Characterization of a Novel DWD Protein that Participates in Heat Stress Response in Arabidopsis

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INTRODUCTION

Plants are subject to a variety of abiotic stresses such as high/low temperature, wound, UV, water deficit and high salts (Bohnert et al., 2006). Although heat (high temperature) stress is becoming one of the most threatening abiotic stresses, it is still not clear how plants cope with this stress (Baniwal et al., 2004). The greenhouse effect is increasing the temperature of biosphere, which has a detrimental effects on the metabolism of living organisms. In the case of plants, a slight elevation in temperature adversely affects crop productivity (Mittler and Blumwald, 2010). Heat stress leads to negative effects on a variety of cellular events including loss of membrane integrity, accumulation of reactive oxygen species, cytoskeletal perturbation, and destruction of RNA and proteins (Kampinga et al., 1995; Ruelland and Zachowski, 2010; Vierling, 1991). To overcome the damage caused by heat stress, plants have developed exquisite mechanisms for thermotolerance in which various signaling components such as protein kinases, transcription factors and chaperones participate. Among them, heat shock proteins (HSP) and heat stress factors (Hsf) play a pivotal role in heat-tolerance (Todaka et al., 2012). Heat shock proteins were initially identified as proteins induced in response to high temperature. Following heat stress, HSP101, HSP90, HSP70, HSP60, HSP40 and sHSP (small HSP) associates with unfolded proteins caused by the stress, preventing their aggregation and facilitating proper refolding, which leads to their functional recovery (Baniwal et al., 2004; Boston et al., 1996). In addition to the contribution heat shock proteins play in survival under heat stress, they also play an important role in plant growth under normal and abiotic stress conditions (Wang et al., 2004). HSP90 has ATP-dependent hydrolysis activity and forms multichaperone complexes with other proteins such as HSP70, HSP70 interacting protein (Hip), HSP70/HSP90 organizing protein (Hop), HSP40 and p23 to enable its function as a chaperone (Wang et al., 2004). In cells, HSP90 are involved in diverse cellular processes such as maturation of signaling components, regulation of protein stability and trafficking, and control of the cell cycle (Richter and Buchner, 2001). In Arabidopsis, seven HSP90 proteins have been reported. The expression of Arabidopsis HSP90 genes are largely affected by cold, salt and heavy metal stresses, as well as heat stress,
implying their complex involvement in abiotic stress signaling (Krishna and Gloor, 2001). Among HSP90 members, AtHSP90-1 is known to be the strongest heat-inducible cytosolic form and to play a major role in thermotolerance (Yabe et al., 1994). One of the most-actively studied proteins involved in heat response is AtHSP101 (also known as cytosolic ClpB1), which is necessary for acclimation to heat stress (Hong and Vierling, 2001). HSP101 is an AAA+ chaperone ATPase that performs ATP-dependent dissocation of protein aggregates with the shsp chaperone system to enable recovery of proteins damaged by heat stress (Lee et al., 2005; Neuwald et al., 1999). Another crucial member, the HSP70 family, has been reported to participate in thermotolerance and resistance against abiotic stresses in plants (Lee and Schöffl, 1996; Ono et al., 2001; Sugino et al., 1999; Sung et al., 2001). Heat stress transcription factors (Hsfs) are key regulators involved in the expression of various heat-shock induced genes, including HSPs. All plant Hsfs commonly possess nuclear localization signal (NLS), DNA binding domains and the oligomerization domain (HR-A/B region). Hsfs use heat stress elements in the promoters of heat-responsive genes as cis-acting elements for gene expression (von Koskull-Döring et al., 2007). Although the master regulator of heat response in Arabidopsis is still elusive, HsfA2 appears to be the dominant form for thermotolerance response since HsfA2 accumulates during heat stress/recovery cycles and its loss leads to marked alteration of the expression of heat-shock responsive genes following heat treatments (Schramm et al., 2006).

