

Transcriptome-based mining and expression profiling of low-Nitrogen-responsive transcription factors in yellowhorn (*Xanthoceras sorbifolia* Bunge)

Jingliang Sun

Dalian Minzu University

Chunyi Jin

Dalian Minzu University

Jixiang Zou

Dalian Minzu University

Xinlin Li

Dalian Minzu University

Mei Wang

Dalian Academy of Agricultural Sciences

Chengchao Yang

Liaoning Provincial Institute of Poplar

Linlin Li

Dalian Minzu University

Hua Jin (✉ jhua@dlmu.edu.cn)

Dalian Minzu University

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Abstract

Nitrogen (N) deficiency affects various pathways in plants, while transcription factors (TFs) play crucial roles in regulating metabolic processes. However, there is restricted information about the activation of TFs in trees subjected to low-N stress. We assessed these functions by identifying and predicting TFs in yellowhorn, a high-quality raw material for the production of biodiesel, under low-N stress. We performed comparative transcriptome analysis to study the overall expression of TFs in yellowhorn under different N concentrations. A total of 56 TF families, including 1,873 TF genes, were identified. Among them, 410 (approximately 22%) TFs were detected at significantly higher or lower differential expression levels in both N deficiency treatments than in normal N conditions. We then sorted eight TF families (593 TFs) related to N stress based on previous studies and determined whether they respond to low-N stress. Further phylogenetic and conservative analyses showed that TFs in yellowhorn were clustered according to their phylogenetic affinity, providing a better understanding of their functional diversity. Protein interaction and Gene Ontology (GO) analyses predicted that TFs mainly functioned in hormone conduction, oxidative stress and defense responses. In addition, some TFs involved in salicylic acid signaling, jasmonic acid signaling and antioxidant stress pathways were significantly expressed, implying that these components may help yellowhorn adapt to N deficiency. This study contributes to a better understanding of the complex mechanism of TFs in response to low-N stress in yellowhorn and lays a foundation for exploring yellowhorn TFs genes involved in low-Nitrogen-responsive.

Introduction

Nitrogen (N) in the soil exist as nitrate (NO_3^-), ammonium (NH_4^+) and other N-containing substances, which is one of the primary limitations to plant growth and productivity (Zhang et al. 2018; Frink et al. 1999). Hence N deficiency affects the growth and development of plants. Under low-N stress, plants grow slowly, leaves are limited in size, become yellow and undergo early senescence, which ultimately affects crop yields (Ding et al. 2005; Zhao et al. 2005). In addition, deficient N supply will lead to a decrease in N-containing compounds, such as glutamine, chlorophyll and proteins, as well as a significant decrease in photosynthetic activity, obstruction of carbohydrate synthesis and accelerated plant aging (Paul et al. 1997; Scheibe et al. 2004; Lin et al. 2016; Fataftah et al. 2018). Previous studies have used RNA sequencing (RNA-Seq) technology to analyze the transcriptomes of plants under low-N stress. Wild barley in Tibet has been shown to undergo changes in hormone signal transduction, flavonoid synthesis, phenylpropane metabolism, carbon (C) and N metabolism and antioxidant stress pathways under low-N conditions (Quan et al. 2016). Further, Yang et al. (2019) found that in the leaves of a low-N-tolerant variety of sugarcane, the specific differentially expressed genes (DEGs) are mainly enriched in photosynthetic and N metabolism pathways under low-N conditions.

Transcription factors (TFs), which are important regulators of various biological processes, play a crucial role in plant development, metabolism and responding to biological and abiotic stresses (Li et al. 2021; Shamimuzzaman et al. 2013; Nath et al. 2019). LBD37, LBD38 and LBD39, which are negative regulators in anthocyanin biosynthesis pathway of *Arabidopsis*, also have inhibitory effects on many N-responsive

genes, including key genes required for NO_3^- absorption and assimilation, thereby changing the NO_3^- content; nitrate reductase activity/activation; protein, amino acid, and starch levels; and the N-related B-type growth phenotype (Rubin et al. 2009). The MYB TF is involved in flavonoid biosynthesis and regulates the N metabolism pathway under N starvation in potato, soybean, lotus japonicus (Razal et al. 1996; Miyake et al. 2003; Supriadi et al. 2016). Moreover, the TF ZmDof1 promotes N assimilation and its transgenic lines show better growth under the conditions of low-N (Yanagisawa et al. 2004). At present, research on TFs can use transcriptome sequencing methods to quickly and effectively identify more important genes involved in plant growth and stress tolerance (Du et al. 2017; Liu et al. 2015; Nath et al. 2019).

