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Genetic foundations of copper stress tolerance in safflower: bridging heavy metal contamination and global warming challenges

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Key message

Integrated phenotyping and multi-omics approaches identified Cu-tolerant safflower genotypes and candidate genes underpinning Cu stress resilience for climate-adaptive breeding.

Abstract

Copper (Cu) contamination threatens agriculture, yet safflower's genetic basis for Cu tolerance remains unexplored—especially as global warming intensifies risks through nighttime warming and soil acidification. We conducted a pot experiment using 94 safflower genotypes under four copper chloride (CuCl_2) concentrations, revealing substantial variation among genotypes and treatments with trait reductions ranging from 1.75% (root-shoot ratio) (RSR) to 57.14% (dry root weight) (DRW). Correlation analysis showed positive trait relationships, while principal component analysis (PCA) grouped genotypes into three clusters. Ten genotypes consistently performed better under Cu stress, indicating strong tolerance potential for climate-resilient breeding. Genome-wide association study (GWAS) detected 180 significant marker-trait associations (MTAs) across eight traits, and gene annotation linked candidate genes to brassinosteroid signaling, ion homeostasis, metal response, and transport. Co-expression analysis revealed a tightly connected tolerance module in which *AT5G01610* and *AT5G14640* shared the same top co-expressed partners ($r > 0.8$), including *HMA4*, *CAX1*, and *SOS1*, suggesting coordinated roles in Cu stress tolerance. Convergent protein-protein interaction (PPI) validation confirmed these candidates as distinct hubs that are physically interacting and transcriptionally co-regulated, supporting their function as Cu tolerance modules. *In silico* heatmap-based expression profiling showed distinct, stress-specific transcriptional responses in *Arabidopsis* roots, with Cu inducing, cadmium (Cd) eliciting mixed, and (Al) aluminum repressing expression. RNA-seq analysis identified seven Cu stress tolerance genes, validated by qRT-PCR. These findings highlight promising genotypes for developing Cu-resilient safflower cultivars, while further investigation of the highly expressed genes is needed to clarify their roles under Cu stress.

Keywords: Genome-wide association study; Stress tolerance index; Candidate genes; Expression visualization; qRT-PCR.

Introduction

Metal toxicity (MT) is a major global environmental concern due to their non-biodegradable nature, causing long-term persistence in ecosystems and posing significant ecological risks (Wan et al. 2024; Vodyanitskii 2016). The toxicity of metals to plants depends on their bioavailability and chemical form in soil (Verdejo et al. 2016). Cu is an essential micronutrient required in trace amounts for plant metabolism but becomes toxic at high levels (Saleem et al. 2020). Excessive use of Cu-based fertilizers and pesticides has led to its accumulation in agricultural soils (Xin et al. 2020; Smolentsev et al. 2024). Elevated Cu disrupts photosynthesis, respiration, enzyme activity, and membrane integrity, leading to oxidative stress through excessive reactive oxygen species (ROS) formation, which damages cells and DNA (Hippler et al. 2018). Cu toxicity also impairs nutrient uptake, lipid membrane stability, and overall growth parameters such as germination, root, leaf, and biomass production (Pätsikkä et al. 2002; Mallick

et al. 2010). Research on crops such as *Boehmeria nivea* (Rehman et al. 2019), *Phyllostachys pubescens* (Chen et al. 2015), and *Brassica napus* (Zlobin et al. 2015) has consistently reported marked reductions in yield under Cu stress conditions. Global warming further intensifies soil heavy-metal risks by enhancing metal mobility and plant uptake, even without new contamination, as rising temperatures and nighttime warming increase root-to-shoot Cu translocation and metal bioavailability via soil acidification (Qi et al. 2024). This “chemical time bomb” effect heightens hazards from legacy pollutants by linking greater metal mobility with altered plant physiology, thereby amplifying Cu stress under climate change (Bibi and Rahman 2023).

Safflower (*Carthamus tinctorius* L.) is a self-pollinated oilseed crop belonging to the Compositae family. It has a haploid genome size of about 1.4 Gb and 24 chromosomes ($2n = 24$) (Kumari et al. 2017). The crop is grown in many parts of the world and is valued for its applications in dye production, edible oil, and traditional medicine (Weiss 2000). Cu stress exerts both dose- and duration-dependent toxic effects in safflower, leading to reduced growth along with activation of antioxidant defense mechanisms. In the variety PBNS-12, lower Cu concentrations (25 μM) have been shown to stimulate root growth, whereas higher concentrations (50–100 μM) result in oxidative damage within 10–20 days (Gautam et al. 2016). At 100 μM Cu, substantial reductions have been reported in root length (82%), shoot length (55%), and tolerance index (62%). The root system is particularly vulnerable, as it is directly exposed to the metal and experiences impaired nutrient uptake (Gautam et al. 2016). Comparable inhibitory effects were also observed by Ahmed et al. (2010), who reported a significant decline in growth at 100 μM Cu. Mitigating Cu stress effects requires identifying tolerant genotypes and their underlying genetic factors. Early growth stages, especially seedling establishment and germination, play a key role in determining final plant performance and yield. For this reason, these stages are widely considered suitable for screening germplasm for stress tolerance (Xu et al. 2024; Thoday-Kennedy et al. 2021; Shaki et al. 2019; Fatahiyan et al. 2025).

Metal tolerance does not follow simple Mendelian inheritance; instead, it is governed by multiple genes acting through complex regulatory networks, which reduces the efficiency of conventional breeding approaches (Mondal et al. 2022). In addition, the effects of stress vary with both intensity and duration (Labudda et al. 2022), making the evaluation of Cu tolerance in genetic panels more challenging. A deeper understanding of the molecular basis of MT is therefore essential for developing Cu-tolerant crops. GWAS offer a powerful means to dissect these complex traits and identify loci associated with MT and other abiotic stress tolerances (Arif et al. 2017). Nevertheless, despite the importance of genetic diversity for Cu tolerance, information on MTAs for Cu response in safflower remains limited. Identifying Cu-responsive genotypes and MTAs is thus a priority for safflower breeding programs aiming to improve Cu tolerance and ensure stable performance under MT. To address this gap, the present study evaluated a safflower genotype panel at the seedling stage under Cu stress using *SilicoDART* markers, with the goal to select Cu-tolerant genotypes based on morphological traits and pinpointing putative candidate genes for marker-assisted breeding.

Materials and methods

Plant material, growth conditions, and trait evaluation

Ninety-four safflower genotypes from 26 countries were obtained from the USDA germplasm collection (Table S1) and grown in a glasshouse under controlled conditions (26-28 °C, 46% humidity, 16/8 h light/dark). The growing substrate consisted of a 1:2 sand-to-soil mixture, passed through a 4 mm sieve and packed into pots at 780 g per container. Soil nutrients were analyzed prior to planting, with micronutrients measured by atomic absorption spectroscopy and macronutrients by flame photometry. Three seeds per pot were sown and later thinned to one seedling. Plants were irrigated with tap water based on genotype requirements. The experiment began on February 20, 2025, following a two-factor randomized complete block design with three replications and four CuCl₂ treatments: T1 (0 µM), T2 (60 µM), T3 (120 µM), and T4 (180 µM). CuCl₂ was applied between 16-35 days after sowing (DAS) in intervals to avoid Cu toxicity shock. All plants were harvested at 35 DAS for morphological measurements. Mean values of each trait across treatments were used for association analysis.

DNA extraction

Genomic DNA was isolated from 14-day-old seedlings using the CTAB method (Doyle and Doyle, 1990) or the DArT protocol (<https://www.diversityarrays.com/services/laboratory-services/>).

Statistical analysis

Phenotypic evaluation and selection of Cu-tolerant genotypes

Phenotypic evaluation was conducted using Statistix 8.1 to obtain descriptive statistics and analysis of variance (ANOVA) results, while data visualization was performed in RStudio version 1.0.153 utilizing the "ggplot2" package for boxplot creation (Wickham 2016), and the "factoextra" package for correlation visualization (Epskamp et al. 2012). PCA was carried out using R version 4.1.3 with the "factoextra" package (Kassambara and Mundt 2017). STI values for each trait under T2, T3, and T4 were calculated using iPASTIC online tool (<https://manzik.com/ipastic>) relative to the control treatment. Mean STI values were computed by averaging trait-wise values within each treatment, followed by an overall genotype STI calculated as the mean of the three treatment-specific values. Data visualization such as bar plots, scatter plots, heatmaps, and histograms was performed in R.