Ubiquitination, a representative post-translational modification by eukaryotes, plays an important role in the regulation of protein stability, enzyme activity, and the cellular localization of proteins. Especially, the chain formation of ubiquitins into substrate functions as a tag to degrade substrate via 26S proteasome complex. In plants, this process is involved in a variety of cellular events including biotic and abiotic stress responses, cell cycle progression and hormone responses (Smalle and Vierstra, 2004; Vierstra, 2009). Ubiquitination sequentially proceeds with three kinds of enzymes: E1 as an ubiquitin-activating enzyme, E2 as an ubiquitin-conjugating enzyme, and E3 as an ubiquitin ligase (Hershko and Ciechanover, 1998). When compared with the numbers of E1 and E2 (6 E1 and 49 E2), Arabidopsis has many more E3 ligases (1300 E3) in its genome, suggesting the importance of E3 in recognition of substrate (Dreher and Callis, 2007; Vierstra, 2009). Cullin4-RING ubiquitin ligase (CRL4) is a type of cullin-RING E3 ligase (CRL) that acts as a multi-subunit E3 ligase complex. CRL4 is composed of RBX1 as a RING finger domain protein for E2 recruitment of DAMAGED DNA BINDING 1 (DDB1) as an adaptor from the complex, and DDB1 BINDING WD40 (DWD) protein as a substrate receptor (Angles et al., 2006; Biedermann and Hellmann, 2010; He et al., 2006; Higa et al., 2006; Jin et al., 2006; Vierstra, 2009). DWD proteins commonly possess the DWD domain, which consists of 16 conserved amino acids within WD40 repeats, and 85 DWD proteins have been reported to exist in Arabidopsis (Lee et al., 2008). The following research for the substrate receptor of CRL4 in Arabidopsis suggested that an additional 34 proteins (WDXR) that only contain a portion of the DWD domain (WDxR region) also act as substrate receptors for CRL4 complex (Zhang et al., 2008). Based on these reports, it is believed that Arabidopsis have 119 potential substrate receptors for CRL4. There have been several studies of the functional involvement of DWD/WDXR in plant abiotic stress signaling. Two DWD proteins, DWD hypersensitive to ABA (DWA 1 and DWA2, have been reported as negative regulators in ABA-mediated drought stress signaling (Lee et al., 2010). dwa1dwa2 double mutants exhibit marked hypersensitivities in response to ABA and salt stress, and drought tolerance. Moreover, DWA1 and DWA2 are responsible for ABA5 degradation via direct interaction with ABA5, a key regulator in ABA signaling. Recently, a WDxR protein, ABA-HYPERSENSITIVE DCAF1 (ABD1), was identified as another negative regulator in ABA-mediated drought tolerance (Seo et al., 2014). UV-B signaling is also mediated by a group of DWD proteins such as COCKAYNE SYNDROME A-LIKE PROTEINS 1A (CSAat1A, also known as ATCSA-1), CSAt1B, and DAMAGE-SPECIFIC DNA BINDING PROTEIN 2 (DDB2), which are reportedly responsible for UV-B-induced damage repair (Biedermann and Hellmann, 2010; Zhang et al., 2010).

Despite emerging efforts to elucidate the functional relationship between abiotic stress signaling and CRL-mediated processes in plants, the role of CRL in heat stress tolerance is poorly understood. Here, we report on heat stress to lerant DWD1 (HTD1), a novel DWD that functions as a negative regulator in thermotolerance.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

Arabidopsis thaliana ecotype Columbia-0 was used as the wild type in this study, htd1-1 (SALK_081295) was obtained from the Arabidopsis Stock Center (http://www.arabidopsis.org/). Arabidopsis seedlings were grown as previously described (Lee et al., 2008) on GM plates or soils under long-day conditions (16 h light/8 h dark) in a controlled-environmental chamber at 22°C.

**Heat stress treatment**

Heat stress treatment was performed as described by Meiri and Breiman (2009), with slight modification. Briefly, three day-old wild type and htd1-1 seedlings cultivated at 22°C were incubated at 37°C for 3 h for acclimation, then allowed 3 days of recovery at 22°C. Next, heat shock treatment at 45°C was applied for 90 min, after which seedlings were grown for an additional 3 days to monitor phenotypes or conduct quantitative real-time PCR (qPCR) and immunoblot analysis.

