Orange protein has a role in phytoene synthase stabilization in sweetpotato

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Carotenoids have essential roles in light-harvesting processes and protecting the photosynthetic machinery from photo-oxidative damage. Phytoene synthase (PSY) and Orange (Or) are key plant proteins for carotenoid biosynthesis and accumulation. We previously isolated the sweetpotato (*Ipomoea batatas*) Or gene (*IbOr*), which is involved in carotenoid accumulation and salt stress tolerance. The molecular mechanism underlying *IbOr* regulation of carotenoid accumulation was unknown. Here, we show that *IbOr* has an essential role in regulating *IbPSY* stability via its holdase chaperone activity both *in vitro* and *in vivo*. This protection results in carotenoid accumulation and abiotic stress tolerance. *IbOr* transcript levels increase in sweetpotato stem, root, and calli after exposure to heat stress. *IbOr* is localized in the nucleus and chloroplasts, but interacts with *IbPSY* only in chloroplasts. After exposure to heat stress, *IbOr* predominantly localizes in chloroplasts. *IbOr* overexpression in transgenic sweetpotato and *Arabidopsis* conferred enhanced tolerance to heat and oxidative stress. These results indicate that *IbOr* holdase chaperone activity protects *IbPSY* stability, which leads to carotenoid accumulation, and confers enhanced heat and oxidative stress tolerance in plants. This study provides evidence that *IbOr* functions as a molecular chaperone, and suggests a novel mechanism regulating carotenoid accumulation and stress tolerance in plants.
chloroplast movement, protein import and translocation, protection of photosynthetic machinery from abiotic stress, and biotic stress tolerance.

Phytoene synthase (PSY) is the most important regulatory enzyme in the carotenoid biosynthetic pathway. *Arabidopsis* contains only one PSY gene, but rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Solanum lycopersicum*), and cassava (*Manihot esculenta*) contain two or more homologs. Multiple PSY genes have tissue-specific expression and unique responses to environmental cues. High light, temperature, drought, salt, ABA, photoperiod, developmental cues, and metabolite feedback affect PSY expression. Li et al. reported that PSY protein level was maximally increased in transgenic potato expressing cauliflower Or, and Zhou et al. reported that Or was a post-transcriptional regulator of PSY. In addition, activation and translocation of PSY are regulated by post-translational effects, and Or-mediated increase in PSY protein level increases PSY activity. Therefore, PSY is controlled by post-transcriptional and post-translational modification, and it regulates the first committed step in carotenoid biosynthesis.

Our previous studies showed that expression of the sweetpotato Or (*IbOr*) transgene in sweetpotato calli resulted in increased carotenoid levels. We observed that transgenic sweetpotato calli overexpressing IbOr had higher carotenoid levels, increased antioxidant activity, and enhanced salt stress tolerance. However, it was unclear how IbOr regulated carotenoid accumulation, although plant DnaJ proteins are reported as heat-shock proteins involved in abiotic stress tolerance and holdase chaperone function. Here, we report the holdase chaperone function of IbOr, which regulates IbPSY stability, enhances carotenoid accumulation, and confers heat stress tolerance in sweetpotato.

**Results**

**IbOr transcripts are induced by heat stress treatment.** We reported previously that the deduced IbOr protein contains a plastid-targeting transit sequence, two transmembrane domains, and a DnaJ-like cysteine-rich zinc finger domain that includes four repeats of the CxxCxGxG motif in the C-terminal region. In plants, DnaJ/Hsp40 proteins are co-chaperones that function as partners of the highly conserved Hsp70, and are required for defense against abiotic stresses such as salinity, drought, and extreme temperatures. Previous work showed that *IbOr* transcript expression was significantly induced in response to NaCl, PEG, and H$_2$O$_2$. To identify a possible functional role for *IbOr* under heat stress conditions, *IbOr* expression was analyzed by quantitative RT-PCR (qRT-PCR) in heat-treated sweetpotato tissues (Supplementary Fig. S1). *IbOr* expression in stem and fibrous root was high at 3 h after heat treatment, and its expression in calli was high at 6 h after treatment. By contrast, *IbOr* expression in leaf decreased after heat treatment. To test the effect of heat treatment on IbOr protein, we purified bacterially-expressed recombinant GST::IbOr protein and evaluated its stability under heat stress conditions. GST::IbOr was stable even at 70 °C, whereas GST was aggregated at 60 °C (Supplementary Fig. S2). These results suggest that IbOr may play an important role in the response to heat stress in sweetpotato.

