

Determining Stress Tolerance from Promoter to Protein of Glym_NAC1 Transcription Factor in Glycine max

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Research Article

Keywords: Copper stress, Glycine max, NAC transcription factors, gene expression, abiotic stress adaptation

Posted Date: September 12th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6912321/v1>

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Additional Declarations: No competing interests reported.

Abstract

Soybean (*Glycine max*) is a pivotal crop species due to its high protein content and agricultural significance. Although heavy metals play a critical role in the growth and development of plants, their excessive accumulation can lead to toxic effects. In this study, the role of Glym_NAC1 transcription factor against copper (Cu^{2+}) stress was investigated. Firstly, the genomic structure of *Glym_NAC1* was analyzed and the exon-intron map of 10 different transcripts was constructed. A subsequent analysis of the promoter region identified 24 cis-regulatory elements, the majority of which were found to be associated with abiotic stress. Furthermore, a protein-protein interaction (PPI) analysis revealed that Glym_NAC1 interacts with proteins associated with laccase and lignin degradation. In physiological experiments, 20 μM and 50 μM Cu^{2+} was applied to Ilksoy and Traksoy soybean cultivars, respectively. It was observed that this stress caused chlorosis, necrosis, and water loss in plants. Relative water content (RWC) and malondialdehyde (MDA) analysis showed that copper stress caused oxidative damage. Gene expression analysis by qRT-PCR revealed that *Glym_NAC1* exhibited increased expression in the Ilksoy cultivar in response to copper stress, while the Traksoy cultivar exhibited a divergent adaptation mechanism. This study makes significant contributions to the field of heavy metal tolerance by elucidating the molecular role of Glym_NAC1 in copper stress tolerance. The findings provide a framework for further research on genetic editing studies aimed at developing plant varieties with enhanced stress tolerance.

INTRODUCTION

Soybean (*Glycine max*) belongs to the *Fabaceae* family, which includes plants that are widely recognized and play a significant role in agriculture worldwide. Among the key characteristics of soybean are its high protein content and rich amino acid profile. These traits make it widely used in food production and animal feed. Additionally, soybean is also rich in oil, fiber, and minerals. Soybean is an economically important crop that is commonly cultivated around the world, and as such, it is extensively studied (Bhat et al., 2019).

Copper (Cu) is an essential micronutrient for plants and plays a critical role in their normal growth and development. Copper functions as a coenzyme required for the occurrence of many enzymatic reactions, and it is involved in vital processes such as oxidative stress management, photosynthesis, cellular respiration, lignin synthesis, and carbohydrate metabolism in plants (Marschner, 2012). Additionally, one of the most important roles of copper in plants is its involvement in the electron transport chain, supporting redox reactions in mitochondrial respiration and photosynthesis, both of which are crucial for cellular energy production (Zhu et al., 2013). Copper also plays a role in strengthening the plant's immune system. Copper deficiency in plants can lead to problems such as stunted growth, chlorosis (yellowing of leaves), and poor root development. However, excessive copper accumulation can cause toxic effects. High copper concentrations can lead to an increase in reactive oxygen species (ROS) and the accumulation of oxidative stress, damaging the cellular structures of plants. This can result in oxidative damage to cell membranes, protein denaturation, and DNA damage, inhibiting plant growth (Maksymiec, 2007). Plants attempt to cope with this stress by developing a range of biochemical and physiological defense mechanisms. These mechanisms include the production of metal-binding proteins that neutralize metal ions, activation of antioxidant enzymes that neutralize oxidative stress, and structural changes that allow copper to be retained in the cell wall. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) help reduce oxidative stress in plants by neutralizing reactive oxygen species (ROS) (Cobbett et al., 2002; Gill et al., 2010). Additionally, plants can limit the bioavailability of metal ions, thereby reducing the toxic effects of these metals, by creating high pH levels and limiting root growth to restrict metal absorption (Yang et al., 2012; Foy et al., 2003; Zhao et al., 2015).

An increase in copper concentration in the environment serves as a stress factor for plants. To maintain homeostasis, plants develop various defense mechanisms in response to environmental stress, and transcription factors (TFs) play a crucial role in regulating these mechanisms. Transcription factors are proteins that initiate or regulate gene expression, coordinating the plant's stress responses. The AP2/ERF family regulates the signal transduction of plant hormones such as

ethylene and abscisic acid, thus playing an important role in oxidative stress and pathogen attacks (Licausi et al., 2013). The bZIP family aids plants in adapting to environmental conditions such as drought and heat stress (Jakoby et al., 2002), while WRKY factors are critical in managing defense against pathogens and oxidative stress (Eulgem et al., 2000). Among the prominent transcription factors, the NAC (No Apical Meristem) family plays an essential role in coping with stress situations such as heavy metal stress and oxidative stress. NAC factors regulate cellular adaptations during stress, enabling plants to develop resistance (Tran et al., 2004). These transcription factors are vital molecules that guide gene expression and help plants survive environmental stress. Genes involved in various transcriptional pathways can exhibit significant behavior depending on the regulations in place. The *NAC1* gene has a significant potential due to its involvement in the molecular mechanisms of stress tolerance. In some studies, the expression of *SNAC1* (Stress-Responsive *NAC1*) is used in relation to this gene's sensitivity to stress. This is because the *NAC1* gene plays a role in regulating tolerance to stress factors such as drought and salt in various plants (Hu et al., 2006; Kurowska & Daszkowska-Golec, 2023). However, the impact of the *NAC1* gene under copper stress in soybean is not yet known. This study is the first to reveal the relationship between the *NAC1* transcription factor and heavy metal stress in soybean both *in vitro* and *in silico*. The aim is to clarify the signal pathways between the *NAC1* gene and heavy metal stress in greater detail and to improve the understanding of adaptation mechanisms in plants.

MATERIALS AND METHODS

Determination of the Exon-Intron Map of the Glym_NAC1 Gene

Genomic, coding, and protein sequences of the *NAC1* gene from soybean were obtained from the NCBI database and saved in FASTA format. The exon and intron structures of the *Gm-NAC1* gene were determined using GSDS (<http://gsds.cbi.pku.edu.cn/>) (Guo et al., 2007).

