Small Heat Shock Proteins Can Release Light Dependence of Tobacco Seed during Germination[1][OPEN]

Hyun Jo Koo[2,3], Soo Min Park[3,4], Keun Pill Kim[3,5], Mi Chung Suh[6], Mi Ok Lee, Seong-Kon Lee[7], Xia Xinli, and Choo Bong Hong*

School of Biological Sciences and Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151–742, Korea (H.J.K., S.M.P., K.P.K., M.C.S., M.O.L., S.-K.L., C.B.H.); and College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, People's Republic of China (X.X.)

ORCID ID: 0000-0001-7753-1502 (C.B.H.).

Small heat shock proteins (sHSPs) function as ATP-independent molecular chaperones, and although the production and function of sHSPs have often been described under heat stress, the expression and function of sHSPs in fundamental developmental processes, such as pollen and seed development, have also been confirmed. Seed germination involves the breaking of dormancy and the resumption of embryo growth that accompany global changes in transcription, translation, and metabolism. In many plants, germination is triggered simply by imbibition of water; however, different seeds require different conditions in addition to water. For small-seeded plants, like Arabidopsis (*Arabidopsis thaliana*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), and tobacco (*Nicotiana tabacum*), light is an important regulator of seed germination. The facts that sHSPs accumulate during seed development, sHSPs interact with various client proteins, and seed germination accompanies synthesis and/or activation of diverse proteins led us to investigate the role of sHSPs in seed germination, especially in the context of light dependence. In this study, we have built transgenic tobacco plants that ectopically express sHSP, and the effect was germination of the seeds in the dark. Administering heat shock to the seeds also resulted in the alleviation of light dependence during seed germination. Subcellular localization of ectopically expressed sHSP was mainly observed in the cytoplasm, whereas heat shock-induced sHSPs were transported to the nucleus. We hypothesize that ectopically expressed sHSPs in the cytoplasm led the status of cytoplasmic proteins involved in seed germination to function during germination without additional stimulus and that heat shock can be another signal that induces seed germination.

Small heat shock proteins (sHSPs) are a large group of proteins that have a highly conserved α-crystallin domain, a short C-terminal sequence, and a highly variable N-terminal region. Their distribution is ubiquitous, with rare exceptions in some *Archaebacteria* spp. (Kappé et al., 2002; Franck et al., 2004; Waters et al., 2008). Monomeric sHSPs range in size from 12 to 42 kD and are often assembled into oligomeric complexes from 9 to more than 24 subunits (Van Montfort et al., 2001; Waters, 2013). sHSPs function as ATP-independent molecular chaperones that prevent cellular proteins from irreversible aggregation and insolubilization, which occur as a result of high denaturation (Eyles and Gierasch, 2010; Tyedmers et al., 2010; Waters, 2013). Most studies that previously addressed the interaction between sHSPs and client proteins revealed that heat-denatured client proteins bind to the hydrophobic surfaces on sHSPs that are mostly newly available after high temperature-induced conformational changes in the proteins. Client proteins that are bound to sHSPs often maintain a folding-competent state that can be renatured by ATP-dependent chaperones and cochaperones (Basha et al., 2012; Fu et al., 2013). Although the production and function of sHSPs have often been referred to in the context of heat stress, expression and function of sHSPs have also been confirmed in fundamental cellular activities, such as protection of transcription, translation, secondary metabolism, cell signaling, and cell division cycles (Van Montfort et al., 2001; Goldfarb and Dalakas, 2009; Graw, 2009; Willis et al., 2009; Basha et al., 2012; Waters, 2013).

[1] This work was supported by the Ministry of Education and Human Resources Development, Korea (BK21 Research Fellowships to H.J.K. and M.O.L.), the Priority Research Institute (grant no. 0413–20130010 to C.B.H.) and the Basic Science Research Program (grant no. NRF–2012–R1A1A2008416 to C.B.H.) supported through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology and the Planta Company (transformation of tobacco and rearing of the transgenic tobacco plants).

[2] Present address: Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037.

[3] These authors contributed equally to the article.

[4] Present address: Experiment Research Station, National Agricultural Products Quality Management Service, Gimcheon-si 740–871, Korea.

[5] Present address: Department of Life Sciences, Chung-Ang University, Seoul 156–756, Korea.

[6] Present address: Department of Bioenergy Science and Technology, Chonnam National University, Gwangju 500–757, Korea.

[7] Present address: Rural Development Administration, Jeonju, Jeonbuk 560–500, Korea.

* Address correspondence to hcb@snu.ac.kr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Choo Bong Hong (hcb@snu.ac.kr).

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.114.252841

In plants, 11 sHSP families have been reported; among them, five are localized in the cytosol, and the other six are localized in the subcellular organelles (i.e. the nucleus, mitochondria, chloroplast, and endoplasmic reticulum). Among the cytosolic sHSPs, class I family members are the most abundant, and their localization has also been detected in the nucleus (Basha et al., 2012). The cytosolic class I sHSPs are expressed not only after exposure to elevated temperatures but also, at particular developmental stages, such as in embryogenesis, microsporogenesis, fruit maturation, and maturing seeds (Wehmeyer et al., 1996; Wang et al., 2014). Function of sHSPs in seed longevity and stress tolerances has also been reported by ectopically expressing heat shock factors in tobacco (*Nicotiana tabacum*) that led to the additional accumulation of sHSPs in the tobacco seed (Personat et al., 2014).

Seed germination mostly requires environmental factors, such as moisture, oxygen, temperature, light, and nutrients, and the breaking of dormancy and the resumption of the growth of the embryo accompany expression of diverse genes and proteins (Miernyk and Hajduch, 2011; Graeber et al., 2012; Rajjou et al., 2012; Wang et al., 2014). The facts that sHSPs accumulate during seed development, sHSPs interact with various client proteins, and seed germination accompanies synthesis and/or activation of diverse proteins led us to investigate the role of sHSPs in seed germination. To understand the function of sHSPs in the seed germination, transgenic lines additionally expressing ectopically introduced sHSP genes were constructed and used to determine the possible function of sHSPs in seed germination, especially in the context of light dependence, because for small-seeded plants, such as Arabidopsis (*Arabidopsis thaliana*), lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), and tobacco, light is an important regulator of seed germination.

RESULTS

Transgenic Tobacco Plants Constitutively Expressing NtHSP

Three cytosolic class I sHSP genes (*NtHSP*s) from tobacco, *NtHSP18.2*, *NtHSP17.6*, and *NtHSP18.3* (Park et al., 2014), were constitutively expressed in tobacco by placing the open reading frames (ORFs) under the control of the *Cauliflower mosaic virus* 35S promoter (P_{35S}; Fig. 1A; Supplemental Fig. S1). *NtHSP18.2* was also expressed in tobacco in the antisense orientation by placing the ORF in the antisense orientation after the promoter (Fig. 1A). After transformation of P_{35S}-*NtHSP* into the tobacco genome and in vitro regeneration of tobacco plants, the transgenicity of T1 generation tobacco plants was confirmed by RNA-blot hybridization with the corresponding *NtHSP* clone as a probe, and the plants were allowed to self-fertilize. The transgenicity of T2 generation plants was also confirmed by RNA-blot hybridization (Fig. 1B; Supplemental Fig. S1). T2 generation tobacco plants carrying P_{35S}-*NtHSP18.2* were also analyzed for expression