**IbOr functions as a holdase chaperone.** Recent work reported that one of the chloroplast development-related proteins, CDF1 containing a DnaJ-like domain and three transmembrane domains, functions as holdase chaperone. IbOr also contains a DnaJ-like domain and transmembrane domains, and has high thermostability. Therefore, we hypothesized that IbOr may function as a molecular chaperone. To test this hypothesis, we examined IbOr for holdase chaperone activity using malate dehydrogenase (MDH) as a substrate in vitro. MDH was incubated at 45 °C for 20 min with increasing amounts of recombinant full-length IbOr protein fused to GST. IbOr prevented thermal aggregation of MDH, and MDH aggregation was completely blocked at a subunit molar ratio of 1 MDH/1 IbOr (Fig. 1b). IbOr conferred greater thermotolerance activity than the positive control AtTrx-h31 (Fig. 1b).

Next, we determined the IbOr region with holdase chaperone activity. We firstly generated two IbOr truncated fragments (IbOr-N and IbOr-C) (Fig. 1a). The N-terminal region of IbOr (IbOr-N) contains the transit sequence and transmembrane domains, whereas the C-terminal region of IbOr (IbOr-C) contains the DnaJ-like cysteine-rich zinc finger domain. Purified IbOr-N protein suppressed thermal aggregation of MDH, whereas IbOr-C protein had no effect. Therefore, we produced two IbOr-N truncated fragments (IbOr-N1 and IbOr-N2) (Fig. 1a). IbOr-N1 retained the transit sequence but deleted the transmembrane domains, whereas IbOr-N2 deleted the transit sequence but retained the transmembrane domains. IbOr-N2 displayed higher holdase chaperone activity than native IbOr, whereas IbOr-N1 did not show any holdase chaperone activity (Fig. 1d). Holdase chaperone activity is reported to be directly proportional with the degree of protein hydrophobicity. Hydrophobicity analysis predicted that the highest hydrophobicity region in IbOr was IbOr-N2 (Supplementary Fig. S3). These results indicate that the N-terminal transmembrane domains are required for IbOr holdase chaperone activity.

**Recombinant IbOr forms a high molecular weight (HMW) protein complex.** HMW complex formation is a conserved feature of holdase chaperone. IbOr is a heat-stable protein (Supplementary Fig. S2) that exhibits holdase chaperone function (Fig. 1b); therefore, we examined the oligomeric status of full-length protein and truncated fragments of recombinant IbOr. Size exclusion chromatography (SEC) analysis showed that full-length IbOr, IbOr-N, and IbOr-N2 primarily consisted of HMW complexes, whereas no HMW complexes were detected for IbOr-C and IbOr-N1 (Fig. 2a). Oligomeric status was confirmed using a silver-stained 10% native PAGE gel (Fig. 2b). The molecular sizes of IbOr, IbOr-N, and IbOr-N2 were too great to penetrate the 10% native polyacrylamide gel matrix, but the sizes were estimated to range up to approximately 1,000 kDa. By contrast, IbOr-C and IbOr-N1 appeared to migrate as trimers. Immunoblotting analysis using GST antibody showed that all IbOr protein fragments produced a single band with the correct theoretical molecular mass (Supplementary Fig. S4). These results suggest that IbOr forms HMW complexes under normal conditions, which are homopolymers consisting of variable numbers of monomers.
We analyzed the effect of heat-shock treatment on IbOr oligomerization status. IbOr protein structure was affected in vitro by incubating the protein above 45 °C (Supplementary Fig. S5). As the temperature increased, the concentration of HMW complexes increased concomitantly with a decrease in the levels of oligomeric proteins.

We examined IbOr hydrophobicity using the fluorescent probe 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS), which binds hydrophobic regions. The fluorescence intensity of protein-bound bis-ANS increased in an IbOr concentration-dependent manner (Fig. 2c). These results suggest that IbOr holdase chaperone activity is determined by its oligomerization status.

