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Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a classical glycolytic enzyme, is involved in cellular energy production and has important housekeeping functions. In this report, we show that a GAPDH from *Arabidopsis*, GAPDHa, has a novel function involved in H_2O_2-mediated cell death in yeast and *Arabidopsis* protoplasts. GAPDHa was cloned along with other plant genes that suppress Bax-induced cell death in yeast. Flow cytometry analyses with dihydrorhodamine 123 indicated that H_2O_2 production mediated by Bax expression in yeast cells was greatly reduced when Bax was coexpressed with GAPDHa. In plants, GAPDHa transcript levels were greatly increased by H_2O_2 treatment. Furthermore, transformation of GAPDHa into *Arabidopsis* protoplasts strongly suppressed heat shock-induced H_2O_2 production and cell death. Together, our results indicate that GAPDH controls generation of H_2O_2 by Bax and heat shock, which in turn suppresses cell death in yeast and plant cells.

Keywords: *Arabidopsis thaliana*; *Cruciferae*; *Saccharomyces cerevisiae*; Glyceraldehyde-3-phosphate dehydrogenase; Bax-induced cell death; Reactive oxygen species; Multifunctional protein

1. Introduction

All living organisms consist of cells that undergo proliferation, differentiation, and cell death during their life span. It has been demonstrated that one type of cell death, known as programmed cell death (PCD), is a genetically controlled process that plays an essential role in the development and physiology of both animals and plants (Dangl et al., 2000; Williams and Smith, 1993). The process of PCD is important for elimination of unwanted, excessive, infected, or damaged cells in response to environmental factors and diseases (Willis et al., 2003; Steller, 1995). In animals, the most well-known component of cell death regulation is the Bcl-2 family. Bcl-2 family members are key regulators of both cell survival and cell death (Gross et al., 1999). Some Bcl-2 proteins (such as the anti-apoptotic family members Bcl-2, Bcl-W, Bcl-X_L, Bfl-1, Mcl-1, and A1) promote cell survival, while others (such as the pro-apoptotic family members Bax, Bak, Bcl-XS, Bid, Bik, Hrk, and Bok) promote cell death.

Although no sequence homologs of the Bcl-2 family have been identified in the plant and yeast genomes, a pro-apoptotic member of the Bcl-2 family, Bax, causes cell death in yeast and plants (Hanada et al., 1995; Greenhalf et al., 1996; Lacomme and Cruz, 1999). Bax-induced PCD in yeast can be also suppressed by coexpression with anti-apoptotic members of the Bcl-2 family, such as Bcl-2

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or Bcl-X_L (Hanada et al., 1995; Greenhalf et al., 1996; Jurgensmeier et al., 1997). In yeast and plants, reactive oxygen species (ROS) are involved in Bax-induced cell death (Moon et al., 2002; Kawai-Yamada et al., 2004; Baek et al., 2004). The phenotype of plant cell death by Bax is similar to the plant hypersensitive response in tobacco (Lacomme and Cruz, 1999) and *Arabidopsis* (Baek et al., 2004; Kawai-Yamada et al., 2001). The carboxyl-terminal transmembrane (TM) domain targets Bax protein to the mitochondria, and deletion of the TM domain of Bax abolishes protein lethality (Baek et al., 2004; Lacomme and Cruz, 1999; Kawai-Yamada et al., 2001). Therefore, targeting of Bax to mitochondria is also crucial for cell death in plants.

Observations on Bax-induced cell death in animals, yeast, and plants suggest that some elements of this mechanism may be conserved among various organisms. We have employed the powerful genetic tool of yeast complementation to identify suppressors of Bax-induced cell death in plants (Moon et al., 2002). A plant cDNA library was co-transformed with the Bax gene into yeast cells, and overexpressed genes that could suppress Bax-induced cell death were isolated. We previously reported the functions of two Bax-inhibiting proteins, sAPX and AtNDPK2 (Moon et al., 2002, 2003). In this report, we characterize the cell death suppression function of *Arabidopsis* glyceraldehyde-3-phosphate dehydrogenase A type (GAPDHa) in detail. We provide evidence that *Arabidopsis* GAPDHa has a novel redox regulatory function in both yeast and plant cells.