Promoter Region Analysis of the Glym_NAC1 Gene

The promoter sequence of *Gm_NAC1* was determined using the Promoter 2.0 database (<https://services.healthtech.dtu.dk/services/Promoter-2.0/>). Based on the identified sequences, the promoter sequence (NC_038254.2:54993595–54998867) was analyzed for regulatory elements using Plantcare (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) from the NCBI shotgun genome. In the analysis, a 2 kb region of the approximately 5 kb promoter region was utilized.

Ontology Analysis of the Glym_NAC1 Gene and Determination of Protein-Protein Interactions

Functional ontologies of the *Glym_NAC1* protein sequences were determined using the Blast2GO program (<http://www.blast2go.com>). For this purpose, the data were initially tested using BLASTP, followed by mapping and ontology algorithms. The molecular functions, cellular components, and biological processes of the proteins were evaluated (Conesa et al., 2005).

To determine the protein-protein interaction (PPI) networks of the *Glym_NAC1* protein, the STRING database (<https://string-db.org/>) was used. The *Glycine max* NAC1 protein was queried in the database, and the PPI network was constructed. Proteins involved in the pathway were identified. The ontological analyses of the obtained data were compared and associated with copper stress.

Motif Analysis and 3D Modeling of the NAC1 Protein

To identify conserved motifs in the NAC1 proteins of *Glycine max*, the Multiple EM For Motif Elicitation Tool (MEME v4.11.1; <http://meme-suite.org/>) was used (Bailey et al., 2009). The identified conserved motifs were validated in the InterPro database (Quevillon et al., 2005). The three-dimensional models of the NAC1 proteins of soybeans were determined using the Phyre2 database.

Plant Growth Conditions and Stress Applications

In this study, the registered soybean varieties obtained from the Trakya Agricultural Research Institute were analyzed, and based on preliminary studies, the Traksoy variety was determined to be resistant to stress, while the İlksoy variety was found to be sensitive to stress. For each variety, at least 10 seeds were used, and the seeds were subjected to surface sterilization. For germination, the seeds were placed on moist filter paper and kept in the dark in Petri dishes for one week. The germinated seeds were then transferred to a soil mixture of perlite/peat in a 1:2 ratio and grown under appropriate conditions (16/8 day cycle, 24°C day / 20°C night temperature, 60% relative humidity in growth chamber). The plants, which were grown under normal watering conditions for 15 days, were classified into three groups: control group, 1st stress group (20 µM copper), and 2nd stress group (50 µM copper). The stress groups were watered with copper-containing solutions for 10 days, while the control group continued to be watered normally. At the end of the experiment, necrosis and color changes were observed, and leaf and root tissues were collected for analysis.

Determination of Relative Water Content

Relative water content was measured using leaf samples. Fresh weights of the leaves were recorded, and the leaves were placed in plastic bags with the undersides in contact with water and kept at 4°C overnight. Afterward, the turgid leaf weight was measured, and the leaves were dried at 60°C for one night and their dry weights were recorded. The relative water content was calculated using the following formula:

$$\frac{FW - DW}{TW - DW} \times 100$$

Here, FW stands for fresh weight, DW stands for dry weight, and TW stands for turgid weight.

Determination of Lipid Peroxidation (Malondialdehyde Levels)

Malondialdehyde (MDA) content, as an indicator of lipid peroxidation, was determined using the thiobarbituric acid (TBA) assay. Samples (leaf, root, and seed) were homogenized with 5% TCA, followed by centrifugation to obtain the supernatant. The resulting supernatant was mixed with an equal volume of a 20% TCA solution containing 0.5% TBA and incubated at 96°C for 25 minutes. After incubation, the mixture was allowed to cool to room temperature and then centrifuged again. The supernatant was measured spectrophotometrically at 532 nm and 600 nm. MDA levels were calculated using the following formulas:

$$\frac{A_{532} - A_{600}}{157000} \times 10^6$$

Gm_NAC1 Gene Expression Analysis

RNA Isolation

RNA was isolated from leaf samples using the Trizol method. The purity and concentration of the RNA were measured using a NanoDrop (Denovix) device. For purity, the OD260/OD280 ratio should range between 1.6–2.1, and the OD260/OD230 ratio should range between 2.0–2.2.

cDNA Synthesis

cDNA synthesis from RNA samples was performed using the Cistrone cDNA Synthesis Kit. The reaction mixture was prepared with dNTPs, 5X Reaction Buffer, RiboLock RNase Inhibitor, RevertAid M-MuLV RT, and RNA MIX. The reverse transcription process was incubated under the conditions recommended by the kit.

Expression Analysis of Glym_NAC1 Gene by qRT-PCR

qRT-PCR analysis was performed using cDNA samples. Analyses were conducted in a 96-well format with Thermo SYBR Green PCR Master Mix. Two technical replicates were performed for each biological replicate and compared to the negative control group. qPCR analyses were carried out by detecting SYBR-Green fluorescence signals and analyzed using the $2^{-\Delta\Delta CT}$ method according to Livak and Schmittgen (2001). *Glym_ACT* gene primers were used for normalization.

Statistical Analysis

One-way ANOVA was performed to assess the significance of all experimental data, and values with $p < 0.05$ were considered statistically significant. Graphs and statistical analyses of the obtained data were performed using GraphPad V9.

RESULTS AND DISCUSSION

There are total of 10 transcripts of the *NAC1* gene in *Glycine max*. Based on the exon-intron maps of the obtained transcripts (Fig. 1), *Glym_NAC1* contains 2 introns, while the 1st, 3rd, 4th, and 7th transcripts of *Glym_NAC1* have 4 introns, and the 2nd, 5th, 6th, and 9th transcripts have 3 introns. The 8th transcript contains no introns.

