Mitochondrial small heat shock protein mediates seed germination via thermal sensing

Wei Ma,1 Xueying Guan,1,2 Jie Li,1,3 Ronghui Pan,4 Luyao Wang,1,2 Fengjun Liu,5 Hongyu Ma,6 Shujin Zhu,7 Jin Hu,7 Yong-Ling Ruan,8 Xiaoya Chen,6 and Tianzhen Zhang1,2

Zhejiang Provincial Key Laboratory of Crop Genetic Resources, Institute of Crop Science, Zhejiang University, 310029 Zhejiang, China; State Key Laboratory of Crop Genetics and Germplasm Enhancement, Nanjing Agricultural University, 210095 Nanjing, China; Department of Plant Protection, Nanjing Agricultural University, 210095 Nanjing, China; School of Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW 2308, Australia; and Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 200032 Shanghai, China

Edited by Maarten Koornneef, Max Planck Institute for Plant Breeding Research, Cologne, Germany, and approved January 18, 2019 (received for review September 19, 2018)

Seed germination is an energy demanding process that requires functional mitochondria upon imbibition. However, how mitochondria fine-tune seed germination, especially in response to the dynamics of environmental temperature, remains largely unknown at the molecular level. Here, we report a mitochondrial matrix-localized heat shock protein GhHSP24.7, that regulates seed germination in a temperature-dependent manner. Suppression of GhHSP24.7 renders the seed insensitive to temperature changes and delays germination. We show that GhHSP24.7 competes with GhCCMH to bind to the maturation subunit protein GhCcmF to form cytochrome C/C (Cyt/C) in the mitochondrial electron transport chain. GhHSP24.7 modulates Cyt/C production to induce reactive oxygen species (ROS) generation, which consequently accelerates endosperm rupture and promotes seed germination. Overexpression of GhHSP24.7’s homologous genes can accelerate seed germination in Arabidopsis and tomato, indicating its conserved function across plant species. Therefore, HSP24.7 is a critical factor that positively controls seed germination via temperature-dependent ROS generation.

Results

GhHSP24.7 Regulates Seed Germination in a Temperature-Dependent Pathway. The mature cotton seed we used contained a fully developed embryo with differentiated meristems, radicle, and cotyledons. The seed-covering layers included testa and endosperm. The embryo was enclosed by a thin layer of endosperm, typically consisting of two layers of cells on the edge. The micropyram endosperm, which formed a cap-like structure covering the radicle tip, had approximately 10 cell layers (Fig. L4 and SI Appendix, Fig. S1A). Testa rupture (TR) and endosperm rupture (ER)

Significance

The propagation of most flowering plant species is determined by the success of seed germination, which is of both economic and ecologic importance. Mitochondria are the energy resource and crucial organelles for plant seed germination. Studying the underlying mechanism is important for us to understand the basic principles of plant development and improve crop yields. Here we identify HSP24.7 as a central activator for temperature-dependent seed germination. HSP24.7 modulates cytochrome C/C production in the mitochondrial electron transport chain and induces the generation of reactive oxygen species, which accelerates seed germination. Our work provides a comprehensive framework of how mitochondria regulate seed germination in response to the dynamics of environmental temperature.
are temporally independent events during seed germination (SI Appendix, Fig. S1B). Cotton seed germination is sensitive to temperature changes. Either low or high temperature delays seed germination. The germination assay at temperatures from 4 °C to 44 °C showed that the conditions below 12 °C and above 44 °C were suppressive to germination (Fig. 1B). The germination efficiency was positively related to temperature in the warm condition at a narrow window between 20 °C and 36 °C, indicating a conserved endogenous thermal system modulating seed germination. We, therefore, attempted to identify the gene(s) response to it. A group of mitochondria I subfamily small heat shock proteins (msHSPs) were proactively expressed during seed germination (10, 11) (SI Appendix, Fig. S1C). Among them, GhHSP24.7 was expressed predominantly in the endosperm after seed imbition (Fig. 1B and SI Appendix, Fig. S1D). The GhHSP24.7 showed high expression correlation ($R = 0.99$) with temperature changes from 4 °C to 36 °C in the seed germination assay (Fig. 1B). These observations suggest that GhHSP24.7 may play an important role in seed germination in response to temperature increase.