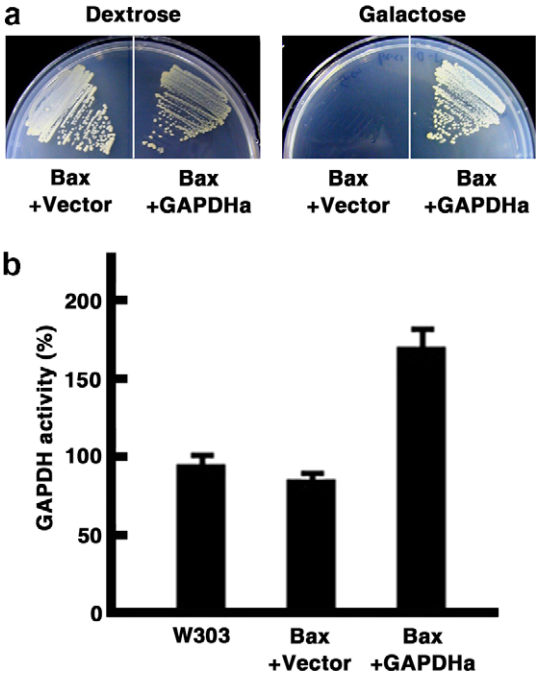

Fig. 1. GAPDHa suppresses Bax-induced cell death in yeast cells. (a) GAPDHa-promoted resistance to Bax lethality. W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 empty vector (left) or pGilda-Bax/pAD4-2.1-GAPDHa (right) were streaked onto plates containing either SD-glucose (Dextrose) or SD-galactose (Galactose). Photographs were taken after culturing at 30 °C for 2 days. (b) Enzyme activity of GAPDHa in yeast. GAPDHa enzyme activity was monitored spectrophotometrically at 340 nm under standard conditions and the amount of NADH/NADPH oxidation was calculated as μmoles min^{-1} μg^{-1} of protein.

2. Results

2.1. Identification of GAPDHa, a suppressor of Bax-induced cell death in yeast

We recently developed a yeast-based genetic screening method to clone plant genes that suppress mammalian Bax-induced cell death in yeast (Moon et al., 2002). Several *PBI* (Plant Bax Inhibitor) genes were isolated; among these, *PBI1* (ascorbate peroxidase) and *PBI2* (nucleotide diphosphate kinase 2) have already been described (Moon et al., 2002, 2003). A third gene, *PBI3*, was identified as *Arabidopsis* GAPDHa (accession no. At3g26650). To verify that GAPDHa suppresses Bax-induced cell death in yeast, we co-transformed the GAPDHa cDNA into yeast containing galactose-inducible Bax. On galactose-free medium, colony formation was observed (Fig. 1a). Colonies formed by cells harboring both Bax and GAPDHa (Bax + GAPDHa) on dextrose-based medium were detected with approximately the same efficiency as were control transformants containing (Bax + vector). However, while colony formation by transformants expressing Bax with the empty vector was completely inhibited on galactose medium, colony formation of transformants expressing Bax with the GAPDHa vector was not. Similar levels of Bax were detected in protein samples from galactose-grown transformants containing either Bax with an empty vector or Bax with GAPDHa, indicating that GAPDHa did not affect the expression of the Bax protein (data not shown). We further confirmed expression of GAPDHa in yeast cells by measuring GAPDH enzyme activity (Fig. 1b). Wild-type cells and transformants containing Bax and the vector only (Bax + vector) showed similar levels of GAPDH enzyme activity. However, yeast cells containing both Bax and GAPDHa (Bax + GAPDHa) showed a greater amount of GAPDH enzyme activity. These findings suggest that GAPDHa suppresses Bax-induced cell death in yeast.

2.2. GAPDHa inhibits ROS generation by Bax

Since the two previous PBIs we identified are involved in cellular redox regulation (Moon et al., 2002, 2003), we investigated whether ROS production by Bax is also altered by expression of GAPDHa. We analyzed the production of ROS during Bax-induced cell death in yeast, using dihydrorhodamine 123 (Schulz et al., 1996) and 2,7-dichlorofluorescein diacetate (Hockenbery et al., 1993). Flow-cytometry analyses indicated that yeast cells expressing Bax accumulated a large quantity of oxygen radicals; this accumulation was significantly inhibited by coexpression with GAPDHa (Fig. 2a and data not shown).