Exon-intron structures of a gene are linked to the adaptation of plants to environmental factors and genetic diversity. The number of exons in *NAC* genes in soybean varies between 1 and 7 (Singh et al., 2021). In this study, however, there are introns between 3 and 4 in the 10 transcripts of the *NAC1* gene. The different exon-intron numbers in *NAC* genes may influence the process of how this gene family responds to various stresses (Hu et al., 2010). Additionally, the exon-intron maps obtained can also affect gene expression. The expression profiles of *NAC* genes during soybean seedling development were found to change significantly (Shamimuzzaman & Vodkin, 2013). Therefore, these findings help us better understand the mechanisms involved in plant development and stress tolerance processes. In the literature it was shown that genes such as *NAC065* and *NAC085* in *Glycine max* play an active role in processes such as plant development, senescence, and stress responses (Melo et al., 2021). The diversity in the exon-intron profiles of *Glym_NAC1* transcripts indicates that they may be effective in various pathways, particularly in stress response. Moreover, many undiscovered features behind introns demonstrate that they possess high transcriptional regulation. For example, untranslated small nuclear RNAs (snoRNAs) encoded in the intronic sequences of some genes have the potential to be influential elements in complex transcriptional networks (Maxwell & Fournier, 1995; Fedorova & Fedorov, 2003).

According to the analysis performed with a 2 kb sequence obtained from the 5' region of the promoter of the *NAC1* gene in *Glycine max*, a total of 24 cis-regulatory elements were identified. These elements include the AAGAA-motif, ABRE, AE-box, ARE, Box 4, CAAT-box, CGTCA-motif, ERE, F-box, G-Box, MRE, MYB, MYB-like sequence, MYC, P-box, STRE, TATA-box, TCA-element, TGA-element, TGACG-motif, Unnamed__4, as-1, and circadian elements. The functions of these elements involve important processes such as abscisic acid response, light response, MeJA response, circadian control, auxin response, salicylic acid response, and gibberellin response.

Copper exposure in plants affects processes such as oxidative stress, hormone signaling, and changes in gene expression. Therefore, many cis-regulatory elements found in the promoter region may play a role in stress responses. For example, abscisic acid responsive elements (ABRE), MYB/MYC regulate ABA-associated defense pathways (Sun et al., 2023; Wang & Xu, 2024). The CGTCA-motif and TGACG motifs (MeJA responsive elements) may contribute to regulating defense genes in response to copper stress by affecting jasmonic acid signaling pathways. Salicylic acid responsive elements (TCA), auxin responsive elements (TGA), and gibberellin responsive elements (P-box) could play roles in oxidative stress tolerance, plant

growth, development, and regulation mechanisms under stress conditions. Additionally, STRE and as-1 elements act as stress-responsive elements and may contribute to the regulation of environmental stress factors like copper stress. The presence of cis-regulatory elements affects how plants respond to various stress conditions. This provides valuable insights into understanding how plants react to and adapt to stress conditions (Zhang et al., 2022; Konieczna et al., 2023).

The ontology data for *Glym_NAC1* were analyzed in three groups: biological process, molecular function, and cellular component. In the biological process category, *Glym_NAC1* protein is involved in the catabolic reactions of organic substances, lignin catabolism, cellular catabolism, SCF-associated proteasomal ubiquitin-mediated protein catabolism, protein ubiquitination, and auxin-related signaling pathways. In terms of molecular functions, this gene/protein has functions such as hydroxyquinone oxygen oxidoreductase activity, ion binding, auxin binding, copper ion binding, and inositol hexakisphosphate binding. As for the cellular component, it is located in the apoplast and SCF ubiquitin ligase complex (Fig. 2).

The data obtained from the ontology analysis show that *Glym_NAC1* gene/protein could be highly effective in both regulating cellular responses and adapting to copper stress. Particularly in terms of molecular function, the copper ion binding function suggests that it plays a role in copper homeostasis. Additionally, other ion binding functions could be crucial in heavy metal detoxification and signaling processes.

According to the PPI interaction of the *NAC1* gene in *Glycine max*, the *NAC1* protein interacts with proteins such as I1J783_SOYBN, I1JGX7_SOYBN, I1JHB0_SOYBN, I1JLR3_SOYBN, I1JV53_SOYBN, I1K9P6_SOYBN, I1LSA5_SOYBN, I1M1I0_SOYBN, I1M7Y1_SOYBN, and I1MAY4_SOYBN (Fig. 3). In this protein network, proteins associated with *NAC1* include laccase, lignin degradation, detoxification of lignin-derived products, and members of the multicopper oxidase family, among other annotations. These findings provide strong evidence that *Glym_NAC1* may play a significant role in the response mechanism to copper stress. Laccase enzymes, members of the multicopper oxidase family, are copper-containing oxidases that oxidize and break down complex phenolic compounds such as lignin. Lignin degradation and detoxification of lignin-derived products could be part of the cell wall remodeling and defense mechanisms against heavy metal stress. Copper ions serve as cofactors for laccase enzymes. Elevated copper levels may directly affect laccase activity, which could alter the plant's lignin metabolism and cell wall composition. Therefore, the *NAC1* transcription factor has specific functions in copper homeostasis in plants. The interactions of *NAC1* with laccase and lignin-related proteins may assist plants in developing a more effective response to copper stress. Consequently, overexpression of *NAC1* in transgenic plants could be used to enhance tolerance to copper stress (Wu et al., 2024).

The three-dimensional structures of proteins include features such as specific DNA binding regions. These specific binding regions are formed by the conformational folding of beta sheets and alpha helices. To gain insights into a protein's functional properties, specific folding motifs that make up the secondary structure help us understand them. According to the data observed in Fig. 4, the fact that all isoforms share the same 3D model indicates that the *NAC1* protein is stable and evolutionarily conserved. Stable proteins' DNA binding stability serves as evidence that they play a key role in stable transcriptional activities.

