https://doi.org/10.17221/12/2025-PPS

Role of the *Arabidopsis At2g21490* dehydrin gene in enhancing tolerance to copper and zinc stress in transgenic tobacco plants

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Citation: Boszorádová E., Švecová M., Nemeček P., Ranušová, P., Karas M., Jana Moravčíková J. (2026): Role of the *Arabidopsis At2g21490* dehydrin gene in enhancing tolerance to copper and zinc stress in transgenic tobacco plants. Plant Protect. Sci.: 229–238.

Abstract: We studied the role of the *Arabidopsis At2g21490* (*DH2*) histidine-rich dehydrin gene in plant responses to copper and zinc stress. Transgenic plants overexpressing the *DH2* gene were generated via *Agrobacterium*-mediated transformation. Progeny from both transgenic and non-transgenic (control) plants were cultivated hydroponically and subjected to short-term stress (100 µM $CuCl_2$ or 200 µM $ZnCl_2$ for 24 h) before analysis. The differences observed between transgenic and non-transgenic plants in the expression of phytochelatin synthase (NtPCS) and certain metal transporters (NtMTP1A, NtMTP1B, NtHMA_A, and NtHMA_B) suggest that the *DH2* gene plays a role in immobilising excess copper, primarily in the roots, thereby mitigating its harmful effects on the aerial parts of the plant. The overexpression of the *DH2* gene influenced the levels of both enzymatic (NtAPX, NtSOD, NtCAT) and nonenzymatic antioxidants, particularly by increasing polyphenolic compounds, such as chlorogenic acid by at least 12-fold and rutin by at least 3-fold. The contribution of the *DH2* gene to zinc stress tolerance appears to be less significant.

Keywords: *Arabidopsis thaliana*; genetic modification; heavy metals; LEA proteins; *Nicotiana tabacum* L.

Dehydrins (Pfam 00257) are thermostable, largely hydrophilic proteins, originally associated with later stages of embryonic development, not only in plants (Soares et al. 2019). Their disordered

Supported by the findings from the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and Slovak Academy of Sciences No. VEGA 1/0230/24 and Cultural and Educational Grant Agency Ministry of Education, Science, Research and Sport of the Slovak Republic No. KEGA 012UCM-4/2025.

https://doi.org/10.17221/12/2025-PPS

structure allows them to bind to specific ligands and perform protective functions within cells (Murray & Graether 2022; Hsiao 2024). Dehydrins exhibit distinctive features given by the presence of conserved sequence motifs, including the K, S, Y, and F segments, along with a Φ segment that is not strictly conserved and displays some degree of randomness (Smith & Greather 2022). A lysine-rich sequence characterises the K segment, which is found in all dehydrins. It likely forms an amphipathic helix that facilitates interactions with cellular components and protects against damage (Xu et al. 2008). The diversity and functional adaptability of dehydrins are determined by the presence or absence of these conserved motifs that categorise them into different types (K_n, SK_n, Y_nSK_n, Y_nK_n, K_nS, F_nK_n, and F_nSK_n), based on the number and combinations of the motifs (Strimbeck 2017; Karas et al. 2024).

Dehydrins are recognised as multifunctional proteins crucial for safeguarding plant molecular and cellular structures, mitigating protein or membrane aggregation, and regulating water balance amid diverse abiotic and biotic stresses (Yang et al. 2019); nonetheless, certain dehydrins exhibit specialised functions, including their involvement in alleviating heavy metal stress (Hara et al. 2010). Some dehydrins may enhance heavy metal tolerance in plants by binding metals, reducing oxidative stress, stabilising cellular structures, activating stress response pathways, or functioning as ROS scavengers (Xu et al. 2008; Liu et al. 2019; Svecova et al. 2023). Several studies have indicated that dehydrins mitigate Cu or Zn toxicity. For instance, the KS-type dehydrin ZmDHN13 has been shown to enhance the tolerance of transgenic tobacco plants by binding Cu ions, thereby reducing ROS accumulation (Liu et al. 2019). Similarly, the overexpression of *Brassica juncea* dehydrins BjDHN2 and BjDHN3 (SK_2 types) improved Cd and Zn tolerance in transgenic plants by reducing lipid peroxidation and maintaining membrane stability (Xu et al. 2008).

The dehydrin At2g21490 (Y_3SK_2 type) possesses histidine (His)-rich regions, which are believed to bind free metal ions, thereby preventing ROS production under stress conditions (Hara 2010). Previous studies showed that the dehydrin At2g21490 is induced during seed development and in response to water and cold stress (Candat et al. 2014; Miura & Tada 2014). Transgenic tobacco (T_0) plants, overexpressing the *DH2* gene, were generated via *Agrobacterium*-mediated transformation. Progeny from three independent transgenic lines of T_0 plants were subjected to short-term stress conditions (100 μM $CuCl_2$ or 200 μM $ZnCl_2$ individually) for 24 h and then analysed. We examined the effect of DH2 overexpression in transgenic plants on the expression of specific enzymatic antioxidants (via qPCR), nonenzymatic antioxidants (using HPLC), and metal transporters (via qPCR) under both normal and stress conditions.