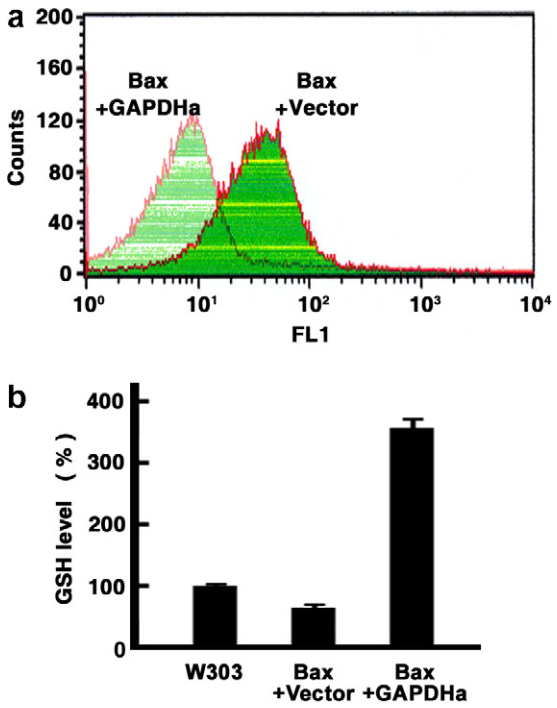

Fig. 2. GAPDHa suppresses ROS generation induced by Bax. (a) Flow cytometric analysis. W303-1a transformants containing pGilda-Bax/pADGal4-2.1 empty vector (Bax + vector) or pGilda-Bax/pAD4-2.1-GAPDHa (Bax + GAPDHa) were grown in galactose medium for 12 h. Cells were further incubated with 50 μM dihydrorhodamine 123 for 15 min for flow cytometric analysis. (b) Analysis of GSH levels in yeast. Yeast cells were cultured in galactose medium for 12 h. Error bars indicate ±SE of three replicates.

Cellular redox levels were further assayed by measuring GSH contents. Compare to wild-type cells and transformants containing (Bax + vector), those containing Bax and GAPDHa (Bax + GAPDHa) showed higher levels of GSH (Fig. 2b and data not shown). These findings suggest that GAPDHa suppresses the hyperproduction of intracellular ROS produced by Bax.

2.3. GAPDHa suppresses ROS generation and cell death in plant protoplasts

To identify the physiological role of GAPDHa in plants, GAPDHa transcript levels were analyzed under H_2O_2 and heat shock conditions. The level of GAPDHa transcripts increased with either H_2O_2 or heat shock treatment alone, indicating that the biological function of GAPDHa is related to oxidative stress (Fig. 3).

We further investigated the role of GAPDHa in plants by using the *Arabidopsis* protoplast system in individual cells (Baek et al., 2004). Protoplasts were isolated from leaf tissues of *Arabidopsis* plants and transformed with GFP alone, pBip::GFP (BiP), or pGAPDHa::GFP (GAPDHa). Twelve hours after transformation, GFP expression was observed, and cell viability was assessed by staining with DAPI. In each experiment, over 50% of the protoplasts expressed the intended protein, and

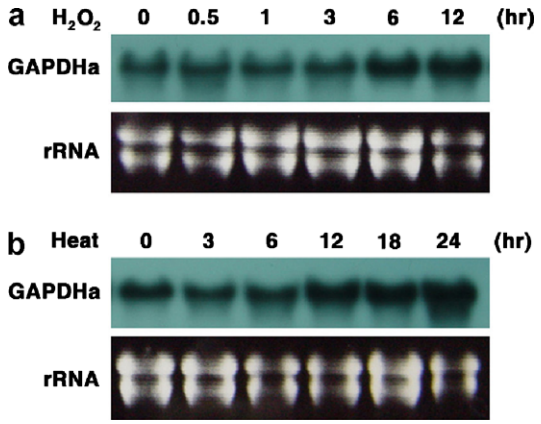

Fig. 3. GAPDHa transcript accumulates during heat shock and oxidative stresses. Total RNA was prepared from 2-week-old *Arabidopsis* seedlings treated with 4 mM H_2O_2 for the lengths of time indicated (a) or treated with heat at 44 °C for 2 h followed by recovery at 22 °C for 0, 3, 6, 12, 18, or 24 h (b). A fixed amount of total RNA (10 μg) was loaded onto each lane. Equal loading for each lane was confirmed by pre-staining gels with ethidium bromide to visualize rRNA. RNA blots were probed with ^{32}P-labeled GAPDHa cDNA.

