RESERCH PAPER

The rice RING finger E3 ligase, OsHCI1, drives nuclear export of multiple substrate proteins and its heterogeneous overexpression enhances acquired thermotolerance

Sung Don Lim, Hyun Yong Cho, Yong Chan Park, Deok Jae Ham, Ju Kyong Lee and Cheol Seong Jang*

Department of Applied Plant Sciences, Kangwon National University, Chuncheon 200–713, Korea

* To whom correspondence should be addressed. E-mail: csjang@kangwon.ac.kr

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Abstract

Thermotolerance is very important for plant survival when plants are subjected to lethally high temperature. However, thus far little is known about the functions of RING E3 ligase in response to heat shock in plants. This study found that one rice gene encoding the RING finger protein was specifically induced by heat and cold stress treatments but not by salinity or dehydration and named it OsHCI1 (Oryza sativa heat and cold induced 1). Subcellular localization results showed that OsHCI1 was mainly associated with the Golgi apparatus and moved rapidly and extensively along the cytoskeleton. In contrast, OsHCI1 may have accumulated in the nucleus under high temperatures. OsHCI1 physically interacted with nuclear substrate proteins including a basic helix-loop-helix transcription factor. Transient co-overexpression of OsHCI1 and each of three nuclear proteins showed that their fluorescent signals moved into the cytoplasm as punctuate formations. Heterogeneous overexpression of OsHCI1 in Arabidopsis highly increased survival rate through acquired thermotolerance. It is proposed that OsHCI1 mediates nuclear–cytoplasmic trafficking of nuclear substrate proteins via monoubiquitination and drives an inactivation device for the nuclear proteins under heat shock.

Key words: Abiotic stress, monoubiquitination, nuclear–cytoplasmic trafficking, rice, RING E3 ligase, thermotolerance.

Introduction

Extreme temperature is a major agricultural problem limiting crop yields worldwide. A transient increase in temperature, usually 10–15 °C above ambient, is generally considered heat shock or heat stress in living organisms, particularly in plants. Heat shock negatively affects plant growth, seed germination, photosynthesis, respiration, water relation, and membrane stability in plants (Wahid et al., 2007). At the cellular and molecular level, heat shock leads to adverse outcomes in plant cell functions, including alterations in cellular composition of membrane fluidity and permeability, enzyme activity, metabolism, production of active oxygen species, and gene expression (Kampinga et al., 1995; Alfonso et al., 2001; Larkindale and Knight, 2002; Larkindale and Huang, 2004; Larkindale et al., 2005). These alterations could cause reduced photosynthesis and carbon gain in plants, thereby leading to decreased growth and reproduction. For example, studies on the relationship between rice crop yields and temperature over the last two decades have demonstrated that grain yields decrease significantly by 10% for each 1 °C increase in the growing-season minimum temperature (Peng et al., 2004).

Investigations into molecular mechanisms underlying thermoprotection have involved genetic and molecular approaches (Iba, 2002; Sung et al., 2003; Ahuja et al., 2010; Qin et al., 2011). Plants generally possess basal and acquired thermotolerance by two heat tolerance mechanisms (Vierling, 1991). Basal thermotolerance is defined as an inherent ability...
to survive high temperatures, whereas acquired thermotolerance is the ability to tolerate an otherwise lethally high temperature after being pre-exposed to a sublethal increased temperature, mimicking an ‘immunization’ against high temperature. Once plants are exposed to high temperature, either basal, acquired, or both, thermotolerance mechanisms may be involved (Larkin 

One of the best-known mechanisms regarding acquired thermotolerance is the induction of heat shock proteins (HSPs; Vierling, 1991). HSPs are molecular chaperone stress-response proteins that protect organisms against various stresses, particularly high temperature. HSPs preserve structural and functional protein integrity by binding to proteins that have become denatured or misfolded as a result of a heat shock (Perez et al., 2009; Sarkar et al., 2009). Plant adaptations to high temperature are not only HSP-based mechanisms but also other components such as phospholipids, the dehydrogenase-responsive element binding protein 2A (DREB2A), and S-nitroglutathione reductase (GSNOR) (Ahuja et al., 2010). For example, the heat stress transcription factor HsfA3 which is transcriptionally induced during heat shock by DREB2A, regulates the expression of an HSP-encoding gene (Schramm et al., 2008). Furthermore, filamentous temperature-sensitive H11 protease and GSNOR activity contribute to plant adaptation to high temperatures (Chen et al., 2006; Lee et al., 2008).

Attachment of ubiquitin molecules (Ub, a small 76-amino acid protein) to target substrates for modification mediates a variety of cellular functions via the Ub/26S proteasome system in higher plants. In this pathway, the conjugation cascade subsequently requires three classes of enzymes: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) (Vierstra, 2009). Approximately 5% of the Arabidopsis proteome is postulated to be involved in the Ub/26S proteasome pathway, and about 1300 genes are predicted to encode E3 ligase components in particular (Smalle and Vierstra, 2004). The E3 ligases specifically interact with target proteins to confer different fates by attachment of ubiquitin molecules. The substrate–ubiquitin structures determine the subcellular localization and different functions of many target proteins (Hicke and Dunn, 2003; Pickart, 2004; Roos-Mattjus and Sistonen, 2004). For example, attachment of single ubiquitin molecules to one or more lysines on target proteins, known as monoubiquitination or multimonoubiquitination, activates a variety of their functions, for example trafficking, subcellular localization, signal transduction, transcription regulation, and DNA repair (Deng et al., 2000; Kaiser et al., 2000; Hicke and Dunn, 2003; Wu et al., 2003). In contrast, polyubiquitinated substrate proteins destined for degradation are usually targeted by the 26S proteasome (Roos-Mattjus and Sistonen, 2004; Vierstra, 2009).