**IbOr interacts directly with IbPSY.** Zhou *et al.* recently reported that AtOr physically interacted with PSY and functioned as the major regulator of active PSY protein abundance. Therefore, we characterized sweetpotato PSY (IbPSY) and examined its interaction with IbOr. We first isolated IbPSY cDNA from the storage roots of orange-fleshed sweetpotato (cv. Sinhwangmi) (Accession no. JX393305). IbPSY had 76–96% sequence homology with several plant PSY genes (Supplementary Fig. S6a). To determine the subcellular localization of IbPSY, a green fluorescent protein (GFP) fusion construct of IbPSY was transiently expressed in *Nicotiana benthamiana* leaves using agroinfiltration. Epidermal cells of infiltrated leaves were examined by confocal laser scanning microscopy. The results showed that GFP fluorescence of IbPSY:GFP was detected in chloroplasts (Supplementary Fig. S6c). These results suggest that IbOr holdase chaperone activity is determined by its oligomerization status.

![Figure 1. Functional analysis of IbOr as a holdase chaperone.](image-url)

(a) Schematic representation of IbOr domains and truncated forms of recombinant IbOr. TP, transit peptide; TM, transmembrane domain; DnaJ-like domain, DnaJ-like cysteine-rich zinc finger domain. (b) Holdase chaperone activity of IbOr in the presence of malate dehydrogenase (MDH) as determined by light scattering. Molar ratios of MDH to IbOr were 1:1 (⚓), 1:0.5 (●), or 1:0.25 (▲). (c) Holdase chaperone activity of the N-terminal region (IbOr-N) and the C-terminal region (IbOr-C). Molar ratios of MDH to IbOr-N (●) or IbOr-C (▲) were 1:1. (d) Holdase chaperone activity of IbOr-N1 and IbOr-N2. Molar ratios of MDH to IbOr-N1 (●) or IbOr-N2 (▲) were 1:1. Thermal aggregation of 1 μM MDH was examined at 45 °C for 20 min in the presence of full-length or truncated IbOr. Reactions performed with 30 μM ovalbumin (○) or 1 μM AtTRX-h3 (◇) instead of IbOr were used as negative and positive controls, respectively.

To determine whether IbOr and IbPSY interact, we first performed bimolecular fluorescence complementation (BiFC) assays in *N. benthamiana* leaves. The N-terminal half of Venus (improved YFP variant) was fused to IbPSY (IbPSY:NV) and the C-terminal half of Venus was fused to IbOr (IbOr:CV), and they were co-expressed in *N. benthamiana* leaf epidermal cells. The results showed strong Venus fluorescence in chloroplasts (Fig. 3a). Next,
we performed luciferase (LUC) complementation imaging (LCI) assays in *N. benthamiana* leaves. LUC activity was detected by combining IbOr:NLUC with CLUC:IbPSY (Fig. 3b), which indicates that IbOr interacts with IbPSY in planta. This interaction was confirmed by *in vitro* pull-down assays (Fig. 3c).

Yeast two-hybrid analysis was performed to define the IbOr domain that interacts with IbPSY. A schematic diagram of the IbOr deletion constructs used in these assays is shown in Fig. 3d. The IbOr DnaJ-like domain was not required for IbPSY interaction, whereas the N-terminal region (1–232 amino acids) interacted with IbPSY (Fig. 3d). Therefore, the interaction between IbOr and IbPSY required the IbOr-N region.

IbOr chaperone activity stabilizes IbPSY. We examined whether IbOr chaperone activity protects IbPSY from heat or oxidative stress-induced denaturation and aggregation. We treated purified recombinant GST:IbPSY protein with heat (45 °C) or oxidative (50 μM H₂O₂) stress in the presence or absence of purified recombinant GST:IbOr protein, and then examined the proteins on SDS-PAGE. IbPSY was aggregated under heat and oxidative stress conditions in the absence of IbOr, whereas the presence of IbOr protected IbPSY from aggregation (Fig. 4a). Next, we evaluated IbOr holdase chaperone activity using IbPSY as a substrate. IbOr prevented thermal aggregation of IbPSY in a concentration-dependent manner, and a molar ratio of 1:3 (substrate:chaperone) completely suppressed IbPSY aggregation (Fig. 4b). We tested whether IbOr prevented IbPSY aggregation induced by oxidative stress in *in vitro*. At 25 °C, IbPSY treatment with 100 μM H₂O₂ for 20 min induced aggregation; however, the presence of IbOr suppressed IbPSY aggregation in a concentration-dependent manner (Fig. 4c). To confirm IbOr holdase chaperone activity for IbPSY *in planta*, IbOr:Flag and IbPSY:GFP fusion constructs were transiently
co-expressed in *N. benthamiana* leaves by agroinfiltration. Then, these plants were subjected to heat stress at 37 °C for 1 h. The infiltrated leaves were detached and total protein extracts were prepared, which were analyzed by SDS-PAGE and immunoblotting. Under normal condition (25 °C), IbPSY levels were essentially equivalent in leaves with or without IbOr co-expression, whereas IbPSY levels were severely reduced in leaves in the absence of IbOr under heat stress conditions at 37 °C (Fig. 4d). Taken together, these results indicate that IbOr has holdase chaperone activity for IbPSY, and IbOr stabilizes IbPSY during heat stress conditions *in planta*.