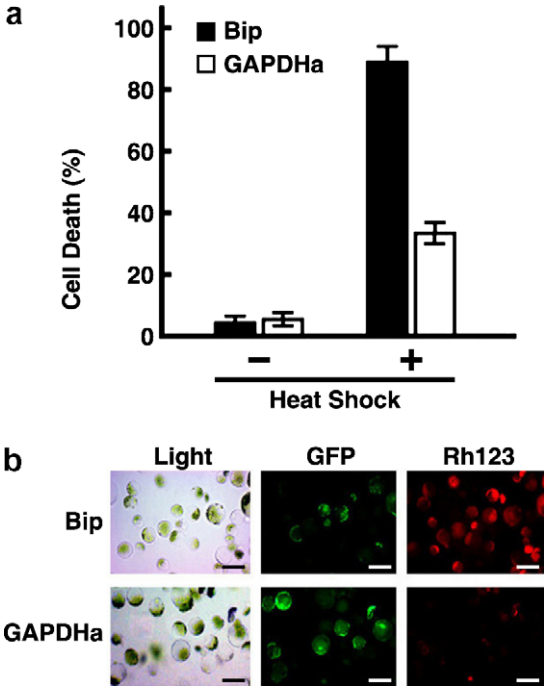

Fig. 4. GAPDHa suppresses ROS-induced cell death in *Arabidopsis* protoplasts. (a) Suppression of heat shock-induced cell death by GAPDHa. Protoplasts isolated from 3-week-old seedlings were transformed with pBiP::GFP (BiP) or GAPDHa::GFP (GAPDHa) reporter gene fusion constructs. Five hours after transformation, the protoplasts were treated at 44 °C for 2 h and then recovered at 22 °C for 24 h. Protoplast cells were treated with 25 μg ml^{-1} 4′,6-diamidino-2-phenylindole diacetate (DAPI) for 5 min and the percentage of viable cells in each population was determined by counting ($n = 250$–300). The data are the average ±SE of four independent experiments. (b) Suppression of heat shock-induced ROS generation by GAPDHa. The protoplast cells recovered after the heat shock treatment described in (a) were incubated with 20 μM dihydrorhodamine 123 for 15 min, and then observed by microscopy. The phase contrast (Light) and green fluorescence (GFP) images of one aliquot of protoplasts from each transformation are depicted. Bar indicates 100 μM.

expression of the introduced proteins did not influence cell viability over 48 h (data not shown). The transformed protoplasts were treated with heat shock, an inducer of PCD in plants (McCabe and Leaver, 2000; Tian et al., 2000; Vacca et al., 2004). Exposing cells to heat shock (44 °C for 2 h) resulted in death of over 98% of the GFP- and BiP-transformed cells (Fig. 4a and data not shown). Non-laddered DNA degradation, as detected by agrose gel electrophoresis, was significant in the heat shock treated cells. Additionally, the dead and dying cells exhibited morphological changes, including vacuolation, disorganization, size shrinkage, and condensation of the organelles on the plasma membrane (data not shown). All these morphological characteristics are considered to be hallmarks of protoplast cells undergoing apoptosis (Baek et al., 2004). However, expression of GAPDHa greatly protected protoplast cells against heat shock-induced cell death (Fig. 4a), indicating that GAPDHa is an efficient suppressor of cell death in plant protoplasts as well as in yeast (Fig. 1).

Because increasing evidence suggests that ROS are effectors of PCD in animals and plants (Jabs, 1999), and GAPDHa suppressed ROS generation in yeast (Fig. 2), the role of GAPDHa in attenuating ROS accumulation in plants was investigated. Protoplasts expressing GFP alone or BiP::GFP exhibited strong fluorescence when incubated with dihydrorhodamine 123, indicating ROS accumulation. In contrast, protoplasts expressing GAPDHa::GFP did not exhibit significant fluorescence (Fig. 4b). Taken together, these results suggest that GAPDHa inhibits the generation of ROS by heat shock, which in turn suppresses heat shock-induced cell death in plant cells.

3. Discussion

GAPDH (EC1.2.1.13) has been considered to be primarily a housekeeping enzyme involved in the glycolytic pathway, catalyzing the NAD-dependent conversion of glyceraldehyde-3-phosphate into 1,3-diphosphoglycerate. However, a variety of studies are now suggesting that GAPDH is a multifunctional protein that is involved in numerous subcellular processes in animals (Sirover, 1999, 2005; Chuang et al., 2005). GAPDH plays a role in gene transcription, DNA replication, nuclear RNA export, endocytosis, microtubule bundling, phosphotransferase, and oncogenesis (Sirover, 2005). In this report, we show that GAPDHa has another novel function that is involved in cellular redox regulation. This conclusion is based on our observations that (1) H_2O_2 induces the transient expression of GAPDHa, (2) GAPDHa suppresses Bax-induced cell death in yeast by inhibiting ROS generation, and (3) expression of GAPDH in plants leads to decreased constitutive ROS levels and enhanced tolerance to heat shock-induced cell death.