MATERIAL AND METHODS

Vector construct, tobacco transformation and regeneration

The sequence (1 037 bp) of the dehydrin gene *At2g21490* (*DH2*) was isolated from genomic DNA of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia as was described by Zimova et al. (2017). The T-DNA of plant transformation vector pDH2 (a derivative of pBinPlus) contained *dCaMV35S/DH2/polyA* sequence (Figure 1). The plasmid was introduced into *Agrobacterium tumefaciens* LBA 4404 and used in transformation experiments.

Tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was transformed as described by Poloniova et al. (2015). Transgenic T_0 plants were regenerated under kanamycin selection at 50 mg/L. Selected transgenic T_0 plants were transferred from *in vitro* culture to soil after acclimatisation and cultivated in pots under greenhouse conditions. The uniformly growing transgenic plant (T_1) were obtained through self-pollination of T_0 plants.

Cu and Zn stress treatments

Surface-sterilised transgenic T_1 seeds were germinated on Murashige and Skoog medium (Murashige & Skoog 1962) supplemented with 1% (*w/v*) sucrose, 0.8% (*w/v*) agar, and 50 mg/L kanamycin. Non-transgenic seeds were germinated without the presence of kanamycin. After 3 weeks, uniformly growing transgenic (T_1) and non-transgenic (NT) plants were transferred to hydroponic conditions in glass jars with 250 mL of ¼ Hoagland solution. After 1 week, whole plants were transferred to ¼ Hoagland solution supplemented with 100 μM $CuCl_2$ or 200 μM $ZnCl_2$ (separately). Non-stressed plants were transferred to glass jars with 250 mL of ¼ Hoagland solution without $CuCl_2$ or $ZnCl_2$.

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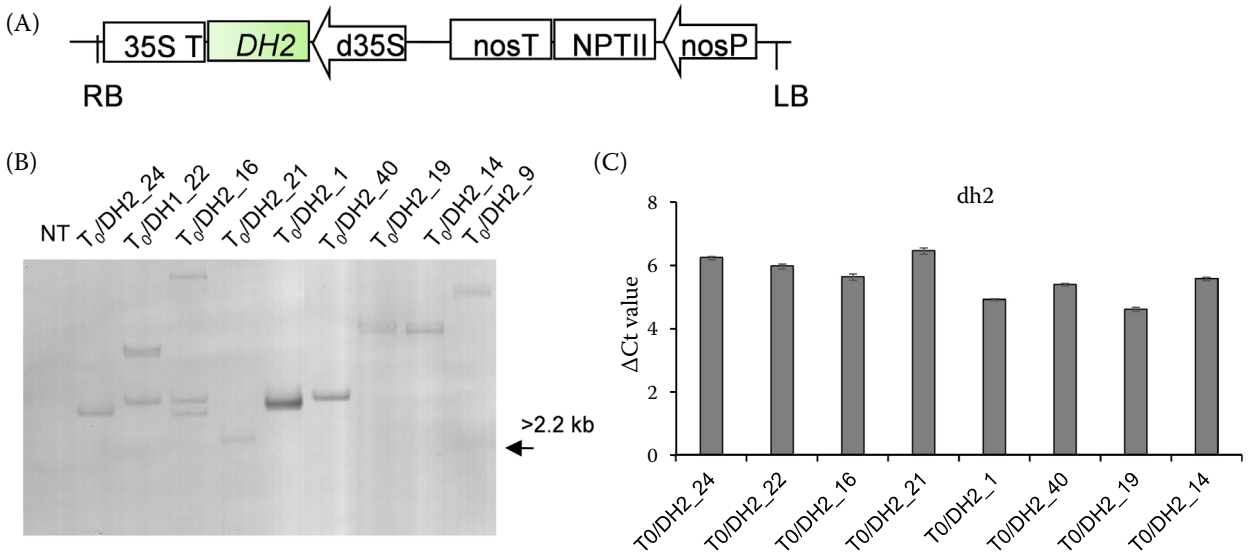

Figure 1. Analysis of transgenic T_0 plants

(A) T-DNA of plant transformation vector pDH2; d35S – *dCaMV35S* promoter, DH2– *DH2* dehydrin gene, 35S T–*35S* terminator, nosP – *nos* promoter, NPTII – selectable *npt*II gene, *nos*T – *nos* terminator; (B) southern blot analysis of T_0/DH2 plants. *Eco*RI-digested DNAs were hybridised with a non-radioactively labelled NPTII-specific probe. Bands (> 2.2 kb) correspond to the number of independent transgene copies; (C) relative expression levels of the *DH2* gene in transgenic T_0 plants, the qPCR analyses were performed using the DH2_F and DH2_R primers corresponding to an internal fragment of the *DH2* gene

Plants were cultivated at 20 ± 2 °C, a 16 h photoperiod, and a light intensity of 50 µmol/m^2.s. After 24 h, the leaves of stressed and non-stressed T_1 and NT plants were sampled and analysed. For each variant, 5 plants per line were used.