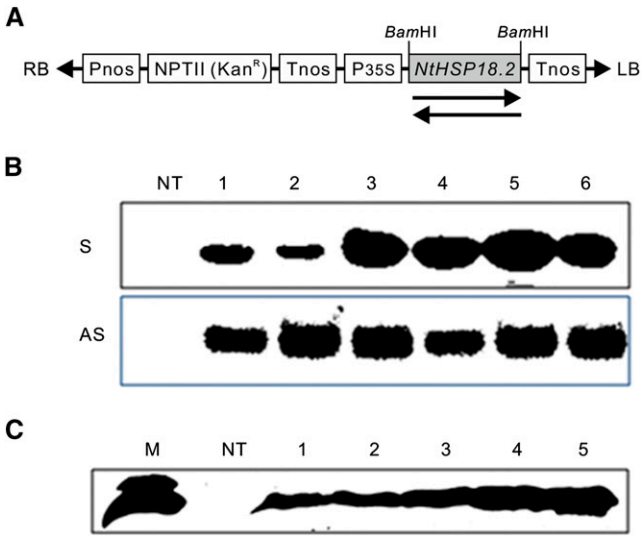

Figure 1. RNA- and protein-blot hybridization results of the transgenic tobacco plants that expressed *NtHSP18.2* and the expression cassette used. A, The ORF of *NtHSP18.2* was placed in sense and antisense orientations under the control of the *Cauliflower mosaic virus* 35S promoter in an expression vector pBKS1-1 (Suh et al., 1994). Arrows indicate the direction of transcription. Kan^R, Kanamycin resistance gene; LB, left border of transferred DNA; NPTII, neomycin phosphotransferase II gene; Pnos, promoter of nopaline synthase gene; RB, right border of transferred DNA; Tnos, terminator of nopaline synthase gene. B, RNA-blot hybridization results of total RNAs from the putative transgenic tobacco plants carrying the ORF of *NtHSP18.2* complementary DNA in the sense (S) and antisense (AS) orientations. In S, the blot was hybridized with the ^{32}P-labeled antisense *NtHSP18.2* riboprobe. In AS, the blot was hybridized with the ^{32}P-labeled sense *NtHSP18.2* riboprobe. Putative transgenic plants are labeled as 1 to 6. NT, Nontransgenic plant. C, Protein-blot hybridization results of the sense transgenic tobacco plants that were confirmed by RNA-blot hybridizations as in B. Polyclonal antibody against NtHSP18.2 was used. M, Purified NtHSP18.2 as a positive control.

of the introduced *NtHSP* to the protein by protein-blot analysis. All of the plants with a positive RNA-blot signal in the sense orientation showed expression of the NtHSP18.2 protein under the normal cultivation temperature (Fig. 1C).

Expression of sHSPs in Tobacco Seeds

Protein-blot analyses for the expression of NtHSPs showed that accumulation of the sHSPs started before desiccation of the seeds was noted and remained detectable after seed browning, whereas no detectable NtHSP existed in the leaves (Fig. 2, A and B). The protein level in the seed decreased during imbibition and germination, and NtHSP was detected until seed imbibition had occurred for 3 d (Fig. 2B). In transgenic tobacco plants with P_{35S}-*NtHSP18.2*, NtHSP18.2 was detected in the dry seeds, although at a very low level, in addition to the normal accumulating NtHSPs, and the level increased as imbibition proceeded (Fig. 2B). Accumulation of NtHSP18.2 as well as the normal accumulating sHSPs in the seeds was again confirmed

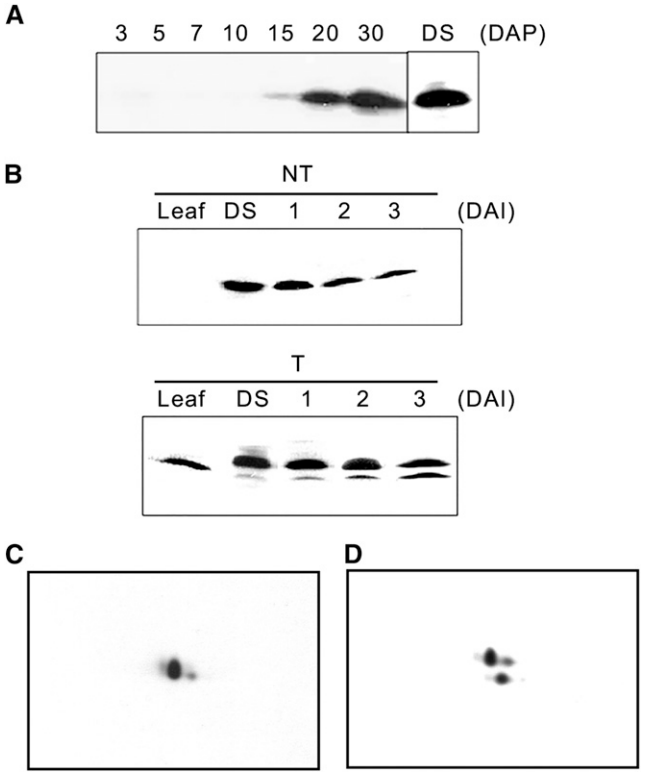

Figure 2. Temporal examination of sHSP accumulation during tobacco seed development. Before opening of the flower, anthers were removed, and ovules were collected 3 to 30 d after pollination (DAP). Proteins were extracted from the ovules, and 50 μg of total protein per lane was separated by SDS-PAGE and transferred to a nitrocellulose membrane. A, Protein-blot analyses for the class I sHSPs accumulated during seed maturation or in the dry seeds (DSs). B, Comparison of the class I sHSPs in the seeds of nontransgenic and transgenic tobacco plants that constitutively expressed NtHSP18.2. DAI, Days after imbibition; NT, wild-type tobacco plants; T, transgenic tobacco plants that constitutively overexpressed NtHSP18.2. C and D, Two-dimensional gel analysis for NtHSPs in the nontransgenic tobacco seeds (C) and the transgenic tobacco seeds that constitutively overexpressed NtHSP18.2 (D). In D, seeds that were imbibed for 3 d were used to increase the level of ectopically expressed NtHSP18.2. Polyclonal antibody against NtHSP18.2 was used.

by protein-blot analysis on two-dimensional gels (Fig. 2, C and D). When the protein spots from the two-dimensional gel protein-blot analysis were compared with the signal of the heat shock-treated nontransgenic tobacco leaves, it was found that sHSPs that accumulated in the seeds probably corresponded to NtHSP17.4 and NtHSP18.0 (Supplemental Fig. S2; Park et al., 2014).

Ectopic Expression of NtHSP(s) Releases Light Dependence of Tobacco Seed Germination

Tobacco seeds had light-dependent germination, and radicle protrusion started after about 24 h under light after imbibition (Fig. 3A). When tobacco seeds of the nontransgenic line or the transgenic lines with *NtHSP18.2* in the sense or antisense orientation were incubated under light after imbibition, all of the lines (i.e. nontransgenic, sense transgenic, and antisense transgenic) germinated to an almost equal extent, although the sense transgenic line might exhibit a slightly enhanced germination, and the antisense transgenic line might show a slightly repressed germination (Supplemental Fig. S3). All of the lines showed full germination after about 40 h of incubation under light after imbibition (Fig. 3A). When nontransgenic, sense transgenic, and antisense transgenic tobacco seeds were incubated in the dark, there was no noticeable germination observed in the nontransgenic and the antisense transgenic lines for up to 4 d, whereas the sense transgenic line showed a good level of germination that reached about 60% germination after 3 d (Fig. 3B). Germination of transgenic seeds carrying other class I *sHSP*s (*NtHSP17.6* or *NtHSP18.3*) was also studied in the dark. After 3 d of imbibition and incubation, germination of the transgenic seeds carrying the additional NtHSP17.6 or NtHSP18.3 showed a similar level of germination compared with the transgenic seeds with NtHSP18.2 in the dark, although slight variation in the germination percentage among the transgenic lines was noticed (Fig. 3C). Brief illumination of the imbibed seeds for 10 and 20 s under fluorescence light induced germination in all of the lines; very strong inductions occurred in the antisense, vector, and nontransgenic lines, and significant induction occurred in the sense lines. Twenty seconds of illumination resulted in about 40% germination in the antisense, vector, and nontransgenic lines (Fig. 4), whereas no statistically meaningful germination was observed without this brief illumination (Fig. 3B).