ROS and cellular redox levels are important mediators of multiple processes in cells such as proliferation, differentiation, and apoptosis. The higher levels of ROS, a by-product of normal metabolic processes, cause damage to lipids, carbohydrates, DNA, and proteins (Byczkowski and Gessner, 1988; Kannan and Jain, 2000). Low levels of ROS, however, play a key role in programmed cell death (PCD) signaling in animals and plants (Jabs, 1999). Thus, antioxidants that scavenge peroxide, such as thiol-reductants like *N*-acetylcystein, and manganese superoxide dismutase (MnSOD) overexpression block or delay apoptosis (Hockenbery et al., 1993; Jacobson and Raff, 1995). Consistent with these results, we found that Bax-induced cell death in yeast and plant protoplasts is correlated with ROS generation, and we observed that cell death was greatly suppressed by exogenous application of *N*-acetyl-L-cysteine or endogenous overexpression of either a ROS scavenging enzyme (sAPX) or a redox regulator (AtNDPK2) (Moon et al., 2002, 2003; Baek et al., 2004). The present study further confirms our earlier observations, and it suggests that ROS is a common element of PCD in animals, plants, and yeasts, where it functions as part of a basic, evolutionarily conserved mechanism.

Because overexpression of GAPDHa in yeast cells resulted in an increase in overall cellular antioxidative capacity (Fig. 2b), it is possible that GAPDHa has direct ROS scavenging activity. However, a role for GAPDHa as a mediator of apoptosis and ROS signaling cannot be ruled out in yeast and plant cells. In animals, GAPDH is translocated to the nucleus during oxidative stress, and overexpression of Bcl-2 prevents nuclear translocation of GAPDH and apoptosis (Dastoor and Dreyer, 2001). GAPDH is also involved in age-induced apoptosis in mature cerebellar neurons (Ishitani et al., 1996; Hara et al., 2006) and in the control of phospholipase D2 activity (Kim et al., 2003). Oxidative stress induced by H_2O_2 modifies cysteine residues at the catalytic site of GAPDH. This modification results in both inactivation of GAPDH enzyme activity and structural changes in GAPDH, allowing it to participate in protein–protein interactions that mediate signaling between H_2O_2 and phopholipase. Although the exact mechanism by which H_2O_2 modifies GAPDH structure/function remains to be determined in plants, several studies indicate that the function of GAPDH is linked to ROS signaling. In *Arabidopsis*, GAPDHa activity is also inhibited by H_2O_2 and this inhibition can be reversed by exogenous application of reduced glutathione (Hancock et al., 2005). Furthermore, expression studies indicate that GAPDH transcript levels are increased by H_2O_2, heat shock (Fig. 3), dehydration, and ABA, all of which are known to increase ROS generation in plants (Velasco et al., 1994). Concluding remarks: As in animals, plant GAPDH might have multiple functions, one of which may be regulation of H_2O_2 signaling in the cell. Further research to identify the specific proteins that directly interact with GAPDH would reveal the role of GAPDH in H_2O_2 signaling in the plant.

4. Experimental

4.1. Media, strains, and plasmids

Saccharomyces cerevisiae strain W303-1a (*MAT*a *ura3-1, leu2-3, 112 his3-11, 15 ade2-1, trp1-1, can1-110*) was grown in minimal medium (0.67% yeast nitrogen base, 2% glucose, 10× amino acid dropout solution). W303-1a was co-transformed with the pGilda-Bax plasmid and an *Arabidopsis* cDNA library using the lithium acetate method as previously described (Moon et al., 2002). GAPDHa cDNA was amplified by PCR with synthetic primers (GAPDHa forward; 5′-TCAGGATCCACTTCTGCAATGGGAAGCAGT-3′, GAPDHa reverse, 5′-GGCCTCGAGCTTCCAGTTGTTGGCAACAAT-3′) and cloned in frame into the pADGal4-2.1 vector.

4.2. Screening strategy

The colonies containing *Arabidopsis* Bax inhibitors (cDNAs that suppressed Bax-induced cell death) were selected as previously described (Moon et al., 2002).