DNA and RNA analyses

Genomic DNA was isolated from leaf tissues following the protocol of Chen et al. (1992).

For Southern blot analyses, 40 µg of DNA was digested with the restriction enzyme *Eco*RI (Fermentas, USA), separated on a 1% (*w/v*) agarose gel and blotted onto a positively charged nylon membrane (Roche, Switzerland). Preparation of the NPTII probe and hybridisation conditions were previously described by Boszoradova et al. (2019).

Total RNA was isolated from tobacco leaves using RNeasy® Plant Mini Kit (Qiagen, Germany), and 1 µg of RNA was reverse-transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

Quantitative expression analyses were performed using the LightCycler® Nano System (Roche, Switzerland). The reaction mixture (10 µL total volume) and the 2-step PCR conditions were prepared according to the manufacturer's instructions (Luminaris HiGreen qPCR Master Mix, Thermo Scientific).

The primers used for detection of the *DH2* gene expression in transgenic T_0 plants were DH2_F 5'-GTTAGCTCCACTCCTCAGCATAAG-3' and DH2_R 5'- GCCGCTACCGAACTTCTCTTTA -3'. The relative expression levels of the *DH2* gene in T_0-DH2 plants were calculated using the formula: $\Delta Ct = Ct_{(dh2)} - Ct_{(actin)}$, where *Ct* indicates cycle threshold.

Relative expression levels of the genes encoding ROS scavenging enzymes [superoxide dismutase *NtSOD* (EU123521.1; NtSOD_F: 5'-CTGGTGATCTTGGTAACATCACAGTTG-3', NtSOD_R: 5'-ATCATCAGGATCAGCGTGAACAACC-3'), catalase 1 *NtCAT1* (U07627.1; NtCAT1_F: 5'- TGGACGATTGGTACTTAACAGGAATAT -3', NtCAT1_R: 5'- GAGAAGCTTGTCCTCCGAATAGTAAAG -3', ascorbate peroxidase *NtAPX* (U15933.1; NtAPX_F: 5'-CAAATGTAAGAGGAAACTCAGAGG-3', NtAPX_R: 5'- CAGCCTTGAGCCTCATGGTACCG -3'), monodehydroascorbate reductase *NtMDHAR* (NM_001326195.1; NtMDHAR_F: 5'-GGATTGACTCTGGTAAGCTGA-3', NtMDHAR_R: 5'-AATGCCTCTTCCACTGAGGATG-3'), dehydroascorbate reductase *NtDHAR* (NM_001326056.1; NtDHAR_F: 5'-TCAAGGCT-

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CACGGACCATATGT-3', NtDHAR_R: 5'-GCACATGACTCAAGCTTTCAGG -3')]; and metal transporters [phytochelatin synthase *NtPCS* (KP136425.1; NtPCS_F: 5'-TTGCCCAGGACTTGATTGTATG-3', NtPCS_R: 5'-CGAAGAGAAATTAGGACGTCAACA -3'), metal tolerance protein 1A *NtMTP1A* (AB201240; NtMTP1A_F: 5'-GGCTCTGTGAGATGGAGGAC-3', NtMTP1A_R: 5'-ACCTTATCCAGCACCGTGTC-3') and metal tolerance protein 1B *NtMTP1B* (AB201241; NtMTP1B_F: 5'-ACCATGAACACGGCCATAAT-3', NtMTP1B_R: 5'- TATGGTGATGGTGTCGGCTA-3'), natural resistance-associated macrophage protein *NtNRAMP1* (AB505625, NtNRAMP1_F: 5'-CATCAGAGACGCATGCAGAT-3', NtNRAMP1_R: 5'- TGAGGAATTGCAGACAGCAC-3'), heavy metal ATPase A *NtHMA_A* (HF675181; NtHMA_A F: 5'-GTTCGGACAAGGAACACGAT -3', NtHMA_A R: 5'- TTGTGCTGTCACAATGAGCA -3') and heavy metal ATPase B *NtHMA_B* (HF937054; NtHMA_B F: 5'- CCAGCATGCAAAACTGCTAA-3', NtHMA_B R: 5'- GGGCTAAATCCAAGCTTTCC -3')] were analysed in T_1 and NT plants under zinc or copper and stress free (control) conditions. The actin gene (GQ339768.1; ACT_F: 5'- GCACCTCTCAACCCAAAG -3', ACT_R: 5'- GGAAAGGACAGCCTGAATAG -3') was used as a reference control. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). The qPCR reactions were performed in triplicate for both biological and technical replicates.