DArTseq markers analysis

All DArTseq-generated images were processed using *DArTsoft* v7.4.7 (DArT P/L, Canberra, Australia). *SilicoDArT* markers, scored as binary data (1 = present, 0 = absent) following Baloch et al. (2017), were filtered based on call rate, polymorphic information content (PIC), and repeatability. Markers with PIC ≥ 0.10, 100% repeatability, and call rate ≥ 80% were retained for further analysis.

Population structure and genome-wide association analysis

Population structure followed Ali et al. (2020a). Marker positions were mapped using the Saf_v1.gff3 reference (Wu et al. 2021). GWAS was conducted in TASSEL 5.0.5 (Bradbury et al. 2007) using a MLM

(Q + K) approach accounting for population structure (Q-matrix) and kinship (K-matrix) following the guidelines of Zia et al. (2020). Significance thresholds were set at $p \leq 0.01$ (Bonferroni and FDR), with p -values indicating association strength and R^2 representing phenotypic variance explained (Jin et al. 2011). Manhattan plots were produced using the "cMplot" package in R 4.0.0.

Systematic identification and characterization of candidate genes

Marker sequences were BLAST searched against TAIR 10 *Arabidopsis* genome (E -values ≤ 0.001) (Swarbreck et al. 2007; Lamesch et al. 2012). Protein sequences of Cu-responsive genes *AT5G14640*, *AT5G01610*, and *AT2G28660* were analysed using InterProScan (<https://www.ebi.ac.uk/interpro/interproscan.html>) for domain/family prediction and WoLF PSORT (https://wolfsort.hgc.jp/_plant_model) for subcellular localization. Gene Ontology (GO) annotations were retrieved from TAIR (<https://www.arabidopsis.org>) and UniProt (<https://www.uniprot.org>), extracting relevant biological process, molecular function, and cellular component terms focused on Cu stress, signalling, and metal binding.

Co-expression, transcriptomic data acquisition and heatmap-based expression profiling

Co-expression analysis was performed using ATTED-II (version 10.0) (Obayashi et al. 2018; <https://atted.jp/>). Pearson correlation coefficients (r) were calculated across publicly available *Arabidopsis* transcriptome datasets. Genes with $r > 0.8$ were considered significantly co-expressed. The highly co-expressed genes were retrieved for each candidate, and overlapping sets were analyzed to identify core regulatory modules. Gene ontology (GO) enrichment analysis was performed using ATTED-II built-in tools with FDR correction.

Expression profiling of eight candidate genes under metal stress were analyzed using publicly available transcriptomic datasets from the NCBI Gene Expression Omnibus (GEO) and EMBL-EBI ArrayExpress (BioStudies) databases (<https://www.ebi.ac.uk/biostudies/arrayexpress>). Root-specific responses to heavy metal rhizo-toxicity were evaluated using the Agilent two-color microarray dataset E-MEXP-1907 (Zhao et al. 2009). Feature extraction software was processed to isolate the LogRatio values representing the log₂ fold-change between metal-treated (Cy5) and control (Cy3) roots. To maintain directional accuracy, log-ratios from dye-swapped arrays (Control-Cy5 vs. Treated-Cy3) were mathematically inverted (multiplied by -1). Data for sodium chloride (NaCl) treatments were excluded to strictly focus on heavy metal toxicity. The extracted LogRatios for Cu, Cd, and Al treatments were then averaged across their respective biological replicates. The datasets underwent independent Z-score normalization across genes to enable standardized cross-platform comparison. All analyses were carried out in R. Data processing was done with the tidyverse package (Wickham et al. 2019), and visualization was performed using ComplexHeatmap. Hierarchical clustering of genes (rows) was based on euclidean distance (Gu et al. 2016). A blue-white-red diverging color scale (Z-score: -2 to +2) was used to represent relative expression levels, with exact zero values masked to improve clarity.

Protein-protein interactions and GO enrichment analysis

Orthologues were used to build PPI networks in STRING 12.0 (confidence ≥ 0.7 , combined score ≥ 400) (Szkarczyk et al. 2019; von Mering et al. 2005), followed by GO enrichment in PANTHER v.14 (Fisher's exact test, Bonferroni correction, $P < 0.01$) (Mi et al. 2019; Thomas et al. 2022) and visualization of biological process in STRING v.12.0 (signal and strength ≥ 0.01).

RNA extraction and qRT-PCR analysis

The RNAprep Pure Plant Kit (TIANGEN, Beijing, China) was used to extract RNA from the plant material. A NanoDrop 2000 C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the RNA concentration of each sample. Genomic DNA was removed from 1 g of RNA per sample using DNase I, prior to its use as a template for reverse transcription to synthesize the required quantity of cDNA (QuantiTect Reverse Transcription Kit; Qiagen, Shanghai, China). For each qRT-PCR, the RNA samples were normalized using the expression level of the actin gene as a reference. Each sample was analyzed in three technical replicates and three biological replicates for qRT-PCR, with *actin* gene serving as the internal control (Li and Leisner 2008). The LightCycler 480 real-time PCR machine (Applied Biosystems, St. Louis, MO, USA) and SYBR Green Master Mix (Novogene, Shanghai, China) were both utilized. The qRT-PCR results were analyzed using the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen 2001).

Results

Soil analysis and phenotypic evaluation

Safflower germplasm was cultivated in a 1:2 sand-soil mixture (Table S2). Two-way ANOVA revealed significant effects of genotype, CuCl₂ treatment, and their interaction on all measured traits (Table 1).

Table 1 Analysis of variance demonstrated significant effects of genotype (G) and CuCl₂ treatment (CC), as well as a significant genotype $\times$ treatment interaction (G $\times$ CC)

Trait	Source of variation	Mean squares	F value	P value
DRW	CC	0.07647	118.2	0.0000
	G	0.00402	6.21	0.0000
	G * CC	0.00079	1.23	0.0181
DSW	CC	0.22036	539.34	0.0000
	G	0.00336	8.22	0.0000

	G * CC	0.0013	3.18	0.0000
	CC	1.52243	1276.35	0.0000
FRW	G	0.07681	64.39	0.0000
	G * CC	0.00509	4.27	0.0000
	CC	5.32124	3231.71	0.0000
FSW	G	0.11159	67.77	0.0000
	G * CC	0.00945	5.74	0.0000
	CC	190.173	657.15	0.0000
NOL	G	1.499	5.18	0.0000
	G * CC	0.344	1.19	0.0375
	CC	112.883	6605.58	0.0000
PH	G	3.091	180.88	0.0000
	G * CC	0.501	29.32	0.0000
	CC	684.507	3918.19	0.0000
RL	G	11.844	67.8	0.0000
	G * CC	2.278	13.04	0.0000
	CC	0.15155	6.2	0.0004
RSR	G	0.25078	10.26	0.0000
	G * CC	0.05025	2.06	0.0000

G = genotype, CC = CuCl₂, G * CC = genotype × CuCl₂ interactions

CuCl₂ treatments significantly influenced all morphological traits examined (Fig. 1). Increasing Cu concentration led to a steady decline in shoot traits. Traits such as FSW, DSW, PH, and NOL decreased from 0.71 g, 0.13 g, 4.08 cm, and 6.09 cm under control conditions (T1) to 0.39 g, 0.06 g, 2.61 cm, and 4.16 under the highest stress treatment (T4), respectively. Root traits showed a similar pattern. Root traits such as RL, FRW, and DRW declined from 9.06 cm, 0.40 g, and 0.07 g in T1 to 5.42 cm, 0.23

g, and 0.03 g in T4. In contrast, RSR did not follow a linear trend. It decreased slightly from 0.57 in T1 to 0.56 in T2, then increased to 0.58 and 0.61 under T3 and T4, respectively (Table 2).