IbOr overexpression enhances abiotic stress tolerance in transgenic plants. Holdase chaperone activity confers heat stress tolerance in plants^{31,32}. To test the physiological role of IbOr in heat-shock tolerance *in vivo*, we subjected the transformed sweetpotato plants with empty vector (*Ib*-EV) or *IbOr* overexpression construct (*Ib*-OX)^{33} to heat stress conditions. IbOr expression levels in transgenic sweetpotato were determined by anti-FLAG immunoblotting analysis (Supplementary Fig. S7). The *Ib*-EV and *Ib*-OX phenotypes were not significantly different under normal growing conditions (25 °C, Fig. 5a) and displayed similar levels of heat stress conditions.
damage in response to 47 °C for 4 h. However, when the heat-stressed plants were allowed to recover at 25 °C for 3 d, the Ib-OX lines showed substantially superior recovery and survival than the Ib-EV lines (Fig. 5b,c).

To confirm IbOr-mediated improvement of heat stress tolerance in other plants, we generated transgenic Arabidopsis lines overexpressing FLAG-tagged IbOr. IbOr expression levels in transgenic Arabidopsis were determined by anti-FLAG immunoblotting analysis (Supplementary Fig. S7). The phenotypes of overexpression lines (At-OX) and empty vector control lines (At-EV) were not significantly different under normal growing conditions (22 °C, Fig. 5d), and were similarly damaged by heat stress at 38 °C for 3 h. When the heat-stressed plants were allowed to recover at 22 °C for 8 d, the At-OX lines displayed enhanced heat-shock tolerance and superior recovery of growth and normal chlorophyll content than the At-EV lines (Fig. 5e,f). When At-OX and At-EV seeds were subjected to heat stress conditions, the germination rate of At-OX seeds was significantly higher than that of At-EV seeds (Fig. 5g).

Oxidative stress was reported as a key factor that exacerbated the detrimental effects of heat stress in plants. Therefore, we investigated the physiological responses of transgenic At-EV and At-OX under oxidative stress conditions. At-OX lines displayed enhanced tolerance to methyl viologen (MV, an inducer of oxidative stress) treatment during germination and seedling growth (Fig. 6a). When detached rosette leaves of At-EV and At-OX were treated with H₂O₂ or MV, the At-OX lines exhibited fewer damage symptoms (Fig. 6b–d). Taken together, these results suggest that IbOr has a crucial role in plant protection from heat and oxidative stress.
Figure 5. IbOr overexpression in transgenic sweetpotato plants enhances heat stress tolerance.
(a) Phenotypes of IbOr-overexpressing (Ib-OX) and empty vector control (Ib-EV) sweetpotato transgenic plants. Seedlings were grown at 25 °C for 7 d after subculture. (b) Thermotolerance of Ib-EV and Ib-OX transgenic plants. A comparison of seedlings on the final day of recovery after heat shock is shown. Scheme of heat shock treatment and recovery of the seedlings is depicted (top panel). (c) Survival rates of Ib-EV and Ib-OX seedlings were determined after recovery. Results are means ± SD from four biological replicates.
(d) Phenotypes of IbOr-overexpressing (At-OX) Arabidopsis plants and empty vector control (At-EV) Arabidopsis plants. Seedlings grown at 22 °C for 12 d are shown. (e) Thermostolerance of At-EV and At-OX transgenic plants. A comparison of seedlings on the final day of recovery after heat shock is shown. Scheme of heat shock treatment and recovery of the seedlings is depicted (top panel). (f) Total chlorophyll contents of At-EV and At-OX seedlings under normal temperature (NT) and after recovery. Results are means ± SD from three biological replicates. (g) Germination assays of At-EV and At-OX transgenic seeds under heat stress conditions. At-OX and At-EV seeds were germinated on MS agar plates with or without heat stress at 47 °C for 4 h. The germination rates were determined 1–8 d after vernalization. Results are means ± SE from three biological replicates. Asterisks indicate a significant difference between EV and OX plants at *p < 0.05 by t-test in (c-f-g).