To investigate the roles of GhHSP24.7, we developed GhHSP24.7 overexpression (GhHSP24.7-OE) and suppression (GhHSP24.7-SE using the antisense strategy) transgenic cotton lines driven by a 35S promoter. Two independent lines of each were selected for detailed analysis (Fig. 1C and SI Appendix, Fig. S1 E and F). A transgenic line with an empty vector (pBI121) and untransformed cotton (accession W0) were used as controls. GhHSP24.7-SE seeds with reduced expression of GhHSP24.7 evidently delayed germination compared with the control groups at 28 °C (Fig. 1C). In contrast, GhHSP24.7-OE seeds germinated more quickly, revealing that GhHSP24.7 plays a positive role in seed germination.

To test if the GhHSP24.7-modulated impacts on seed germination are temperature dependent, a germination assay was performed at 20 °C, 28 °C, and 36 °C (Fig. 1D and SI Appendix, Fig. S2). It took about 86 h to reach 50% seed germination (ER) at 20 °C for the control groups, but only about 40 h at 36 °C. For the GhHSP24.7-SE seeds, the delayed germination was not influenced by the temperature changes from 20 °C to 36 °C, suggesting that temperature-dependent germination was disturbed due to the suppression of GhHSP24.7 (Fig. 1E). By contrast, the GhHSP24.7-OE seeds showed a fast-germination phenotype, which resembled the fast germination of the control groups at high temperature (Fig. 1D). The above data indicated the GhHSP24.7 regulates seed germination in a temperature-dependent manner. Here, the behavior of GhHSP24.7-OE seeds in germination mimicked that of the control seeds under warm conditions, while the behavior of GhHSP24.7-SE seeds represented that of the control seeds under cold conditions.

Abscisic acid (ABA) and gibberellins (GAs) are known to be major regulators of seed dormancy and germination. To examine whether the function of GhHSP24.7 is dependent on the hormones, we applied ABA, fluridone (ABA biosynthesis inhibitor), and GAs to the germinating seeds, respectively (SI Appendix, Fig. S34). The fast-germination phenotype of GhHSP24.7-OE seeds was not suppressed by ABA. Neither was the delayed germination of the GhHSP24.7-SE seeds recovered by fluridone. All of the transgenic and control seeds exhibited similar responses to GAs. In addition, there was no significant difference in the endogenous ABA and GA contents between these transgenic and control seeds (SI Appendix, Fig. S3B). These data suggest that GhHSP24.7 regulates seed germination via a pathway which might be independent of the ABA and GA signaling.

**GhHSP24.7 Induces Endosperm Weakening.** Seed dormancy and germination are balanced between the resistance of the seed-covering layers (testa and endosperm) and the embryo growth potential (12). The latter determines embryo growth by water uptake and can be quantified using solutions with different concentrations of polyethylene glycol (PEG) (5). GhHSP24.7 did not affect the embryo growth potential at any tested ambient water potential (SI Appendix, Fig. S3C). The resistance of the seed-covering layers was further tested by measuring the puncture force of endosperm (PFE). A high PFE represents strong resistance of the seed-covering layers, which is a suppressive force for seed germination, or vice versa (13). We found that the PFE decreased along with the progress of ER. The GhHSP24.7-SE seeds, which exhibited delayed germination, showed consistently high PFE (Fig. 1F). The PFE of the controls dropped quickly from 20 °C to 36 °C (SI Appendix, Fig. S2E), which was in agreement with the fast germinating dynamics under relatively warm conditions. However, the GhHSP24.7-SE seeds still exhibited a high PFE even at 36 °C (SI Appendix, Fig. S2E). Taken together, these results suggest that GhHSP24.7 plays a unique role in the decay of the endosperm during seed germination.
GhCcmF, is the Client of GhHSP24.7. HSPs are known chaperones to protect their client proteins against stress conditions. To determine the client of GhHSP24.7, a yeast two-hybrid (Y2H) screen was performed using a cotton leaf cDNA library. A CytC maturation protein Fc (CcmFc) was identified as an interactor of GhHSP24.7 (Fig. 2A). CytC is one of the essential components of the mETC (14), and transfers electrons from complex III to IV (15). The maturation of CytC is activated and stabilized by binding with heme, which is synthesized from different modules in plant, namely CytC maturation A-I (CcmABCDEFGH) (16). CcmF delivers heme to apocytochrome C with the assistance of CCMH. GhHSP24.7::GFP fusion signals were detected in punctate spots that overlapped with the mitochondria marker AtPGN::RFP (17) (Fig. 2B). Similarly, GhCcmFc was also localized on mitochondria (Fig. 2B). The interaction between GhHSP24.7 and GhCcmFc, was confirmed by bimolecular fluorescence complementation (BiFC) (Fig. 2C) and in vitro protein coimmunoprecipitation (Co-IP) assays (Fig. 2D).

