylamide gel, according to Laemmli (1970). After gel electrophoresis, the proteins were blotted onto nitrocellulose (Schleicher & Schüll, Dassel, Germany). The membrane was blocked with 2% (w/v) nonfat dry milk in PBS and incubated with an antibody raised against Hsp17.6 class I (1:2000 in PBS) obtained from E. Vierling, Hsp70 (1:2000) obtained from D. Neumann (Institut für Pflanzenbiochemie, Halle, Germany), or Hsp90 (1:2000) obtained from P. Krishna, respectively. Detection was performed using an anti–rabbit antibody conjugated to alkaline phosphatase, according to the manufacturer's instructions (Boehringer, Mannheim, Germany).

DNA Gel Blot Analysis

Integration of the *luc* gene into the Arabidopsis genome was confirmed by DNA gel blot analysis (Southern, 1975). Four micrograms of genomic DNA isolated according to Ausubel et al. (1993) was digested overnight with EcoRI, BamHI, and EcoRI and BamHI, separated on a 0.8% (w/v) agarose gel, and blotted onto a nylon filter (Hybond N^+, Amersham Buchler GmbH, Braunschweig, Germany) by overnight capillary transfer. The DNA was UV cross-linked to the membrane and hybridized for 16 hr at 62°C according to standard procedures (Ausubel et al., 1993), with a probe coding for the *luc* gene. Nonbound probe was removed by washing the filter at 55°C in the presence of 0.1% (w/v) SDS and 0.1 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate).

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