**Genomic DNA extraction and genotyping analysis**

Genomic DNA was extracted with Edwards buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) and then used for PCR analysis with LbB1.3 primer and the forward and reverse primers for HTD1. The DNA primer sequences used for genotyping analysis are listed in Supplementary Table S1.

**RNA isolation and semi-quantitative RT-PCR analysis**

Total RNA was obtained using PureLink™ RNA mini kits (Ambion). Semi-quantitative RT-PCR analyses were performed as previously described (Lee et al., 2008). Amplicons produced with RPN6a Forward Primer and RPN6a Reverse Primer were used as internal controls. DNA sequences for the primer pairs used in semi-quantitative RT-PCR are presented in Supplementary Table S1.

**Quantitative real-time PCR (qPCR) analysis**

Quantitative PCR assay was conducted using a SolQ™ 2+ Real-Time PCR Smart mix (SolGent) according to the manufacturer’s instructions and the Rotor-Gene Q system (Qiagen). Transcript amounts of various genes from each sample were obtained using the comparative CT method and normalized against ACTIN2 expression. All assays were performed in-
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4500 (NP_001065677) and Z. mays LOC100217083 (NP_001136925). The WD40 domains are underlined with black lines and the DWD domains with a red line. The red asterisks represent the conserved tryptophan, aspartate and arginine residues with the WDxR motif. The regions with a high-level of conservation are indicated with black shade (100%), a middle-level with dark gray (80%) and a low level with light gray (60%).

dependently at least three times. DNA sequences for the primer pairs used in qPCR are presented in Supplementary Table S2.

**Generation of the 35S:HTD1/htd1-1 transgenic lines and the HTD1-MYC/FLAG-DDB1b/ddb1a transgenic plants**

For the 35S:HTD1/htd1-1 transgenic lines, the entire coding region for HTD1 was introduced into the binary vector pJIM19 (Gentamycin) under control of the cauliflower mosaic virus 35S promoter (Lee et al., 2010). The generated construct was then introduced into Agrobacterium strain GV3101 by electroporation and into Col-0 plants by the floral-dip method (Clough and Bent, 1998). The resulting T1 seeds were selected on MS plates containing 200 mg L–1 gentamycin to obtain T1 transgenic lines (Clough and Bent, 1998). In the case of HTD1-MYC/FLAG-DDB1b/ddb1a transgenic plants, the coding region of HTD1 cDNA was fused in frame to the 5’ end of nine copies of MYC that had already been subcloned in pJIM19 (Gentamycin) under control of the cauliflower mosaic virus 35S promoter. Finally, the generated construct was transformed into FLAG-DDB1b/ddb1a transgenic plants (Lee et al., 2008) via the GV3101 strain.

**Protein isolation and Western blot analysis**

Protein isolation and Western blot analysis were performed as previously described (Lee et al., 2010). Arabidopsis samples were extracted with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1× complete protease inhibitor (Roche). Extracted proteins were fractioned by SDS-PAGE and transferred to nitrocellulose membranes. The resulting blot was incubated in blocking buffer (1× PBS buffer including 0.1% Tween 20 and 5% dried nonfat milk) for 1 h at room temperature. Membranes were then incubated with the appropriate primary antibody for 2 h at room temperature or overnight at 4°C. Next, samples were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG) for 1 h at room temperature. Proteins were then visualized using a western blotting detection system (EZ-Capture II, ATTO). The assays were performed with anti-FLAG (Sigma-Aldrich), anti-MYC (Cell Signaling), anti-RPN6 (Chen et al., 2006), anti-HSP90-1 (Agrisera), anti-HSP70 (Agrisera), anti-HSP101 (Antibodies) or anti-α-tubulin (Sigma-Aldrich).