4.3. Enzyme assay

The enzyme activity of purified GAPDH protein was assayed at 25 °C by following the absorbance change resulting from NAD(P)H oxidation at 340 nm (extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1}). The reaction mixture contained 100 mM Tris–Cl buffer, pH 8.0, 10 mM $MgCl_2$, 10 mM GSH, 5 mM ATP, 0.2 mM NADPH, 2 units of 3-phosphoglyceric phosphokinase, and 2 mM 3-phosphoglyceric acid.

4.4. Flow cytometric analysis

W303a yeast cells transformed with either pGilda-Bax/pADGal4-2.1 vector (Bax + vector) or pGilda-Bax/pADGal4-2.1-GAPDHa (Bax + GAPDHa) were grown in minimal medium containing glucose. To induce cell death by Bax protein, yeast cells were grown under the conditions previously described (Moon et al., 2002). To measure intracellular ROS in yeast, cells were incubated with 50 μM dihydrorhodamine 123 (Molecular Probes) and 50 μM 2,7-dichlorofluorescein diacetate (Molecular Probes) for 15 min and analyzed using a flow cytometer (Becton-Dickinson) as described elsewhere (Moon et al., 2002, 2003).

4.5. Determination of GSH level

W303a yeast cells containing either pGilda-Bax/pADGal4-2.1 empty vector (Bax + vector) or pGilda-Bax/pADGal4-2.1-GAPDHa (Bax + GAPDHa) were grown to an OD_{600} of 1×10^7 cells ml^{-1} and washed two times using phosphate-buffered saline (PBS) buffer. The cells were resuspended in ice-cold extraction buffer (8 mM HCl, 1.3% 5-sulfosalicyclic acid). To estimate the amount of free thiols, the cells were broken by sonication and centrifuged at 12,000 rpm for 15 min. The pellet was resuspended in 2 M sodium phosphate buffer (pH 7.5). To determine the GSH level, the amount of free GSH was measured spectrophotometrically at 412 nm in the reaction mixture between GSH and DTNB (5,5-dithiobis-2-nitrobenzoic acid). The content of GSH was calculated following subtraction of the blank (buffer and cell suspension).

4.6. Northern blot analysis

Total RNA was purified from 2-week-old *Arabidopsis* seedlings treated with 4 mM H_2O_2 for the amounts of time indicated in Fig. 3. To purify total RNA from heat-treated *Aarbidopsis* seedlings, plants were treated with heat stress at 44 °C for 2 h and then recovered at 22 °C for the amounts of time indicated. Samples (10 μg per lane) were analyzed by electrophoresis on a 1.2% formaldehyde agarose gel and transferred to an Immobilon-Ny^+ transfer membrane (Millipore). Membranes were UV cross-linked and hybridized to gene-specific probes in Church buffer (1% BSA, 1 mM EDTA, 0.25 M $NaHPO_4$, pH 7.2, 7% SDS) at 65 °C. Following incubation with gene-specific probes, membranes were washed twice with 2 × SSC/0.1% SDS for 10 min, then rewashed with 0.1 × SSC/0.1% SDS at 65 °C for 10 min (Moon et al., 2003).

4.7. Transient assays in protoplasts

The *Arabidopsis* GAPGHa cDNA was isolated from pADGal4-2.1-GAPDHa using synthetic primers (GAPDHa-forward; 5′-TCTAGACCACTACAATGGATGATG-3′, GAPDHa-reverse; 5′-GGATCCGAGATGGTGCACGAT-3′) and cloned into a p326-smGFP vector with GFP fusion protein (Baek et al., 2004) for protoplast expression. GFP fusion protein was placed under CaMV35S promoter and followed by NOS3 terminator in pUC plasmid. The Bip, targeting marker protein in ER, is constructed in p326-smGFP vector. Protoplasts were isolated from leaf tissues of 3-week-old *Arabidopsis* and transformed with plasmid DNA using polyethylene glycol (Baek et al., 2004). To determine intracellular ROS levels, transformed protoplasts ($\sim 2 \times 10^5$ ml^{-1}) were incubated with 20 μM dihydrorhodamine 123 (Molecular Probes) for 15 min. The images were monitored with a Zeiss Axioplan fluorescence microscope (Carl Zeiss). The filter sets used were XF116 (exciter, 474AF20; dichroic, 500DRLP; and emitter, 605DF50) and XF33/E (exciter, 535DF35; dichroic, 570DRLP; emitter, 605DF50; Omega Inc.) for GFP and Rhodamine 123, respectively. To count the percentage of cells killed by heat stress, transformed protoplasts were stained with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Molecular Probes) for 5 min and visualized under a fluorescence microscope using UV absorbance (excitation, 364 nm; emission, 454 nm).

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