The mitochondrial gene coded CcmFc protein is conserved in plant (SI Appendix, Fig. S4 A and C). CmFc, CCMH, and CcmF form a complex to synthesize holocytochrome, which transfers electrons from complex III to complex IV on the mETC (14). The direct interaction between CcmF and CCMH widely exists in prokaryotic cells such as Rhodobacter capsulatus (18). We employed BiFC, Co-IP, and Y2H (Fig. 2 C, E, and F) assays to confirm their interaction in vitro. On the other hand, the expression of both GhCCMH and GhCcmFc (SI Appendix, Fig. S4 E and F) was synchronized with the expression of GhHSP24.07 (SI Appendix, Fig. S4G). We did not detect any direct interaction between GhHSP24.7 and GhCCMH (Fig. 2F and SI Appendix, Fig. S5D). A competitive protein pull-down assay showed that the binding efficiency of GhCCMH and GhCcmFc was reduced with increasing amounts of GhHSP24.7, indicating that GhHSP24.7 and GhCCMH antagonistically interact with GhCcmFc (Fig. 2G). These data demonstrate that GhCcmFc is the client of GhHSP24.7 in mitochondria. The GhHSP24.7 competes with GhCCMH to form the GhCcmFc-GhHSP24.7 complex, preventing the GhCcmFc-GhCCMH interaction.

To study the membrane association and topology of GhHSP24.7, GhCcmFc, and GhCCMH in the mitochondria, the soluble and nonsoluble fractions of mitochondrial extraction were isolated (19) (SI Appendix, Fig. S6A). As shown in SI Appendix, Fig. S6B, GhHSP24.7::GFP was detected only in the soluble fractions after each treatment, indicating that GhHSP24.7 is not a mitochondrial membrane protein. In contrast, GhCcmFc::GFP and GhCCMH::GFP were detected only in the insoluble fractions after each treatment (SI Appendix, Fig. S6B), indicating that GhCcmFc and GhCCMH are integral proteins of the mitochondrial membrane. We further dissected the localization and topology of GhHSP24.7::GFP, GhCcmFc::GFP, and GhCCMH::GFP by a set of protease protection experiments with thermolysin, which degrades proteins on the surface of the organelles, and trypsin, which can access the intermembrane space (19). The matrix protein GhHSP24.7::GFP was protected from both enzymes (SI Appendix, Fig. S6 C and D). However, GhCcmFc::GFP and GhCCMH::GFP were resistant to thermolysin but disrupted by trypsin (SI Appendix, Fig. S6 C and D), indicative of a topology in which GhCcmFc and GhCCMH are anchored to the mitochondrial inner membrane with their C terminals facing the intermembrane space (SI Appendix, Fig. S6D), as occurs with CCMH in Arabidopsis (20). To identify the domains in GhCcmFc and GhCCMH responsible for interaction, a series of truncated constructs were generated for Y2H assays. GhCcmFc and GhCCMH were divided into five and two sections according to the transmembrane domain, respectively (SI Appendix, Fig. S6E). GhHSP24.7 does not contain transmembrane domains, consistent with its localization in the mitochondrial matrix. As shown in SI Appendix, Fig. S6F, only subsection GhCcmFc-4 was able to interact with GhHSP24.7 and GhCCMH but not with GhCCMH-1, suggesting that the GhCcmFc-4 is necessary and sufficient for the interaction of GhHSP24.7 and GhCCMH. Together, these results indicate GhHSP24.7 binds with the C terminal of GhCcmFc in competition with GhCCMH in the matrix site of the mitochondrial inner membrane.