**Yeast two-hybrid assay**

Yeast two-hybrid assays were performed according to the

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Identification of a possible CRL4 substrate receptor related to heat stress response

To determine if a fraction of CRL4 complexes participates in heat stress response, we monitored mRNA levels of 119 CRL4 substrate receptor genes (DWD and WDxR) with or without heat stress treatments using microarray data from the AtGenExpress Visualization Tool (AVT) (Kilian et al., 2007). Among them, one DWD gene (At2g19540) was upregulated by about 3.2 times in response to heat stress (3.0 h of 38°C heat stress followed by 1.0 h recovery at 25°C). This gene was named HTD1. Our qPCR analysis confirmed that the transcript of HTD1 was increased by heat shock treatment, indicating possible involvement of HTD1 in cellular responses to heat stress (Fig. 3A). HTD1 is a 1407 bp region encoding 469 amino acids. A DWD domain of HTD1 is included in the WD40 domain region, similar to other DWD proteins (Figs. 1A and 1C).

RESULTS AND DISCUSSION

Identification of a possible CRL4 substrate receptor related to heat stress response

To determine if a fraction of CRL4 complexes participates in heat stress response, we monitored mRNA levels of 119 CRL4 substrate receptor genes (DWD and WDxR) with or without heat stress treatments using microarray data from the AtGenExpress Visualization Tool (AVT) (Kilian et al., 2007). Among them, one DWD gene (At2g19540) was upregulated by about 3.2 times in response to heat stress (3.0 h of 38°C heat stress followed by 1.0 h recovery at 25°C). This gene was named HTD1. Our qPCR analysis confirmed that the transcript of HTD1 was increased by heat shock treatment, indicating possible involvement of HTD1 in cellular responses to heat stress (Fig. 3A). HTD1 is a 1407 bp region encoding 469 amino acids. A DWD domain of HTD1 is included in the WD40 domain region, similar to other DWD proteins (Figs. 1A and 1C). To investigate if the heat-inducibility of HTD1 is functionally related to heat stress response, its T-DNA insertion homozygous mutant, htd1-1 (SALK_081295), was obtained and analyzed for HTD1. T-DNA was inserted 279 bp upstream from the CDS start site of this gene (Fig. 1A). RT-PCR analysis showed that HTD1 expression was significantly decreased in htd1-1 (Fig. 1B), indicating that the insertion line could be useful for further study. We identified several HTD1 homologs from various plant species in the GenBank database, including Glycine max (soybean), Solanum lycopersicum (tomato), Oryza sativa (rice) and...
Zea mays (maize). All selected proteins exhibited high similarity (more than 74.9%) at the amino acid level when compared to Arabidopsis HTD1 based on the EMBOSS Pairwise Alignment Algorithms at EMBL-EBI (http://www.ebi.ac.uk/Tools/emboss; Rice et al., 2000). All five proteins shared highly conserved WD40 and DWD regions and exactly matched the WDxR motif, which is a crucial site for binding to the adaptor protein of CRL4 complex (Fig. 1D). These results indicate that HTD1 and its related proteins may be highly conserved in the plant kingdom and play shared roles from dicots to monocots. Thus, it is expected that the results of the Arabidopsis HTD1 study could be applied to other useful crops such as rice, maize and tomato.

The decrease in HTD1 expression leads to heat stress tolerance

To elucidate the possible role of HTD1 in heat stress tolerance, three day-old wild type and htd1-1 seedlings were exposed to heat treatment at 37°C for 3 h, followed by 3 days recovery time (for acclimation) before exposure to 45°C heat shock for 90 min. (Fig. 2A). Following heat shock, seedlings were grown for an additional 3 days to monitor phenotypic differences between wild type and htd1-1. Although a large portion of wild type seedlings collapsed and became bleached after heat shock, htd1-1 was much less affected by this treatment, indicating that it was thermotolerant (Fig. 2B). Indeed, the rate of bleached cotyledons was about three times lower in htd1-1 than the wild type (Fig. 2C). Next, to confirm whether the decrease in HTD1 expression was correlated with such tolerance phenotypes, we generated transgenic lines complementing htd1-1. Two independent complementation lines (35S:HTD1 in the background of htd1-1) showed thermotolerance levels similar to wild type (Fig. 2D). Therefore, the expression level of HTD1 functions as a negative regulator in thermotolerance in Arabidopsis. As shown in Fig. 1B, T-DNA insertion 279 bp upstream from the translation start site of HTD1 failed to knock-out HTD1 expression in htd1-1. Thus, the generation of transgenic lines that exhibit stronger repression of HTD1 expression would be required to generate plants with greater tolerance to heat stress, which would be useful in elucidation of the mechanism of HTD1 in heat stress response in plants in greater detail.