Discussion

IbOr is a key protein involved in carotenoid accumulation and environmental stress tolerance in sweetpotato7,33, but the molecular mechanism of IbOr function was previously unknown. Here, we report a novel molecular function for IbOr in stabilizing chloroplastic IbPSY via its holdase chaperone activity. Further, we demonstrate that IbOr enhances abiotic stress tolerance in transgenic plants. Our results provide new insights into the molecular mechanism of Orange protein function, which post-translationally regulates IbPSY and thereby affects carotenoid biosynthesis and accumulation. Our results are summarized in the model shown in Fig. 7.

In Arabidopsis, AtOr directly interacts with AtPSY in plastids24. PSY levels strongly increase in AtOr-overexpressing lines and dramatically decline in ator and ator-like double mutants, without any transcriptional change in PSY expression24. However, the mechanism of Or-mediated PSY regulation remained undetermined. PSY is reported to regulate carotenoid biosynthesis under abiotic stress conditions26,27, but IbPSY aggregates under heat and oxidative stresses (Fig. 4a). This result suggested that IbPSY may receive protection from partner protein(s) during abiotic stress conditions. We found that IbOr directly interacted with IbPSY in the chloroplast, similar to the interaction between AtOr and AtPSY. Our results also determined that IbPSY is protected by IbOr holdase chaperone activity under heat and oxidative stress conditions. PSY stability also was enhanced by Or transgene expression in cold-storage potato tuber34. The combined evidence indicates that IbOr has a role in post-translational regulation of IbPSY, and thereby controls carotenoid biosynthesis and accumulation and abiotic stress responses.

Plant Or proteins contain an N-terminal unknown region, transmembrane domains, and a C-terminal DnaJ-like domain. These domains are highly conserved among plant species7,10. CDF1 protein contains a DnaJ-like domain and three transmembrane domains40. Both CDF1 and IbOr have holdase chaperone activity. CDF1 required both the DnaJ-like domain and the transmembrane domains for holdase chaperone function, whereas IbOr only required the transmembrane domains that exhibited the strongest holdase chaperone activity. In Arabidopsis, AtPSY interacts with the AtOr N-terminal unknown region24. In sweetpotato, IbPSY interacted with the IbOr-N fragment (1–232 amino acids), which contains the N-terminal unknown region (30–153 amino acids) and the transmembrane domains (154–232 amino acids). Both the IbOr N-terminal unknown region and the IbOr C-terminal DnaJ-like domain have been reported to be involved in protein-protein interactions, suggesting that Or may be multi-functional protein28,35. The Orange protein N-terminal region interacts with PSY in the chloroplast and is involved in regulating the homeostasis of photosynthesis and carotenoid biosynthesis48, whereas the C-terminal DnaJ-like domain interacts with eRF1–2 in the nucleus and controls leaf petiole elongation35. IbOr also is mainly localized in the nucleus (Supplementary Fig. S8, top panel), and IbOr localization prominently changes to the chloroplast in response to heat stress (Supplementary Fig. S8, bottom panel). This suggests that IbOr might translocate to the chloroplast during heat stress to protect IbPSY from heat stress-induced aggregation. Subcellular protein translocation in response to oxidative stress condition has been reported in plants46. The potential subcellular translocation of IbOr in response to environmental stress conditions is consistent with Or function. These combined results indicate that Or is a multi-functional protein involved in plant growth, development, and abiotic stress responses.