Germination of Nontransgenic Tobacco Seeds in the Dark after Heat Treatment

Because the ectopic expression of *NtHSP*s in tobacco seeds led to germination in the dark, the effect of heat shock treatment on the seeds was examined in the dark. After imbibition of nontransgenic tobacco seeds for 2 d at 25°C in the dark, the seeds were exposed to a high temperatures of 35°C, 38°C, or 42°C for 4 h in the dark and placed at 25°C for 2 d in the dark; then, the germination percentage was determined. From the heat-shocked nontransgenic tobacco seeds, high levels of germination (i.e. 68% at 35°C, 73% at 35°C, and 79% at 42°C) were observed, whereas seeds kept at 25°C without heat treatment did not germinate (Fig. 5A). When the same treatment was applied to the *NtHSP18.2* sense and antisense transgenic seeds (i.e. imbibition for 2 d at 25°C, 42°C heat shock for 4 h, and additional incubation for 2 d at 25°C all in the dark), almost complete germination in the sense transgenic line and about 60% germination in the antisense transgenic line were observed (Fig. 5B; Supplemental Fig. S4).

Localization of the Ectopically Expressed NtHSP18.2 in Transgenic Tobacco Seeds

Immunohistochemical analyses of the imbibed P_{35S}-*NtHSP18.2* transgenic tobacco seeds showed a strong

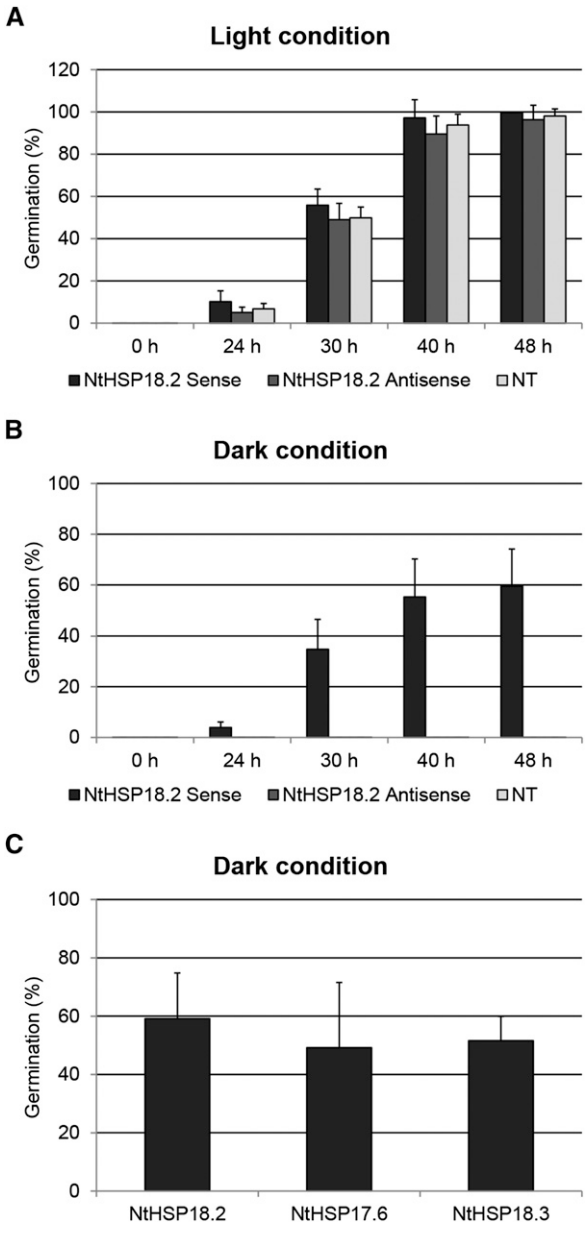

Figure 3. Comparison of seed germination under light or in the dark. Seeds were imbibed for 1 d in the dark and further incubated either under light (A) or in the dark (B) for 24, 30, 40, or 48 h. Antisense, Transgenic plant with *NtHSP18.2* in the antisense orientation; NT, nontransgenic plant; Sense, transgenic plant that overexpressed *NtHSP18.2* in the sense orientation. C, Comparison of seed germination percentages in the dark between transgenic tobacco plants that constitutively overexpressed *NtHSP18.2*, *NtHSP17.6*, or *NtHSP18.3*. Germination percentages were checked at 3 d after imbibition. Each data point represents an average of 10 measurements, and error bars represent SD. Each measurement was on different tobacco lines, and 50 seeds were used.

red signal from the embryo over the faint background signal in the endosperm. The signal was evenly detected throughout most of the cytosol (Fig. 6B). The same assay for the imbibed nontransgenic tobacco seeds revealed the presence of NtHSPs in the embryo as well as the endosperm (Fig. 6A). Because the immune-response signal for the ectopically expressed NtHSP18.2 was very strong, the incubation time to reveal the red signal was shortened to distinguish the signal in each cell, and this resulted in a very weak signal in the endosperm (Fig. 6B). Hybridization of the seed sections with preimmunized serum could not distinguish the transgenic seeds from the nontransgenic seeds (i.e. there was low-level background staining in both samples; Fig. 6, C and D).

Subcellular Localization of NtHSPs under Heat Shock Conditions

Intracellular localization of the NtHSPs in the seeds after heat shock treatment was also closely analyzed by immunohistochemical assays. Nontransgenic tobacco seeds were imbibed for 2 d at 25°C in the dark and then heat shocked at 42°C for 10 min, 20 min, 30 min, 1 h, and 4 h. After the heat shock treatment, the seeds were incubated at 25°C for 1, 6, 12, and 24 h. Thin sections of the treated seeds were hybridized to an anti-NtHSP18.2 polyclonal antibody and then, a biotinylated anti-rabbit bridge antibody. The signal was primarily detected in the cytoplasm of the embryo within 10 min of heat shock treatment; then, NtHSPs quickly moved into the nucleus, and at 30 min, NtHSPs were mainly detected in the nucleus (Fig. 7A). Prolonged heat shock treatment over 30 min did not show noticeable difference on the localization of NtHSPs from the localization pattern at 30 min; that is, most of the NtHSPs remained in the nucleus (data not shown). During the recovery period at 25°C, nuclear localization of NtHSPs was diminished, and overall presence of NtHSPs in the cytoplasm gradually decreased. After 24 h at 25°C, presence of NtHSPs was hardly noticed (Fig. 7A). Translocation of NtHSPs into the nucleus from the cytosol was again

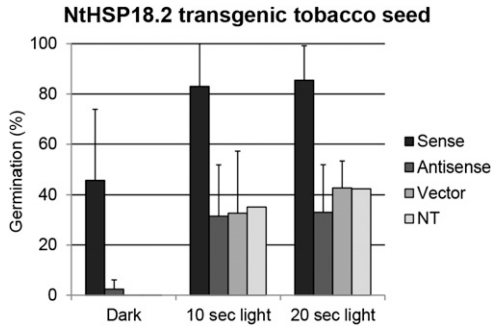

Figure 4. Effect of brief illumination on seed germination. *NtHSP18.2* sense transgenic tobacco seeds, *NtHSP18.2* antisense transgenic tobacco seeds, empty vector transgenic tobacco seeds, and nontransgenic tobacco seeds were used for the test. The dark condition consisted of 3 d of imbibition in the dark. After 1 d of imbibition in the dark, 10 or 20 s of fluorescent lighting was given, and the seeds were incubated for 2 d in the dark. Each data point represents an average of 10 measurements, and the error bars represent SD. Each measurement was on different tobacco lines, and 50 seeds were used. NT, Nontransgenic plant.