The Ub/26S proteasome pathway is an important mechanism of tolerance against high temperature. For example, seedlings of Prosopis chilensis, which is a leguminous tree, are able to survive at 50 °C after germination at 35 °C (Medina and Cardemil, 1993). P. chilensis showed higher relative accumulation rates of free Ub, conjugated Ub, and HSP70 than cultivated Glycine max (soybean) under heat stress, suggesting that the ubiquitinated-proteolytic pathway is an important heat tolerance mechanism (Ortiz and Cardemil, 2001). In addition, small ubiquitin-like modifiers (SUMOs) that are ubiquitin-like polypeptides also attach to various target substrates and, thus, modify their cellular functions. In Arabidopsis, the findings that SUMO1/2 conjugates were highly accumulated by repeated heat shock, while HSP70-overexpressing plants showed fewer SUMO1/2 conjugates during heat shock, suggested that the accumulation of SUMO1/2 conjugates is relevant to thermotolerance (Kurepa et al., 2003).

Plant single-subunit E3 ligases are generally classified into three groups based on the presence of the homologous E6-AP C-terminus, U-box, and RING domain (Smalle and Vierstra, 2004). Of these, the RING domain of the really interesting new gene was the first to be identified as a novel cysteine-rich sequence (Freemont et al., 1991). The proteins harbouring a RING domain are believed to play E3 ligase for recognizing and ubiquitylation of substrate proteins. Subsequently, a number of RING E3s have been reported to play crucial roles in post-translational regulation of plant hormone signalling pathways, for example abscisic acid (ABA), and environmental stresses. For example, the RING E3 ligase ABI3-interacting protein 2 is a negative regulator of ABA signalling by promoting degradation of ABSCISIC ACID-SENSITIVE 3 (AB13; Zhang et al., 2005). Another outstanding example is the KEEP ON GOING E3 ligase, which also regulates the protein level of ABI5, a basic domain/leucine zipper transcription factor, by 26S proteasomal degradation in an ABA-dependent manner (Stone et al., 2006). The Arabidopsis RING E3 ligases DREB2A-interacting protein 1 and 2 negatively modulate the expression of drought stress-response genes (Qin et al., 2008). Hot pepper RING membrane-anchor 1 homologue 1 (Rma1H1) functions as an E3 ligase plasma membrane aquaporin, PIP2;1, under water-deficient conditions (Lee et al., 2009). In Arabidopsis, the high expression of osmotically responsive gene 1 (HOS1) harbouring a RING-like domain negatively regulates cold signal transduction (Lee et al., 2001a). Additionally, salt- and drought-induced ring finger 1 E3 ligase is believed to enhance salt stress-responsive ABA signalling (Zhang et al., 2007). However, RING E3 ligase and its substrate proteins on heat shock response via ubiquitination still remain unknown in plants.

This study identified the molecular functions of a rice RING domain E3 ligase, OsHClII (Oryza sativa Heat and Cold Induced 1), which is highly induced under heat and cold stress conditions. Studies with a Golgi-localized OsHClI-EYFP fusion protein showed that OsHClI dynamically moved from the cytoplasm to the nucleus along cytoskeletal tracts under heat shock conditions. To shed light on the molecular function of this gene, this study performed a yeast-two hybrid (Y2H) screen, a bimolecular fluorescence complementation (BiFC) assay, and an in vitro ubiquitination assay. The results demonstrated that OsHClI interacted with six substrate proteins and mediated subcellular trafficking of nuclear proteins to the cytoplasm via monoubiquitination. Furthermore, Arabidopsis overexpressing OsHClI-EYFP
exhibited a heat-tolerant phenotype, suggesting an important role of this protein in the regulation of heat-generated signals in plants.

Materials and methods

Plant materials and heat shock treatments

Seeds of rice (O. sativa L. cv. Donganbyeo) were grown on mesh supported in plastic containers with 1/2 Murashige and Skoog (MS) nutrient solution in a growth chamber (16/8 light/dark cycle at 25 °C with 70% relative humidity). Two-week-old seedlings were exposed to high salinity (250 mM NaCl), dehydration, cold (4 °C), and heat (45 °C). The high-salinity and dehydration stress treatments were performed as described by Lim et al. (2010). Two-week-old seedlings were transferred to fresh MS nutrient solution with each of ABA (0.1 mM), jasmonic acid (0.1 mM), and salicylic acid (1 mM). For ethylene treatments, seedlings were moved into air-tight plastic containers with fresh MS solution for the ethylene treatment. Ethylene gas (50 μl l−1) was injected into the plastic boxes using a syringe (Wuriyanghan et al., 2009). Leaf tissues were sampled at 0, 1, 6, 12, 24, and 48 h after the stress treatment. Healthy samples without stress treatment were harvested as controls at the same times. All leaf samples were ground using liquid nitrogen and immediately stored at −80 °C until total RNA extraction.

Dry seeds of A. thaliana ecotype Columbia were grown, and two constructs of 35S:EYFP (EV) and 35S:OsHCl1-EYFP were transformed via Agrobacterium tumefaciens GV3101 using the floral dip method (Zhang et al., 2006). The assessment of segregation of kanamycin resistance in T1 transformants was conducted less than 1 month after harvest for the assay. Three independent OsHCl1-overexpressing lines and a control plant (35S:EYFP) were tested according to Larkindale et al. (2005) to observe the heat shock effect. Transgenic Arabidopsis plants were grown on MS agar for 7 days, then dipped into water baths at either 38 or 45 °C, as appropriate. Basal thermotolerance treatments were performed by heating the plants in sealed plates at 45 °C for 1 h. Acquired thermotolerance treatments were conducted by heating the plants initially to 38 °C for 90 min, and then they were moved to a growth chamber (24 °C) for 120 min before finally heating to 45 °C for 3 h. Both heat shock treatments were performed in the dark. Heat-treated plants were recovered in a growth chamber at 24 °C for 5 days in the light.

To evaluate the expression patterns of six interacting protein genes with OsHCl1, rice plants were grown on MS agar for 14 days. Then basal or acquired heat treatments were performed as described above. Leaves were sampled at different time points using liquid nitrogen and immediately stored at −80 °C until total RNA extraction.

