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# $Cu^{2+}$ triggers reversible aggregation of a disordered His-rich dehydrin MpDhn12 from *Musa paradisiaca*

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**Peiqiang Mu*, Dongru Feng*, Jianbin Su, Yang Zhang, Jinran Dai, Honglei Jin, Bing Liu, Yanming He, Kangbiao Qi, Hongbin Wang† and Jinfa Wang**

State Key Laboratory for Biocontrol and Key Laboratory of Gene Engineering of Ministry of Education, School of Life Sciences, Sun Yat-sen University, 510275, Guangzhou, P. R. China

†Hongbin Wang, School of Life Sciences, Sun Yat-sen University, 510275, Guangzhou, P. R. China. Tel/Fax: +862084039179, email: Wanghb@mail.sysu.edu.cn

*These authors contributed equally to this work.

**Copper is an essential nutrient, but it is toxic in excess. Here, we cloned and characterized a His-rich low molecular weight dehydrin from *Musa paradisiaca*, *MpDhn12*. Analysis by circular dichroism (CD) spectra and a thermal stability assay showed that MpDhn12 is an intrinsically disordered protein, and immobilized-metal affinity chromatography (IMAC) analysis revealed that MpDhn12 can bind $Cu^{2+}$ both *in vitro* and *in vivo*. Interestingly, MpDhn12 aggregated under excess $Cu^{2+}$ conditions, and the aggregation was reversible and impaired by histidine modification with diethylpyrocarbonate (DEPC), while the disordered structure of another dehydrin ERD14 (as a control) was not changed. Furthermore, MpDhn12 could complement the copper-sensitive phenotype of yeast mutant Δ*sod1*. These results together suggested that MpDhn12 may take part in buffering copper levels through chelation and formation of aggregates in excess $Cu^{2+}$ conditions. To the best of our knowledge, it is the first report that a dehydrin interchanged between disordered and aggregated state triggered by copper.**

*Keywords*: aggregation/copper homoeostasis/$Cu^{2+}$ binding/dehydrin/disorder.

*Abbreviations*: AAS, atomic absorption spectra; CD, circular dichroism; Cu-IMAC, IMAC column charged with $Cu^{2+}$; Free-IMAC, IMAC column charged no metal; IMAC, immobilized metal ion affinity chromatography; MpDhn12, *Musa Paradisiaca* dehydrin 12 kDa; MTs, metallothioneins; PCs, phytochelatins.

Copper is essential for the nutrition of living organisms, since it acts as a co-factor in numerous proteins (*1*, *2*). However, excessive copper can produce reactive oxygen species (ROS) through the Haber–Weiss reaction (*3*, *4*), which subsequently damages macromolecules (*4*, *5*). Because of this, tightly controlled homoeostatic conditions for copper concentrations are crucial (*2*, *6*, *7*).

Excessive copper can be chelated by low molecular weight proteins, polypeptides, organic acids and amino acids (*8*, *9*). Among them, metallothioneins (MTs) and phytochelatins (PCs) are well-characterized (*10–13*). MTs are a class of ubiquitous low molecular weight Cys-rich proteins that protect cells against the toxic effects of excessive metals, including copper, by chelating them via their Cys thiol groups (*12*, *14*). PCs are biosynthesized polypeptides which are characterized by the amino acid sequence $(\gamma\text{-Glu-Cys})_n$ Gly, where *n* ranges from 2 to 11 (*15*, *16*). Both MTs and PCs are believed to play important roles in copper detoxification via chelating it to Cys thiol groups (*12*, *14*, *16*, *17*). However, bioinformatic and proteomic analysis suggest that copper-binding ligands prefer His (*18–20*). Recently, several members of the dehydrin family were reported to have metal-binding activities including copper. The dehydrin family proteins are featured with three typical conserved domains: K-segment, Y-segment and S-segment, and less conserved regions (Φ-segments) (*21*, *22*). Based on the numbers and order of the three conserved domains, dehydrins are divided into five subgroups: $Y_nSK_n$, $Y_nK_n$, $SK_n$, $K_n$ and $K_nS$ (*23*, *24*). The Φ-segments are usually rich in fairly polar and charged amino acids and show considerable variation between different dehydrins (*21*, *22*). It was proposed that the polar and charged amino acids may contribute to metal binding (*25*, *26*). In *Arabidopsis*, LTI30 was reported to bind $Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Zn^{2+}$ (*27*); RAB18, LTI29 and COR47 were reported to bind $Cu^{2+}$ and $Ni^{2+}$ but not $Co^{2+}$ or $Zn^{2+}$ (*27*). ITP from *Ricinus communis* was found to bind $Fe^{3+}$ preferentially, and also complexes with $Cu^{2+}$, $Zn^{2+}$ and $Mn^{2+}$, but not $Fe^{2+}$ (*25*). CuCOR15 from *Citrus unshiu* was shown to bind $Cu^{2+}$ preferentially but also $Ni^{2+}$, $Co^{2+}$, $Zn^{2+}$, $Fe^{3+}$ with different affinities *in vitro* (*26*). Although there is some specificity for different metal ions, almost all the identified metal-binding dehydrins have copper-binding activity. As dehydrins are usually high in His content, which ranges from 3.2 to 13.5% in *Arabidopsis*, and His was reported to be preferentially involved in copper binding (*18*, *20*), the high affinity of copper to dehydrins may be related to the high His content. However, little work has been done with respect to the structural changes after binding to copper, and the function of copper-binding activity for dehydrins is still unclear.