HPLC analyses

HPLC analyses were conducted following the procedures outlined by Svecova et al. (2023).

Statistical analyses

One-way ANOVA was used to evaluate all binary factors (e.g. stressed/non-stressed). The Duncan post hoc test was used for multilevel factors (e.g., $CuCl_2$ stress, $ZnCl_2$ stress, no stress). All results with $P \leq 0.05$ were considered significant. Statistical analyses were performed using the IBM SPSS software (version 19.0).

RESULTS

Generation and verification of transgenic tobacco plants

Leaf discs were transformed with *A. tumefaciens* LBA 4404 carrying the pDH2 (Figure 1A). Transgenic plants were regenerated under kanamycin selection pressure. The transgenic status of T_0 plants was verified by Southern blot analysis using an NPTII probe (Figure 1B). The expression of the dehydrin DH2 in T_0 plants was confirmed by qPCR using the primers DH2_F and DH2_R, which amplify an internal fragment of the *DH2* gene (Figure 1C). T_0 plants with the highest *DH2* gene expression (T_0_21, T_0_24, T_0_40) were transferred to greenhouse conditions to grow to maturity and obtain T_1 seeds through self-pollination. The T_1 seeds were subsequently used in stress experiments.

Three-week-old transgenic T_1 and non-transgenic NT plants were transferred to hydroponic conditions and exposed to 100 µM $CuCl_2$ or 200 µM $ZnCl_2$ (separately). Following 24 h, leaves of Cu- and Zn-stressed T_1-DH2 transgenic lines and non-transgenic NT plants and their control counterparts were analysed. The data of the individual lines (T_1-DH2_21, T_1-DH2_24, T_1-DH2_40) were expressed as averages.

Effect of the *DH2* gene on the expression levels of the genes encoding ROS scavenging enzymes

Leaves of Cu- and Zn-stressed T_1-DH2 transgenic lines and non-transgenic NT plants and their control counterparts were analysed by qPCR for activity of the genes encoding CuZnSOD superoxide dismutase (*NtSOD*), catalase 1 (*NtCAT*), and the enzymes of the glutathione-ascorbate cycle [ascorbate peroxidase (*NtAPX*), monodehydroascorbate reductase (*NtMDHAR*) and dehydroascorbate reductase (*NtDHAR*)].

The overexpression of the dehydrin gene *DH2* in T_1 plants (under standard conditions) resulted in the upregulation of mRNA transcripts for the genes *NtAPX, NtCAT, NtDHAR*, and *NtMDHAR* by at least 1.4-fold, compared to non-stressed NT plants ($P \leq 0.05$) (Figure 2). We did not detect any significant differences in NtSOD gene expression between non-stressed T1 and NT plants.

In T_1 plants, stress did not significantly affect the mRNA levels of NtSOD, NtAPX, NtMDHAR, and NtDHAR compared to their non-stressed counterparts. However, the NtCAT mRNA transcript in Cu-T_1 plants was downregulated by 1.4-fold. In contrast, the mRNA transcripts of the studied genes (*NtSOD, NtAPX, NtCAT*, *NtDHAR* and *NtMDHAR*) were significantly (at least 1.8-fold) upregulated in Cu-stressed NT plants.