HTD1 negatively regulates the expression of various heat-inducible genes

Since htd1-1 exhibited enhanced heat stress tolerance (Fig. 2), we investigated whether the decrease in HTD1 expression altered the expression pattern of heat-inducible genes. As shown in Fig. 3B, four heat-inducible genes, HSP14.7, HSP21, At2g03020 and WRKY28 were hyper-induced in response to heat stress, indicating that HTD1 could play a negative role in the expression of various heat-inducible genes. These results imply that the selected heat-stress inducible genes may contribute to enhancement of thermotolerance of htd1-1. Since small HSPs function as molecular chaperones that prevent misfolding and aggregation of
their client proteins caused by heat stress (Waters, 2013), the enhanced expression of our selected shSP genes (HSP14.7, HSP21 and At2g03020) in htd1-1 suggest the involvement of genes in the heat tolerance of htd1-1. WRKY28 has been shown to participate in the activation of SA (salicylic acid) signal-related genes and defense responses against pathogens in Arabidopsis (van Verk et al., 2011). Additionally, when co-expressed with AtbHLH17, Arabidopsis WRKY28 is responsible for resistance against various abiotic stresses such as those caused by NaCl, mannitol and oxidation, indicating its versatile role in plant biotic/abiotic stress responses (Babitha et al., 2013). Furthermore, several WRKY genes (WRKY25, WRKY 26 and WRKY 33) that show heat-inducibility have been reported to regulate the expression of heat-inducible genes in response to heat shock and to be responsible for thermotolerance (Li et al., 2011). Based on the above reports together with the heat-inducibility of WRKY28 (Fig. 3B), WRKY28 hyper-induced in htd1-1 might be involved in modulation of the thermotolerance process in Arabidopsis.

HTD1 may be a component of CRL4 complex
Since HTD1 possesses a putative DWD domain within WD40 repeats, we wondered if HTD1 could act as a part of CRL4 complexes. Therefore, we checked the interaction between DDB1a and HTD1 using a yeast two hybrid assay (Fig. 4A). Although the empty vector and green fluorescent protein (GFP) as prey showed weak binding activities with DDB1a as bait, HTD1 as prey exhibited much stronger binding activities with DDB1a than the above negative controls. To confirm that HTD1 is a component of the CRL4 complex in planta, we introduced HTD1-MYC constructs into FLAG-DDB1b/ddb1a plants, after which in vivo co-immunoprecipitation (co-IP) assays were performed to check the physical association between DDB1 and HTD1. As shown in Fig. 4B, MYC-tagged HTD1 proteins were successfully precipitated with FLAG-tagged DDB1b. Taken together, these results indicate that HTD1 interacts with the adaptor of the CRL4 complex and HTD1 may play its role as a possible component of CRL4 complex.

