

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

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Cullin4-RING ubiquitin ligase (CRL4) is a family of multi-subunit E3 ligases. To investigate the possible involvement of CRL4 in heat stress response, we screened T-DNA insertion mutants of putative CRL4 substrate receptors that exhibited altered patterns in response to heat stress. One of the mutants exhibited heat stress tolerance and was named heat stress tolerant DWD1 (*htd1*). Introduction of *HTD1* gene into *htd1-1* led to recovery of heat sensitivity to the wild type level, confirming that the decrease of *HTD1* transcripts resulted in heat tolerance. Therefore, *HTD1* plays a negative role in thermotolerance in *Arabidopsis*. Additionally, *HTD1* directly interacted with *DDB1a* in yeast two-hybrid assays and associated with *DDB1b* *in vivo*, supporting that it could be a part of a CRL4 complex. Various heat-inducible genes such as *HSP14.7*, *HSP21*, *Atg03020* and *WRKY28* were hyper-induced in *htd1-1*, indicating that *HTD1* could function as a negative regulator for the expression of such genes and that these genes might contribute to thermotolerance of *htd1-1*, at least in part. *HTD1* was associated with *HSP90-1*, a crucial regulator of thermotolerance, *in vivo*, even though the decrease of *HTD1* did not affect the accumulation pattern of *HSP90-1* in *Arabidopsis*. These findings indicate that a negative role of *HTD1* in thermotolerance might be achieved through its association with *HSP90-1*, possibly by disturbing the action of *HSP90-1*, not by the degradation of *HSP90-1*. This study will serve as an important step toward understanding of the functional connection between CRL4-mediated processes and plant heat stress signaling.

## 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,

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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 dissolution 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)/WDxR protein as a substrate receptor (Angers 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 ABI5 degradation via direct interaction with ABI5, 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), CSAat1B, 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 process 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

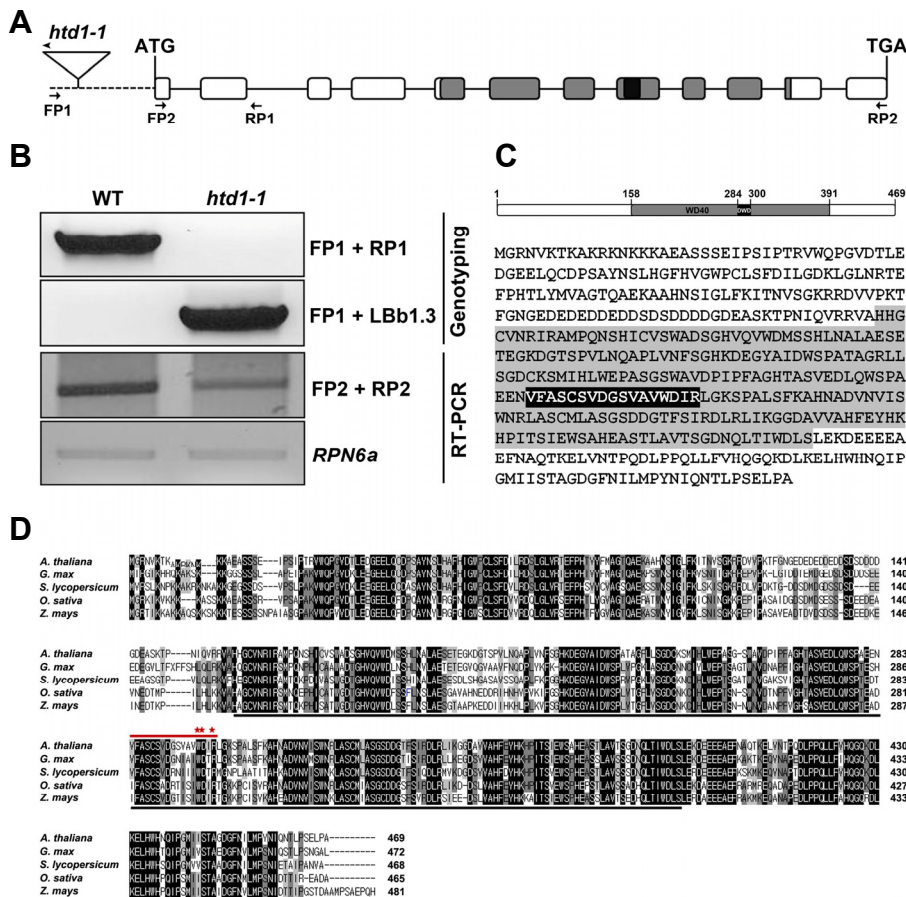
Genomic DNA was extracted with Edwards buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) and then used for PCR analysis with LBb1.3 primer and the forward and reverse primers for *HTD1*. The DNA primer sequences used for genotyping analysis are listed in Supplementary Table S1.