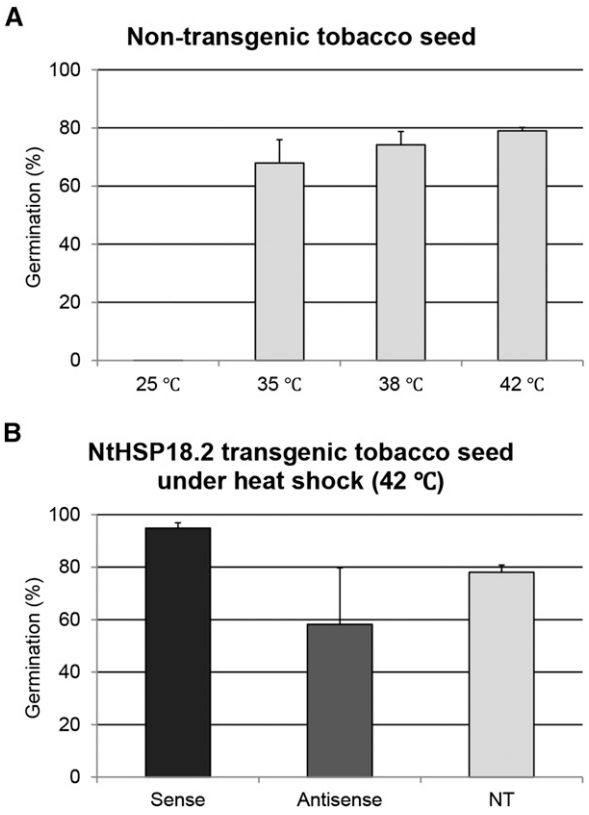

Figure 5. Effect of heat shock on seed germination. A, Nontransgenic tobacco seeds were imbibed for 2 d at 25°C in the dark and then exposed to 35°C, 38°C, or 42°C for 4 h in the dark. The heat-stressed seeds were incubated at 25°C in the dark for 2 d, at which time germination percentages were checked. Each data point represents the average of 10 measurements, and error bars represent SD. B, *NtHSP18.2* sense transgenic tobacco seeds, *NtHSP18.2* antisense transgenic tobacco seeds, and nontransgenic tobacco seeds were imbibed for 2 d in the dark at 25°C followed by heat shock treatment at 42°C for 4 h. Heat-shocked seeds were incubated for 2 d in the dark at 25°C. Each data point represents the average of 10 measurements, and error bars represent SD. Each measurement was on different tobacco lines, and 50 seeds were used. NT, Nontransgenic plant.

confirmed by confocal microscopy. Nontransgenic tobacco seeds that were imbibed for 2 d with synthesized NtHSPs with 42°C heat stress revealed that the majority of NtHSPs was located in the nucleus after 1 h of heat shock treatment (Fig. 7B; Supplemental Fig. S5), which is similar to the results shown in Figure 7A. Cytoplasmic location of NtHSP18.2, translocation of NtHSP18.2, and probably, other NtHSPs induced upon heat shock into the nucleus were also apparent in the NtHSP18.2 sense transgenic tobacco plants (Fig. 7B; Supplemental Fig. S5). Localization of the NtHSPs was again assayed using electron microscopy of immunogold-labeled NtHSP18.2. Wild-type tobacco seeds were imbibed for 48 h at 25°C and heat shocked at 42°C for 30 min.

Electron microscopy of the heat-shocked tobacco seeds confirmed translocation of the NtHSPs from the cytoplasm to the nucleus under the heat stress conditions (Supplemental Fig. S6).

DISCUSSION

A seed contains an embryo in the dormant stage that becomes the next generation of plant after the conditions are favorable for growth. Even in severe stress conditions, most seeds can survive for prolonged periods. Seed germination involves the breaking of dormancy and resumption of embryonic growth: gene transcription is resumed, protein synthesis begins, and the rates of respiration and intermediary metabolism increase (Weitbrecht et al., 2011; Rajjou et al., 2012). In many plants, germination is simply triggered by imbibition of water, but in addition to water, different seeds have their own distinct set of germination requirements regarding temperature, air, light, nitrate, oxygen, smoke, and allelochemicals (Graeber et al., 2012). During plant seed development, sHSPs accumulate in seeds (class I cytocolic sHSPs are primarily expressed during seed development), are first detected during midmaturation, and are most abundant in dry seeds. It has been suggested that sHSP expression in seeds is part of the normal development during late seed maturation, and it has been postulated that sHSP presence has adaptive significance for plant reproduction by preventing aggregation of nonnative proteins in developing tissues or protecting a broad range of cellular proteins from the low-water content environment of the seed to maintain their native structure

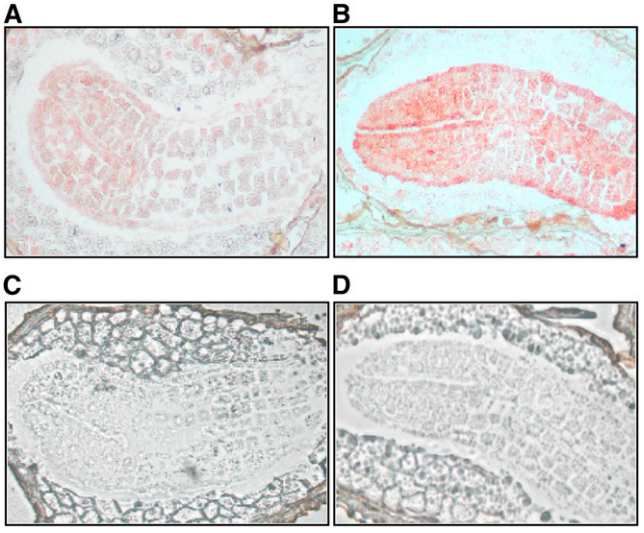

Figure 6. Localization of NtHSPs, including ectopically expressed NtHSP18.2, in the imbibed tobacco seeds. Longitudinal sections of a nontransgenic tobacco seed (A) and a transgenic tobacco seed that constitutively overexpressed NtHSP18.2 (B) were hybridized with anti-NtHSP18.2 polyclonal antibody. C and D show protein-blot hybridization results for the nontransgenic tobacco seeds (C) and transgenic tobacco seeds with preimmunized serum (D). NtHSPs were visualized in red by immunohistochemical staining with streptavidin-conjugated horseradish peroxidase. Because the red signal was much stronger in the transgenic seeds, the length of the coloring reaction for the peroxidase was shortened to one-quarter in B compared with A.