Rice protoplast isolation and transfection

Protoplasts were isolated from 2-week-old seedlings (Kim et al., 2012). Seeds of rice were grown on 1/2 MS nutrient solution in a growth chamber (16/8 light/dark cycle at 25 °C with 70% relative humidity). Young leaves and sheaths were chopped and dipped in enzyme solution (0.5 M mannitol, 1.5% cellulose RS (Yakult Honsa, Tokyo, Japan), 0.75% macerozyme R10 (Yakult Honsa), 1 mM CaCl2, and 0.1% BSA) with carbenicillin (100 μg ml-1). This mixture was incubated on a shaking incubator for 16 h at room temperature then filtered through Miracloth. Protoplasts were pelleted by centrifugation for 4 min at 300 g and resuspended in an equal volume of W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM glucose, and 1.5 mM MES, adjusted to pH 5.7) and incubated on ice for 5 h. Protoplasts were centrifuged and resuspended in MMg solution (0.4 M mannitol, 15 mM MgCl2, and 4.7 mM MES, adjusted to pH 5.7). Plasmid DNA (10 or 20 μg) was added to the protoplast solution and transected with 40% polyethylene glycol (PEG) solution (40% PEG 4000, 0.4 M mannitol, and 100 mM Ca(NO3)2) for 20 min at room temperature. W5 solution was added stepwise to dilute the PEG solution and discarded. Transfected protoplasts were incubated overnight at room temperature and then observed under confocal microscopy.

Gene expression study

Total RNA was extracted using TRIzol regent, according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis from 500 ng total RNA was conducted using a cDNA Synthesis kit (Takara-Bio, Ohtsu, Japan). Semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously (Lim et al., 2010). Gene-specific primers were designed using Primer-BLAST (NCBI, www.ncbi.nlm.nih.gov/tools/primer-blast/). Reliable genes such as Os18S-rRNA (Os09g00999) was used as an internal control (Kim et al., 2003). Primers with restriction enzyme sites used in this study are listed in Supplementary Table S1 (available at JXB online).

Yeast two-hybrid (Y2H) screening and Y2H assays

A full-length coding sequence of OsHCl1 was amplified and cloned in-frame with the GAL4 DNA binding domain of theGBK7-BD vector to generate the GAL4 DNA-BD fusion construct. A rice cDNA library was generated from 14-day-old seedlings treated with salt stress (250 mM NaCl). Then, yeast transformation and library screening were conducted in accordance with the recommended procedures (Make Your Own ‘Mate & Plate’ Library System; Matchmaker Gold Yeast Two-Hybrid System; Yeastmaker Yeast Transformation System 2, Clontech, Palo Alto, CA, USA). The full-length OsHCl1 coding sequence was fused to the yeast GAL DNA-binding domain and used as a bait protein for screening. A rice cDNA library from salt-treated seedlings was fused to the yeast GAL4 activation domain as a prey protein. A total of 280 yeast transformants were selected on a synthetic defined medium lacking Leu and Trp supplemented with 40 μg ml−1 X-α-Gal and 70 ng ml−1 aureobasidin A (AbA) (DDO/X/A) and repatched on synthetic defined medium lacking Ade, His, Leu, and Trp with 40 μg ml−1 X-α-Gal and 70 ng ml−1 AbA (QDO/X/A).

Six full-length interaction partners were amplified by RT-PCR using primers listed in Supplementary Table S1 to confirm a positive interaction with OsHCl1. Each PCR product was digested with the appropriate restriction enzyme and introduced into the pGADT7 vector. These constructs with pGBK7-T7-OsHCl1 were co-transformed into the Y2H Gold yeast strain. Transformed yeast cells were separately grown onto synthetic defined/−Leu/−Trp and synthetic defined/−Ade/−His/−Lue/−Trp/X-α-Gal/Aba with 70 ng ml−1 AbA for 5 days at 30 °C. All experiments were repeated three times.

Subcellular localization

Two fluorescence protein constructs were prepared for the subcellular localization assay. For the 35S:EYFP and 35S:DsRed2 constructs, the coding sequence of the EYFP and DsRed2 were amplified using a high-fidelity Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) from pEYFP-C1 and pDsRed2-C1 (Clontech) as templates, respectively, with primers harbouring multiple cloning sites (Supplementary Table S1). The PCR products were cloned into the pBIN35S binary vector under the control of the CaMV 35S promoter. The coding region of the full-length cDNA of OsHCl1 was amplified from rice cDNA with

Supplementary Table S1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full-length cDNA sequence</th>
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<tr>
<td>OsHCl1</td>
<td>5'-CTGGATCCATGAGTTCGCTTCGGTTTCTGCTGGT-3'</td>
</tr>
<tr>
<td>Os18S-rRNA</td>
<td>5'-GAGCTCGAGCTACCTTCTTCTGCTGGTCTGCTGGT-3'</td>
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appropriate primer pairs and then inserted into the pBIN35S-EYFP vector between the XbaI and KpnI sites for the subcellular localization study. A single amino acid substitution (OsHCl1<sup>C172A</sup>) in the RING domain of OsHCl1 was generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) with the OsHCl1/C172A-F and OsHCl1/C172A-R primer pair. Additionally, full-length cDNAs of the six OsHCl1 interacting partners were cloned into the pBIN35S-DsRed2 vector with appropriate enzyme sites, respectively. The plasmid containing an organellar marker for the Golgi apparatus (Nelson et al., 2007) was kindly provided by the Arabidopsis Biological Resource Center.

**BiFC assay**

The full-length OsHCl1 cDNAs and the six interacting partners were amplified by PCR using appropriate primers to generate BiFC constructs. PCR products were digested and then ligated into 35S-HA-SPYCE(M) and 35S-c-myc-SPYNE(R)173 vectors, respectively (Waadt et al., 2008). The primers and restriction enzymes used for cloning are presented in Supplementary Table S1. A. tumefaciens GV3101 harbouring each construct was inoculated for 16h at 28 °C for transient expression. These cells were harvested and resuspended in infiltration buffer (10 mM MES, 10 mM MgCl₂, 0.2 mM aceto-syringone, pH 5.6) to a final concentration at an optical density of 600 nm of 0.5. Equal volumes of different combinations of the Arabidopsis strains were mixed and coinfiltrated into 5-week-old Nicotiana benthamiana leaves with a syringe. Infiltrated plants were placed at 25 °C for 3 days to detect YFP fluorescence.