In this study, to validate the results obtained from *in silico* data, copper treatment was applied to the *Ilksoy* and *Traksoy* varieties at two different concentrations (20 μ M and 50 μ M). Then, the effects of copper were analyzed morphologically, and relative water content, malondialdehyde (MDA) levels, and *Glym_NAC1* gene expression data were determined. According to the obtained data, it was observed that copper accumulated morphologically in the leaves, and as copper concentration increased, necrosis and chlorosis occurred (Fig. 5).

A color change was observed in the soybean leaves exposed to copper stress, depending on the concentration. The leaves in the control group remained healthy and green, while the leaves exposed to 20 μ M copper showed slight yellowing, and those exposed to 50 μ M copper displayed more intense yellowing and necrosis. These observations indicate that increased copper concentration leads to physiological stress symptoms in soybean plants. The color change could have resulted

from the loss of photosynthetic pigments or cellular damage caused by heavy metal stress. These findings suggest that high copper concentrations could have negative effects on plant health (Fig. 5).

The relative water content (RWC) results of soybean varieties exposed to copper stress show a decreasing trend from the control group to the 50 μM copper concentration. This clearly demonstrates the water imbalance and stress effects caused by copper accumulation in the physiological processes of plants. The control group plants had the highest RWC levels, indicating that under normal conditions, the plants' capacity to maintain cellular water balance was functioning properly. However, the 20 μM and especially 50 μM copper treatments led to a significant decrease in the plants' relative water content. Several key mechanisms underlie the increased water loss in plants due to copper stress. Firstly, copper accumulation increases the production of reactive oxygen species (ROS), leading to oxidative stress. Oxidative stress can damage cell membranes through lipid peroxidation, which increases the permeability of cell membranes and facilitates water loss. Secondly, high copper concentrations can have toxic effects on root cells, reducing their ability to absorb water. This results in the plants' inability to take up sufficient water from the soil, leading to a loss of turgor pressure in leaf cells. Thirdly, copper binding to cellular enzymes and proteins disrupts metabolic processes, reducing water potential and disturbing cellular water balance. The decrease in RWC triggers a chain reaction in the plants' metabolic processes, negatively affecting their growth and development. This results in leaf wilting, necrosis formation, and overall deterioration of plant health. The reduction observed at the 50 μM copper concentration further confirms that higher copper concentrations have more severe physiological and morphological effects on plants (Fig. 6).

In this study, the effect of different copper concentrations applied to soybean varieties on malondialdehyde (MDA) levels was examined. Compared to the control group, both *İlksoy* and *Traksoy* varieties showed a gradual and significant increase in MDA levels with 20 μM and 50 μM copper treatments. This increase was particularly prominent in the 50 μM copper treatment and was considered as an indication of oxidative stress in both varieties. MDA is a product formed by the oxidation of unsaturated lipids in plant cell membranes, and it is an important biochemical marker of oxidative stress. The increasing concentrations of copper in this study led to the excessive production of reactive oxygen species (ROS), which exacerbated lipid peroxidation. This indicates that the structural integrity of plant membranes was compromised and cellular functions were jeopardized. The higher MDA levels observed in the *İlksoy* variety suggest that this variety is more sensitive to copper stress. On the other hand, the lower MDA levels in *Traksoy* may indicate that this plant develops more effective antioxidant mechanisms or is better at detoxifying toxic ions in response to copper stress. In plants exposed to copper stress, the management of oxidative stress is associated with balancing reactive oxygen species through antioxidant enzymes (e.g., superoxide dismutase, catalase, and peroxidase). The results of this study suggest that *İlksoy* has a lower antioxidant defense capacity, leading to a more severe accumulation of oxidative damage. In contrast, *Traksoy* seems to control ROS levels better and sustain less membrane damage due to a more efficient antioxidant defense mechanism. In conclusion, different copper concentrations increased MDA levels in the soybean varieties, and this increase was a result of oxidative stress. Significant differences were observed between the varieties in managing this stress. These findings suggest that *Traksoy* is more tolerant to copper stress, while *İlksoy* is more vulnerable under such stress. This points to the need for future studies to explore the molecular and biochemical defense mechanisms of these varieties in detail (Fig. 7).

In this study, the effects of different copper concentrations (20 μM and 50 μM) on the expression levels of *Glym_NAC1* gene in *İlksoy* and *Traksoy* varieties were evaluated using qPCR analysis. The data obtained from fold change graphs clearly demonstrate that the molecular responses of *İlksoy* and *Traksoy* varieties to copper stress are distinct from each other.

In the *İlksoy* variety, a gradual increase in *Gm_NAC1* gene expression was observed with increasing copper application concentrations. Compared to the control group, a significant increase in gene expression started with the 20 μM copper application, and this increase reached its highest level at the 50 μM copper concentration. This suggests that *İlksoy* activates the *Glym_NAC1* gene more in response to copper stress as an adaptation strategy. Transcription factors like

Gm_NAC1 play critical roles in regulating cellular defense mechanisms and enhancing the expression of antioxidant genes in response to heavy metal stress. The observed increase in İlksoy may reflect a defense mechanism indicative of the variety's survival effort under stress conditions.

In the Traksoy variety, however, a decrease in *Glym_NAC1* gene expression was recorded in the control group when exposed to the 50 μ M copper application. This decline suggests that Traksoy has developed a different response mechanism to copper stress. The lower *Glym_NAC1* gene expression levels in Traksoy may indicate that this variety can detoxify copper ions more effectively at the cellular level or that other genes responsible for stress tolerance play a more dominant role in this process. Additionally, this decline may suggest that the *Gm_NAC1* gene has a less critical role in stress management in Traksoy, or that this variety does not need to increase gene expression due to experiencing less oxidative stress.

These differences indicate that the responses of İlksoy and Traksoy to copper stress are closely related to their genetic backgrounds. The increased *Glym_NAC1* expression in İlksoy suggests that this variety is more vulnerable to heavy metal stress and has increased gene expression levels to maintain cellular balance. In Traksoy, the decrease in *Glym_NAC1* expression suggests that the variety is more tolerant and uses different molecular pathways to manage stress (Fig. 8).