Transcript levels of several plant DnaJ genes targeted to the chloroplast are induced by abiotic stresses23. We reported previously that IbOr expression also responds to abiotic stresses including salt, drought, and oxidative stress7. In this study, we found that IbOr transcript expression was induced by heat stress in sweetpotato stem, fibrous root, and calli, but not in leaves. Zhou et al.25 recently reported that AtOr transcript levels were greatly reduced in psy co-suppressed plants. Similarly, heat stress may severely suppress IbPSY expression in leaves and lead to reduced IbOr transcript levels. In heat-stressed leaves, IbOr translocated to the chloroplast to protect IbPSY (Supplementary Fig. S8). These results indicate that IbOr displays tissue-specific responses to heat stress.

DnaJ proteins belong to a large protein family that is characterized by different subcellular localizations57. However, the majority of DnaJ proteins (including Or) are localized in the chloroplast58. Chloroplast-targeted DnaJ proteins have important roles in photosynthesis because they are involved in maintaining PSII function23, protecting Rubisco activity24, chloroplast development21,30, PSI accumulation39, and optimizing photosynthetic reactions32. Carotenoids are essential for photosynthesis, and PSY catalyzes the rate-limiting step of carotenoid biosynthesis5. Because Or regulates PSY, Or is involved in photosynthesis via regulation of carotenoid biosynthesis. The Arabidopsis ator and ator-like double mutants exhibited a pale green phenotype with reduced carotenoid contents due to the loss of chlorophyll and disruption of carotenoid homeostasis29. Transgenic sweetpotato and Arabidopsis plants overexpressing IbOr displayed enhanced heat stress tolerance and higher chlorophyll contents than those of control plants transformed with empty vector. Stress tolerance of IbOr-OX plants is likely caused by enhanced stability of photosynthetic proteins and controlled homeostasis of chlorophyll and carotenoids. Photosynthesis is sensitive to heat stress. The protection of photosynthetic enzymes and cofactors protects photosynthetic reactions and accessory pathways, and thereby enhances stress tolerance24. IbOr-OX Arabidopsis
plants also displayed enhanced oxidative stress tolerance. Recent reports show that IbOr overexpression enhances abiotic stress tolerance in sweetpotato calli, alfalfa, and potato. These results suggest that IbOr has a crucial role in maintenance of photosynthesis, which thereby confers stress tolerance.

Conclusively, our results indicated that IbOr plays a role in stabilization of IbPSY in response to heat and oxidative stresses. In addition, holdase chaperone function of IbOr is involved in carotenoid biosynthesis by protection of IbPSY and tolerance to environmental stress in plant. This work will provide a new strategy to develop plants with enriched carotenoids contents and enhanced environmental stress tolerance.

Methods

Plant materials, growth conditions, and stress treatments. Orange-fleshed sweetpotato plants [Ipomoea batatas (L.) Lam. cv. Sinhwangmi], sweetpotato transgenic lines overexpressing empty vector (Ib-EV) and IbOr (Ib-OX), Arabidopsis thaliana (ecotype Columbia-0), and Nicotiana benthamiana were used in this study. Orange-fleshed sweetpotato plants were obtained from the Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration, Korea. Plants were cultivated in plastic pots filled with soil in a growth room at 25 or 22°C under 16 h light/8 h dark photocycles. Three-week-old sweetpotato plants were
used for IbOr expression analysis under heat stress conditions. Sweetpotato calli were induced from storage roots and cultured on MS42 medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose, and 0.4% Gelrite (MS1D). Calli were proliferated on MS1D media with 21 d subculture intervals and incubated at 25 °C in the dark. Sweetpotato calli 10 d after subculture were used for IbOr expression analysis under heat stress conditions. Sweetpotato transgenic lines, Ib-EV and Ib-OX, were cultivated on MS plates with 21 d subculture intervals and incubated in a growth room at 25 °C under 16 h light/8 h dark photocycles. To test heat-shock tolerance, Ib-EV and Ib-OX were grown on MS plates for 1 week after subculture, subjected to heat treatment at 47 °C for 4 h, and then returned to 25 °C for recovery. The plants’ ability to recover growth following heat shock was then analyzed.