**In vitro ubiquitination assay**

Full-length OsHCl1 cDNA was amplified by PCR with primer pairs (Supplementary Table S1). The amplicon was digested with NorI and BamHI and then ligated into a digested pMAL-c5X vector (New England BioLabs, Ipswich, MA, USA) with the same enzymes. Recombinant MBP-OsHCl1, MBP-OsHCl1<sup>C172A</sup>, and non-recombinant MBP (negative control) were expressed in <i>Escherichia coli</i> BL21 (DE3) pLysS (Promega, Madison, WI, USA), purified by affinity chromatography using amylose resin (New England BioLabs), and used for the <i>in vitro</i> self-ubiquitination assay. The full-length cDNAs of AtUBC10 and AtUBC11 were amplified and then introduced into the pET-28a(+) vector (Novagen, Gibbstown, NJ, USA) with a 6×His-tag. The fusion 6×His-tagged AtUBC10 and AtUBC11 were expressed in <i>E. coli</i> BL21 (DE3) pLysS and purified using the Ni-NTA Purification System (Invitrogen).

The <i>in vitro</i> self-ubiquitination assay was conducted as described previously by Hardtke et al. (2002) with some modifications. Purified MBP-OsHCl1 (250 ng) was mixed with 50 ng yeast E1 (Boston Biochemicals, Cambridge, MA, USA), 250 ng purified Arabidopsis E2 (AtUBC10 and AtUBC11), and 10 μg bovine ubiquitin (Sigma-Aldrich, St. Louis, MO, USA) incubated in ubiquitination reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 0.05 mM ZnCl₂, 1 mM ATP, 0.2 mM DTT, 10 mM phosphocreatine, and 0.1 unit of creatine kinase (Sigma-Aldrich)). After 3 h incubation at 30 °C, the reaction was halted at different time points by adding 2×SDS sample buffer followed by 5 min of boiling at 95 °C. Each reaction (10 μl) was analysed via 12% SDS-PAGE and then transferred to a nitrocellulose membrane. Immunoblot analyses were conducted using anti-ubiquitin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with a secondary goat anti-rabbit IgG peroxidase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for HRP and imaged on X-ray film (Kodak, Rochester, NY, USA). To confirm that OsHCl1 mediated ubiquitination of the six interacting partners, OsPGLU1, OsbHLH065, OsGRP1, and OsPOX1-His-Trx fusion proteins were affinity-purified, and 200 ng purified protein was incubated together with purified MBP-OsHCl1 in the ubiquitination mixture for 3 h. The mixture was then subjected to 10% SDS-PAGE and immunoblot analysis.

Confocal microscopy and imaging

Transformed tobacco leaves were cut 3–5 days after infection for microscopic analyses. Fluorescent images were obtained using a Multiphoton confocal laser scanning microscope (model LSM 510 META NLO and LSM 780 NLO, Carl Zeiss, Oberkochen, Germany) at the Korea Basic Science Institute, Chuncheon Center. Excitation/emission wavelengths were 514/553 590 nm for EYFP and BiFC constructs and 543/565 615 nm for the DsRed2 and mCherry construct. All images were acquired using either a C-Apochromat ×40/1.2 water immersion) objective. To prevent cross-talk between EYFP and mCherry (or DsRed2) signals, the spectral images were acquired using the lambda mode. Scanned images were captured as single optical sections or as a z-series of optical sections. Image processing was carried out using an LSM 5 Image Browser (Zeiss) and Photoshop 9.0 software (Adobe, Mountain View, CA, USA).

**Results**

OsHCl1 is upregulated by heat and cold

This research group previously defined expression diversity of members of the rice RING finger protein genes based on their expression profiles via *in silico* analysis (Lim et al., 2010). Subsequently, in an effort to isolate RING finger protein gene(s) that play a critical role in extreme temperature, 48 RING finger protein genes were randomly selected and examined for their expression patterns via semi-quantitative RT-PCR (data not shown). Interestingly, one gene (Os10g30850) was highly induced at 1–48 h after heat treatment (45 °C), whereas OsHsp90-1 used for validation of the stress treatment was similarly induced by the stress (Hu et al., 2009) (Fig. 1A). Consequently, the gene was named <i>O. sativa</i> heat and cold inducible gene 1 (OsHCl1). The expression patterns of the gene were further examined against other abiotic stresses such as cold (4 °C), salinity, and dehydration (Fig. 1A). The gene was upregulated at 12–48 h by cold stress, whereas LIP19 (Shimizu et al., 2005) was induced at 1–48 h. However, both the salinity and dehydration stresses exhibited no induction of the gene through 48 h after the treatments. Two reliable stress-inducible genes were employed as quality control, OsSalT (Claes et al., 1990) and OsbZIP23 (Xiang et al., 2008) for salinity and dehydration, respectively. High induction of OsSalT and OsbZIP23 served as evidence that the plants had been subjected severe stresses, supporting no response of <i>OsHCl1</i> to either stress. The transcript levels of <i>OsHCl1</i> were further confirmed via quantitative real-time PCR, which revealed high expression patterns under heat and cold stresses but not under salt and drought stresses (Supplementary Fig. S1).

When plants are subjected to heat shock, phytohormones including ABA, salicylic acid, and ethylene act as key signals (Larkindale and Knight, 2002). Therefore, this study further examined phytohormonal regulation during <i>OsHCl1</i> gene expression (Fig. 1B). Under 0.1 mM ABA treatment, <i>OsHCl1</i> was induced at 3 h, the highest transcript level occurred at 12 h, and then gradually decreased to 48 h, whereas OsSalT exhibited an increase at 3 h and then steady expression until 48 h. In the case of jasmonic acid, OsPZ21, which is inducible by hormone treatment (Lee et al., 2001b), showed a slight induction at 3 h and then a subsequent increase up to 48 h, whereas <i>OsHCl1</i> exhibited a somewhat slight induction at 3–24 h. In
addition, OsHCII gene expression increased at 3 h, reached its highest transcript level at 12 h, and then showed no induction until 48 h. However, OsPR1b (Agrawal et al., 2000) was induced at 6 h and gradually and slightly increased until 24 h. Additionally, the transcription level of OsHCII under 50 μl l⁻¹ ethylene treatment increased at 6 h then reached its highest level at 12 h. Collectively, the OsHCII expression patterns under phytohormonal treatments were induced gradually at 12 h then its transcript levels decreased until 24 h. These results indicate that OsHCII rapidly responds to hormone treatments.