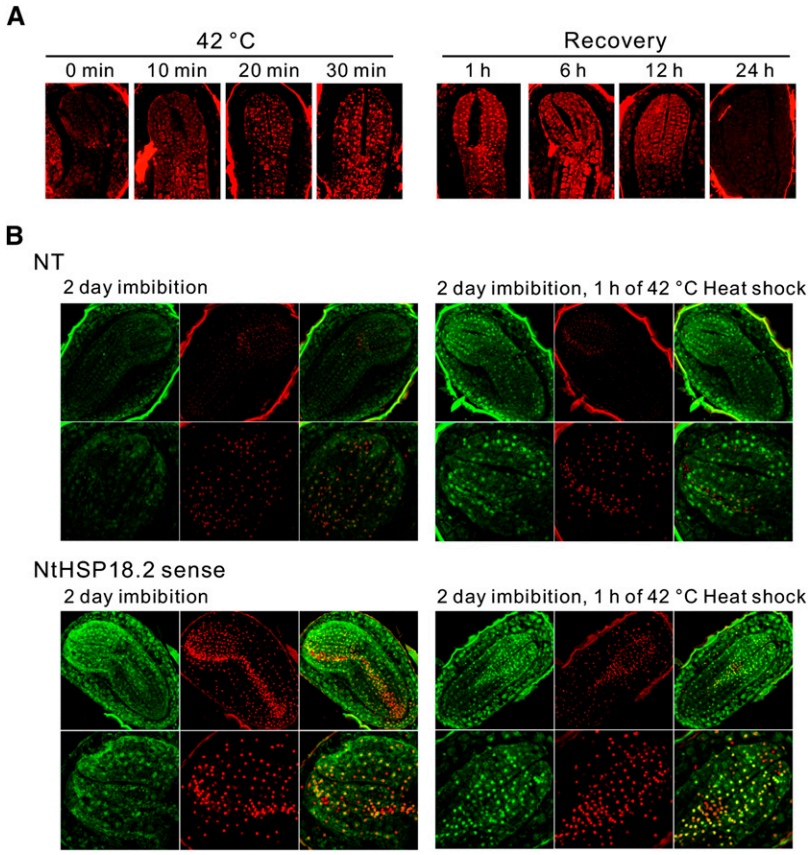

Figure 7. Effect of heat shock treatment on the distribution of NtHSP(s) in the seeds. A, Localization of the sHSPs in the nontransgenic tobacco seeds. Seeds were imbibed for 2 d at 25°C in the dark, heat shocked at 42°C for 10, 20, or 30 min, and placed at 25°C for 1, 6, 12, or 24 h. Anti-NtHSP18.2 polyclonal antibody and biotinylated anti-rabbit bridge antibody were used for immunohistochemical staining in red. B, Confocal microscopy images for nontransgenic tobacco seeds and *NtHSP18.2* sense transgenic tobacco seeds with NtHSP18.2 polyclonal antibody. Seeds were imbibed for 2 d followed by heat shock treatment at 42°C for 1 h. In each condition, upper depicts the entire seed, including embryo and endosperm, and lower shows the magnified embryo in upper. In each condition, left shows detections of NtHSP18.2 polyclonal antibody with fluorescein isothiocyanate, center shows detections of nucleus with propidium iodide, and right shows emerged images of both. NT, Nontransgenic.

(Wehmeyer et al., 1996; Gallardo et al., 2001; Wang et al., 2014).

Protein-blot analyses of tobacco seeds in this study also showed that sHSPs significantly accumulate in developing and dried seeds. Additionally, as in other species, a portion of cytosolic class I sHSPs (i.e. NtHSP17.4 and NtHSP18.0) accumulated in the seeds, whereas seven cytosolic class I sHSPs were observed in vegetative tissue upon heat stress. These sHSPs started to be detected in the seeds about 2 weeks after pollination occurred; the level of these sHSPs increased as the embryo developed into dry seeds and disappeared during imbibition and germination, and the sHSPs were no longer detectable after 3 d of imbibition (Fig. 2). The level of ectopically expressed NtHSP18.2 was low in the dry seeds, and the level increased during imbibition and germination, which is different from the developmentally expressed NtHSPs (Fig. 2). In the immunocytological analysis with the antibody against NtHSP18.2, the signal was much stronger from the transgenic seeds, although the amount of endogenous NtHSPs (i.e. NtHSP17.4 and NtHSP18.0) in the seeds was much larger than the amount of the ectopically expressed NtHSP18.2 at the same time point (Fig. 2B). Although it is only possible to speculate at present, NtHSP17.4 and NtHSP18.0 might have formed tighter complexes with the proteins in the seed, which may have occurred during seed development compared with the ectopically expressed NtHSP18.2. Thus, less is available to the antibody binding during the immunohistochemical assays. In the sense transgenic plants, the ectopically expressed NtHSP18.2 was mainly located in the embryo as well as throughout the embryonic cells, and it was probably mostly in the cytoplasm (Figs. 6 and 7; Supplemental Fig. S5). Thus, NtHSP18.2 that was ectopically overexpressed in the transgenic seeds may interact with cellular proteins in the cytoplasm in a different manner from the endogenous and developmentally expressed NtHSPs.

Germination percentages between the transgenic tobacco seeds with constitutively overexpressed *NtHSP18.2* in the sense or antisense direction and the nontransgenic tobacco seeds were compared. All of the transgenic and nontransgenic tobacco seeds germinated well at a similar level under the light, although the sense transgenic tobacco seeds showed a slightly higher germination percentage and the antisense transgenic tobacco seeds showed a slightly lower germination percentage than the other seeds (Fig. 3A). However, in the dark, only the sense transgenic tobacco seeds that constitutively overexpressed NtHSP18.2 germinated. That is, neither the nontransgenic seeds nor the antisense transgenic seeds could germinate in the dark (Fig. 3B). When different NtHSPs (i.e. NtHSP17.6 or NtHSP18.3) were ectopically expressed in tobacco and the same assay to evaluate the effect of the ectopic expression on the seed germination in the dark was conducted, it was revealed that ectopic expression of the sHSP commonly released light dependence

in tobacco seed germination (Fig. 3C). Brief illumination (10 or 20 s) on the seeds significantly enhanced germination (Fig. 4), although the germination percentages did not reach to a complete level as the case under the continuous illumination (Fig. 3A). The nontransgenic empty vector and antisense seeds germinated about 40% under the effect of brief illumination. This result showed that the light-dependent seed germination in tobacco is a result of light reception that activated signal transduction that lead to necessary changes in seeds for germination to occur. The brief illumination also significantly influenced the sense transgenic seeds in a positive way. About a 2-fold increase (i.e. from approximately 40% to approximately 80%) in germination in the sense transgenic seeds indicates that the light-dependent signal transduction leading to germination adds to the effect of the ectopically expressed NtHSP(s) on seed germination.