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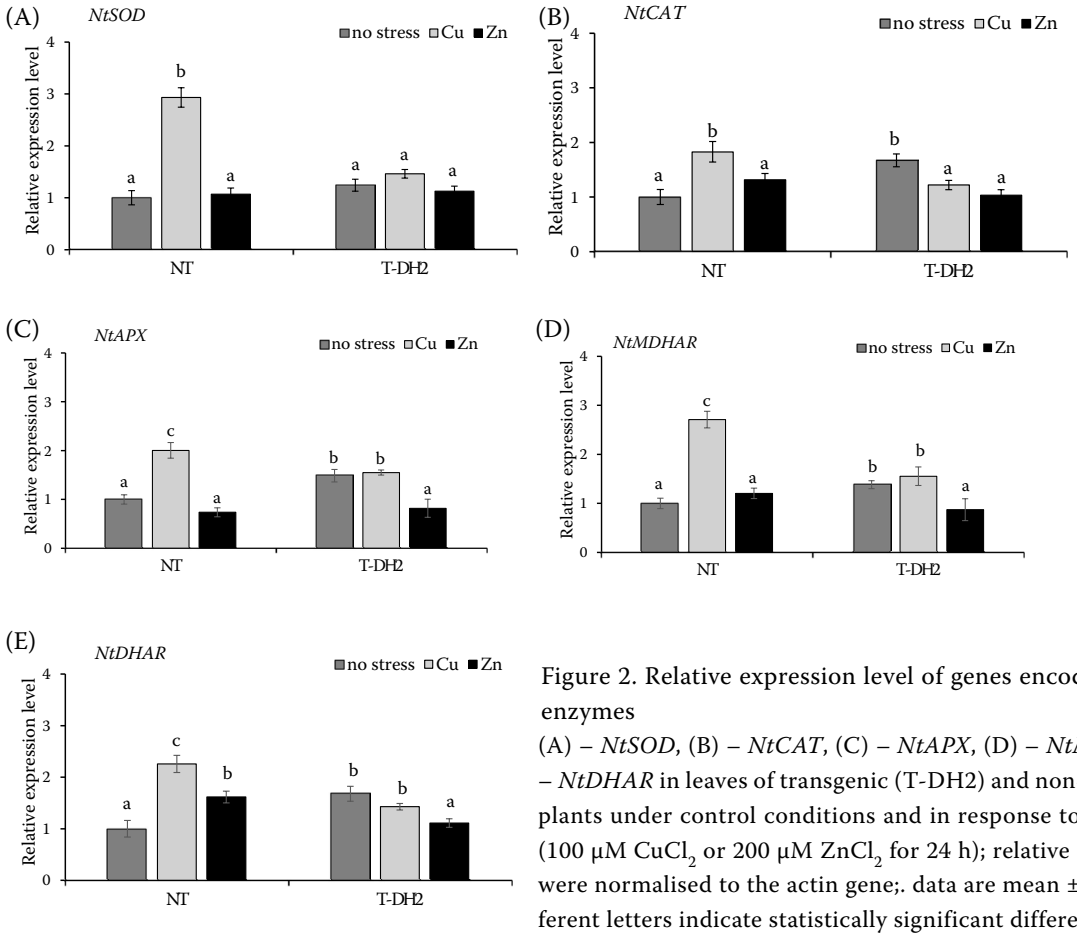

Figure 2. Relative expression level of genes encoding antioxidant enzymes

(A) – *NtSOD*, (B) – *NtCAT*, (C) – *NtAPX*, (D) – *NtMDHAR*, and (E) – *NtDHAR* in leaves of transgenic (T-DH2) and non-transgenic (NT) plants under control conditions and in response to Cu or Zn stress (100 µM $CuCl_2$ or 200 µM $ZnCl_2$ for 24 h); relative expression levels were normalised to the actin gene;. data are mean ± SE (n = 15); different letters indicate statistically significant differences at $P \leq 0.05$

Zn stress in T_1 plants resulted in downregulation (at least 1.5-fold) of the mRNA transcripts of *NtAPX, NtCAT, NtDHAR* and *NtMDHAR*, while the mRNA transcript of *NtSOD* remained unchanged, compared to their non-stressed counterparts. Except for *NtDHAR*, no significant changes were observed in the expression levels of the genes *NtSOD, NtAPX, NtCAT*, and *NtMDHAR* between Zn-stressed and non-stressed NT plants. The mRNA transcript of *NtDHAR* increased by 1.6-fold in Zn-stressed NT plants.

Effect of the *DH2* gene on the contents of nonenzymatic antioxidants

HPLC analyses (Figure 3) were conducted to assess the content of nonenzymatic antioxidants, including chlorogenic acid (CGA), quercetin, vanillin, and rutin in the leaves of T_1-DH2 transgenic lines, as well as non-transgenic NT plants, and their counterparts.

Under standard conditions, T_1 plants exhibited significantly higher contents of CGA and rutin (12.3-fold and 3.5-fold, respectively) compared to NT plants ($P \leq 0.05$). Following Cu stress, the content of CGA remained unchanged in transgenic T_1 plants, whereas in non-transgenic NT plants it increased 21-fold compared to their non-stressed counterparts. The rutin content significantly decreased (1.7-fold) in Cu-stressed T_1 plants, whereas in Cu-stressed NT plants, it increased by 1.6-fold.

Zn stress did not significantly impact the contents of both CGA and rutin in T_1 plants compared to their non-stressed counterparts. In contrast, Zn stress led to a significant increase in the contents of CGA (9.2-fold) and rutin (1.6-fold) in non-transgenic NT plants.

Quercetin and vanillin levels were undetectable (below the limit of detection, LOD) in all analysed plant samples.

Effect of the *DH2* gene on the expression levels of the genes encoding heavy metal transporters

The expression of heavy metal transporter genes (*NtPCS, NtMTP1A, NtMTP1B, NtNRAMP1, NtHMA_A*, and *NtHMA_B*) was investigated