**Dynamics of OsHCII-EYFP subcellular localization**

It is generally believed that subcellular localization of a protein of interest is crucial to understand its cellular function. To examine subcellular localization of the OsHCII protein, this study constructed a binary vector harbouring the enhanced yellow fluorescence protein (EYFP) under the control of a CaMV 35S promoter. Transient expression of 35S:EYFP was diffuse in both the cytosol and the nucleus in tobacco epidermal cells (Fig. 2A, upper panel). This study further generated a 35S:OsHCII-EYFP construct, which is transiently expressed in tobacco leaves. OsHCII fluorescence displayed a punctate pattern; the fluorescence appeared to localize in the dispersed organization of Golgi stacks in most (about 93%) tobacco cells (Fig. 2A, lower panel). In contrast, about 7% of the transformed tobacco cells showed an additional reticulate fluorescence with a punctate pattern, which seemed to target endoplasmic reticulum network patterns (Supplementary Fig. S2A, C). To confirm whether the destination of the OsHCII protein alone was the Golgi apparatus, this study employed the G-rk-mCherry organelle marker localized to the Golgi body. Both constructs, OsHCII-EYFP and G-rk-mCherry, were transiently co-expressed with p19 in tobacco cells (Nelson et al., 2007). The OsHCII-EYFP signal was closely overlapped by that of G-rk-mCherry (Fig. 2D), indicating that the final destination of OsHCII was the Golgi complex. The endoplasmic reticulum localization may represent newly synthesized OsHCII-EYFP protein that has not yet been transported to the Golgi stack. Furthermore, the punctate patterns of OsHCII-EYFP fluorescence were also displayed around the nuclear envelope (Fig. 2A lower panel and D). In addition, there was dynamic movement in which the OsHCII-EYFP fluorescent signals moved rapidly and extensively along the cytoskeleton of leaf epidermis cells (Supplementary Fig. S2B and Supplementary Movie S1).

The finding that the subcellular localization of fluorescently tagged fusion proteins is changed by environmental stress (von Arnim and Deng, 1994; Lee et al., 2001a) led to the question as to whether the Golgi localization of the OsHCII protein could be altered by heat shock. Thus, 35S:EYFP and 35S:OsHCII-EYFP was transiently expressed in tobacco leaves, which were then incubated for 1 h at 38 or 45 °C. Interestingly, strong OsHCII-EYFP signals were found in the nucleus (Fig. 2B and C, lower panel). Because of a concern that heterogeneous expression of OsHCII caused protein mislocalization and functional diversity, the constructs were subsequently expressed in rice protoplasts, which were then incubated for 15 min at 38 or 45 °C, resulting in a similar expression pattern compared to that of tobacco (Fig. 2E). These results support the previous finding (i.e. there is no significant difference in protein localization between tobacco and rice cells). Under
moderate heat treatment (38 °C), approximately 55.0% of cells exhibited a nuclear localized pattern of the OsHCI1-EYFP protein and approximately 35.0% of cells displayed this pattern in both the Golgi and nucleus. However, approximately 10.0% of cells still showed only the Golgi-localized pattern of rice protoplasts (Fig. 2F). Similarly, approximately 66.6% and 21.6% of cells showed nuclear localization and both the Golgi and nucleus pattern, respectively, under severe heat treatment (45 °C). By contrast, approximately 11.6% of cells only displayed a Golgi-localized pattern at 45 °C.

Expression pattern and subcellular localization of proteins interacting with OsHCI1

A Y2H screen was performed to identify proteins that interact with OsHCI1. Twenty-four positive clones were selected, sequenced, and their α-galactosidase activity was measured (Supplementary Fig. S3). To confirm these positive interactions with OsHCI1, full-length coding sequences of the top six genes, which exhibited strong α-galactosidase activity, were cloned into GAL4 activation domain, respectively. Full-length OsHCI1 and each interacting protein were co-transformed into the Y2H Gold strain and grown on QDO/X/A medium (Supplementary Fig. S4). The six interacting protein genes were 20S proteasome subunit α7 (named OsPSA7, Os01g59600), periplasmic beta-glucosidase (OsBGLU1, Os03g53800), ethylene-responsive protein (OsbHLH065, Os04g41570, Li et al., 2006), glycine-rich cell-wall structural protein (OsGRP1, Os05g02770), peroxidase (OsPOX1, Os07g48020), and 14-3-3 protein (Os14-3-3, Os11g34450).

This study also examined the expression patterns of the interacting partner genes with OsHCII under two different
heat stresses via semi-quantitative RT-PCR with rice seedlings treated by basal or acquired heat shock treatments (Fig. 3). The results showed that the OsHClI transcript was highly induced by basal heat treatment (45 °C for 24 h) and its transcript level was downregulated when seedlings were recovered at 24 °C for 2 h. For acquired heat shock treatment, OsHClI was slightly induced by mild heat treatment at 38 °C for 90 min and downregulated at 24 °C for 2 h. The OsHClI transcript was highly accumulated by re-heat shock treatment at 45 °C for 24 h and downregulated by 24 °C for 2 h.

Next, the expression patterns of the six interacting partner genes were evaluated under both heat shock conditions (Fig. 3). OsPSA7 and Os14-3-3 transcript levels were stable under both heat stress conditions. In contrast, OsPGLU1, OsbHLH065, OsGRP1, and OsPOX1 showed strikingly decreased transcript levels at 45 °C during the basal and acquired heat treatments. In addition, OsPGLU1 and OsbHLH065 displayed a slight decrease at 38 °C. Expression patterns of OsPGLU1, OsbHLH065, and OsGRP1 were likely to have a reverse correlation with that of OsHClI. Three genes, OsPGLU1, OsbHLH065, and OsGRP1, were downregulated at 45 °C during basal and acquired heat treatments; their transcript levels were upregulated following the recovery period at 24 °C. These results suggest that heat shock results in high expression of the OsHClI transcript or protein, which can affect the transcript levels of its interacting genes.