Table 2 Summary statistics of traits measured in safflower genotypes under varying CuCl₂ concentrations

Treatment		T1				T2				T3				T4				Mean data			
Variable	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	
DRW	0.07	0.03	0.01	0.11	0.05	0.02	0.01	0.09	0.05	0.03	0.01	0.21	0.03	0.01	0.01	0.07	0.05	0.02	0.01	0.11	
DSW	0.13	0.04	0.04	0.32	0.1	0.02	0.06	0.17	0.08	0.02	0.05	0.12	0.06	0.01	0.01	0.09	0.09	0.02	0.06	0.14	
FRW	0.4	0.1	0.17	0.63	0.34	0.09	0.14	0.52	0.29	0.09	0.13	0.47	0.23	0.08	0.09	0.43	0.32	0.08	0.14	0.49	
FSW	0.71	0.13	0.37	1.02	0.62	0.11	0.33	0.92	0.51	0.1	0.27	0.77	0.39	0.09	0.17	0.63	0.56	0.1	0.3	0.77	
NOL	6.09	0.55	5	8	5.46	0.49	4.33	6.67	4.91	0.43	4	6	4.16	0.33	3.33	5	5.16	0.35	4.17	6.17	
PH	4.08	0.67	2.6	5.7	3.68	0.57	2.47	5.3	3.21	0.57	1.6	4.67	2.61	0.66	0.6	4.1	3.4	0.51	2.14	4.79	
RL	9.06	1.59	4.8	12.6	7.87	1.15	5.4	10.73	6.74	1.04	4.3	9.4	5.42	1.14	3	8.33	7.27	0.99	5.19	9.45	
RSR	0.57	0.12	0.29	0.92	0.56	0.13	0.2	0.92	0.58	0.16	0.25	1.06	0.61	0.23	0.23	1.46	0.57	0.13	0.26	0.93	

DRW = dry root weight, DSW = dry shoot weight, FRW = fresh root weight, FSW = fresh shoot weight, NOL = number of leaves, PH = plant height, RL = root length, RSR = root shot ratio.

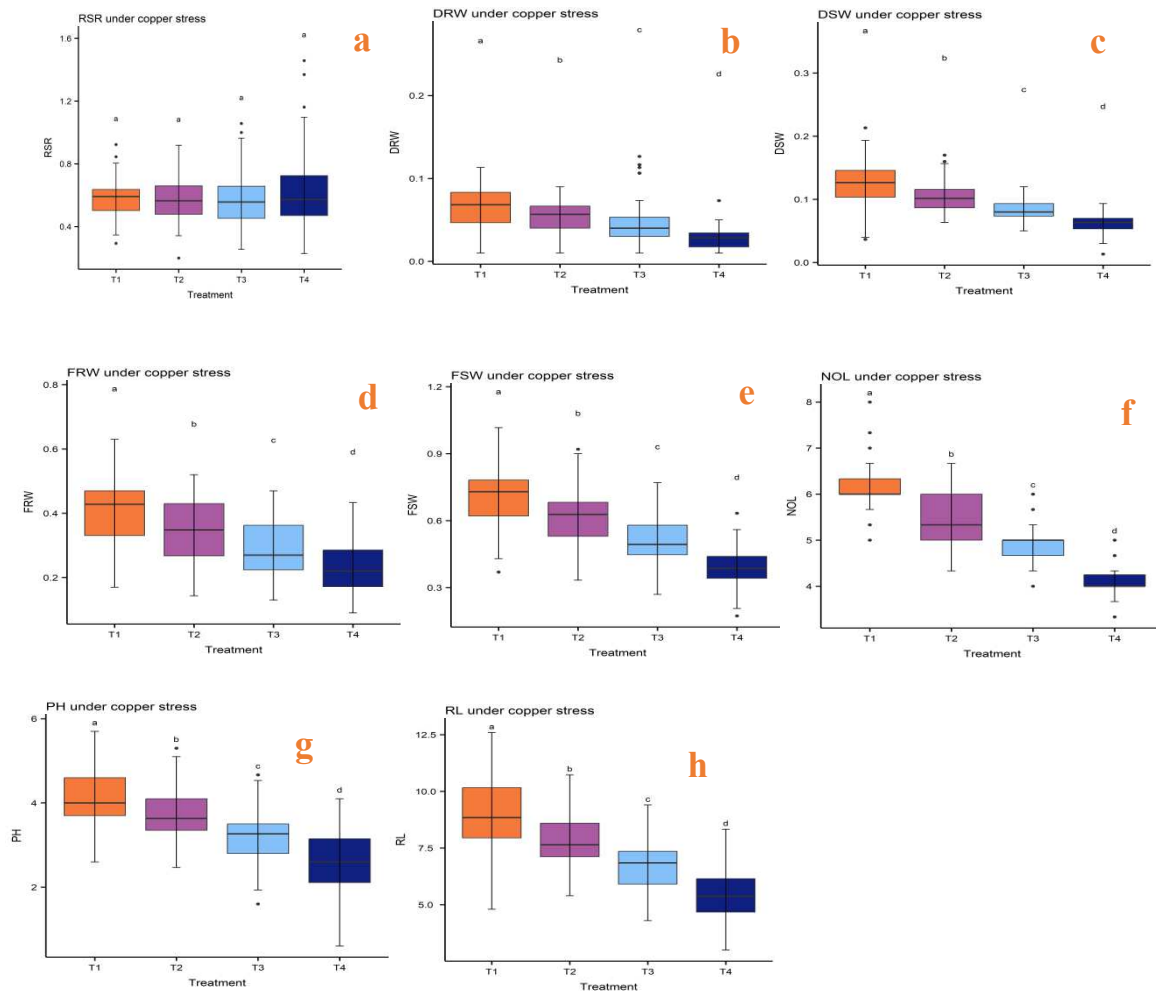


Fig. 1 Boxplot analysis showed significant treatment differences. (a) RSR = root shoot ratio, (b) DRW = dry root weight, (c) DSW = dry shoot weight, (d) FRW = fresh root weight, (e) FSW = fresh shoot weight, (f) NOL = number of leaves, (g) PH = plant height, (h) RL = root length

Correlation analysis showed significant positive associations among most traits, with the exception of RL and RSR. RL was correlated only with plant height, while RSR showed significant associations with DRW, FRW, and FSW (Fig. 2). The first two principal components, each with eigenvalues greater than 1, together explained 37.8% of the total phenotypic variation (PC1 = 27.2% and PC2 = 10.6%). Biplot analysis grouped the genotypes into three distinct clusters (Fig. 3). Considerable variation in Cu stress tolerance was evident among the 94 safflower genotypes, reflected in their STI values. The broad distribution of STI values suggests considerable variation among genotypes, with highly tolerant lines representing valuable genetic resources for developing Cu-tolerant safflower cultivars (Fig. 4).