In conclusion, these qPCR data show that there are distinct *Glym_NAC1* gene expression regulation in response to copper stress between the two varieties, and these regulations significantly impact the plants' stress tolerance levels. These differences may serve as an important foundation for future research on genetic modifications and stress tolerance. Moreover, the findings of this study highlight that the physiological and molecular effects of copper stress differ markedly between varieties, emphasizing the importance of stress management and variety selection in plant production processes. In this context, the integration of genetic approaches and environmental stress factors emerges as a critical area for enhancing plant resilience.

The data obtained from the combined evaluation of *in silico* and *in vitro* analyses suggest that the *Glym_NAC1* gene is highly effective in relation to copper stress. This process shows that *Glym_NAC1* is an effective candidate gene that may facilitate the plant's adaptation to copper stress.

Declarations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

Declaration Statement

TUBITAK 2209 Project no: 1919B012438324.

*This study has a data availability statement

*Ethics, Consent to Participate, and Consent to Publish declarations: The *Glycine max* varieties used in this study were cultivated in a controlled experimental field at [Trakya Agricultural Research Institute], and no wild collection was performed. The study complied with all relevant institutional, national, and international guidelines. No specific permits were required for the use of these cultivated plant materials.

* Clinical Trial: Not applicable.

Author Contribution

Idea/Concept – FŞG, FE; Design – FŞG, FE; Consultancy –FE; Data Collection and/or Processing –FŞG, EÇ; Analysis and/or Interpretation – FŞG, EÇ, FE; Literature Review – FŞG, EÇ; WritingThe Article -FŞG, EÇ, FE; Critical Review – FŞG, FE.

Acknowledgement

Clinical Trial: Not applicable.

Data Availability

*This study has a data availability statement

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Figures

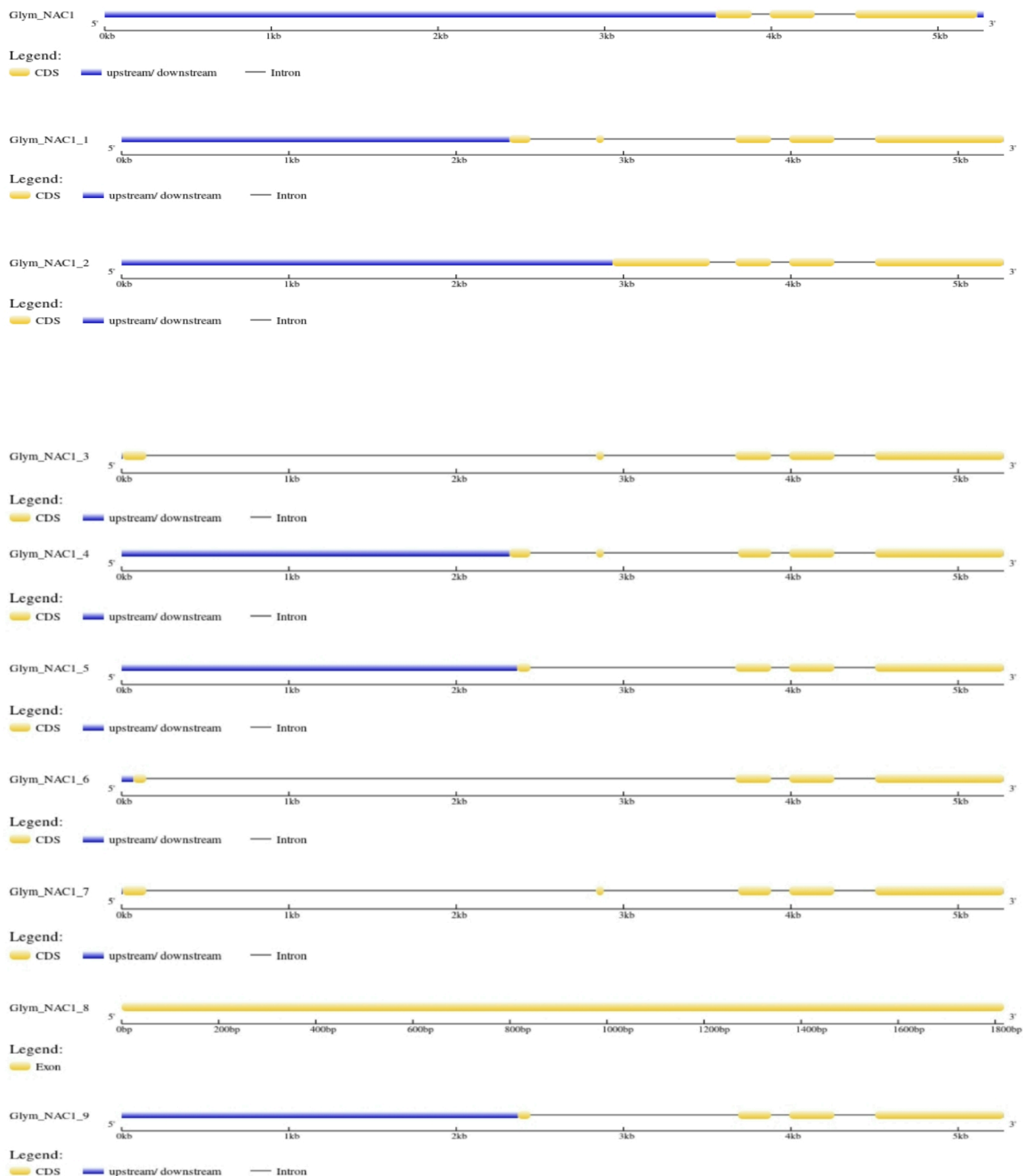


Figure 1

Exon-intron maps of *Glym_NAC1* transcripts

Biological Process (Gene Ontology)					
GO-term	description	count in network	strength	signal	false discovery rate
GO:1901575	Organic substance catabolic process	9 of 3089	1.17	1.5	2.36e-07
GO:0046274	Lignin catabolic process	5 of 64	2.6	4.31	5.53e-09
GO:0044248	Cellular catabolic process	9 of 2435	1.27	1.79	4.75e-08
GO:0031146	SCF-dependent proteasomal ubiquitin-dependent protein catabolic ...	4 of 127	2.2	2.64	5.71e-06
GO:0016567	Protein ubiquitination	4 of 1680	1.08	0.56	0.0428
GO:0009734	Auxin-activated signaling pathway	4 of 518	1.59	1.37	0.00082