This study questioned the subcellular localization of each OsHClI interaction partner that showed dynamic subcellular localization. The six interaction partners were tagged with DsRed2, and each construct was transiently expressed together with p19 in tobacco leaves. A series of DsRed2 signal z-stack images were captured and merged after 5 days of agro-infiltration. As shown in Fig. 4, DsRed2 fluorescent signals of the OsPGLU1, OsbHLH065, and OsGRP1 proteins were only associated with the nucleus, whereas OsPSA7-DsRed2 was found in both the cytoplasm and the nucleus. In contrast, OsPOX1-DsRed2 was observed in a punctuate/dot form pattern (Fig. 4E), and Os14-3-3-DsRed2 was localized to the cytoplasm and cytoskeleton (Fig. 4F).

Subcellular localization of the complex of OsHClI and each interacting protein

BiFC technology was employed to visualize the interactions between OsHClI and each of the interaction partners in living cells (Waadt and Kudla, 2008). Full-length coding sequences of OsHClI and each of the six interacting protein genes were cloned into the 35S-HA-SPYCE(M) and 35S-c-myc-SPYNE(R)173

![Fig. 3. Expression patterns of the response of interacting protein genes with OsHClI under heat treatment. Two-week-old rice seedlings were exposed to basal (A) or acquired heat stress (B) and then placed to normal temperature for 2 h. Each leaf sample was harvested at different time points.](https://academic.oup.com/jxb/article-abstract/64/10/2899/542627)
vectors, respectively. After 5 days of agro-infiltration, we observed YFP signals of all BiFC complex formations in tobacco cells. All of the YFP signals except that of OsPSA7 appeared to associate with the cytoplasm and nucleus (Fig. 5); however, the OsPGLU1-, OsbHLH065-, and OsGRP1-DsRed2 alone protein signals were detected only in the nucleus (Fig. 4B–D). In contrast, the OsHCI1 BiFC complex with OsPSA7 was localized to the cytoplasm with a punctuate complex (Fig. 5A).

OsHCI1 functions as an E3 ligase and mediates ubiquitination of interacting proteins

OsHCI1 encoded a 246-amino acid protein with a predicted molecular mass of 28.8 kDa and harboured a single RING-HC domain in its C-terminal region (Supplementary Fig. S5). It is generally believed that many proteins harbouring the RING-HC domain function are Ub E3 ligases (Stone et al., 2005). An in vitro ubiquitination assay was used to test whether the OsHCI1 protein has E3 Ub ligase activity. A purified MBP-OsHCI1 fusion protein was mixed with ubiquitin, ATP, yeast E1 activating enzyme, and Arabidopsis E2 conjugating enzymes (AtUBC10 and AtUBC11) and then incubated at 30 °C for 3 h. An immunoblot analysis with anti-Ub showed that ubiquitinated proteins were detected in the presence of all of these components (Fig. 6A). Furthermore, clearer ubiquitinated proteins were observed in the presence of the AtUBC10 enzyme but not AtUBC11 (Fig. 6A, lanes 6 and 7). In time-course experiments, MBP-OsHCI1 began to cause high-molecular-mass ubiquitinated ladders after 30 min that gradually reached their highest level after 2 h incubation (Fig. 6B). However, no ubiquitinated ladders were found at 0 h. These results suggest that the OsHCI1

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**Fig. 4.** Subcellular localization of six interacting proteins. The full-length OsPSA7 (A), OsPGLU1 (B), OsbHLH065 (C), OsGRP1 (D), OsPOX1 (E), Os14-3-3 (F), and empty EYFP (G) were tagged with DsRed2 and transiently expressed with p19 in Nicotiana leaves. Images were captured and merged by z-series optical sections after 5 days of agro-infiltration.

**Fig. 5.** BIFC assay for six substrate proteins confirms the interaction with OsHCI1 in living cells. Full-length OsPSA7 (A), OsPGLU1 (B), OsbHLH065 (C), OsGRP1 (D), OsPOX1 (E), and Os14-3-3 (F) were cloned into pSPYNE(R) and OsHCI1 was cloned into pSPYCE(M). Combinations of each construct and SPYNE(R):empty (G, negative control) with OsHCI1:SPYCE(M) were transiently expressed with p19 in Nicotiana leaves. Images were captured and merged by z-series optical sections after 5 days of agro-infiltration.
OsHCI1 drives nuclear export of substrates for thermotolerance

The six interaction proteins were fused with His and Trx tags to determine whether OsHCI1 mediated ubiquitination of the six interacting proteins. The recombinant fusion proteins were expressed in E. coli BL21 (DE3) pLysS. However, His- and Trx-tagged OsPSA7 and Os14-3-3 fusion proteins were not expressed well in this E. coli system. Therefore, an in vitro ubiquitination assay was conducted with OsPGLU1, OsbHLH065, OsGRP1, and OsPOX1 as substrates. In the presence of E1, E2, and MBP-OsHCI1 as E3 ligases, an additional higher-molecular-weight band was detected by anti-Trx immunoblot analysis (Fig. 6C–F). Interestingly, nuclear-localized OsPGLU1, OsbHLH065, and OsGRP1 proteins had one additional ubiquitin monomer, whereas the OsPOX1 protein had polyubiquitinated chains on the original fusion protein bands. Collectively, the OsHCI1 protein was a functional E3 ligase and, mediated multiple substrate mono- and polyubiquitination.

OsHCI1 translocates nuclear substrate proteins into the cytoplasm

The findings that the Golgi-localized OsHCI1 protein relocated to the nucleus along the cytoskeleton under heat shock and that it mediated monoubiquitination of each of the three nuclear-localized substrates in an in vivo ubiquitination assay led to the hypothesis that OsHCI1 E3 translocates its substrate proteins for heat-stress regulation. To test this hypothesis, this study first investigated whether nuclear substrate proteins of OsHCI1 could be relocated by themselves under a heat shock condition in tobacco leaves. Nuclear localization of the OsPGLU1-, OsbHLH065-, and OsGRP1-DsRed2 signals was not significantly different between normal and heat shock conditions (Fig. 4 and Supplementary Fig. S6). Next, a single amino acid substitution (OsHCI1 C172A) in the RING domain of OsHCI1 was generated to obtain a non-functional RING E3 ligase. MBP-OsHCI1 C172A did not show self-ubiquitination activity in vitro (Supplementary Fig. S7). In addition,
subcellular localization of OsHCl1C172A-EYFP was highly similar to that of wild-type OsHCl1-EYFP in tobacco leaves under normal and heat shock conditions (Supplementary Fig. S8). Combinations of each of the OsHCl1-EYFP, OsHCl1C172A-EYFP, and empty-EYFP constructs were transiently co-expressed with OsbHLH065-DsRed2 in tobacco leaves. The fluorescence signal of OsbHLH065-DsRed2 was detected in the cytoplasm and in the nucleus when co-expressed with OsHCl1-EYFP under heat shock and normal conditions (Fig. 7 and Supplementary Fig. S9). In contrast, no alterations in their subcellular localizations were observed when the OsHCl1C172A-EYFP or empty-EYFP construct was co-expressed (Fig. 7B and C). Furthermore, co-expression of OsPGLU1- and OsGRP1-DsRed2 with OsHCl1-EYFP showed the same patterns of fluorescence signals as OsbHLH065-DsRed2 in the cytoplasm and in the nucleus (Supplementary Fig. S10).