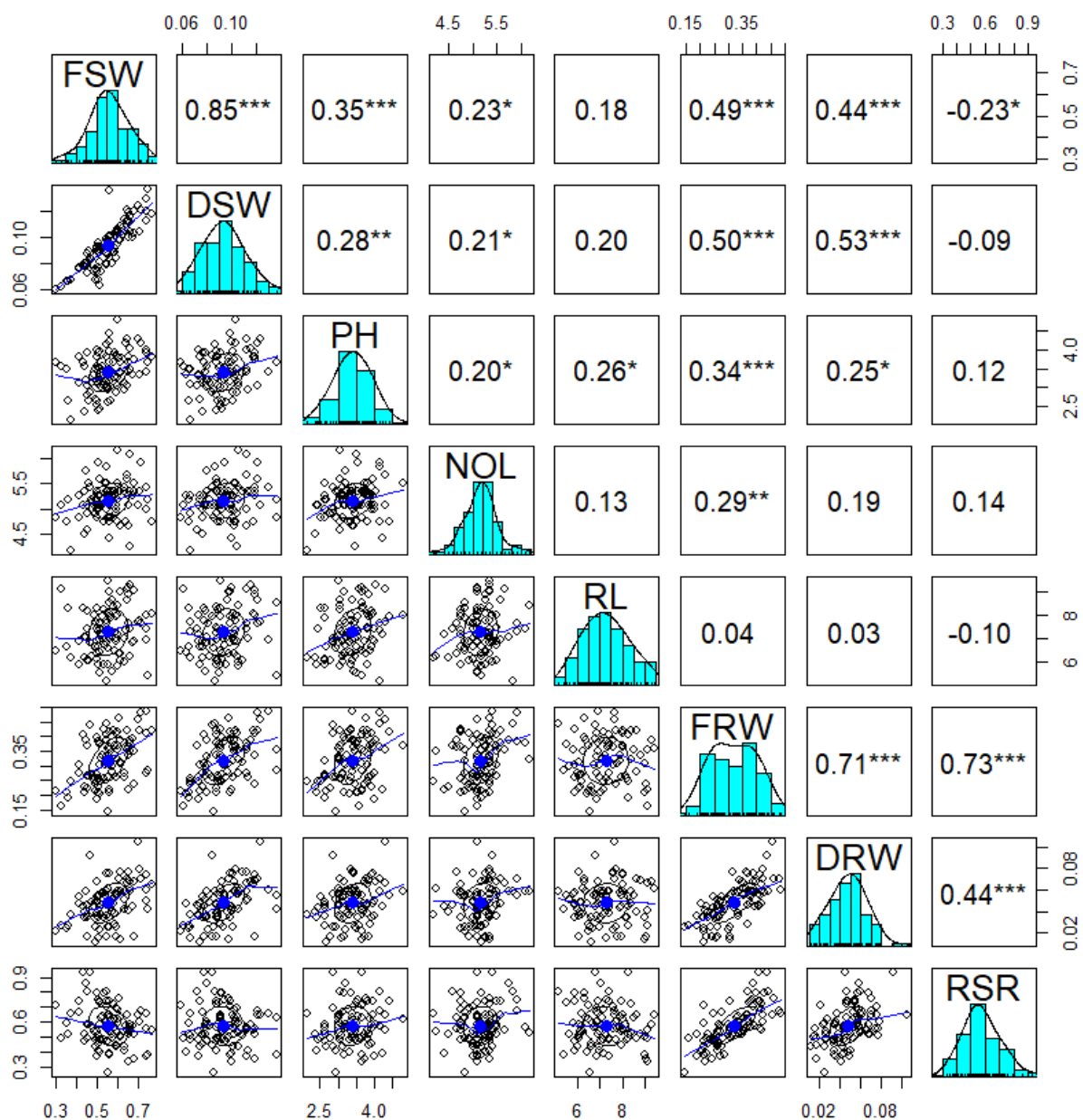


Fig. 2 Correlation matrix of studied traits calculated from treatment means ($n = 4$). DRW = dry root weight, DSW = dry shoot weight, FRW = fresh root weight, FSW = fresh shoot weight, NOL = number of leaves, PH = plant height, RL = root length, RSR = root shoot ratio

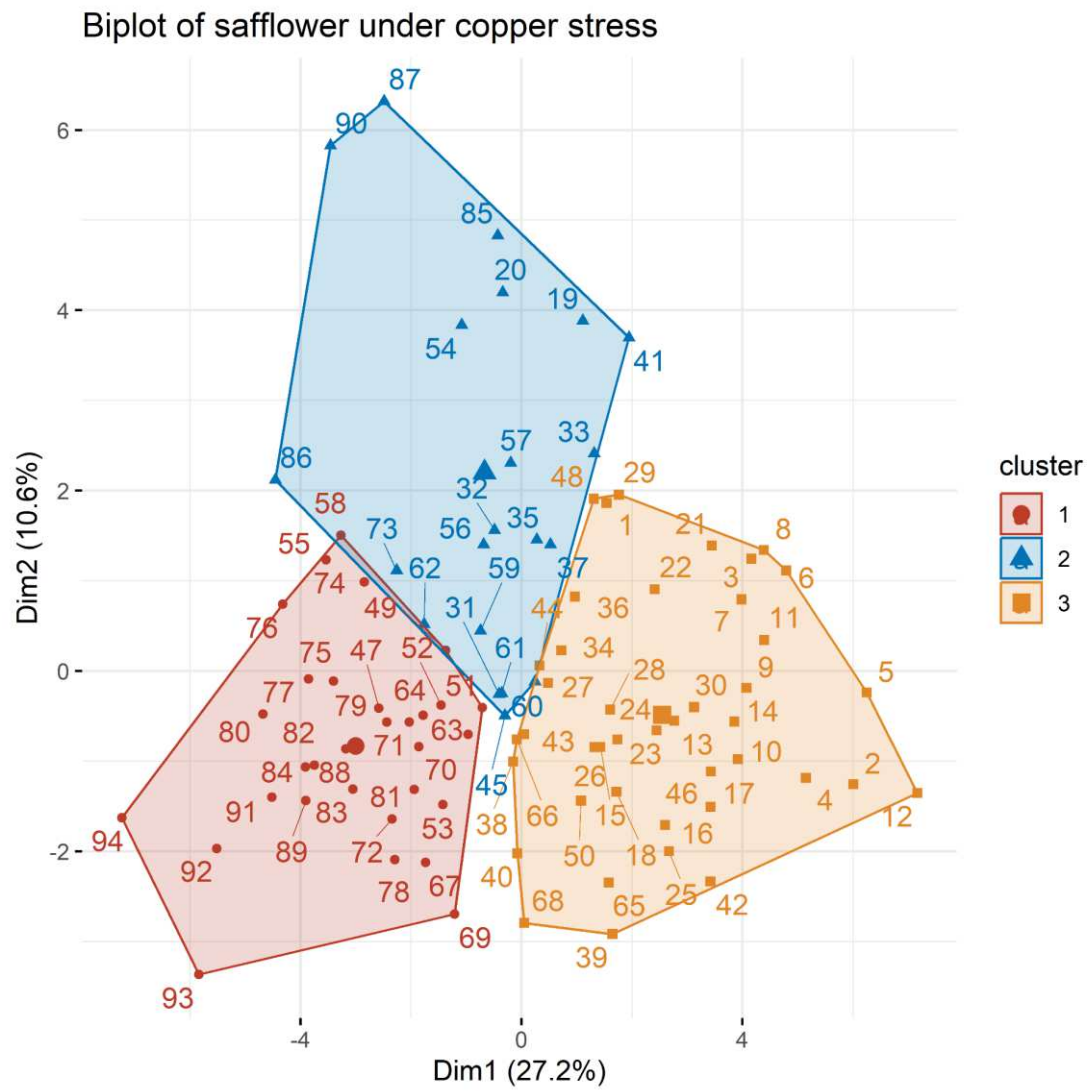


Fig. 3 PCA-based classification of safflower genotypes clustered into three groups using averaged treatment data.