Because the ectopic expression of sHSPs significantly affected tobacco seed germination with regard to light illumination, nontransgenic tobacco seeds were heat shocked after imbibition and checked for seed germination in the dark (Supplemental Fig. S4). Heat shock-released light dependence in nontransgenic seed germination occurred at a comparable level with the level in the transgenic tobacco seeds with an NtHSP in the sense orientation (Fig. 5). However, immunohistochemical assays on the heat-shocked seeds showed a significantly different spatial pattern of sHSP presence compared with the ectopically expressed NtHSP18.2. Although ectopically expressed NtHSP18.2 was mainly located in the cytoplasm and still affected seed germination that resulted in germination in the dark, most of the newly synthesized sHSPs under heat shock conditions quickly moved into the nucleus. At 20 min, almost all of the sHSPs were located in the nucleus, which was maintained under prolonged heat shock treatment (Fig. 7; Supplemental Figs. S5 and S6). After the heat shock was removed from the seeds, nuclear localization of the sHSPs was no longer defined (Fig. 7A), which could be from either dissipation of the sHSPs from the nucleus to the cytoplasm or translation of the heat shock-induced sHSP transcripts that resulted in the accumulation of the sHSPs in the cytoplasm while most of the nucleus-localized sHSPs degraded.

The fact that ectopic expression and heat shock-induced expression of *NtHSP* can lead to seed germination without light shows that the light-dependent signal transduction pathway for seed germination is broadly intermingled with the NtHSP function in seed germination. The linking may occur in the cytosol and/or nucleus. However, localization and function of NtHSPs related to seed germination in the cytosol and the nucleus as well may be not needed for seed germination. As the effect of the ectopic expression of *NtHSP18.2* that was mainly in the cytosol showed, only cytosolic functioning of NtHSP(s) seems to be sufficient for seed germination.

Extensive studies on the function of sHSPs commonly depict them as ATP-independent molecular chaperones that prevent cellular proteins from irreversible aggregation and insolubilization, which occurs as the result of high-degree denaturation (Eyles and Gierasch, 2010; Tyedmers et al., 2010). Most studies that previously addressed the interaction between sHSPs and client proteins revealed that heat-denatured client proteins bind to the hydrophobic surfaces on the sHSPs that are often recently available after high temperature-induced conformational changes occur in the proteins. Similar conformational change of proteins can occur under severe dehydration conditions, such as in dry seeds. Presence of sHSPs in the dry seeds has been considered in the context of molecular chaperone activity of the sHSP(s) for the denaturing proteins under this severe dehydration condition. The presence of sHSP(s) in the dry seeds does not lead to seed germination in tobacco, which is shown in Figure 4. It is more likely that the endogenous sHSPs protect viable proteins and possibly, subcellular structures during seed development and allow the seed to start germination after the conditions become favorable, including proper light illumination. Ectopically expressed NtHSPs, including NtHSP18.2, NtHSP17.6, and NtHSP18.3, apparently have different functions compared with the endogenous NtHSPs, including NtHSP17.4 and NtHSP18.0, although they are structurally very similar. Thus, the following is possible: it is not the NtHSP presence itself that leads to tobacco seed germination in the dark. De novo synthesis of NtHSP18.2 in the sense transgenic seeds upon imbibition and de novo synthesis of NtHSPs in the nontransgenic seeds upon imbibition and heat shock treatment do lead to seed germination in the dark (i.e. release of light dependence in seed germination).

As previously reported (Bae and Choi, 2008; Seo et al., 2009; Zhou et al., 2012; Fu et al., 2013) and shown in Figures 3 and 4, light is a key environmental factor for inducing seed germination. As shown in Figure 5A, heat shock may be another environmental factor that induces seed germination. Fast translocation of NtHSP(s) into the nucleus upon heat shock may be another supporting fact for heat shock as an environmental factor that induces seed germination in tobacco plants, although it is unlikely that NtHSP moving into the nucleus is the activator that turns on the series of events leading to seed germination. Rather, it is more likely that the NtHSP moving into the nucleus affects the activator(s) of seed germination. Although NtHSPs in the nucleus may function in seed germination indirectly by influencing chromosome structure and/or transcription factors, ectopically expressed NtHSPs may more directly affect seed germination. The chemical nature of NtHSPs that can chaperone and/or interact with very various proteins (Basha et al., 2012; Park et al., 2014) should also work in the cytosol during imbibition, and the result can overrule requirements for seed germination, such as light. In other words, proteins that are involved in tobacco seed germination await the activation of the light-dependent signal transduction pathway, and the ectopically expressed NtHSPs in the cytoplasm interact with the proteins that are involved in seed germination and change the status of the proteins, such that they

are ready to work for seed germination without an additional stimulus.

NtHSP(s) in the nucleus and cytoplasm may interact with phytochrome- or other photoreceptor-mediated signal transduction pathways in germination. Likewise, NtHSP(s) may interact with GA signaling and abscisic acid signaling, which has an effect on seed germination that had been closely approached in Arabidopsis (Bae and Choi, 2008; Holdsworth et al., 2008; Seo et al., 2009; Weitbrecht et al., 2011). Because the germination process accompanies extensive synthesis and activation of proteins, such as proteins in the basic biochemical pathways (including glycolysis, tricarboxylic acid cycle, transport of amino acids and nucleotides, DNA repair, lipid degradation, translation machinery, proteases, and proteasome machinery; Van Montfort et al., 2001; Jiao et al., 2007; Goldfarb and Dalakas, 2009; Graw, 2009; Willis et al., 2009; Angelovici et al., 2010; Basha et al., 2012; Zhou et al., 2012; Fu et al., 2013), many client proteins could be associated with the ectopically expressed NtHSPs and heat shock-induced NtHSPs. This interaction leads to tobacco seed germination, the process that can be dissected into testa rupture and endosperm rupture that lead to radicle protrusion, in the dark and is a fundamental issue that should be addressed in the future. Because our work just followed the emergence of the radicle to visualize seed germination, in future work, impact of NtHSP(s) either ectopically expressed or heat shock induced on each step of seed germination (i.e. testa rupture and endosperm rupture) also needs to be addressed.

MATERIALS AND METHODS

Plant Growth Conditions, Heat Stress Treatments, and Determination of Seed Germination Percentage

Tobacco (*Nicotiana tabacum* 'WI 38') was grown in a greenhouse at 25°C ± 2°C under natural lighting with additional fluorescent lighting to maintain a 16-h photoperiod. Young tobacco plants with five expanded leaves were heat stress treated for 4 h at 48°C in an incubator (Robbins Scientifics) in the dark. The relative humidity in the incubator was maintained at higher than 90%. To evaluate germination, 50 seeds from each tobacco line (i.e. wild-type, T2 generation transgenic lines that constitutively expressed sense or antisense *NtHSP18.2*, *NtHSP17.6*, or *NtHSP18.3*) and lines carrying the empty vector (i.e. just the expression vector without *NtHSP*) were imbibed on two layers of Whatman Filter Paper 2 in a petri dish under a 16-h photoperiod with fluorescent lighting of 150 μmol m^{-2} s^{-1} or in the dark at 25°C. Seeds with an emerged radicle that follows endosperm rupture in tobacco (Weitbrecht et al., 2011) were counted 24, 30, 40, or 48 h after imbibition began, and the percentage of seeds with emerged radicle among 50 seeds was used as the rate of germination. To evaluate the effect of brief illumination of light, after 1 d of imbibition at 25°C in the dark, seeds were illuminated with fluorescent lighting of 150 μmol m^{-2} s^{-2} for 10 to 20 s. After an additional imbibition at 25°C for 2 d in the dark, the germination percentage was determined. To evaluate the effect of heat shock in seed germination, tobacco seeds were imbibed at 25°C for 2 d in the dark, and heat stress treatment occurred at 35°C, 38°C, or 42°C for 4 h in the incubator in the dark. For electron microscopy, the imbibed seeds were heat stress treated at 42°C for 30 min to locate the HSP during the transfer from the cytosol to the nucleus. The heat-stressed tobacco seeds were transferred to 25°C for 48 h in the dark, and the percentage of germination was determined as described above.