This study questioned whether regulation of dynamic translocation under heat shock misleads through heterogeneous expression. In an effort to verify the mechanism in rice cells, the nuclear-localized OsbHLH065-DsRed2 was transformed in rice protoplasts. The fluorescent signal of OsbHLH065-DsRed2 was associated with the nucleus under normal conditions (Fig. 8A). However, the signal displayed both the nucleus and cytoplasm as punctuate formations under heat shock (Fig. 8B). Subsequently, OsbHLH065-DsRed2 and OsHCl1-EYFP were co-transformed into rice protoplasts and then by heat shock. The OsHCl1-EYFP fluorescence clearly moved from the cytoplasm to the nucleus, whereas the OsbHLH065-DsRed2 signal was displayed in both the nucleus and cytoplasm (Fig. 8C).

OsHCl1-overexpressing Arabidopsis enhances heat shock tolerance

The distinct induction of OsHCl1 expression by temperature extremes and the dynamics of its subcellular translocation under heat treatment conditions suggest a crucial role of the gene in thermotolerance. To test this possibility, several independent Arabidopsis transgenic lines (T3) were developed with strong OsHCl1 gene expression and compared to plants without the gene (35S:EYFP), which served as controls (Fig. 9A). Plants were tested for basal heat treatment by heating directly to 45 °C for 1 h, which resulted in no recovery (0%) in all tested control lines, whereas transgenic lines showed approximately 4–7% of survival rates at 5 days after treatment (Fig. 9B). For acquired heat treatment, the plants were subjected to heating to 38 °C for 90 min and subsequently cooled for 2 h at room temperature (24 °C). After pretreatment, plants were subjected to heating to 45 °C for 3 h.
OsHClI drives nuclear export of substrates for thermotolerance

and then allowed to recover for 5 days at 24 °C (Fig. 9B). The OsHClI-overexpressing lines showed strikingly high survival rates of approximately 55–65%; however, most control plants did not recover (Fig. 9C).

Discussion

This study’s findings regarding dynamic movement of OsHClI under heat shock, translocations of target proteins co-expressed with OsHClI, and acquired thermotolerance via heterogeneous overexpression might provide some clues regarding a new molecular mechanism for the heat stress-regulated RING E3 ligase. RING E3 ligases have been recently reported as major players in plant responses to environmental stresses. For example, HOS1 RING E3 ligase is a negative regulator of plant cold responses by mediating degradation of ICE1, which binds the CBF promoter and induces its transcription (Dong et al., 2006), and Rma1H1 RING E3 ligase functions in the downregulation of plasma membrane aquaporin levels as a response to drought stress (Lee et al., 2009). However, the role of RING E3 ligases in the adaptation to heat shock in plants has remained largely unknown.

The finding that OsHClI gene expression patterns were specifically and somewhat rapidly increased by heat and cold stresses but not by salt and drought stresses indicates that the gene is associated closely with thermal stress in rice (Fig. 1A and Supplementary Fig. S1). Subcellular localization of OsHClI was mainly associated with the Golgi apparatus and these punctuate signals rapidly moved to the nucleus under heat shock (Fig. 2). Wild-type OsHClI-EYFP expression effectively moved its nuclear target substrate proteins to the cytoplasm (Figs. 7 and 8 and Supplementary Fig. S10) and attachment of the ubiquitin molecule on the nuclear substrates by OsHClI fusion protein via in vitro ubiquitination assay might support this translocation of nuclear substrate proteins to the cytoplasm (Fig. 6). In addition, heterogeneous overexpression of OsHClI in Arabidopsis resulted in rising survival rates through acquired heat treatment (Fig. 9). These results suggest that the OsHClI E3 ligase might function in the heat shock response in plants.

A hypothesis regarding E3 ligase translocation for functional activation might be postulated by several findings. For example, the COP1 RING E3 ligase is localized to the nucleus in the dark but translocates to the cytoplasm under light signals (von Arnim and Deng, 1994; Deng et al., 2000). Similarly, the Arabidopsis HOS1 protein exhibits nucleocytoplasmic partitioning in response to cold stimuli (Lee et al., 2001a). Recently, two alternative splicing forms of Arabidopsis XBAT35 RING E3 ligase have been reported that display dual targeting of this E3 ligase to the nuclear and cytoplasmic compartment, suggesting a novel player in ethylene-mediated regulation of the apical hook curvature (Carvalho et al., 2012). The OsHClI protein, whose localization is confined to the Golgi stack under control conditions, accumulated in the nucleus in response to heat shock (Fig. 2). In addition, OsHClI protein interacts with substrate proteins localized in both the nucleus and the cytoplasm and relocates nuclear substrate proteins to the cytoplasm (Figs.
These findings suggest that the nucleo-cytoplasmic partitioning of E3 ligases is an extensive regulatory mechanism to control cellular responses to environmental stimuli. However, the OsHCl1 protein can also interact with its substrate proteins and relocates them to the cytoplasm under both normal and heat shock conditions (Fig. 5 and Supplementary Fig. S9). It is possible that overexpression of OsHCl1 might lead to interaction with its nuclear proteins under normal conditions. However, further studies are necessary to test this possibility.