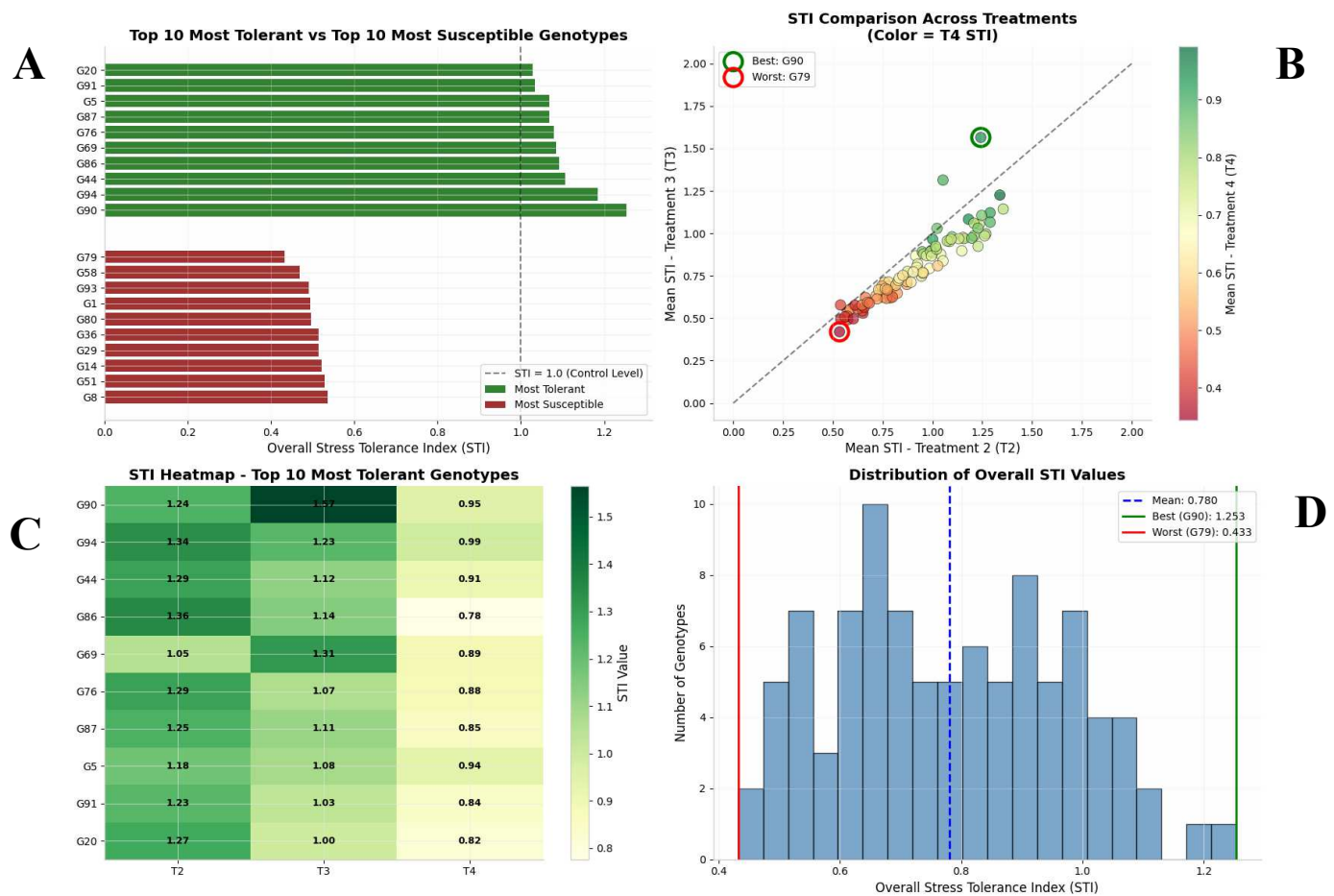


Fig. 4 STI analysis was used to distinguish Cu-tolerant and susceptible genotypes. Four complementary approaches were applied based on mean STI values across the three stress treatments (T2–T4) relative to the control (T1). **(A)** A bar plot ranked the top 10 tolerant and top 10 susceptible genotypes. A dashed line at STI = 1 represents average performance, with values above 1 indicating tolerance and values below 1 indicating susceptibility. **(B)** A scatter plot comparing STI values of T2 and T3, with points colored according to T4. The diagonal line represents equal performance across treatments, while points above the line indicate improved performance under higher stress. **(C)** A heatmap displaying the top 10 tolerant genotypes across treatments. Darker shades represent higher tolerance and highlight stability across different stress levels. **(D)** Histogram of STI distribution (n=94). Vertical lines: mean (blue dashed), max (green), min (red)

Trait-associated markers identification

A total of 12,232 highly informative DArTseq markers were analyzed using the mixed linear model (MLM; Q + K) across four treatments for genome-wide association mapping. This approach identified 180 significant MTAs across all treatments. Trait-specific MTAs were as follows: 23 for DRW, 34 for

DSW, 20 for FRW, and 34 for FSW. Additionally, 16, 14, 18, and 21 MTAs were detected for NOL, PH, RL, and RSR, respectively (Table S3; Fig. 5).

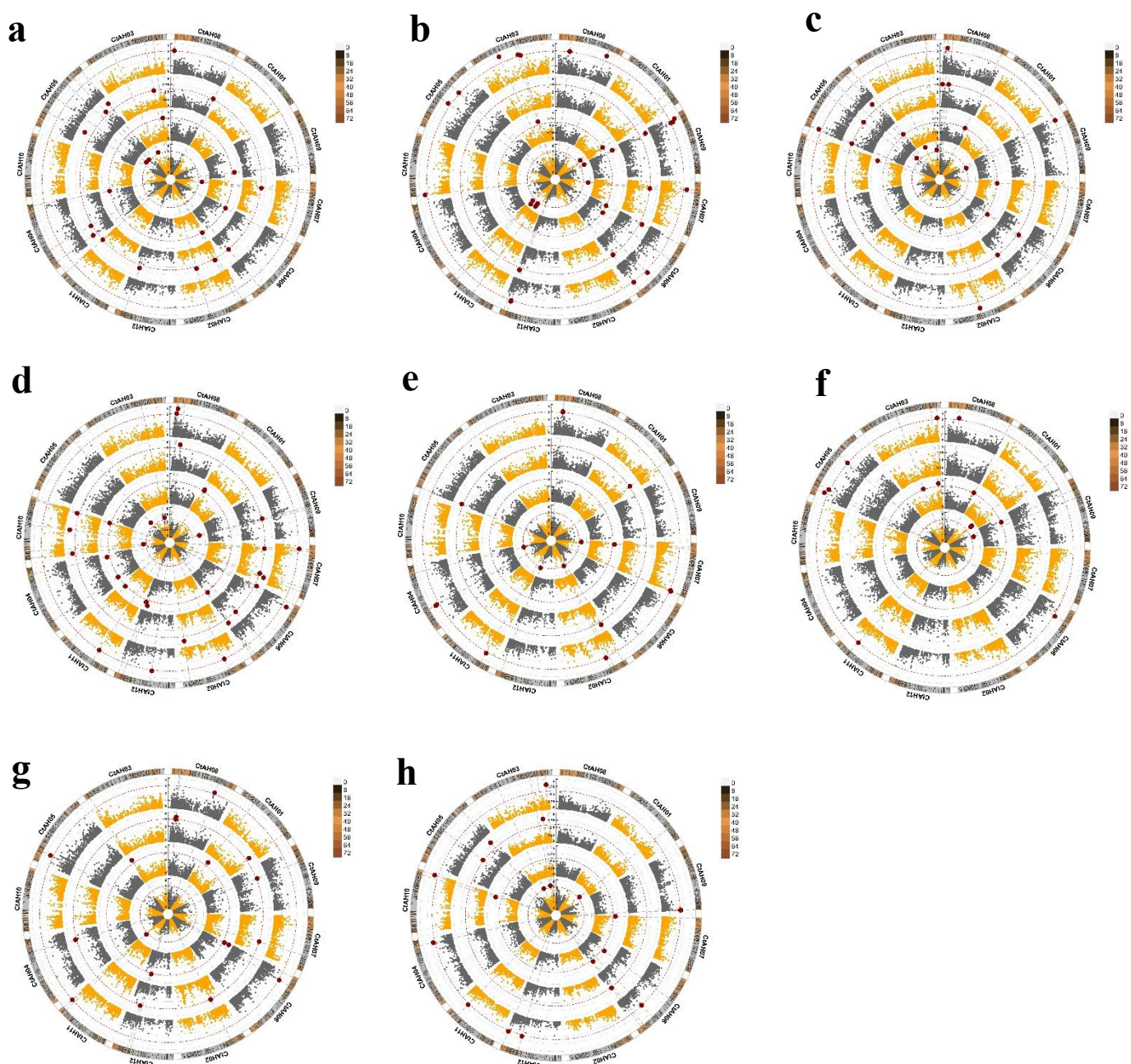


Fig. 5 Circular manhattan plots for (a) DRW, (b) DSW, (c) FRW, (d) FSW, (e) NOL, (f) PH, (g) RL, and (h) RSR. Concentric circles represent treatments T1 (inner) through T4 (outer). The dotted red line denotes significance at $p < 0.001$ ($-\log_{10} \geq 3$), with significant associations highlighted in red. LOD thresholds are scaled between chromosomes 1–12. SNP density gradients (grey to pink) are indicated by colored boxes

Candidate gene identification and *in silico* functional characterization

We identified genomic loci associated with significant GWAS markers and determined their *Arabidopsis thaliana* orthologues through BLAST analysis to elucidate the molecular basis of Cu tolerance in safflower (Table 3). InterProScan identified *AT5G14640* as containing glycogen synthase kinase-3 alpha family and phosphorylase kinase/transferase(phosphotransferase) domain; *AT5G01610* as T31J12.3 protein-related family with *At5g01610*-like domain; and *AT2G28660* as Chloropl_CC family with heavy-metal-associated domain. WoLF PSORT predictions assigned *AT5G14640* to the cytoplasm, *AT5G01610* to the nucleus, and *AT2G28660* to the chloroplast. TAIR/UniProt GO terms associated *AT5G14640* with hyperosmotic/salt stress response, signal transduction, ATP binding, and protein kinase activity; classified *AT5G01610* as unknown/putative regulatory function; and annotated *AT2G28660* with nucleotidyltransferase activity, metal ion/nucleotide binding, and chloroplast targeting.