Isolation of RNA and RNA-Blot Hybridization

Total RNA was isolated from tobacco leaves and subjected to RNA-blot hybridization (Sambrook et al., 1989). The complementary DNA clones *NtHSP18.2*, *NtHSP18.3*, and *NtHSP17.6* were labeled with ^{32}P-dCTP and used as the probe. RNA-blotted Hybond-N membrane was prehybridized and hybridized in 50% (v/v) formamide, 5× sodium chloride/sodium phosphate/EDTA, 5× Denhardt's solution, 1% (w/v) SDS, and 50 μg mL^{-1} denatured salmon sperm DNA at 42°C. The membrane was washed several times in 0.2× sodium chloride/sodium phosphate/EDTA and 0.1% (w/w) SDS at 42°C and then exposed to an x-ray film (Sambrook et al., 1989).

Protein Gel Electrophoresis and Blot Analysis

Tobacco leaves or seeds were ground in liquid nitrogen and resuspended in an extraction buffer (50 mM for leaves or 15 mM for seeds; sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 8.0, 0.1% [v/v] *N*-lauroyl sarcosine, and 0.1% [v/v] Triton X-100). After 5 min of incubation at 4°C, the debris was removed by centrifugation for 10 min at 12,000 rpm in a microcentrifuge; 50 μg of soluble protein from each sample for leaves and 100 μg of soluble protein from each sample for seeds were separated on a 15% (w/w) SDS polyacrylamide gel for one-dimensional gel electrophoresis. For two-dimensional gel electrophoresis, a ReadyStrip IPG Strip and PROTEAN IEF Cell (Bio-Rad, 1997) were used. Briefly, immobilized nonlinear pH gradient strips were rehydrated for 12 h at 50 V at 20°C. Isoelectrofocusing was performed at 20°C for 30 min at 300 V and then at 5 kV for 12 h. Before running the two-dimensional electrophoresis, the IPG Strips were equilibrated with SDS-PAGE equilibration buffer (6 M urea, 0.375 M TrisCl, pH 8.8, 2% [w/w] SDS, and 20% [v/w] glycerol) with 2% (v/v) dithiothreitol for 10 min and again with SDS-PAGE equilibration buffer containing 2.5% (w/w) idoacetamide for 10 min. Equilibrated strips were placed on top of 15% (w/v) vertical polyacrylamide gels, and sealing solution (1% [w/v] agarose, 0.4% [w/w] SDS, and 60 mM TrisCl, pH 6.8) was placed onto the strips. After either one- or two-dimensional electrophoresis, proteins on the polyacrylamide gel were electroblotted onto a nitrocellulose membrane (Amersham Biosciences) in Gly electrode buffer (12.5 mM TrisCl, pH 8.3, and 96 mM Gly). After preincubation with 5% (v/v) nonfat milk in Tris-buffered saline (TBS; 10 mM TrisCl, pH 7.5, and 150 mM NaCl), the membrane was incubated with anti-NtHSP18.2 antiserum diluted 1:2,000 in TBS for 1 h at 25°C on an orbital shaker (Sambrook et al., 1989). After washing three times for 10 min each in TBS containing 0.1% (v/v) Tween 20, the membrane was incubated for 1 h in goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences) diluted 1:2,000 in TBS. The membrane was then washed three times for 15 min in TBS containing 0.1% (v/v) Tween 20 and developed with an ECL Kit according to the manufacturer's instructions (Amersham Biosciences). The antibody used for the blot was a polyclonal antibody against NtHSP18.2 (Joe et al., 2000) and showed similar affinity to all NtHSPs, yielding the same intensity signal for different NtHSPs in the protein-blot hybridizations.

Immunohistochemical Analyses

After imbibition and heat treatments, seeds were fixed in a fixing solution (50% [v/v] ethanol and 5% [v/v] acetic acid) containing 10% (v/v) formaldehyde. Dehydration was done in a graded series of ethanol (25%, 50%, 75%, and 100% [v/v] for 1 h each), and the seeds were embedded in paraffin. Paraffin-embedded tissue was sectioned at 7-μm thickness; the sections were then affixed to slides with polylysin and dried on a hot plate at 45°C. Sections were deparaffined with xylene, rehydrated in a graded series of ethanol (100%, 75%, 50%, and 25% [v/v] for 1 h each), and, finally, soaked in water for 5 min (Muhitch et al., 1995). For immunolocalization of NtHSPs, a biotinylated anti-rabbit bridge antibody against NtHSP18.2 (Joe et al., 2000) and streptavidin-conjugated horseradish peroxidase (Dako LSAB) were used. The primary antiserum and preimmunized antiserum were diluted 1:10,000. The sections were examined under a light microscope (Nikon).

Confocal Microscopy

Seeds were imbibed for 1 d in 1.5-mL tubes with 50 μL of sterilized water in the dark, incubated in an water bath at 45°C, rapidly frozen in liquid nitrogen, and cryosectioned by microtome (model HM525; MICROM International GmbH) with optimum cutting temperature compound (Sakura) to 30-μm thickness. The sections were attached to HistoBond adhesive microscope slides (Marienfeld), incubated for 1 h in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS; 138 mM NaCl and 2.7 mM KCl, pH 7.4), and washed three times in PBS. After 1 h of incubation in blocking solution (0.3% [v/v] Triton X-100 and

1% [w/v] bovine serum albumin [BSA] in PBS), the sections were incubated with H_6NtHSP18.2 antiserum diluted 1:100 in PBS containing 1% (w/v) BSA for 1 h, washed four times for 10 min each in PBS containing 0.3% (v/v) Triton X-100, and incubated for 1 h in goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (Jackson Immuno Research) diluted 1:50 in PBS containing 1% (w/v) BSA. Confocal images were collected using a confocal laser-scanning microscopy system (model Radiance 2100; Bio-Rad, 1997).

Immunoelectron Microscopy

After imbibition and heat treatments, seeds were fixed in 0.1 M sodium phosphate buffer (pH 7.4) containing 3.5% (v/v) paraformaldehyde, 0.2% (v/v) glutaraldehyde, 1% (w/v) Suc, and 3 mM $CaCl_2$ for 2 h. Fixed seeds were rinsed three times with 0.1 M sodium phosphate buffer (pH 7.4) and dehydrated through a graded ethanol series (25%, 50%, and 70% [v/v] for 1 h each). The samples were infiltrated with LR-White resin dilute series (25%, 50%, and 70% [v/v] in ethanol for 2 h each), placed in 100% (v/v) resin in a conical BEEM capsule with cap (Pelco), and polymerized for 48 h at 55°C in a dry oven. The sample block was ultrathin sectioned to 80- to 90-nm thickness, and the samples were collected on uncoated nickel grids and completely dried at 37°C for 2 h in a dry oven. The grids were then transferred to drops of 1:200 diluted anti-NtHSP18.2 polyclonal antibodies in PBS containing 0.1% (v/v) Tween 20 and 1% (w/v) BSA and incubated in an enclosed humidity chamber to avoid drying of tissue at 4°C overnight. The grids were then washed five times in the same buffer and incubated with 1:50 diluted gold (10 nm in diameter)-conjugated polyclonal antibody (Amersham Biosciences) for 1 h at 4°C. The grids were washed on top of the drops of the same buffer to prevent nonspecific binding of the antibody. The specimens were then postfixed in 0.5% (v/v) glutaraldehyde in PBS buffer for 10 min. Sections were stained with 5% (w/v) uranyl acetate in 18 MΩ deionized water and then visualized with a transmission electron microscope (model JEM-2000EXII; JEOL) at an acceleration voltage of 80 kV.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. RNA-blot hybridization results of the transgenic tobacco plants that expressed *NtHSP17.6* or *NtHSP18.3* and the expression cassette used.