Plants and other organisms have the intrinsic ability to acquire thermotolerance for survival under lethally high temperatures. It is generally known that the ability accelerates transcription and translation of HSPs and decreases normal protein synthesis (Vierling, 1991; Barnabas et al., 2008). Thus, translational modifications of transcription factors might be...
necessary to decrease synthesis of normal proteins under heat shock. A number of studies regarding the transcriptional regulation of targets via post-translational modification of transcript factors, such as ABI3, ABI5, DREB2A, and ICE1 by E3 ligases and 26 proteasomes have been reported (Zhang et al., 2005; Dong et al., 2006; Stone et al., 2006; Qin et al., 2008). Similarly, this study provides evidence to support that OsHClI interacts with multiple substrates including the OsbHLH065 transcription factor with a basic helix-loop-helix transcription factor, which is highly downregulated by heat shock treatment (Fig. 3). Interestingly, the transcription levels of three nuclear-targeted partner genes displayed a significant decrease following overexpression of OsHClI in rice protoplasts (Supplementary Fig. S11). These results lead to the hypothesis that OsHClI plays a crucial role in the thermotolerance mechanism via post-translational modifications. Significant future work on target protein degradation by OsHClI via the 26S proteasome is warranted.

A large body of evidence demonstrates the role of E3 ligase in the differential control of mono- versus polyubiquitination of target proteins. For example, ubiquitination by Mdm2, an oncogenic E3 ligase, causes two alternative p53 fates depending on Mdm2 levels. When Mdm2 levels are high, Mdm2 drives p53 degradation via polyubiquitination, whereas low levels promote p53 nuclear exclusion via monoubiquitination (Li et al., 2003). Human Nedd4-1, an E3 ligase, catalyses monoubiquitination of hDCNL1, which drives its nuclear export (Wu et al., 2011). The current study observed that OsHClI drives two different ubiquitination types depending on the target proteins (Fig. 6). In addition, co-expression of each of three nuclear-localized proteins and wild-type OsHClI promoted nuclear export of target proteins to the cytoplasm, while non-functional OsHClIC172A did not affect (Fig. 7). Collectively, the findings suggest that OsHClI may mediate a nuclear–cytoplasmic translocation of nuclear target substrates via monoubiquitination, demonstrating an inactivation device of nuclear proteins in this compartment under heat shock (Li et al., 2003). An alternative hypothesis is that translocation of the target proteins drives another cellular program to mediate thermotolerance mechanisms in plant cells (Mihara et al., 2003). However, much work is needed to rule out this hypothesis. Why OsHClI drives different ubiquitination processes depending on the target protein localization is a mystery. A simple hypothesis may be that different fates of the target proteins exist under heat shock.

The finding that heterogeneous OsHClI overexpression in Arabidopsis enhances heat shock tolerance suggests that this gene is involved in acquired thermotolerance (Fig. 9). An outstanding report suggested that the protection mechanism against heat-induced oxidative damage involves phytohormones such as ABA, salicylic acid, and ethylene in Arabidopsis (Larkindale and Knight, 2002). As shown in Fig. 1B, phytohormone treatment (i.e. ABA) causes a rapid increase in OsHClI transcripts, which suggests that the gene is related to the ABA-dependent pathway involved in temperature stress responses (Yamaguchi-Shinozaki and Shinozaki, 2006). Furthermore, induction of OsHClI by salicylic acid and ethylene treatments might be consistent with the previously reported relationship among salicylic acid, ethylene, and thermotolerance (Dat et al., 1998; Wang and Li, 2006). This study tested whether OsHClI is related to the ABA-dependent pathway involved in acquired thermotolerance. However, constitutive expression of OsHClI did not confer sensitivity or insensitivity to ABA during seed germination, cotyledon greening, or root growth (Supplementary Fig. S12), suggesting that the OsHClI E3 Ub ligase is involved in the ubiquitination of unidentified proteins, which might function in the heat response in transgenic Arabidopsis plants in a ABA-independent manner.

This study demonstrated the specific expression patterns of the OsHClI transcript and dynamic movement of OsHClI-EYFP under normal and heat shock conditions. In addition, OsHClI functions as an E3 ligase that mediated ubiquitination of substrate proteins in vitro. OsHClI-overexpressing Arabidopsis showed higher tolerance than control plants under heat shock conditions. These results demonstrate that accumulation of the OsHClI RING E3 ligase by heat shock mediates nuclear–cytoplasmic trafficking of nuclear substrate proteins via monoubiquitination to improve heat tolerance as an inactivation mechanism. The results are an excellent example of the post-translational regulation of the heat tolerance mechanism via the Ub/26S proteasome system in plant cells.

**Supplementary material**

Supplementary data are available at JXB online.

Supplementary Table S1. Primer list.

Supplementary Fig. S1. Quantitative real-time PCR analysis of OsHClI in 2-week-old rice plants subjected to heat, cold, NaCl, and dehydration.

Supplementary Fig. S2. Subcellular localization of the OsHClI-EYFP fusion protein.

Supplementary Fig. S3. Positive clones from yeast two-hybrid screening.

Supplementary Fig. S4. Identification of OsHClI interaction with six proteins.

Supplementary Fig. S5. Sequence analysis of OsHClI.

Supplementary Fig. S6. Subcellular localization of nuclear localized OsPGLU-, OsbHLH065-, and OsGRP1-DsRed2 fusion proteins under heat shock.

Supplementary Fig. S7. The ubiquitination reaction contains E1, E2 (Arabidopsis UBC10), MBP-OsCTR1, Ub, and ATP.

Supplementary Fig. S8. Subcellular localization of the OsHClIC172A-EYFP fusion protein.

Supplementary Fig. S9. OsHClI protein mediates nuclear–cytoplasmic trafficking of OsbHLH065 at normal temperature.

Supplementary Fig. S10. OsHClI protein mediates nuclear–cytoplasmic trafficking in tobacco leaves.

Supplementary Fig. S11. Expression patterns of interacting protein genes in overexpression OsHClI-EYFP in rice protoplast.

Supplementary Fig. S12. Phenotypes of 35S:EYFP and 35S:OsHClI-EYFP plants in response to different
concentrations of ABA during seed germination and seedling growth.

Supplementary Movie S1. Dynamic movement of the Golgi-localized OsHCl1-EYFP fusion protein along the actin cytoskeleton.

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