The Elevated GhHSP24.7 Reduces the Production of CytC/C1. Both CcmFc and CCMH are required for holocytochrome assembly of CytC/C1 in mitochondria (16) (Fig. 3A). So, the destabilization of the CcmFc and CCMH complex might impair CytC/C1 maturation and substantially influence the mETC components. Given GhHSP24.7 is a competitor of GhCCMH for GhCcmFc binding (Fig. 2G), we wondered whether the elevated expression of GhHSP24.7 during seed germination could affect CytC/C1 in mETC. Western blotting analysis revealed that the CytC/C1 levels were significantly lower in the GhHSP24.7 OE lines than in the controls constantly after imbibition (Fig. 3B). Interestingly, the AOX level was similar in both the transgenic lines and the controls at 3 h postimbibition, but was higher in the GhHSP24.7 OE lines than in the controls at 9, 15, and 21 h postimbibition (Fig. 3B). However, the levels of Nad9 (complex I), Cox2 (complex IV), and α-ATPase (complex V) were similar in both the transgenic lines and the controls (Fig. 3B).

One of the initial changes during the early stages of seed germination is the resumption of respiratory activity. The dynamics of the respiration, represented by the oxygen uptake at 28 °C, displayed an S-shaped curve for increase in respiration during cotton seed germination (SI Appendix, Fig. S8). GhHSP24.7 OE exhibited a similar total respiration level to the control groups in terms of...
oxygen uptake (Fig. 3C). However, the total respiration level was lower in the GhHSP24.7-OE lines than the controls (Fig. 3C). In plants, the respiration is usually regulated by two pathways in mitochondria, the cytochrome oxidase pathway (COX) and the alternative oxidase pathway (AOX) (21) (Fig. 3). Western blots show major components of the mETC during the seed germination over a time course at 3, 9, 15, and 21 h postimbibition at 28 °C. (C) Histograms show the oxygen uptake rate in the 3-, 9-, 15-, and 21-h imbibed seeds at 28 °C. NaN3 is an inhibitor of COX. SHAM is an inhibitor of the AOX. Data are means ± SEM of n = 3 (*P < 0.05; **P < 0.01 compared with the control by Student’s t test).

**GhHSP24.7 Mediates ROS Release in Seed Germination.** Complex III in the ETC produces ROS upon the leakage of electrons caused by reduced production of mature CytC (22). ROS play a positive role in endosperm germination. After 12 h imbibition at 28 °C, the endosperm and micropylar endosperm maintained a fine cell structure (Fig. 5A). The two layers of cells in the endosperm began to separate, representing the decay of the cell structure of micropylar endosperm. However, in the GhHSP24.7-OE seeds the endosperm layer was distinctively ruptured with deformed cells in micropylar endosperm (Fig. 5A). The PFE was examined following treatment with H2O2 and NAC. H2O2 decreased the PFE of the controls to the level of the GhHSP24.7-OE lines (Fig. 5B), while NAC rescued the PFE of the GhHSP24.7-OE seeds to the level of GhHSP24.7-SE seeds. Therefore, the endogenous ROS play a positive role in endosperm weakening during seed germination.