Molecular Function (Gene Ontology)					
GO-term	description	count in network	strength	signal	false discovery rate
GO:0052716	Hydroquinone:oxygen oxidoreductase activity	5 of 64	2.6	4.45	2.97e-09
GO:0043167	Ion binding	10 of 14898	0.53	0.4	0.0044
GO:0010011	Auxin binding	4 of 13	3.19	4.68	2.97e-09
GO:0005507	Copper ion binding	5 of 257	1.99	2.93	4.28e-07
GO:0000822	Inositol hexakisphosphate binding	4 of 42	2.68	3.81	6.88e-08

Cellular Component (Gene Ontology)					
GO-term	description	count in network	strength	signal	false discovery rate
GO:0048046	Apoplast	5 of 606	1.62	1.86	3.05e-05
GO:0019005	SCF ubiquitin ligase complex	4 of 166	2.09	2.29	2.43e-05

Figure 2

Glym_NAC1 proteins ontology results

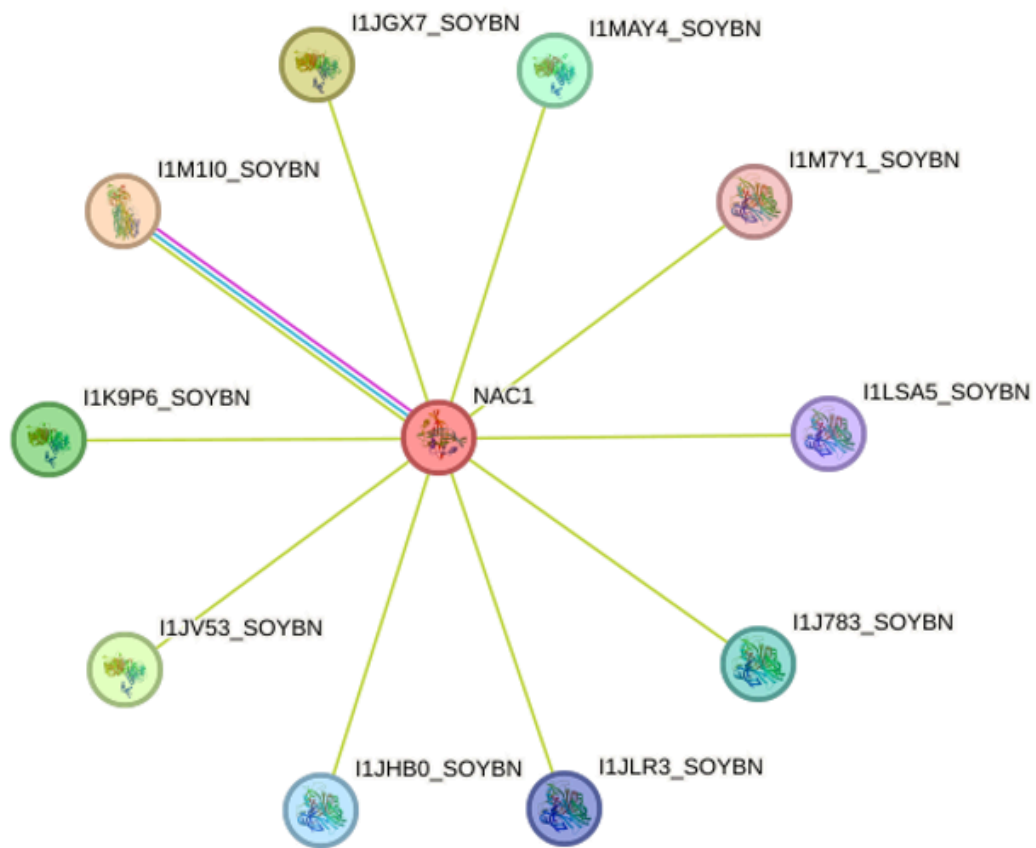


Figure 3

Glym_NAC1 protein-protein interaction map

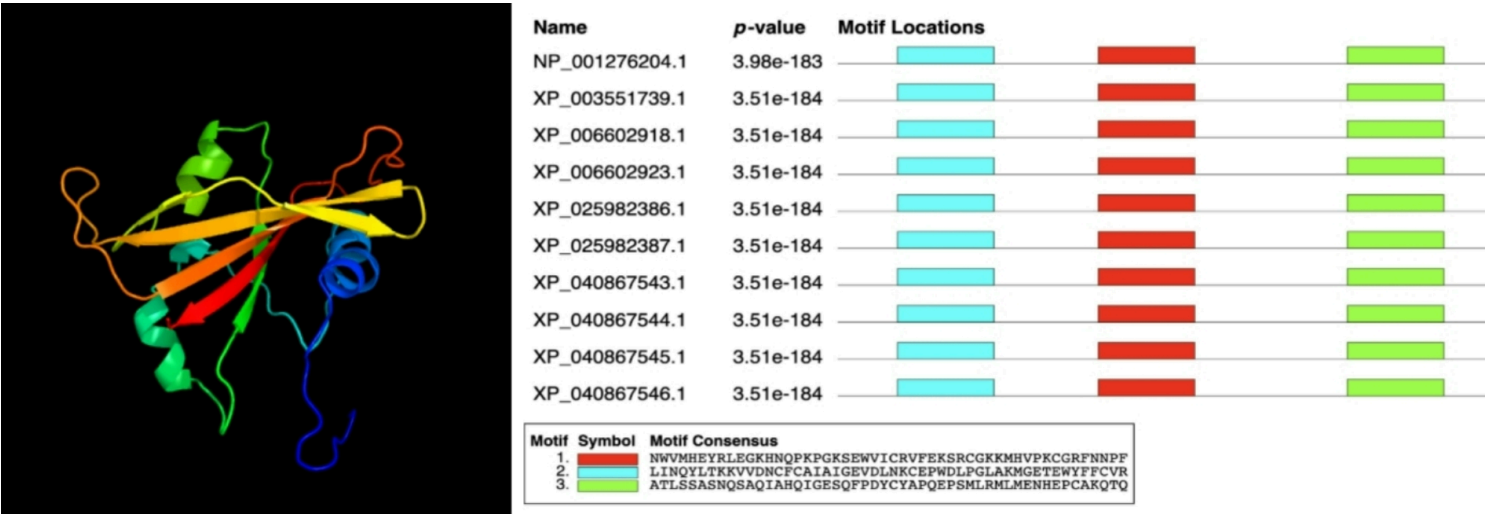