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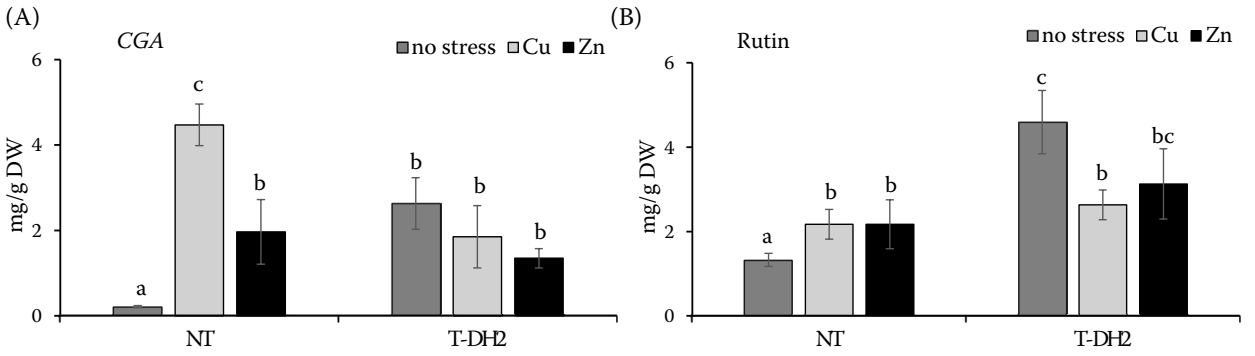

Figure 3. Chlorogenic acid and rutin levels in leaves of transgenic (T-DH2) and non-transgenic (NT) plants under control conditions and in response to Cu or Zn stress (100 µM CuCl2 or 200 µM ZnCl2 for 24 h)

(A) – chlorogenic acid (CGA), and (B) – rutin levels in leaves of transgenic (T-DH2) and non-transgenic (NT) plants under control conditions and in response to Cu or Zn stress (100 µM $CuCl_2$ or 200 µM $ZnCl_2$ for 24 h); levels are expressed as mg/g dry weight; data are mean ± SE (n = 15); different letters indicate statistically significant differences at $P \leq 0.05$

in the leaves of non-stressed and Cu-stressed or Zn-stressed transgenic T_1 and non-transgenic NT plants through qPCR (Figure 4).

Under standard conditions, T_1 plants exhibited significant upregulation of NtPCS mRNA and downregulation of NtHMA_A mRNA, with increases of 1.9-fold and decreases of 1.6-fold, respectively. The mRNA transcripts of the remaining metal transporters showed no changes compared to NT plants. After Cu stress, the expression levels of *NtPCS, NtMTP1A, NtMTP1B, NtNRAMP1*, and *NtHMA_B* remained unaltered, while the mRNA

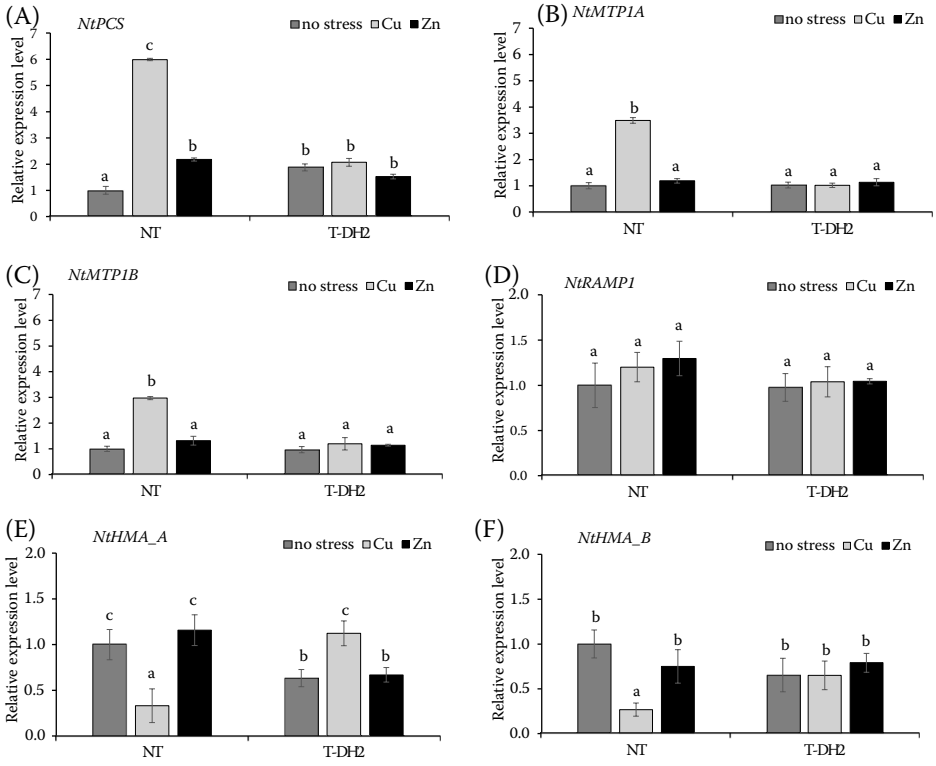

Figure 4. Relative expression level of genes encoding metal transport enzymes

(A) – *NtPCS*; (B) – *NtMTP1A*; (C) – *NtMTP1B*; (D) – *NtNRAMP1*; (E) – *NtHMA_A*; and (F) – *NtHMA_B* in leaves of transgenic (T-DH2) and non-transgenic (NT) plants under control conditions and in response to Cu or Zn stress (100 µM $CuCl_2$ or 200 µM $ZnCl_2$ for 24 h); data are mean ± SE (n = 15); different letters indicate statistically significant differences at $P \leq 0.05$

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transcript of *NtHMA_A* increased by 1.7-fold, compared to non-stressed counterparts.