Plantain (*Musa paradisiaca* L.; ABB Group) has been considered to be tolerant to various types of

environmental stresses (*28*). In our previous studies, two abiotic stress response genes *MpRCI* and *MpAsr* and two biotic stress response genes *MpChi-1* and *MpGlu* were identified from plantain. The *MpRCI* and *MpAsr* confers cold tolerance and osmotic tolerance respectively (*29*, *30*), and the *MpChi-1* and *MpGlu* can inhibit the growth of *Gloeosporium musarum* Cke and Massee and *Fusarium oxysporum* fsp. *cubense* respectively (*31*, *32*). In this study, a dehydrin gene *MpDhn12* was cloned from plantain, which encodes a low molecular weight $K_nS$ type dehydrin with $Cu^{2+}$-binding activity *in vitro* and *in vivo*. We subsequently focused on the structural characteristics when exposed to excess $Cu^{2+}$ and the biological implications of the structural changes triggered by $Cu^{2+}$ is discussed.

## Materials and Methods

### Plant materials and growth conditions

Plantain seedlings and tobacco were grown in a greenhouse under 16 h light/25°C and 8 h dark/20°C. Detached leaves of 8-week-old T2 transgenic tobacco were used for $Cu^{2+}$-binding assays and detection of MpDhn12 aggregates *in vivo*.

### Cloning and construction of expression plasmids for MpDhn12 and ERD14

The cDNA for *MpDhn12* was obtained by the rapid amplification of cDNA ends (RACE) method (*30*) using the GeneRacer TM Kit (Invitrogen, USA). The open reading frames (ORF) of *MpDhn12* and *ERD14* were amplified by RT–PCR and subcloned into pGEX4T-1(EcoRI, XhoI) to construct the recombinant expression vector, and into the binary vector pCAMBIA2301(HindIII, SpeI) to construct the tobacco expressing vector.

### Protein expression, purification and polyclonal MpDhn12 anti-serum production

The expression of recombinant proteins (GST-MpDhn12 and GST-ERD14) was induced in *Escherichia coli* BL21 (DE3). GST fusion proteins were purified by Glutathione sepharose 4B (GE healthcare, USA) affinity chromatography first, and the GST tag was then removed by on-column thrombin (GE healthcare) digestion according to the handbook of the GST gene fusion system (18-1157-58, GE healthcare). For details, see Materials and Methods section in the Supplementary Data.

Recombinant MpDhn12 protein was used to produce polyclonal anti-serum in rabbits according to the methods described by Hu *et al.* (*33*). The titres of the anti-serum were determined by indirect ELISA according to standard procedures (*34*).

### Circular dichroism spectroscopy

Circular dichroism (CD) measurements were carried out using a CD Spectrometer J810 (JASCO, Japan). The protein concentration was 5 μM in sodium phosphate buffer (10 mM $K_2HPO_4$, 20 mM NaCl, pH 7.4. Analytical conditions were as follows: temperature, 25°C; path length, 1.0 cm; band width, 2.0 nm; sensitivity, 20 mdeg; response, 4 s; wave length, from 250 to 190 nm; scan speed, 50 nm/min; step resolution, 0.1 nm; accumulation, 4. Finally, noise reduction by smoothing was applied to the spectrum.

### Trypsin proteolysis and thermal stability analysis

Protease sensitivity was tested with trypsin. MpDhn12 or ERD14 (2 μg) was treated with 200 ng trypsin (Sigma Aldrich, USA) in the absence or presence of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM $CuCl_2$. The reactions were carried at 37°C for 30 min and stopped by adding SDS–PAGE loading buffer and heated to 100°C for 5 min. The samples were analysed by 15% SDS–PAGE.

For analysis of thermal stability, MpDhn12, ERD14 (2 μg, 20 μl) and BSA (2 μg, 20 μl) were boiled at 100°C for 10 min and centrifuged at 20 000 *g* for 20 min at 25°C. ERD14 (2 μg, 20 μl) and BSA (2 μg, 20 μl) were used as controls. The supernatants (17 μl) were analysed on a 12% SDS–PAGE gel.

### Chemical modification of histidines by Diethylpyrocarbonate

Diethylpyrocarbonate (DEPC) modification of MpDhn12 was carried out as described by Miles (*35*). Briefly, 5 μM purified MpDhn12 proteins in PBS buffer (PH 7.4) were incubated with DEPC (dissolved in absolute ethanol) at a final concentration of 0 (control), 10, 50, 250 μM, 1, 5 and 10 mM at 25°C for 15 min. The number of modified histidine residues was determined from the difference in absorbance at 240 nm between protein solutions containing DEPC and those with the same amount of ethanol (control tubes), using the molar absorption coefficient, 3200/cm/M.

### $Cu^{2+}$-binding assay

$Cu^{2+}$ binding of MpDhn12 or His modified MpDhn12 (treated with MpDhn12) *in vitro* were analysed by IMAC according to the procedure described by Ueda *et al.* (*36*) and an ultrafiltration method described by Nishikawa *et al.* (*37*). $Cu^{2+}$ binding of MpDhn12 *in vivo* was analysed by IMAC together with western blot. For details, see Materials and Methods section in Supplementary Data.

### Detection of aggregates triggered by $Cu^{2+}$

The aggregation of recombinant MpDhn12 or DEPC treated recombinant MpDhn12 were detected *in vitro* by limited trypsin digestion, CD spectra and precipitation analysis. The aggregates of MpDhn12 *in vivo* were detected by western blot. For details, see Materials and Methods section in Supplementary Data.