Arabidopsis transgenic lines overexpressing empty vector (At-EV) or IbOr (At-OX) were generated as follows. The pGWB11 or pGWB11-IbOr-Wt plant expression vector was transformed into Agrobacterium tumefaciens and introduced into Arabidopsis using the flower-dipping method. IbOr protein expression was evaluated by immunoblotting analysis. For the Arabidopsis heat-shock tolerance experiment, At-EV and At-OX were grown on MS plates for 12 d at 22 °C under 16 h light/8 h dark photocycles, heat treated at 38 °C for 3 h, and then returned to 22 °C for recovery. The plants’ ability to recover following heat shock was then analyzed. For the heat-shock tolerance test of transgenic seed germination, stratified (3 d in the dark at 4 °C) T3 lines of At-EV and At-OX seeds were subjected to 47 °C for 4 h (or no heat treatment for control), sown on MS plates, and incubated for 8 d at 22 °C under 16 h light/8 h dark photocycles. Germination was assessed every 24 h and defined as emergence of the radicle. For the oxidative stress resistance experiment, At-EV and At-OX were germinated and grown for 21 d at 22 °C under 16 h light/8 h dark photocycles on MS plates containing 0.25 μM methyl viologen, and then seedling phenotypes were analyzed. All values are averages of at least three independent measurements.

**Determination of holdase chaperone activity.** In vitro holdase chaperone activity was evaluated using MDH and GST:IbPSY as substrates. The substrates were incubated in 50 mM HEPES-KOH (pH 8.0) buffer at 45 or 50 °C or 50 or 100 μM H2O2 with various concentrations of GST:IbOr or IbOr truncated fragments. Substrate stability was determined by SDS-PAGE, and substrate aggregation was determined by monitoring the turbidity (light scattering) at A340 as described previously. In planta holdase chaperone activity was evaluated using IbPSY:GFP as substrate. Three-week-old N. benthamiana plants were used for Agrobacterium-mediated transient expression; pMDC83-IbPSY, pCAMBIA1300-multi-GUS, and pGWB11-IbOr or pGWB11 (EV) were transformed into Agrobacterium tumefaciens GV3101, and Agrobacterium-mediated transient expression was performed. Three days after infiltration, N. benthamiana plants were subjected to 38 °C for 1 h, and then total proteins were extracted. Substrate stability was determined by immunoblotting with anti-GFP, anti-FLAG, and anti-GUS.

**Size exclusion chromatography, polyacrylamide gel electrophoresis (PAGE), and immunoblot analysis.** SEC was performed at 25 °C using HPLC (Dionex, Sunnyvale, CA USA) and a TSK G4000SWXL
column equilibrated with 50 mM HEPES-KOH (pH 8.0) buffer containing 100 mM NaCl as described previously. SDS-PAGE, native PAGE, and immunoblot analysis were performed as described previously.

**Hydrophobicity analysis.** IbOr hydrophobicity was determined spectrophotometrically using the SFM25 spectrophluorometer (Kontron, Basel, Switzerland). The binding of bis-ANS was measured in the presence of increasing IbOr concentrations, which revealed exposure of the IbOr hydrophobic domain.

**Laser scanning confocal microscopy.** Constructs were introduced into Agrobacterium tumefaciens EHA105 for Agrobacterium-mediated transient expression. Three days after infiltration, N. benthamiana plants were treated with 38 °C for 1 h (or no treatment for control), and then leaves were cut off into small squares. The cut leaves were fixed and stained with DAPI (to label nuclei) as described previously. The samples were examined for fluorescent protein expression by confocal microscopy as described previously.

**Bimolecular fluorescence complementation (BiFC) assay.** Constructs were transformed into Agrobacterium tumefaciens EHA105, and Agrobacterium-mediated transient expression was performed. Three days after infiltration, N. benthamiana leaves were cut off into small squares. The samples were examined for Venus fluorescence by confocal microscopy as described previously.

Detailed procedures of cloning and preparation of recombinant proteins, oligomerization status analysis, thermostability test, qRT-PCR analysis, firefly luciferase complementation imaging assay, pull-down assay, yeast two-hybrid assay, total chlorophyll content measurement, and ion leakage analysis are described in Supplementary Information.

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S.P., H.S.K., Y.J.J., J.C.J. and S.-S.K. designed the research, and S.P., H.S.K., Y.J.J., S.H.K., C.Y.J. and Z.W. performed the research. J.C.J., H.-S.L., S.Y.L. and S.-S.K. analyzed the data, and S.P., H.S.K., Y.J.J. and S.-S.K. wrote the paper.

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