Figure 4

The 3D structure of the *Glym_NAC1* protein (a), the locations of protein motifs (b), and the protein motifs (c)

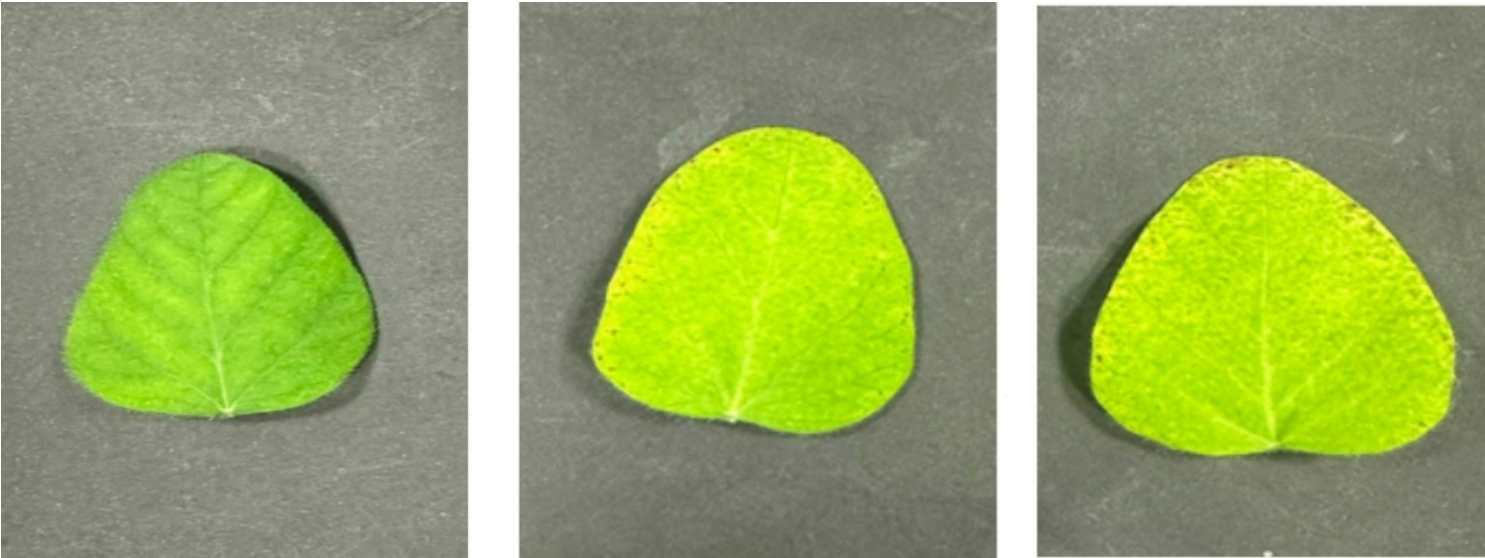


Figure 5

Leaf appearances of soybean varieties subjected to control, 20 μ M, and 50 μ M copper treatments, respectively. In the control group, the leaves exhibited a healthy green color, while the leaves exposed to 20 μ M copper showed a yellowish color change. In the leaves exposed to 50 μ M copper, the color change became more pronounced, shifting towards yellow tones.

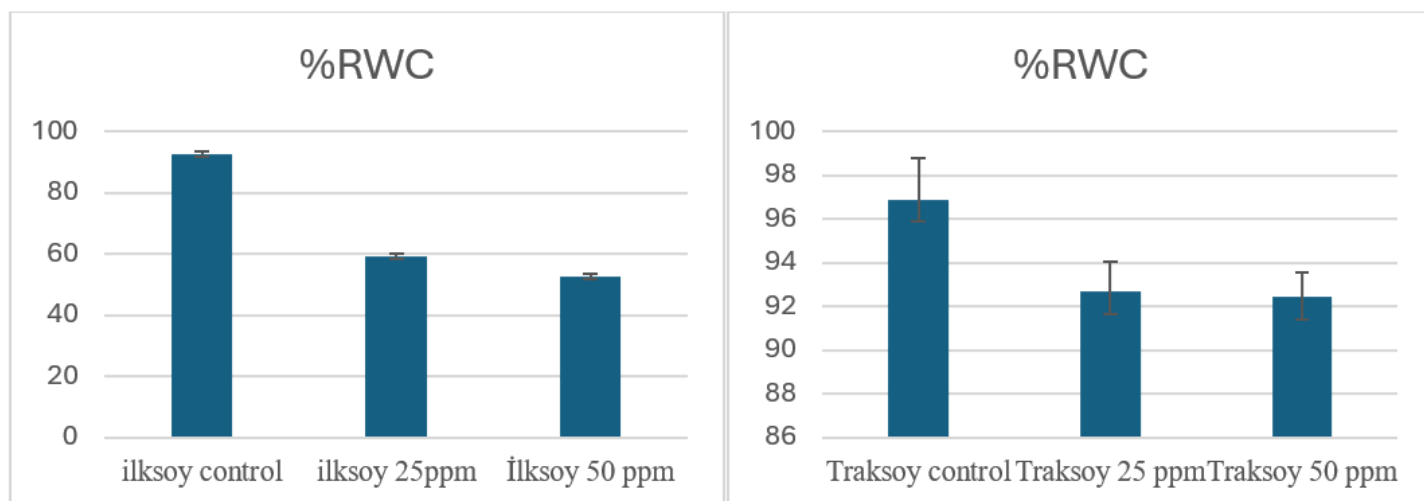


Figure 6

Relative water content (RWC) values of soybean leaves exposed to copper stress. Differences were observed between the control, 20 μ M, and 50 μ M copper treatments. An increase in copper concentration led to a significant decrease in relative water content.