### Copper tolerance assay in yeast

The genotypes of *Saccharomyces cerevisiae* strains used are listed in Supplementary Table S1. The ORF encoding MpDhn12, AtMT1a, AtMT2b or AtMT3 were cloned into the pYES2 expression vector (Invitrogen, USA) and transformed into yeast strains using the LiAc method (*38*). For the copper tolerance test, the overnight cultures of yeast strains were diluted 20-fold in a fresh liquid synthetic complete medium (SC medium) and incubated at 30°C until the optical density ($OD_{600}$) reached 1.0. Three microlitres of serially diluted cultures were spotted on the SC medium plates supplemented with 0 or 150 μM $CuSO_4$. The plates were incubated at 30°C for 3 days and photographed.

## Results

### MpDhn12 is rich in His containing copper-binding motifs and is intrinsically disordered

We isolated the *M. Paradisiaca* dehydrin-like gene, *MpDhn12* (GenBank accession No: JF502066) from the cDNA library of plantain leaves (*30*). The full-length cDNA is 639-bp long with an ORF of 321 bp (Fig. 1A). The deduced amino acid sequence showed that MpDhn12 is a $K_nS$ type dehydrin which contains a typical K-segment of the dehydrin family at positions 55–72 and a tract of Ser residues (S-segment) at the C terminus (Fig. 1A). Importantly, the His content of MpDhn12 is as high as 16.8%. Dehydrins usually contain a high proportion of His, which ranges from 3.2% to 13.5% in the *Arabidopsis* dehydrins. Additionally, the His distribution of MpDhn12 presents as a pattern of His-$X_n$ ($n = 0$, 3, 4, 5, 6, 14, 15)-His (Fig. 1B). The His-$X_n$-His sequences ($n$ can be potentially ranged from 0 to 12) were previously shown to be preferentially involved in copper binding (*18*, *20*, *26*, *39*), suggesting that MpDhn12 may bind copper.

Furthermore, MpDhn12 was predicted to be a disordered protein by PrDOS (http://prdos.hgc.jp/cgi-bin/top.cgi) and RONN (http://www.strubi.ox.ac.uk/RONN) (Fig. 1C). The CD spectra also showed that recombinant MpDhn12 had a strong negative peak at 198.2 nm, which was characteristic of the coil conformation (Fig. 1E) (*40*) and indicated that MpDhn12 is a typical disordered protein. The disordered structure of