### 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 Solg™ 2x 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-



**Fig. 1.** Characterization of *htd1-1* and HTD1. (A) Genomic structure and T-DNA insertions in *HTD1* and HTD1 protein structure. Exons are shown as boxes, introns are represented by lines and the 5'-untranslated region is depicted by dotted lines. Grey and black boxes indicate the WD40 region and DWD domain, respectively. The arrows indicate the position and direction of primers for genotyping and RT-PCR analysis. The arrowhead represents the direction of the LBB1.3 primer for genotyping analysis. (B) Genotyping and semi-quantitative RT-PCR analysis of wild type and *htd1-1*. *RPN6a* was used as an internal control for semi-quantitative RT-PCR assays. (C) Amino acid sequence of HTD1. The gray-shaded region represents the WD40 repeats and the black-shaded region indicates the DWD domain. (D) Alignment of the HTD1 protein and their homologues in various plant species.

The alignment was performed with HTD1 and the proteins that showed the highest homology with HTD1 from each species. The corresponding proteins are *A. thaliana* HTD1 (NP\_179544), *G. max* glutamate-rich WD repeat-containing protein 1-like (XP\_003531198), *S. lycopersicum* glutamate-rich WD repeat-containing protein 1-like isoform 2 (XP\_004243762), *O. sativa* Os11g013

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%).

independently 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

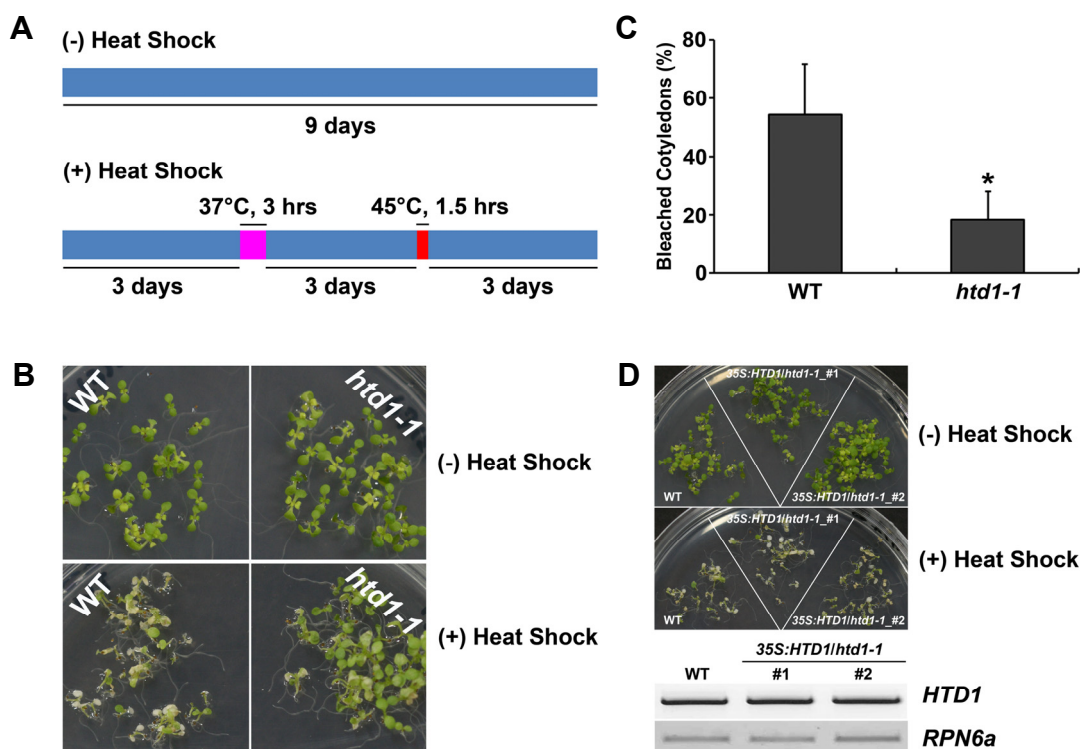
For the 35S:HTD1/*htd1-1* transgenic lines, the entire coding region for *HTD1* was introduced into the binary vector pJIM19 (Gentamycin) under control of the cauliflower mosaic virus 35S promoter (Lee et al., 2010). The generated construct was then introduced into *Agrobacterium* strain GV3101 by electroporation 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<sup>-1</sup> 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, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1× complete protease inhibitor (Roche). Extracted proteins were fractionated 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- $\alpha$ -tubulin (Sigma-Aldrich).