Cu stress in non-transgenic NT plants led to an increase in mRNA transcripts of *NtPCS* (6-fold), *NtMTP1A*, and *NtMTP1B* (at least 3-fold), while causing a decrease in mRNA transcripts of *NtHMA_A* and *NtHMA_B* (at least 3-fold). Cu stress did not significantly affect the expression level of *NtNRAMP1* in non-transgenic NT plants.

In transgenic T_1 plants, Zn stress did not induce significant changes in mRNA levels of all the studied genes (NtPCS, NtMTP1A, NtMTP1B, NtNRAMP1, NtHMA_A, and NtHMA_B) compared to their non-stressed counterparts. In Zn-stressed non-transgenic NT plants, the expression level of *NtPCS* in Zn-stressed NT plants was upregulated by 2.2-fold.

DISCUSSION

We aimed to explore the role of the His-rich *Arabidopsis* Y_3SK_2-type dehydrin At2g21490 (DH2) in mitigating Cu and Zn toxicity. Transgenic tobacco plants overexpressing the dehydrin DH2 were generated via *Agrobacterium*-mediated transformation, and their offspring were exposed to short-term (Cu and Zn) stress in hydroponics (separately). Cu and Zn are essential micronutrients for plants, serving as cofactors for numerous enzymes and participating in various metabolic processes, and contributing to plant growth and development (Liscakova et al. 2022). However, high concentrations of Cu or Zn can be toxic to plants, leading to oxidative stress due to increased ROS levels and disrupting plant growth and development. Our first aim was to investigate the extent to which overexpression of the dehydrin DH2 in transgenic tobacco plants influenced the activity of selected enzymatic antioxidants NtSOD (Cu/Zn form), NtAPX (cytosolic form), NtCAT1 (strongly expressed in photosynthetic tissues), NtDHAR and NtMDHAR as well as the accumulation of nonenzymatic antioxidants, such as chlorogenic acid (CGA) and rutin. SOD is considered the first line of defence in the plant's response to ROS. It catalyses the removal of superoxide radicals (O_2^-) by dismutating them into O_2 and H_2O_2 (Chung 2017). Both enzymes APX and CAT catalyse the decomposition of H_2O_2, converting it into H_2O. While APX scavenges H_2O_2 using ascorbate as a reducing agent, CAT is effective at relatively high H_2O_2 levels (Gechev et al. 2006). MDHAR and DHAR are enzymes involved in the ascorbate-glutathione cycle (Martinez & Araya 2010). We observed altered expression of the genes encoding NtAPX, NtCAT1, NtMDHAR, and NtDHAR in DH2-overexpressing plants under standard conditions. Similarly, Liu et al. (2015) reported upregulation of ROS-scavenging enzymes in tomato plants overexpressing the dehydrin ShDHN. Furthermore, we observed a significant increase in the levels of the nonenzymatic antioxidants CGA and rutin in DH2-overexpressing plants. Tobacco is generally rich in polyphenols, with chlorogenic acid (CGA) and rutin emerging as the primary compounds (Wang et al. 2008). Both CGA and rutin are synthesised via the phenylpropanoid biosynthetic pathway, which is activated in response to abiotic or biotic stresses (Mei et al. 2020). They act as scavengers of highly reactive hydroxyl radicals (Cardenas-Manriquez et al. 2016). The CGA and rutin contents in Cu-stressed DH2-overexpressing plants remained unchanged for CGA and decreased for rutin, whereas in non-transgenic (Cu-stressed) plants they significantly increased, particularly for CGA. Similarly, the expression of genes encoding antioxidant enzymes either remained unchanged (*NtSOD, NtAPX, NtDHAR*, and *NtMDHAR*) or was suppressed (*NtCAT*) in Cu-stressed DH2-overexpressing plants. In contrast, in Cu-stressed non-transgenic plants, the expression of all analysed antioxidant enzymes significantly increased. The observed disparities suggest that Cu ions might already be detoxified in root cells of DH2-overexpressing plants. Consequently, Cu ions might not have been translocated to the upper parts of the transgenic plants, thereby preventing leaves from being exposed to higher Cu concentrations, unlike in non-transgenic plants. The dehydrin DH2 belongs to the family of His-rich proteins. It contains 14 histidine (His) residues and 3 His-related residues, including $(His\text{-}X_3\text{-}His)_1$ and $(His\text{-}His)_2$ motifs. Several studies have demonstrated that certain His-rich dehydrins can function as metal-binding proteins with varying affinity to metal ions (Hara et al. 2005, 2011). For instance, Hara et al. (2005) showed that the citrus dehydrin CuCOR15 binds Fe3+, Co2+, Ni2+, Cu2+, and Zn2+, but not Mg2+, Ca2+, or Mn^{2+}. In another study (Svecova et al. 2023), the overexpression of the His-rich *Arabi-*