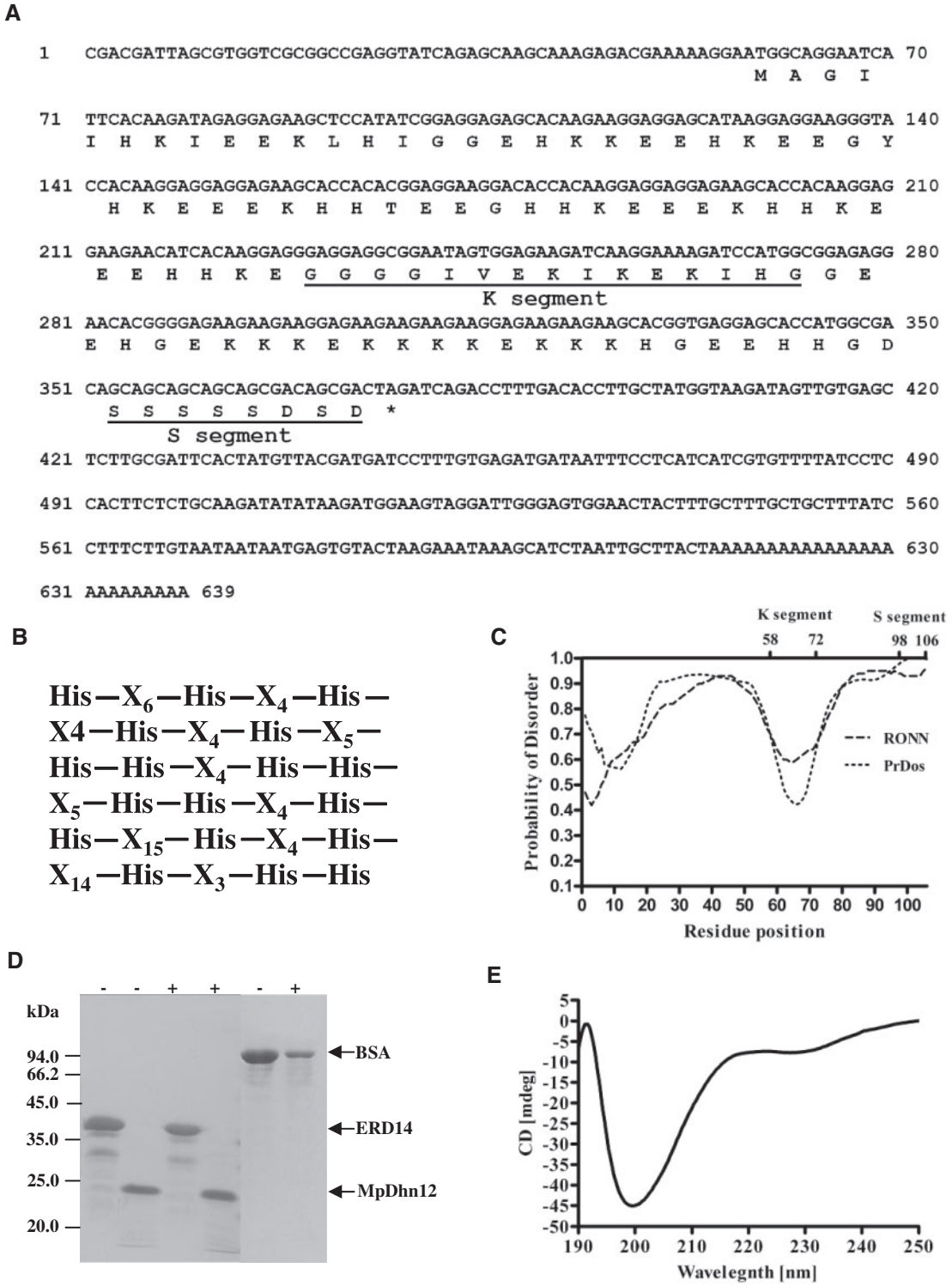


**Fig. 1 Sequences and disordered structure of MpDhn12.** (A) Nucleotide and deduced amino acid sequence of *MpDhn12*. (B) His distribution of MpDhn12. X represents any amino acids other than His. (C) Prediction of protein disorder *in silico* by PrDOS or RONN. (D) Thermal stability analysis of MpDhn12. Proteins were (+) or were not (−) incubated at 100°C for 10 min and centrifuged. The supernatants were analysed by SDS–PAGE. (E) CD spectra analysis of MpDhn12.

MpDhn12 was further determined by thermal stability analysis. After boiling for 10 min, MpDhn12 and ERD14 (positive control) were still dissolved in the supernatants, while most of the BSA (negative control) aggregated into precipitates (Fig. 1D). It is worth noting that MpDhn12 showed low mobility on an SDS–PAGE gel, which ran at ~22 kDa (Fig. 1E and Supplementary Fig. S1A), whereas the calculated molecular mass is 12.17 kDa (ProtParam, http://www.expasy.org/tools/protparam.html). The apparent high

molecular mass caused by highly hydrophilic residues is also a characteristic of disordered proteins (*41*, *42*). Taken together, the deduced protein of *MpDhn12* is a low molecular weight intrinsically disordered and thermally stable $K_nS$ type dehydrin which contains His-rich copper-binding motifs.

### *Cu$^{2+}$-binding property of MpDhn12*

The His-$X_n$ ($n = 0$–12)-His sequences of MpDhn12 suggested that it may be able to bind $Cu^{2+}$. We first analysed the $Cu^{2+}$-binding activity by IMAC. Recombinant MpDhn12 was retained on a $Cu^{2+}$ immobilized IMAC column (Cu-IMAC) but not on an IMAC column without immobilized metal (free-IMAC) (Fig. 2A). The Cu-IMAC trapped MpDhn12 could be eluted with the histidine solution or ethylenediamine tetraacetic acid (EDTA) solution (Fig. 2A), suggesting that MpDhn12 can bind $Cu^{2+}$ *in vitro* and His may be involved in the $Cu^{2+}$ binding. To test whether the $Cu^{2+}$ binding of MpDhn12 is mediated by histidine, we treated MpDhn12 by DEPC which modifies histidine (*35*). The calculation of the number of histidine residues modified after treatment with DEPC is shown in Supplementary Table S2. As the concentration of DEPC increasing, the His modification rate of MpDhn12 increased. Interestingly, although the DEPC concentration was elevated to 10 mM, only 71.71% of the total His residues were modified. The incomplete His modification by DEPC was also observed in another disordered dehydrin CuCOR15 (*26*). We subsequently chose a moderate concentration of DEPC (1 mM) to treat MpDhn12 and examined its