Co-expression and *in silico* heatmap-based expression profiling

Co-expression analysis using ATTED-II revealed that *AT5G01610* (T8018.5, heavy metal homeostasis) and *AT5G14640* (*SK13*/ASKepsilon) share identical highly co-expressed genes ($r > 0.8$), forming a tight Cu tolerance module (Table 4). This module was significantly enriched for transition metal ion transport (GO:0046915, FDR = 2.3×10^{-8}) and Cu ion response (GO:0010038, FDR = 1.2×10^{-6}). Top partners included *HMA4* ($r = 0.92$), *CAX1* ($r = 0.89$), and *SOS1* ($r = 0.87$) - established heavy metal transporters indicating strong transcriptional coordination. *In silico* heatmap-based expression profiling of GWAS- and co-expression identified candidate genes revealed distinct transcriptional responses under Cu, Cd, and Al stress in *Arabidopsis thaliana* roots. Under Cu stress, most genes showed moderate to strong upregulation, with *AT5G01610*, *AT4G32870*, and *AT5G54780* exhibiting consistently elevated expression, while Cd stress induced more heterogeneous patterns including both repression (*AT5G14640*, *AT3G14280*) and strong induction (*AT5G66250*). Al stress led to reduced expression in most of the candidate genes, including *AT2G02960*, *AT4G32870*, and *AT5G54780*, while *AT5G14640* and *AT3G14280* showed clear upregulation. Hierarchical clustering distinguished genes induced by Cu from those suppressed under Al stress, and grouped Cd-responsive genes into a separate cluster, indicating distinct regulatory patterns under different metal stresses (Fig. 6).

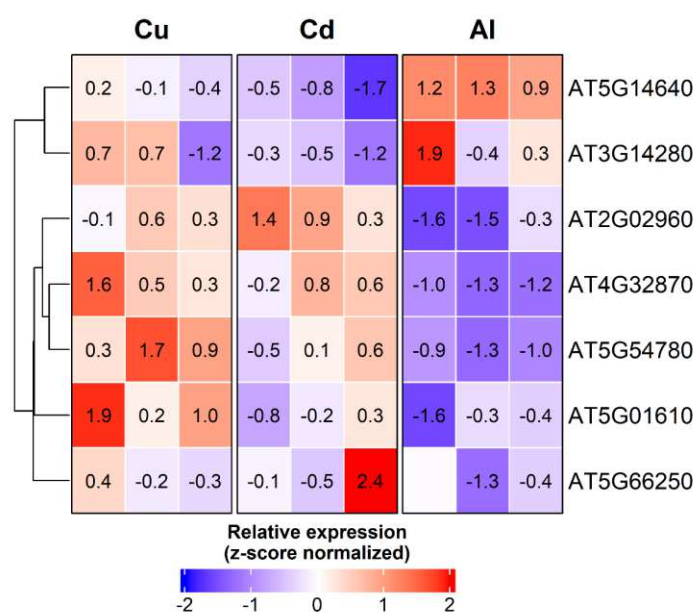


Fig. 6 *In silico* heatmap-based expression profiling of candidate genes in *Arabidopsis thaliana* roots under Cu, Cd, and Al stress conditions. Expression values were z-score normalized, where red colour indicates upregulation and blue colour indicates downregulation. Hierarchical clustering groups genes based on similarity in expression patterns

Table 3 *Arabidopsis* orthologs of the genes

Marker name	Associated trait	AGI	Gene abbreviation	Gene name	Sequence similarity (%)	E value
Treatment: T4						
<i>DArT</i> -15672192	DRW4	<i>AT5G14640</i>	<i>SK13</i>	(SHAGGY-LIKE KINASE 13) (Primary Symbol), ATSK13 (SHAGGY-LIKE KINASE 13), ASKepsilon	58.1	0.000000004
<i>DArT</i> -100007602	DSW4	<i>AT2G28660</i>	<i>ATHMP18</i>	ATHMP18 (HEAVY METAL ASSOCIATED PROTEIN 18, Chloroplast-targeted copper chaperone protein)	39.2	0.001
Treatment: T2, T3, T4						
<i>DArT</i> -15670616, <i>DArT</i> -15670616, <i>DArT</i> -15670616	DSW3, DSW4, FSW2	<i>AT5G01610</i>	<i>DUF538</i>	hypothetical protein (Protein of unknown function, DUF538)	40.1	0.001

Table 4 Target genes used in root-specific expression profiling

Gene ID	Gene number/name	Function
<i>AT5G54780</i>	<i>AT5G54780</i>	Ypt/Rab-GAP domain of gyp1p superfamily protein
<i>AT4G32870</i>	<i>AT4G32870</i>	Polyketide cyclase/dehydrase and lipid transport superfamily protein
<i>AT3G14280</i>	<i>AT3G14280</i>	LL-diaminopimelate aminotransferase
<i>AT2G02960</i>	<i>AT2G02960</i>	RING/FYVE/PHD zinc finger superfamily protein
<i>AT5G66250</i>	<i>AT5G66250</i>	Kinectin-like protein
<i>AT5G14640</i>	<i>SK13</i> (SHAGGY-LIKE KINASE 13), <i>ASKepsilon</i>	Serine/threonine protein kinase
<i>AT5G01610</i>	<i>DUF538</i>	Hypothetical protein, Domain of Unknown Function 538
<i>AT2G28660</i>	<i>ATHMP18</i>	Heavy Metal Associated Protein 18, chloroplast-targeted copper chaperone

PPI networks and GO enrichment reveal convergent Cu tolerance mechanisms

To elucidate the molecular mechanisms underlying Cu tolerance, we constructed PPI networks for candidate genes identified through DArT marker-trait associations. Three markers - *DArT-15672192* (associated with DRW in T4), *DArT-100007602* (associated with DSW in T4), and *DArT-15670616* (linked to DSW in T3, DSW in T4, and FSW in T2) - were selected for orthologue identification, yielding 2, 4, and 9 orthologues, respectively (Table S4; Fig. 7-9). PPI network analysis revealed that ASK5 (an SKP1-like protein; orthologue of *DArT-15672192*) interacted with 10 proteins (Fig. 7), T8018.5 (orthologue of *DArT-100007602*) interacted with six proteins (Fig. 8), and LOC108797327 (orthologue of *DArT-15670616* across all three trait-timepoint combinations) interacted with 10 proteins (Fig. 9), highlighting these hub genes as key candidates underlying Cu tolerance through distinct molecular pathways.

We performed GO enrichment analysis on protein clusters from PPI networks to determine their biological significance (Fig. S1–S3). GO enrichment of PPI clusters revealed protein-specific pathways: ASK (*Arabidopsis* SKP1-like) proteins featured key role in brassinosteroid mediated signalling pathway and signal transduction (Fig. S1). T8018.5 involved in metal ion homeostasis, detoxification of metal ion, transition metal ion homeostasis, and response to metal ion (Fig. S2). LOC108797327 involved in multiple processes such as golgi to plasma membrane transport, exocytosis, recognition of pollen, pollination, cell communication, vesicle tethering involved in exocytosis, vesicle docking involved in exocytosis, and seed coat development (Fig. S3). These networks reveal interconnected pathways mediating safflower Cu responses. Convergent validation revealed that GWAS-derived orthologs T8018.5 (*AT5G01610*) and LOC108797327 (*AT5G14640*) form distinct PPI hubs (6–10 stress partners each) while sharing identical ATTED-II co-expression partners ($r > 0.8$), including *HMA4*, *CAX1*, and

SOS1. These genes physically interact in PPI networks and are transcriptionally coregulated across stress conditions.