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dopsis dehydrin AtLTI30 in transgenic tobacco plants improved their tolerance to Cd. Most of the absorbed Cd was retained within the roots and did not translocate to the aerial parts of the AtLTI30 overexpressing tobacco plants. It seems plausible that dehydrin DH2 may exhibit a similar activity pattern. This underscores that, while the expression of several genes encoding metal transporters increased in the leaves of non-transgenic plants under Cu stress, it remained unchanged in Cu-stressed DH2 overexpressing plants. The notable upregulation of phytochelatin synthase (NtPCS), responsible for the biosynthesis of phytochelatins (PCs) (Faizan et al. 2024), suggests a higher concentration of Cu in the leaves of non-transgenic plants. Metal stress-inducible PCs play a crucial role in detoxifying heavy metals, including Cu and Zn, by forming stable complexes with them and then sequestering these complexes into the vacuoles (Singh et al. 2019; Shabbir et al. 2020). Furthermore, in Cu-stressed non-transgenic plants, the expression of metal transporters (NtMTP1A and NtMTP1B) belonging to the MTP family, responsible for heavy metal sequestration in the vacuole (Ricachenevsky et al. 2013), was upregulated. This may be related to the natural detoxification mechanism tobacco uses to cope with elevated copper levels in its leaves. To our knowledge, the role of these metal transporters in Cu sequestration in tobacco has not been studied in detail. In tobacco, heavy metal P-type ATPases (HMAs) NtHMA_A and NtHMA_B (orthologous to AtHMA2 and AtHMA4, later denominated as NtHMA4.1 and NtHMA4.2) are export transporters highly expressed in roots rather than shoots (Hermand et al. 2014; Kozak & Antosiewicz 2023). They are responsible for loading metals into xylem vessels, thereby facilitating their efficient translocation to the shoots (Bahmani et al. 2019). In Cu-stressed DH2 overexpressing plants, the expression of NtHMA_A increased, while that of NtHMA_B was not affected. The downregulation of both NtHMA_A and NtHMA_B in Cu-stressed non-transgenic plants suggests a nutritional imbalance, as indicated by Kozak and Antosiewicz (2023). The NtNRAMP1, a member of the NRAMP (Natural Resistance Associated Macrophage Proteins) family, is a plasma membrane protein involved mainly in Fe uptake (Sano et al. 2012). Our expression analysis indicates that the metal transporter NRAMP1 probably did not play a significant role in overall Cu uptake in tobacco, and that overexpression of the *DH2* gene did not have a significant effect on it either.

Unlike copper stress, zinc did not significantly affect the expression of the analysed metal transporters in either DH2-overexpressing plants or non-transgenic plants. However, the expression of the gene encoding phytochelatin synthase (*NtPCS*) was significantly increased in Zn-stressed non-transgenic plants, whereas in Zn-stressed DH2 overexpressing plants, it remained unchanged. Analysis of Zn-stressed DH2-overexpressing plants revealed a decrease in the expression of genes encoding enzymatic antioxidants (*NtAPX*, *NtCAT*, *NtDHAR*, and *NtMDHAR*). In contrast, Zn stress in non-transgenic plants altered the expression of only NtDHAR. Furthermore, the contents of CGA and rutin in Zn-stressed DH2-overexpressing plants remained unchanged, whereas in non-transgenic plants subjected to Zn stress, they significantly increased, particularly CGA. The less pronounced reaction of non-transgenic plants to excess zinc can be attributed to the fact that tobacco is one of the plant species capable of tolerating higher levels of zinc (Kozak & Antosiewicz 2023).

Previous studies have suggested a role for dehydrin (At2g21490, DH2) in seed development and germination (Candat et al. 2014), particularly in response to water deficit and low temperature (Miura & Tada 2014). Our results indicate that the *DH2* gene contributes to the response to Cu stress in at least two ways: *(i)* by influencing the expression of specific metal transporters and *(ii)* by modulating the levels of both enzymatic and nonenzymatic antioxidants, thereby enhancing the plant's ability to scavenge free radicals efficiently. The differences observed between transgenic and non-transgenic plants in the expression of certain metal transporters suggest that the *DH2* gene plays a role in immobilising excess Cu, primarily in the roots, thereby mitigating its deleterious effects on the aerial parts of the plant. Our findings contribute to enhancing our understanding of the role of His-rich dehydrins in plants under Cu and Zn stress.

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Received: January 21, 2025
Accepted: December 15, 2025
Published online: May 20, 2026