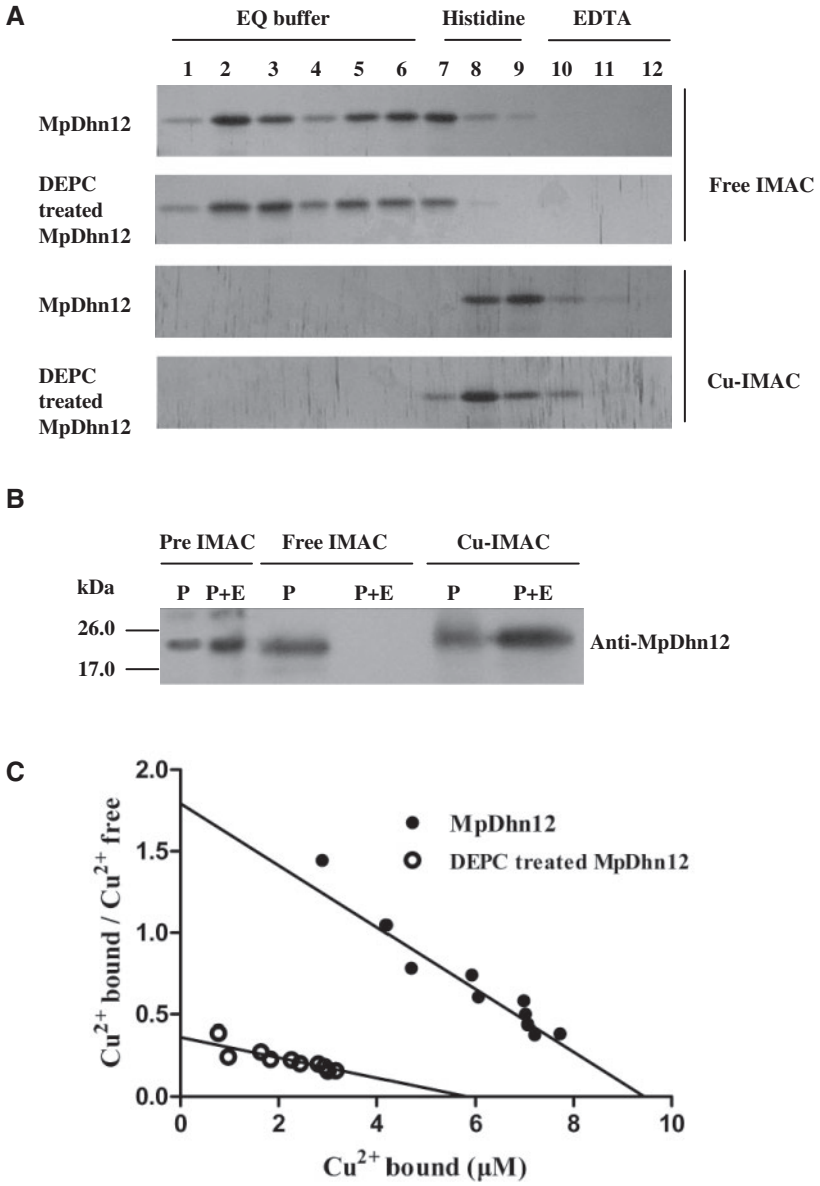


**Fig. 2 Investigation of $Cu^{2+}$-binding activity of MpDhn12.** (A) Investigation of *in vitro* $Cu^{2+}$-MpDhn12 binding by IMAC. Numbers indicate serially washed fractions by the indicated solution (1.5 ml each). (B) Scatchard plots of $Cu^{2+}$-MpDhn12 binding. (C) Investigation of $Cu^{2+}$-MpDhn12 binding *in vivo* by IMAC. Proteins extracted from *MpDhn12* overexpressing tobacco by PBS buffer plus EDTA (P+E) or not (P) were used for $Cu^{2+}$-MpDhn12 binding *in vivo* through IMAC and western blot.

$Cu^{2+}$-binding activity after His modification. Interestingly, MpDhn12 treated by 1 mM DEPC (60.86% of the total 18 histidines were modified, Supplementary Table S2) could also be trapped by Cu-IMAC (Fig. 2A), we guess the reserved $Cu^{2+}$-binding activity of DEPC treated MpDhn12 may be caused by the incomplete His modification.

To quantitate the metal-binding capacity of MpDhn12, a $Cu^{2+}$-binding assay combined with ultrafilitration and atomic absorption spectra (*37*) was carried out. Equilibrium binding was observed when solutions contain 50 μM MpDhn12 protein and varying concentrations of $CuCl_2$ (0.1–1 mM). The Scatchard plot showed that one molecule of MpDhn12 could bind 9.4 atoms with a dissociation constant of 5.08 μM (Fig. 2C). In the presence of 1 mM DEPC, only 5.6 $Cu^{2+}$ molecules bound to a molecule of the MpDhn12 protein with a dissociation constant of 15.55 μM (Fig. 2B). The results indicated that the MpDhn12 protein has nine $Cu^{2+}$-binding sites per molecule, and His is involved in the $Cu^{2+}$ binding.

To determine whether MpDhn12 bind to $Cu^{2+}$ in plant, a method similar to co-immunoprecipitation and pull-down in investigating protein–protein interactions based on the IMAC was devised as described in Materials and Methods section of Supplementary Data. In consistent with recombinant MpDhn12 from *E. coli*, the Cu-IMAC column could also trap MpDhn12 from proteins extracted from *MpDhn12* overexpressing tobacco as detected by anti-MpDhn12 (Fig. 2C). Interestingly, MpDhn12 was trapped in the free-IMAC when the proteins were extracted by EDTA-free PBS buffer, but not by PBS buffer plus EDTA (Fig. 2C), indicating that MpDhn12 had already bound to $Cu^{2+}$ *in vivo*.

### $Cu^{2+}$ triggers aggregation of MpDhn12

We next investigated whether binding of $Cu^{2+}$ can trigger conformational changes in MpDhn12. The limited trypsin digestion patterns of MpDhn12 (24 trypsin cleavege sites, http://expasy.org/tools/peptidecutter/) in the absence or presence of 0–1 mM $CuCl_2$ were examined. In the absence of $CuCl_2$, MpDhn12 was almost completely digested, while the digestion was gradually inhibited by increasing concentration of $CuCl_2$ (Fig. 3A). As a control, ERD14 was almost completely digested regardless of the presence of $CuCl_2$ or not (Fig. 3B). These results indicated that $Cu^{2+}$ may trigger a conformational change in MpDhn12.

To verify this hypothesis, we studied the structures of MpDhn12 and ERD14 in the absence or presence of $CuCl_2$ by CD spectra. $Cu^{2+}$ only up to 250 μM could trigger a minor transition of disordered to ordered structure of MpDhn12 (Fig. 3C). Surprisingly, the CD signal decayed sharply when $Cu^{2+}$ was present in the MpDhn12 solution (Fig. 3C), while $Cu^{2+}$ had almost no effect on the CD spectra of ERD14 which was used as a control (Fig. 3D). The sharp decay of CD signal suggested that MpDhn12 may be aggregated in the presence of $Cu^{2+}$.