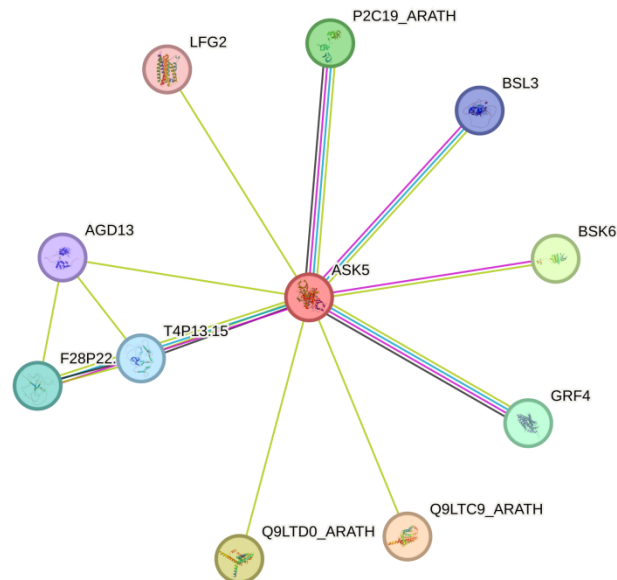


Fig. 7 PPI network analysis was performed for loci associated with the SNP marker linked to DRW under treatment T4, which involved exposure to 180 μ M CuCl₂. The PPI networks were generated using the STRING database

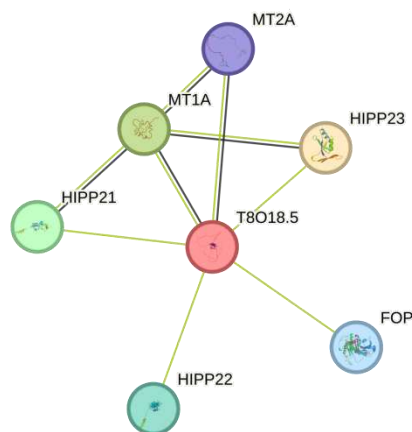


Fig. 8 PPI network analysis was performed for loci associated with the SNP marker linked to DSW under treatment T4, which involved exposure to 180 μ M of CuCl₂. The PPI networks were generated using the STRING database

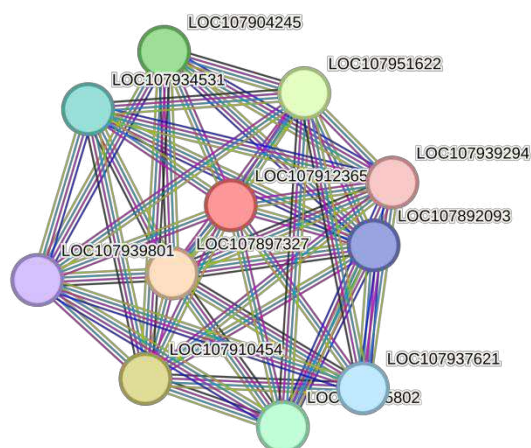


Fig. 9 PPI network analysis was performed for loci associated with the SNP marker linked to DSW under treatment T3 and T4, and FSW in T2, which involved exposure to 60, 120, and 180 μ M of CuCl₂. The PPI networks were generated using the STRING database

qRT-PCR-based gene expression visualization

The primer sequences used for qRT-PCR-based gene expression visualization are detailed in Table 5. Candidate genes exhibited distinct upregulation under Cu stress, with *AT5G01610*, *AT4G32870*, and *AT5G54780* showing the strongest induction at higher Cu concentrations (120–180 μ M). These genes showed clear increases in expression with rising Cu levels, along with marked upregulation of *AT5G66250*, *AT2G02960*, and *AT3G14280*. The observed patterns suggest that they act together in Cu homeostasis and detoxification processes (Fig. 10).

Table 5 List of the primers used in this study

Genes	F/R	Primer Sequence 5' to 3'
<i>AT5G54780</i>	F	TGCGGGAAGTACGAGAGAGA
	R	TCATTGCACCAGTACAGGCTT
<i>AT3G14280</i>	F	AAGCCACAGTTTCACAGCCT
	R	TGTTTGAATCGCTCAGCTCCT
<i>AT2G02960</i>	F	TGTTTCAGCGTTGGTGCAATG
	R	CCAACGCAAACGTGTAGCC
<i>AT5G66250</i>	F	GGCTAGCTGCTTTAGGGGTT
	R	GGCCATCATTTTGCCTGCT
<i>AT5G14640</i>	F	AAGCGTACACCTCCAGAAGC
	R	AACATTGTTTTCGGGCGTGG
<i>AT5G01610</i>	F	ACTGGTTAGGCCAAAAGGCT
	R	TCGTAGTTTGTAGCATCTCCCCG

<i>At4g32870</i>	F	TGCAACGTCCAAGAGTGGTT
	R	TCCACTGGCGTTACTTGGAC
<i>Actin</i>	F	CAACCGGTATTGTGCTGGATTCTGG
	R	GCAAGGTCAAGACGGAGGATGG

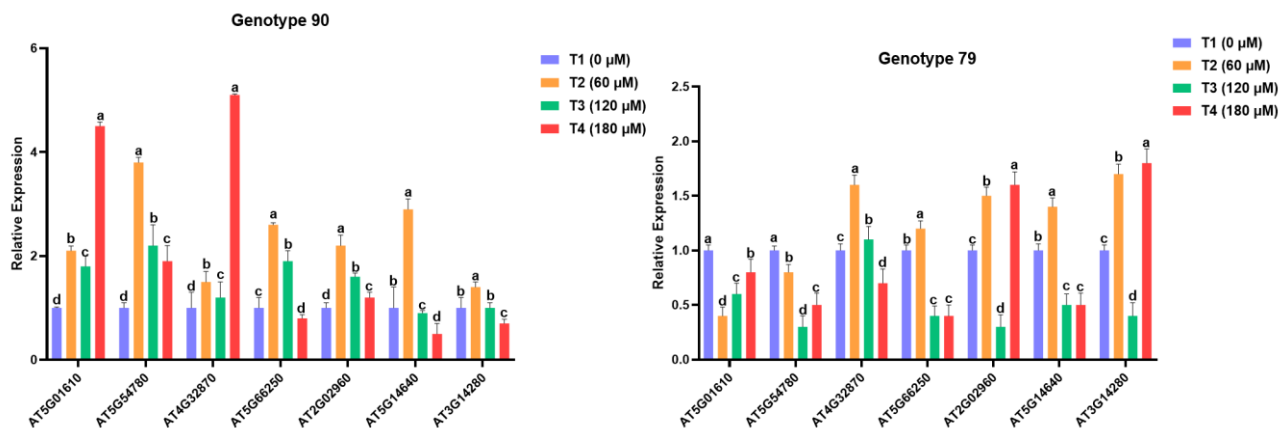


Fig. 10 Expression patterns of selected genes associated with Cu stress tolerance, shown as mean values with standard errors ($\pm$ SE). Different letters indicate significant differences at $p \leq 0.05$. Genotype 90 represents the resistant line, while genotype 79 represents the susceptible line

Discussion

Breeding for Cu toxicity tolerance is difficult because the trait is controlled by many genes, and the genomic regions involved in safflower are still not well defined. To address this, a diverse set of genotypes was evaluated at the seedling stage to identify promising lines and candidate genes associated with Cu tolerance.

Phenotypic variation and tolerant genotype selection

ANOVA revealed significant effects of genotype, CuCl_2 treatment, and their interaction for all traits (Table 1), indicating substantial genetic variation (Ali et al., 2025, 2026). T2 (60 mM) caused minimal growth reduction, whereas T4 induced maximum inhibition (Fig. S4). Compared with the control, DRW, DSW, FRW, FSW, NOL, PH, and RL decreased by 28.57%–57.14%, 23.08%–53.85%, 15.00%–42.50%, 12.68%–45.07%, 10.34%–31.69%, 9.80%–36.03%, and 13.13%–40.18%, respectively, across T2–T4. RSR decreased 1.75% in T2 but increased 1.75% and 7.02% in T3 and T4, indicating altered biomass allocation (Fig. S4). These patterns demonstrate moderate Cu tolerance in safflower (Gautam et al.,

2016). Cu ≥ 50 $\mu\text{g/g}$ affects lateral root development (Li et al. 2015a), with roots more sensitive than shoots (Houshmandfar and Moraghebi 2011). Sayyad et al. (2009) showed Cu stress reduced safflower biomass by only 6% versus 12% in wheat, suggesting higher relative tolerance. Pronounced genotypic variation under elevated Cu makes moderate-to-severe stress optimal for screening.

Correlation analysis revealed strong positive associations among traits, with FSW and FRW most strongly correlated ($r = 0.89^{***}$), indicating coordinated growth (Gautam et al. 2016). PH correlated moderately with FSW and FRW, consistent with documented stress effects (Houshmandfar and Moraghebi 2011). RL showed weak correlations with biomass parameters ($r = 0.03\text{--}0.12$), suggesting Cu-induced root thickening decouples elongation from accumulation (Li et al. 2015). PCA explained 37.8% of variation and clustered genotypes into three groups based on FSW, DSW, and FRW with mean values of 0.614 g, 0.104 g, 0.356 g. STI analysis identified genotypes 90, 94, 44, 86, 69, 76, 87, 5, 20, and 91 as Cu-tolerant (Fig. 4). These genotypes were clustered into group 1 (86, 87, 76, 90, 91, and 94), group 2 (20, 44, and 69), and group 3 (5). Elevated STI indicates superior stress tolerance (Ali et al. 2026), so these genotypes represent valuable parents for breeding Cu-tolerant cultivars.