#### Yeast two-hybrid assay

Yeast two-hybrid assays were performed according to the



**Fig. 2.** Correlation between *HTD1* and heat stress phenotype. (A) Scheme of heat shock treatment in this study. (B) The phenotypes of wild type (WT) and *htd1-1* plants in response to heat shock treatments. Three day-old wild type and *htd1-1* seedlings grown at 22°C were incubated at 37°C for 3 h for acclimation, then allowed 3 days of recovery at 22°C. Heat shock treatments at 45°C for 90 min were then applied, after which the seedlings were grown for an additional 3 days in order to monitor phenotypes. The plants were photographed 3 days after 45°C heat shock. (C) Rate of bleached cotyledons of wild type and *htd1-1* seedlings under heat stress. Seedlings with more than 50% bleached cotyledons were counted from both samples. Values are the means  $\pm$  SD ( $n = 3$ ). Statistically significant differences between the wild type and *htd1-1* were identified by a Student's *t*-test; the single star indicates  $P < 0.05$ . (D) The phenotypes of two *HTD1*-complementation lines in *htd1-1* (*35S:HTD1/htd1-1*) in response to heat shock treatments. The condition for heat treatment was the same as that in (B). For semi-quantitative RT-PCR analysis to monitor *HTD1* expressions, 9-day-old wild type and *35S:HTD1/htd1-1* seedlings grown in the absence of heat shock were used. Analysis was conducted with FP2 and RP2 primers. *RPN6a* was used as an internal control.

MATCHMAKER LexA Two-Hybrid System manual (Clontech). p80p-lacZ and the gene constructs cloned with pB42AD or pLexA were introduced into yeast strain EGY48. The transformants were then confirmed by growth on SD/-His/-Trp/-Ura plates. Interactions between proteins were monitored on SD/Gal/Raf/-His/-Trp/-Ura plates including 80 mg L<sup>-1</sup> X-Gal.

#### In vivo co-immunoprecipitation assay

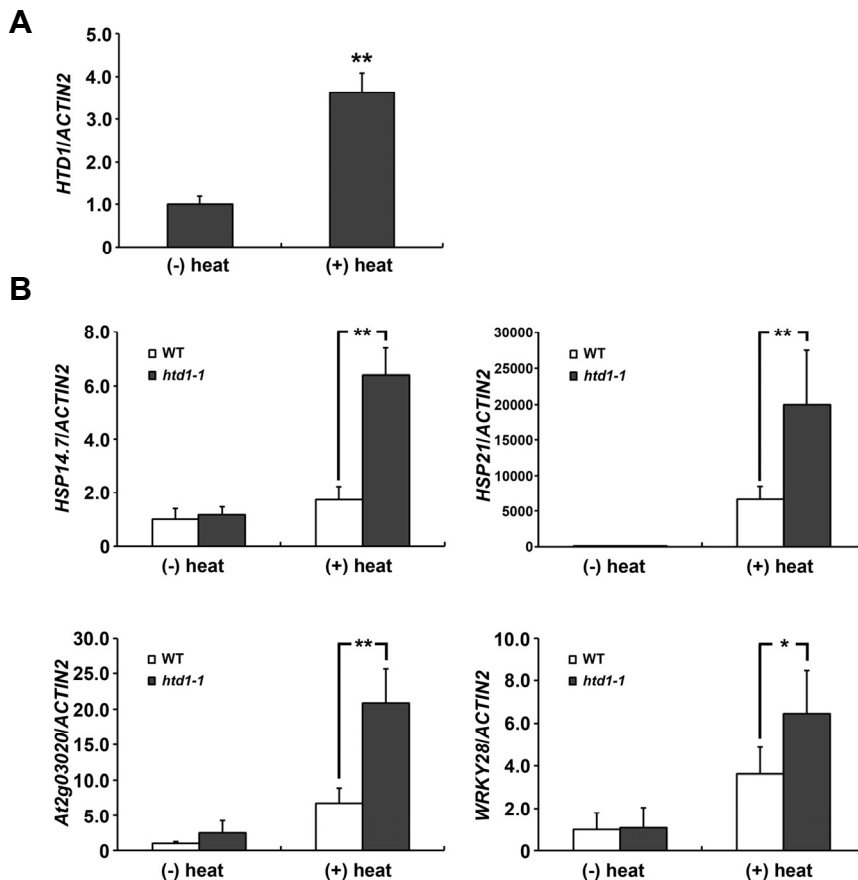
*Arabidopsis* seedlings were resolved in protein extraction buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 1 $\times$  complete protease inhibitor (Roche)]. Next 30-50  $\mu$ l of anti-MYC affinity matrix (COVANCE) were added to 1-5 mg of protein extracts. The mixture was then incubated overnight at 4°C and washed at least three times with protein extraction buffers. For elution, beads with immunoprecipitated proteins were resuspended in Laemmli sample buffer (BioRad) and boiled for 5 min.