To test this possibility, a precipitation analysis was carried out (*43*). The amount of soluble MpDhn12 decreased gradually with increasing concentrations of $Cu^{2+}$, while the soluble ERD14 remained constant (Fig. 3E). Additionally, the aggregated MpDhn12 could be resolubilized through diluting the $Cu^{2+}$ concentration with $Cu^{2+}$ free PBS buffer or through chelating by addition of EDTA (Fig. 3F). Taken together, these results indicated that $Cu^{2+}$ could trigger the reversible aggregation of MpDhn12 *in vitro*.

The aggregation of MpDhn12 was also detected *in vivo* by western blot. The detached leaves of *MpDhn12* overexpressing tobacco were treated with a series of concentrations of $CuCl_2$, and the abundance of MpDhn12 in total, soluble and insoluble fractions were detected. The total amount of MpDhn12 in each $CuCl_2$ treated samples remained constant (Fig. 3G). The soluble amount of MpDhn12 in the $CuCl_2$-treated samples decreased gradually as $CuCl_2$ concentrations increased (Fig. 3G). Correspondingly, the amount of insoluble MpDhn12 increased gradually (Fig. 3G). These results suggested that $Cu^{2+}$ could also trigger aggregation of MpDhn12 *in vivo*. Overall, the results above demonstrated that $Cu^{2+}$ was able to trigger the aggregation of MpDhn12 both *in vitro* and *in vivo*.

### $Cu^{2+}$ triggered aggregation of MpDhn12 is impaired by His modification with DEPC

Since $Cu^{2+}$ could bind to MpDhn12, we investigated whether this binding was involved in the $Cu^{2+}$ triggered aggregation by modifying the His residues of MpDhn12 with DEPC. The $Cu^{2+}$-induced antagonism to trypsin digestion, and the CD spectra signal decay and precipitation of MpDhn12 were impaired with increasing amounts of DEPC treatment (Fig. 4). These results indicated that the His-mediated $Cu^{2+}$ binding was involved in the $Cu^{2+}$ triggered aggregation of MpDhn12.

### MpDhn12 rescues the yeast copper sensitive phenotype of Δsod1

To test whether MpDhn12 plays a role in copper homoeostasis, we performed functional complementation assays using yeast mutants deficient in copper tolerance. The copper sensitive yeast strains Δ*cup2 and* Δ*sod1* were used for functional complementation assays (*44–47*). The growth of the two mutants on SC plates were inhibited by 150 μM $CuSO_4$ compared to wild-type (BY4742), and MpDhn12 restored the copper tolerance of Δ*sod1* to nearly the wild-type level, comparable with the effect of *Arabidopsis* MTs which were used as positive controls (Fig. 5A). Unexpectedly, MpDhn12 could not restore the copper tolerance of Δ*cup2*, while *Arabidopsis* MTs partially restored it (Fig. 5B). These results suggested that MpDhn12 may be a member of copper homoeostasis regulator which functions in chelating excess $Cu^{2+}$.

## Discussion

Copper is a cofactor in numerous proteins, particularly in those which are involved in the photosynthetic and the respiratory electron transport chains (*1*, *2*). When plants were exposed to environmental stresses, the photosynthetic and the respiratory rate would