HTD1 can be associated with HSP90-1 in planta
As shown in Figs. 2 and 3, HTD1 negatively regulates the thermotolerance process and heat-induction of various heat-inducible genes. For this action, HTD1 may directly interact with signaling component proteins involved in heat stress tolerance and negatively regulate their activities or stabilities since HTD1 is expected to function as the substrate receptor CRL4 E3 ligase (Figs. 4A and 4B). Three heat shock proteins were selected as the candidate substrates of CRL4<sup>HTD1</sup> in heat stress response, HSP70, HSP101 and HSP90-1 (an inducible form of HSP90), since they play a central role in thermotolerance in plants (Wang et al., 2004). To check whether CRL4<sup>HTD1</sup> is directly involved in the decrease of activities or stabilities of such HSP proteins via UPS (ubiquitin-proteasome system), we investigated the in vivo association pattern between HTD1 and the HSPs. The co-IP assays demonstrated that HTD1 was associated with HSP90-1, but not with HSP70 and HSP101, in planta (Fig. 4B), implying the functional connection between HSP90-1 and HTD1. However, in our yeast two hybrid assay, HSP90-1 did not directly interact with HTD1 (Fig. 4C). Therefore, it is thought that the negative regulation of HTD1 in thermotolerance may be related to the indirect physical association between HTD1 and HSP90-1, at least in part, and that this association seems to require the mediation of an unknown factor. Based on this association, we hypothesized that HTD1 could be involved in degradation of HSP90-1. Actually, it was reported that taxotere (a cancer chemotherapeutic drug) reduced the function of HSP90 by ubiquitination and subsequent proteasomal degradation of HSP90 in HUVEC, indicating that HSP90 could be a target of ubiquitination and degraded via UPS (Murtagh et al., 2006). However, when we compared HSP90-1 protein levels following heat stress in wild type and htd1-1, the levels of HSP90-1 proteins from both plants were comparable (Supplementary Fig. S1). These results indicate that the CRL4 complex mediated by HTD1 might play a different role from a typical E3 ubiquitin ligase complex, which is responsible for the substrate recognition and degradation via UPS. It was recently reported that REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2 function as negative regulators in both processes of photomorphogenesis.

**Fig. 4.** Interaction patterns of various proteins with HTD1. (A) Direct interaction between HTD1 and the adaptor of the CRL4 complex based on yeast two-hybrid assays. Assays were performed with HTD1 protein as prey and DDB1a as bait to check their interactions. Empty vector (B42) and GFP proteins (B42-GFP) were used as negative controls. (B) Co-immunoprecipitation (Co-IP) assay of HTD1 with DDB1b and various HSP proteins. Transgenic plants overexpressing FLAG-DDB1b or FLAG-DDB1b/HTD1-MYC were used for these assays. The immunoblot used anti-RPN6 as a loading control. Total, 5% of the crude extracts used for Co-IP assays. (C) Interaction pattern between HTD1 and HSP90-1 in yeast two hybrid assay.

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and stress acclimation mediated by UV-B in Arabidopsis (Gruber et al., 2010). These genes commonly possess the conserved DWD domains and have therefore been categorized into the Arabidopsis DWD protein group (Lee et al., 2008), despite lack of evidence of their direct interactions with DDB1. They inhibit the interaction between UV RESISTANCE LOCUS 8 (UVR8) and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) via competition with COP1 for binding of UVR8, which is essential for the UV-B signal transduction pathway. This competition leads to facilitation of UVR8 redimerization, which makes UVR8 inactive. This report implies that a portion of the proteins categorized into the DWD group play an atypical role rather than the role as E3 ligase for substrate degradation. Arabidopsis ROF1 (AtFKBP62) has been reported to associate with HSP90-1-HsfA2 and play a role in long term acquired thermotolerance by modulating the expression levels of HsfA2-dependent sHSPs, which are responsible for heat stress tolerance (Mein and Breiman, 2009), whereas ROF2 is a ROF1 homologous protein that interacts directly with ROF1 and negatively functions in expression of sHSPs by joining in the COP1-HSP90-1-HsfA2 complex and sequestering ROF1 from the complex during recovery from heat stress (Mein et al., 2010). Similar to ROF2, the possible function of association/sequestration of HTD1 might be to disturb the role of a co-chaperone (like ROF1) that helps HSP90-1 function, which would explain the connection between HTD1/HSP90-1 association and the hyper-induction of sHSPs in htt1-1 observed in our study (Figs. 3B and 4B). Alternatively, HTD1 might interfere with the action of HSP90-1 in the heat tolerance process by associating with HSP90-1 via an unknown adaptor that links between HTD1 and HSP90-1, and then keeping HSP90-1 away from the formation of multichaperone complexes. However, we cannot exclude the possibility that HSP90-1 can be monoubiquitinated by CRL4

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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