Characterization of Cu tolerance genes

Identifying loci controlling key morphological traits is essential for marker-assisted breeding under climate change. Few studies have reported markers associated with morphological traits in safflower (Ali et al. 2026), and none under Cu stress. Here, 16 markers showed associations with multiple traits or consistent associations across Cu stress levels: 15 for DSW, 8 for FRW, 6 for RSR, 4 for DRW, 2 for NOL, and 1 each for FSW and RL (Table S3). These stable markers should be validated and converted into KASP assays to accelerate breeding for Cu tolerance. Quantitative traits are influenced by genetic and environmental factors (Yang et al. 2021). Comparable results include 11 stable markers in wheat (Safdar et al. 2020) and 18 in safflower under salt stress (Ali et al. 2026), supporting the genetic basis of stress resilience.

Arabidopsis orthologues *AT5G14640* (GSK-3 kinase, cytoplasm), *AT5G01610* (nucleus), and *AT2G28660* (heavy-metal-associated domain, chloroplast) of safflower GWAS loci encode proteins involved in Cu sensing, signaling, and detoxification (Table 3). Their co-expression ($r > 0.8$) with transporters *HMA4*, *CAX1*, and *SOS1*, plus GO enrichment for transition metal ion transport ($\text{FDR} = 2.3 \times 10^{-8}$) and Cu ion response ($\text{FDR} = 1.2 \times 10^{-6}$), reveals a conserved network underpinning Cu homeostasis through signalling, nuclear regulation, and plastid-level chaperoning. Complete overlap in co-expressed genes between *AT5G01610* and *AT5G14640* suggests co-regulation by shared cis-regulatory elements (del Pozo et al. 2010; Wang et al. 2025). Module enrichment for metal transport and Cu response, with top partners *HMA4*, *CAX1*, and *SOS1*—mediating plasma membrane exclusion (Courbot et al. 2007; Zimmermann et al. 2009), vacuolar sequestration (Oh et al. 2010), and long-distance transport (Oh et al. 2009)—indicates that these genes act as hubs integrating detoxification pathways (del Pozo et al. 2010; Zhao et al. 2005) (Table 4). This convergence of GWAS, PPI, and co-expression validates these as functional Cu tolerance hubs, reducing false-positive risk (Agrahari et al. 2021; Wang et al. 2025).

This study demonstrates that GWAS and co-expression identified candidate genes exhibit distinct stress-specific transcriptional responses, with Cu stress activating defense and detoxification pathways, Cd stress eliciting mixed induction and repression patterns, and Al stress predominantly causing gene repression. The strong upregulation of genes like *AT5G66250* under Cd and the specialized induction of *AT5G14640* and *AT3G14280* under Al suggest their involvement in metal-specific tolerance mechanisms. Hierarchical clustering supports the existence of coordinated regulatory networks responding to different metal stresses, while the consistency between GWAS identification and stress-responsive expression strengthens the functional relevance of these candidates. These findings provide valuable insights into molecular mechanisms of heavy metal tolerance and highlight key genes for future functional validation and crop improvement strategies (Fig. 6).

ASK5 (*DarT-15672192*) in PPI network encodes a flower-specific SKP1-like protein and SCF E3 ubiquitin ligase component. Unlike stress-responsive paralogs ASK1 and ASK2, ASK5 interacts with limited F-box proteins, indicating a specialized stress role (Zhao et al. 2003). ASK5 forms SCF complexes with CUL1 and belongs to rapidly evolving Clade 3, showing lower conservation than stress-associated Clade 1 members (Dezfulian et al. 2012). ASK5 is predominantly flower-expressed (Zhao et al. 2003). Though experimentally unvalidated, ASK5 may protect reproductive organs by degrading damaged proteins via ubiquitination, regulating floral development, and maintaining fertility. Through SCF-mediated hormonal signaling (auxin, jasmonic acid, gibberellin) and ubiquitin–proteasome activity, ASK5 could target oxidatively damaged or misfolded proteins to support stress adaptation in reproductive tissues (Fig. 7).

T8O18.5 (*AT2G28660*) in PPI network encodes a Cu chaperone family protein implicated in heavy metal (Cu, Pb) stress tolerance. It contributes to metal chelation and detoxification, particularly in roots, and interacts with metallothioneins and HIPP proteins within a broader metal defense network (Auguy et al. 2016; Schiavon et al. 2007; Dezfulian et al. 2012). Although less characterized than metallothioneins or COPT transporters, T8O18.5 represents a key component of the plant's heavy metal detoxification system (Fig. 8).

LOC107897327 (AT5G01610) in PPI network encodes a *DUF538* domain-containing protein (170 amino acids) localized to cytoplasm and nucleus, classified within the heavy metal transport/detoxification superfamily (Li and Lan 2015). Structural analysis reveals a β -complex fold (PDB: 1YDU) typical of metal-binding proteins (Szakasits et al. 2009). In *Gossypium hirsutum*, orthologs exhibit dense co-expression with exocyst subunits Sec8, Sec5A, Sec10b, and vacuolar sorting components (Zou et al. 2019), suggesting roles in vesicle-mediated trafficking of metal transporters and vacuolar sequestration. Though direct Cu binding evidence is limited, domain architecture and network topology support *AT5G01610* function in metal detoxification via membrane trafficking and compartmentalization (Fig. 9).

This study identifies key Cu stress tolerance genes enhancing safflower growth through defense-related expression modulation. *AT5G01610 (DUF538)*, *AT4G32870 (CAX1)*, and *AT5G54780 (HMA4)* showed significant expression variation under Cu stress (Fig. 10). Though typically linked to Cr

tolerance, their Cu responsiveness was notable. *AT5G01610* lacks direct Cu stress evidence; its DUF538 domain suggests stress adaptation potential but remains uncharacterized for Cu homeostasis (Gholizadeh 2011). *CAX1* sequesters toxic cations into vacuoles, preventing oxidative damage (Ahmadi et al. 2018). *HMA4*, a P-type ATPase, mediates Cu⁺ transport and detoxification—conserved across plants, with *Arabidopsis* mutants showing Cu sensitivity (Guo et al. 2024). Differential expression likely stems from transcription factor variation, epigenetic modifications, and post-transcriptional regulation (Wang et al. 2022), corroborated by GWAS, co-expression analysis, and *in silico* heatmaps. Their highest expression in tolerant genotypes makes these genes promising targets for engineering Cu tolerance in safflower.

Conclusion

This study provides a detailed pheno-genotypic evaluation of safflower seedling traits under different concentrations of CuCl₂ and sheds light on how the crop responds to elevated Cu levels. The results indicate that 120 µM CuCl₂ is an effective concentration for screening Cu-tolerant genotypes. Several candidate genes linked to Cu tolerance were identified, and their expression was confirmed through qRT-PCR analysis. Further molecular studies are needed to clarify the mechanisms underlying Cu tolerance and how these genes interact with key metabolic pathways in contrasting genotypes. Including a broader range of safflower germplasm in future work would also improve the genetic understanding of this trait. These findings can serve as a useful basis for developing Cu-tolerant and environmentally resilient safflower cultivars.

Authorship contributions

Fawad Ali: Conceptualization, Writing – original draft, Formal analysis. **Arif Ali:** Writing – original draft. **Obaid Ullah Shah:** Writing – original draft, Writing – review & editing. **Mian Abdur Rehman Arif:** Conceptualization, Writing – original draft, Writing – review & editing. **Muhammad Azhar Nadeem:** Writing – original draft, Writing – review & editing. **Faheem Shehzad Baloch:** Writing – original draft, Supervision. **Syed Mansoor Jan:** Writing – original draft. **My Khanh Tran Thi Ha:** Writing – original draft, Writing – review & editing, Visualization. **Yong Suk Chung:** Writing – original draft, Project administration, Writing – review & editing, Supervision.

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Data availability

Data will be made available on request.

Declarations

The authors declare no competing interests.

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