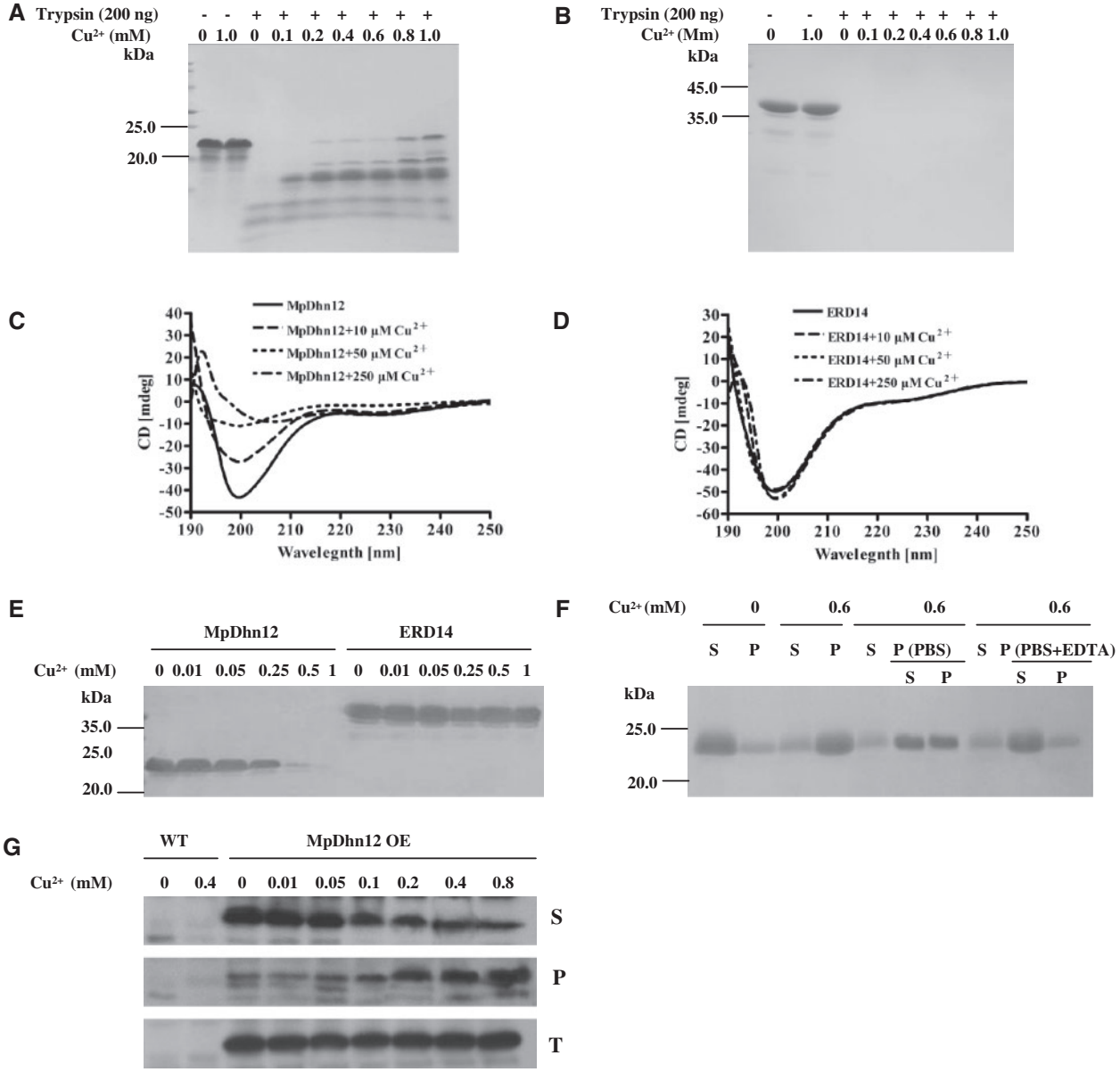


**Fig. 3 $Cu^{2+}$ triggers aggregation of MpDhn12.** (A and B) Digestion of $Cu^{2+}$ treated MpDhn12 (A) or ERED14 (B) by trypsin. (C and D) CD spectra of MpDhn12 (C) or ERD14 (D) in the absence or presence of indicated concentrations of $Cu^{2+}$. (E) Precipitation analysis of MpDhn12 or ERD14 in the presence of $Cu^{2+}$ at the indicated concentrations. (F) Resolubility analysis of the MpDhn12 precipitates. S: supernatants after centrifugation P: precipitates after centrifugation. P (PBS): precipitates resolublized by PBS buffer. P (PBS+EDTA): precipitates resolublized by PBS buffer plus EDTA. (G) Detection of MpDhn12 aggregation *in vivo*. Relative abundance of MpDhn12 protein detected by anti-MpDhn12 in total (T), soluble fraction (S) and insoluble fraction (P) in *MpDhn12* overexpressing tobacco treated with the indicated concentrations of $Cu^{2+}$. WT: wild-type tobacco. MpDhn12 OE: T2 *MpDhn12* overexpressing tobacco.

decrease, which may result in the down regulation of the copper proteins and releasing copper ions (*48–50*). Thus, the binding of free copper ions is important for alleviating the damage caused by environmental stresses. Several dehydrins have been shown to have copper-binding activity (*23*). However, little work has been done with respect to the structural changes of these proteins after binding to copper and the function of copper-binding activity for dehydrins is still unclear.

In this study, MpDhn12 was shown to be intrinsically disordered under physiological conditions and be thermal stable (Fig. 1C–E), but aggregate under excess copper conditions (Fig. 3). Interestingly, copper has almost no effect on the conformation of *Arabidopsis* dehydrin ERD 14 (Fig. 3). From analysing the sequence of MpDhn12 and ERD14, we found that MpDhn12 has a high His content (16.8%), while ERD14 has a low His content (3.2%). Thus, the aggregation of MpDhn12 under excess copper may be correlated with the His content and/or some other factors. Copper triggered aggregation of small copper-binding proteins have also been found in other organisms. PhoCutA from *Pyrococcus horikoshii* can bind copper, resulting in multimerization and

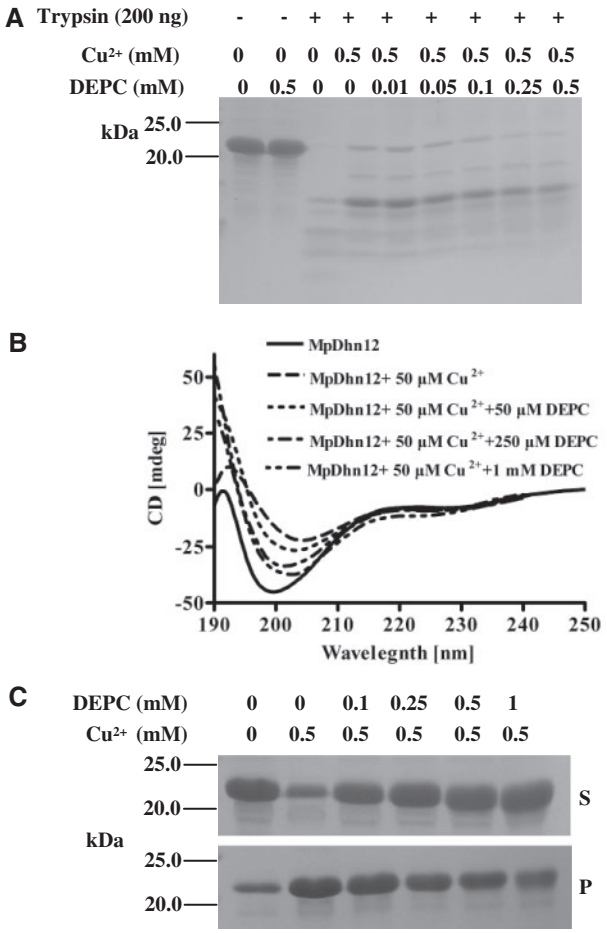


**Fig. 4 Effect of DEPC on the aggregation of MpDhn12 triggered by $Cu^{2+}$.** (A) Digestion of DEPC and $Cu^{2+}$ treated MpDhn12 by trypsin. (B) Effect of DEPC on CD spectra of MpDhn12 changed by $Cu^{2+}$. (C) Precipitation analysis of DEPC treated MpDhn12 in the presence of $Cu^{2+}$. S, supernatants after centrifugation; P, precipitates after centrifugation.