**Fig. 4.** GhHSP24.7 regulated seed germination by fine tuning ROS release. (A) DCFH-DA staining on vertical dissections of radicles of the 24-h imbibed seeds at 28 °C. Abbreviations are the same as in Fig. 2. (B) Quantified fluorescence intensity of A. (C and D) Upper shows H2O2 concentration in the seeds and mitochondria of transgenic lines over time after imbibition at 28 °C. Lower shows H2O2 concentration in the 3-h imbibed seeds and mitochondria at the indicated temperature. (E) Photographs show the germination status of seeds treated with H2O2, MV, and NAC 48 h after imbibition at 28 °C. (F) ER percentage of seeds treated with H2O2, MV, or NAC 48 h after imbibition at 28 °C. For all panels, data are means ± SEM of n = 3 (*P < 0.05; **P < 0.01 compared with the control by Student’s t test).
Mitochondrial biogenesis is also involved in seed germination (27). Therefore, the peptidase activity of Cytbc1 may also contribute to seed germination. The importance of Cytbc1 in seed germination and how each of its functions contributes to seed germination are very interesting topics in future studies. GhHSP24.7 is identified as an inhibitor for the formation of the GhCcmFc and GhCCMH complex instead of protecting this client protein. The GhHSP24.7 level is within a gentle and comfortable dynamic during the seed germination. This finding expands our understanding of HSPs’ functions in general and sheds a light on the mechanism by which sHSPs regulate seed germination under favorable conditions.

**Discussion**

**Fine Tuning of mETC Activity During Seed Germination.** Here, we discovered that a mitochondrion-localized plant sHSP, GhHSP24.7, which acts in cooperation with its in vivo client GhCcmFc, to modulate proper ROS production from the mETC for seed germination (Fig. 7). The CytC/C1 maturation subunit protein GhCcmFc is a client of GhHSP24.7. It is known that cytochrome bc1 (Cytbc1) complex is bifunctional in plants, as it also contains the mitochondrial processing peptidase required to remove the targeting signals from proteins imported into mitochondria (26). Hence, Cytbc1 has an important role in mitochondrial biogenesis.
germination, especially in response to the temperature changes is not clear. Based on the data obtained from this study, we conclude that GhHSP24.7 abundance is sensitive to the dynamic of temperature during seed germination. In response to warm conditions, GhHSP24.7 level is elevated to inhibit the COX pathway and block the electron transport from complex III in mitochondria, leading to the release of additional ROS, which promotes seed germination (Fig. 7). When the COX pathway is reduced, the AOX pathway is activated, which has a decreasing effect on ROS levels to some extent, but the effect is not enough to suppress ROS levels in the GhHSP24.7-OE lines to match levels in the controls. Hence, the higher ROS levels in the GhHSP24.7-OE lines accelerate endosperm rupture to promote seed germination. Moreover, the elevated ROS level may also affect other mechanisms sensitive to ROS in the mitochondria, such as iron-sulfur assembly (31), which likely relate to seed germination. The function of GhHSP24.7 in seed germination control is widely conserved in the plant kingdom. GhHSP24.7 therefore can fine tune seed germination by manipulating ROS release. Our study led to the discovery of a mechanism to regulate ROS production in mitochondria during seed germination.

Materials and Methods

Details about plant materials and growth conditions are described in SI Appendix, Materials and Methods. Seed germination, puncture force measurement, gene expression, plant transformation, yeast two-hybrid system, BiFC assay, pull-down assay, ROS detection, oxygen consumption measurement, and mitochondrial assays are also described in SI Appendix, Materials and Methods.

ACKNOWLEDGMENTS. We thank Prof. Jiawei Wang (Shanghai Institute of Plant Physiology and Ecology) and Prof. Yuxian Zhu (Wuhan University) for their critical comments; and Prof. Baocai Tan (Shandong University) for providing the antibodies of mitochondrial complexes. This work was financially supported in part by grants from the National Natural Science Foundation of China (U1503284), the National Key Research and Development Program (2016YFD0100605), the Distinguished Discipline Support Program of Zhejiang University, and the Australian Research Council (DP180103834).

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