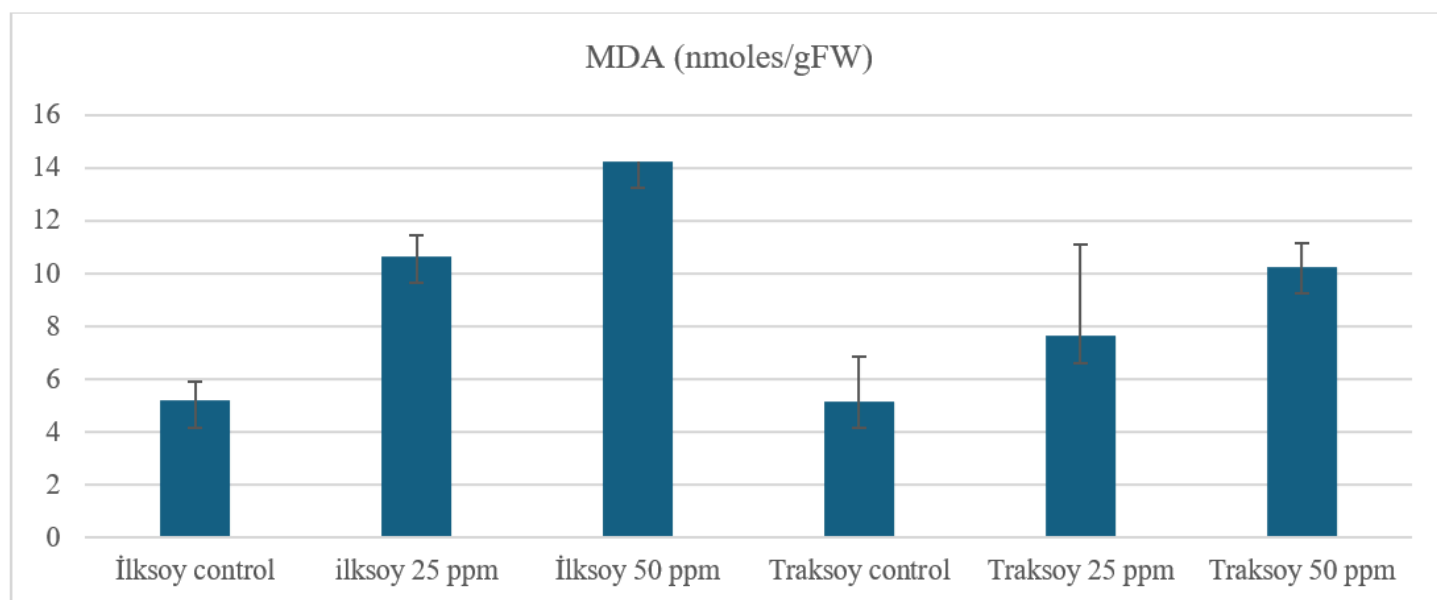


Figure 7

Comparative analysis of malondialdehyde (MDA) levels in leaf tissues of *ilksoy* and *Traksoy* varieties exposed to control, 20 μ M, and 50 μ M copper (Cu^{+2}) treatments.

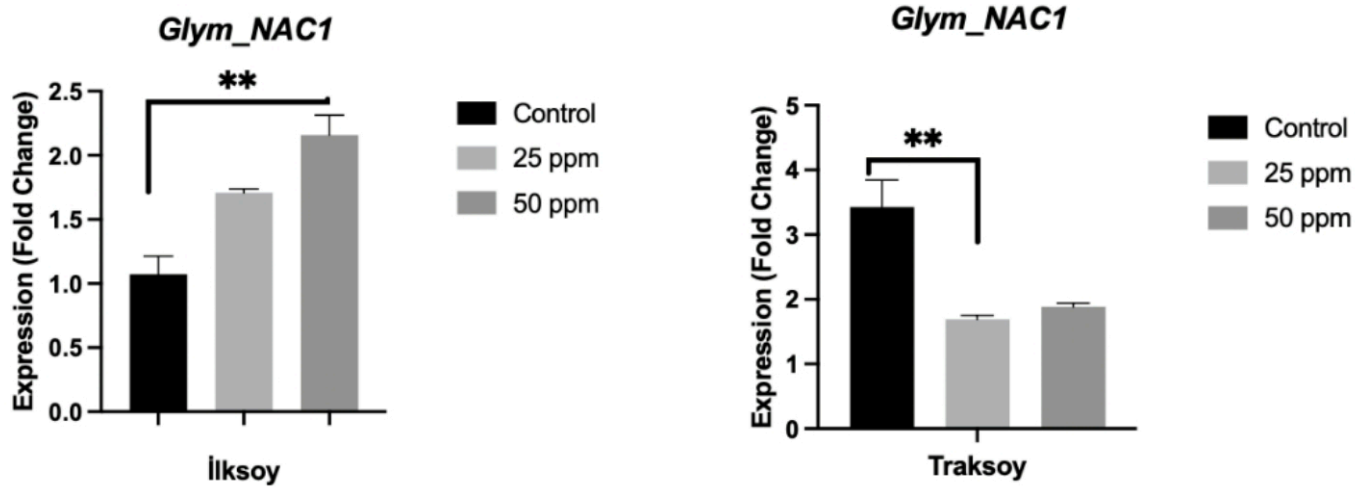


Figure 8

Change in *Glym_NAC1* gene expression in response to copper (Cu) stress in *Ilksoy* and *Traksoy* varieties.