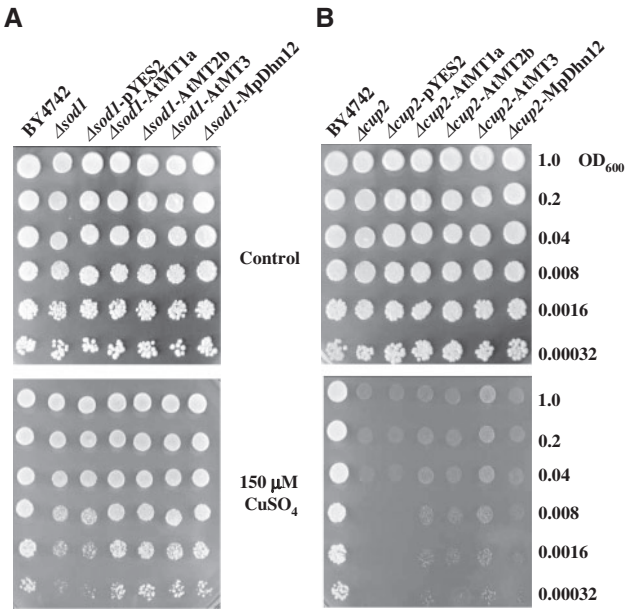


**Fig. 5 MpDhn12 complements the copper sensitive phenotype of yeast mutant Δ*sod1*.** Yeast strains expressing MpDhn12 or *Arabidopsis* MT1a, MT2b, MT3 in the Δ*sod1* (A), or Δ*cup2* (B) background were grown on SC medium containing 0 or 150 μM $CuSO_4$. Numbers at the right indicate the dilution factors.

precipitation, suggesting that it plays a role in intracellular metal sequestration by capturing and precipitating intracellular heavy metals (*43*). Another example of a protein conferring metal resistance by precipitation of intracellular heavy metals is Cus, from *Mycobacterium* (*51*). In addition, it should be taken into account that the aggregation of MpDhn12 was reversible when the excess copper was diluted to a low concentration or chelated by EDTA (Fig. 3). Moreover, MpDhn12 could rescue the yeast copper sensitive mutant Δ*sod1*, comparable with the activity of the *Arabidopsis* MTs (Fig. 5). Considering the copper-binding activity and tendency of MpDhn12 to aggregate, it is possible that MpDhn12 may function in copper homoeostasis through binding and precipitating excess copper *in vivo*.

The major identified copper chelators in plants and yeast which help to buffer copper levels are MTs (*2*, *12*). MTs were shown to bind to copper via Cys thiol groups (*12*). However, there is no Cys in MpDhn12, but it is rich in His (Fig. 1). The high His content is ubiquitous in dehydrins (*23*), and it was proven to be involved in metal binding of dehydrins (*26*, *27*). His residues are usually rare in proteins, with ~2% of the amino acid content (*36*), and were reported recently to be preferentially bound to copper (*18*, *20*). The His residues content of MpDhn12 is as high as 16.8% (Fig. 1), and His was shown to be involved in the copper binding and triggering aggregation of MpDhn12 (Figs 2 and 5). Therefore, we propose that MpDhn12 is a novel type of copper chelator, functioning in copper homoeostasis which uses His as binding ligands.

Increasing number of experiments showed that dehydrins possess multifunctional property (*21*, *23*). Although it is not clear which functions are common in many dehydrins and which are restricted to specific dehydrins. At present, binding to acidic phospholipids, binding to ions, and cryoprotection are supposed to the common functions of dehydrins (*23*). The cryoprotective activity of MpDhn12 with a lactate dehydrogenase (LDH) assay was also tested in this study. The LDH cryoprotective activity was compared to the known cryoprotectants BSA, Sucrose, and ERD14. The results indicated that MpDhn12 has a strong cryoprotective activity to protect LDH activity under freeze–thaw cycles treatment, which was comparable with ERD14 and BSA (Supplementary Fig. S3). Thus, MpDhn12 may be also a multifunctional dehydrin at least binding $Cu^{2+}$ and cryoprotective activity.

In summary, an intrinsically disordered His-rich $K_nS$ type dehydrin, MpDhn12, from *M. paradisiacal* was found to bind $Cu^{2+}$ in a His-related manner. Furthermore, MpDhn12 was shown to reversibly aggregate with copper and could complement the yeast copper sensitive mutant Δ*sod1*, which is a new characteristic of dehydrins. To the best of our knowledge, this

is the first report which provides evidences for copper triggering reversible aggregation of dehydrin which may implicate a function in buffering copper levels.

## Supplementary Data

Supplementary Data are available at *JB* Online.

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### Conflict of interest

None declared.

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