Supplemental Figure S2. Localization of NtHSPs in the leaves of tobacco after heat shock treatment.

Supplemental Figure S3. Protein-blot hybridization results of the transgenic tobacco plants that expressed *NtHSP18.2* in antisense or sense orientation.

Supplemental Figure S4. Comparison of germination in nontransgenic tobacco seeds and *NtHSP18.2* sense transgenic tobacco seeds.

Supplemental Figure S5. Confocal microscopy images for nontransgenic tobacco seeds and *NtHSP18.2* sense transgenic tobacco seeds with NtHSP18.2 polyclonal antibody.

Supplemental Figure S6. Electron microscopy of immunogold-labeled NtHSP18.2 in nontransgenic tobacco seeds.

Received October 30, 2014; accepted January 16, 2015; published January 20, 2015.

LITERATURE CITED

Angelovici R, Galili G, Fernie AR, Fait A (2010) Seed desiccation: a bridge between maturation and germination. Trends Plant Sci **15:** 211–218

Bae G, Choi G (2008) Decoding of light signals by plant phytochromes and their interacting proteins. Annu Rev Plant Biol **59:** 281–311

Basha E, O'Neill H, Vierling E (2012) Small heat shock proteins and α-crystallins: dynamic proteins with flexible functions. Trends Biochem Sci **37:** 106–117

Bio-Rad (1987) Bio-Rad Protein Assay. Bio-Rad Technical Bulletin, No. 1069. Bio-Rad Laboratories, Hercules, CA

Eyles SJ, Gierasch LM (2010) Nature's molecular sponges: small heat shock proteins grow into their chaperone roles. Proc Natl Acad Sci USA **107:** 2727–2728

Franck E, Madsen O, van Rheede T, Ricard G, Huynen MA, de Jong WW (2004) Evolutionary diversity of vertebrate small heat shock proteins. J Mol Evol **59:** 792–805

Fu X, Shi X, Yin L, Liu J, Joo K, Lee J, Chang Z (2013) Small heat shock protein IbpB acts as a robust chaperone in living cells by hierarchically activating its multi-type substrate-binding residues. J Biol Chem **288:** 11897–11906

Gallardo K, Job C, Groot SPC, Puype M, Demol H, Vandekerckhove J, Job D (2001) Proteomic analysis of Arabidopsis seed germination and priming. Plant Physiol **126:** 835–848

Goldfarb LG, Dalakas MC (2009) Tragedy in a heartbeat: malfunctioning desmin causes skeletal and cardiac muscle disease. J Clin Invest **119:** 1806–1813

Graeber K, Nakabayashi K, Miatton E, Leubner-Metzger G, Soppe WJJ (2012) Molecular mechanisms of seed dormancy. Plant Cell Environ **35:** 1769–1786

Graw J (2009) Genetics of crystallins: cataract and beyond. Exp Eye Res **88:** 173–189

Holdsworth MJ, Bentsink L, Soppe WJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytol **179:** 33–54

Jiao Y, Lau OS, Deng XW (2007) Light-regulated transcriptional networks in higher plants. Nat Rev Genet **8:** 217–230

Joe MK, Park SM, Lee YS, Hwang DS, Hong CB (2000) High temperature stress resistance of *Escherichia coli* induced by a tobacco class I low molecular weight heat-shock protein. Mol Cells **10:** 519–524

Kappé G, Leunissen JA, de Jong WW (2002) Evolution and diversity of prokaryotic small heat shock proteins. Prog Mol Subcell Biol **28:** 1–17

Miernyk JA, Hajduch M (2011) Seed proteomics. J Proteomics **74:** 389–400

Muhitch MJ, Felker FC, Taliercio EW, Chourey PS (1995) Immunolocalization of a unique form of maize kernel glutamine synthetase using a monoclonal antibody. Plant Physiol **107:** 757–763

Park SM, Kim KP, Joe MK, Lee MO, Koo HJ, Hong CB (2014) Tobacco class I cytosolic small heat shock proteins are under transcriptional and translational regulations in expression and heterocomplex prevails under the high-temperature stress condition in vitro. Plant Cell Environ 10.1111/pce.12436

Personat JM, Tejedor-Cano J, Prieto-Dapena P, Almoguera C, Jordano J (2014) Co-overexpression of two *Heat Shock Factors* results in enhanced seed longevity and in synergistic effects on seedling tolerance to severe dehydration and oxidative stress. BMC Plant Biol **14:** 56–67

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D (2012) Seed germination and vigor. Annu Rev Plant Biol **63:** 507–533

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Seo M, Nambara E, Choi G, Yamaguchi S (2009) Interaction of light and hormone signals in germinating seeds. Plant Mol Biol **69:** 463–472

Suh MC, Hong CB, Kim SS, Sim WS (1994) Transgenic tobacco plants with *Bacillus thuringiensis* δ-endotoxin gene resistant to Korean born budworms. Mol Cells **4:** 211–219

Tyedmers J, Mogk A, Bukau B (2010) Cellular strategies for controlling protein aggregation. Nat Rev Mol Cell Biol **11:** 777–788

Van Montfort R, Slingsby C, Vierling E (2001) Structure and function of the small heat shock protein/α-crystallin family of molecular chaperones. Adv Protein Chem **59:** 105–156

Wang WQ, Ye JQ, Rogowska-Wrzesinska A, Wojdyla KI, Jensen ON, Møller IM, Song SQ (2014) Proteomic comparison between maturation drying and prematurely imposed drying of *Zea mays* seeds reveals a potential role of maturation drying in preparing proteins for seed germination, seedling vigor, and pathogen resistance. J Proteome Res **13:** 606–626

Waters ER (2013) The evolution, function, structure, and expression of the plant sHSPs. J Exp Bot **64:** 391–403

Waters ER, Aevermann BD, Sanders-Reed Z (2008) Comparative analysis of the small heat shock proteins in three angiosperm genomes identifies new subfamilies and reveals diverse evolutionary patterns. Cell Stress Chaperones **13:** 127–142

Wehmeyer N, Hernandez LD, Finkelstein RR, Vierling E (1996) Synthesis of small heat-shock proteins is part of the developmental program of late seed maturation. Plant Physiol **112:** 747–757

Weitbrecht K, Müller K, Leubner-Metzger G (2011) First off the mark: early seed germination. J Exp Bot **62:** 3289–3309

Willis MS, Schisler JC, Portbury AL, Patterson C (2009) Build it up-Tear it down: protein quality control in the cardiac sarcomere. Cardiovasc Res **81:** 439–448

Zhou Y, Chen H, Chu P, Li Y, Tan B, Ding Y, Tsang EWT, Jiang L, Wu K, Huang S (2012) NnHSP17.5, a cytosolic class II small heat shock protein gene from Nelumbo nucifera, contributes to seed germination vigor and seedling thermotolerance in transgenic Arabidopsis. Plant Cell Rep **31:** 379–389