## 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



**Fig. 3.** Expression patterns of various heat-inducible genes in wild type and *htd1-1*. (A) Heat-inducibility of *HTD1*. Quantitative real-time PCR analysis was performed with wild type in the presence or absence of heat shock. The condition for heat treatment was the same as that in Fig. 2B. Relative amounts of *HTD1* were normalized to the levels of *ACTIN2* in the same sample. Values are the means  $\pm$  SD ( $n = 3$ ). Statistical analysis was conducted by a Student's *t*-test; \*\* $P < 0.05$ . (B) Hyper-induction of various heat-inducible genes in *htd1-1*. Quantitative real-time PCR analysis was performed with wild type and *htd1-1* seedlings in the presence or absence of heat shock. The conditions for heat treatment were the same as described in Fig. 2B. Relative amounts of *HSP14.7*, *HSP21*, *At2g03020* and *WRKY28* were normalized to the levels of *ACTIN2* in the same sample. Values are the means  $\pm$  SD ( $n = 6$ ). Statistical analysis was conducted by a Student's *t*-test; \* $P < 0.05$ ; \*\* $P < 0.01$ .

*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.

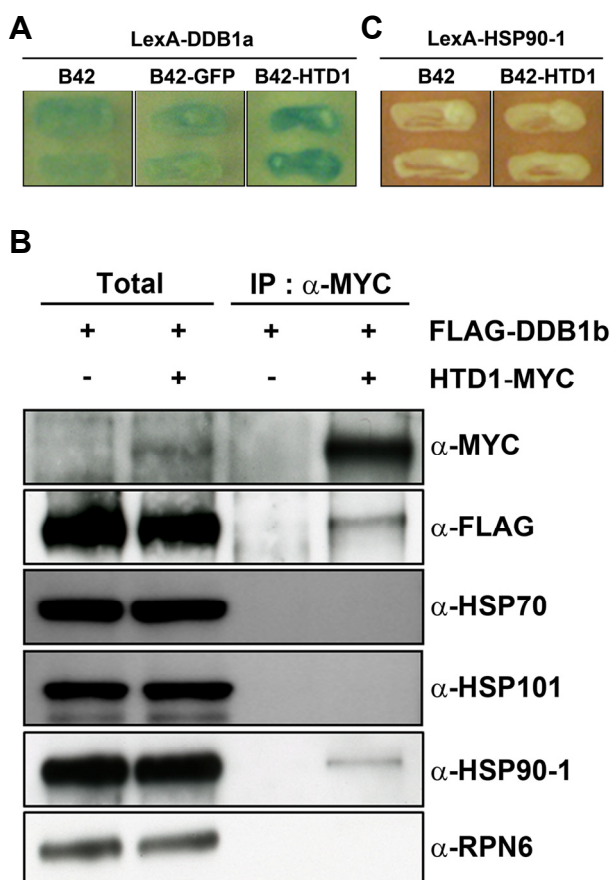
#### The decrease in *HTD1* expression leads to heat stress tolerance

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* is highly related to sensitivity to heat stress. Taken together, 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* (At5g47600), *HSP21* (At4g27670), *At2g03020* (as Heat shock protein HSP20/alpha crystallin family protein) and *WRKY28* (At4g18170), 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



**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.

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*, *WRKY26* and *WRKY33*) 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* lines to generate HTD1-MYC/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 ubiquitylation 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

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 destruction. *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 (Meiri 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 ROF1-HSP90-1-HsfA2 complex and sequestering ROF1 from the complex during recovery from heat stress (Meiri 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 *htd1-1* 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<sup>HTD1</sup>, which leads to the alteration of HSP90-1's activity independent of proteolysis. Therefore, further investigation of the ubiquitination pattern of HSP90-1 under the presence/absence of HTD1 would be required for the detailed elucidation of the relationship between HSP90-1 and HTD1. Moreover, since it may be possible that CRL4<sup>HTD1</sup> negatively regulates the stability of unidentified positive regulator(s) in heat stress signaling, additional investigations of the binding partners of HTD1 as potential substrates should be conducted to determine if a possible CRL4 complex mediated by HTD1 could exert E3 ligase activity for protein degradation.

Here, we report a new type of DWD protein that negatively regulates the thermotolerance process in *Arabidopsis*. Although a decrease of *HTD1* expression results in enhanced thermotolerance and increased expression of various heat-inducible genes, detailed information regarding the role of HTD1 in heat stress response was not fully provided by this study. Further evidence of the role of HTD1 in the thermotolerance process could be obtained by genetic combination between *htd1-1* and various heat stress-related mutants such as *rof1* and knockout mutants related to *HSP* and *Hsf* genes. Furthermore, based on the link between HTD1 and HSP90 observed in the present study and reports of the versatile role of HSP90 involved in biotic and abiotic stresses (Botër et al., 2007; Krishna and Gloor, 2001), investigation of the potential role of HTD1 in other stress signaling pathways would serve as a platform for elucidation of the detailed action mechanism of HTD1 in plants.

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

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