With the continuous development of human beings, energy resources are continuously consumed, followed by environmental deterioration. It is urgent to find alternative fuels based on renewable energy and clean energy. In recent years, biodiesel, as an environmentally friendly energy with high cleanliness, has attracted extensive attention (Lang et al. 2001; Duffy et al. 2009; Yao et al. 2013). Biodiesel has the characteristics of sulfur-free, non-toxic, oxygenated and biodegradable, which can greatly reduce the emission of unburned hydrocarbons, sulfur dioxide, carbon monoxide and other harmful gases, effectively curb the further deterioration of the global environment, and is the best substitute for traditional fossil fuel (Sendzikiene et al. 2006; Li et al. 2012). At present, soybean oil is the main raw material for the preparation of biodiesel in the United States, while rapeseed oil is the raw material in Europe (Li et al. 2012; Halim et al. 2011). According to the national conditions of our country, large-scale cultivation, planting non-edible oil plants with no occupation of cultivated land and high oil content as raw materials for the development of biodiesel industry is the development directions (Shao et al. 2008; Zhong et al. 2010).

Yellowhorn (*Xanthoceras sorbifolia* Bunge) is a kind of woody oil species peculiar to China with strong root systems and environmental adaptability. It is an ideal tree species for desert greening and barren mountain management and is widely planted in some desert, semi-arid and arid areas in northwest China (Yao et al. 2013; Guo et al. 2012; Ruan et al. 2017). The oil content of Yellowhorn seeds is more than 50%, the content of unsaturated fatty acid is very high, and the acid value is low (0.5–0.7 mg KOH/g) (Li et al. 2012). It is a high-quality raw material for the production of biodiesel (Li et al. 2005; Li et al. 2010). In addition, it can also be used to produce lubricants, paints, soap, hair wax and other chemical products, which plays an important role in industry (Wan et al. 2013; Zang et al. 2021).

In this study, our aim was to identify and predict possible functions of yellowhorn TFs under low-N stress. We sequenced yellowhorn samples were based on three N treatment concentrations (2 mM, 0.2 mM and 0 mM) and identifies TFs. Further, we selected eight TF families for phylogenetic, conserved domain, Gene Ontology (GO) and protein interaction analyses. We thus aimed to understand the main response pathways and identify the key candidate TFs of yellowhorn. Our results will contribute to the understanding of the TFs involved in the regulation of low-N stress in yellowhorn and through the exploration of internal regulatory mechanisms, we expect key TFs of yellowhorn to affect resistance to low-N stress.

Materials And Methods

Plant material and transcriptome sequencing

Yellowhorn seeds were obtained from the Chifeng Forestry Bureau of Inner Mongolia. The seeds were soaked in water at 50 °C for 3 days, then wrapped with a damped cotton cloth to keep them moist until they germinated. Sprouted seeds were transplanted into flowerpots filled with sand and cultivated for 2 months. All reagents were purchased from Shanghai Sangon. Hoagland's nutrient solution ($\text{Ca}(\text{NO}_3)_2$ and KNO_3 replaced by CaSO_4 and K_2SO_4 , with NH_4NO_3 as the sole N source) was used for watering every 3 days. Three N concentrations were set as follows: 2 mM NH_4NO_3 (CK), 0.2 mM NH_4NO_3 (low nitrogen, LN) and 0 mM NH_4NO_3 (no nitrogen, NN). After 15 days, the top tender leaves were collected and frozen in liquid nitrogen; RNA extraction (B511311, Trizol, Chinese laborers) and RNA-seq (Illumina HiSeq) were then performed by Sangon Biotech (Shanghai). Treatments were conducted in triplicate.

Physiological Index Determination And Phenotype Observation

After 15 days of treatment, control (CK) and stressed (LN, NN) seedlings were harvested for the following measurements, which were performed with three independent biological replicates. The average fresh weight, plant height, root length and lateral root length of seedlings under normal condition and low-N-stress were measured using three randomly selected seedlings for each group. The amount of soluble protein content of seedlings treated under normal condition and low-N-stress was measured by Bradford (1976) using bovine serum albumin as the standard.

Identification Of Yellowhorn Tfs

All CDS sequences were obtained from the low N transcriptome database and were compared with those in the plant TF database by sequencing (Plant TFDB v4.0) to identify the TFs of yellowhorn (Jin et al. 2017). Gene expression values were calculated on the basis of transcripts per million (TPM) (Wagner et al. 2013). To determine whether there was differential expression among the three N concentration treatments, the TFs with a TPM value > 0 were used to identify up-regulated or down-regulated expression based on 5 fold-change (FC) ($|\text{FC}| \geq 1$ was defined as differential expression, $p \leq 0.05$). Using the BMK Cloud Online Tool v1.0 (Becker et al. 1988), the expression data were visualized as a heat map of clustering FC values.

Phylogenetic Analysis And Conserved Domains

To infer phylogenetic relationships, we used ClustalW with default settings to sequence the amino acids of the TFs of yellowhorn and *Arabidopsis thaliana*. Subsequently, MEGA v7.0 software (Kumar et al. 2016) was employed to build an unrooted phylogenetic tree based on the neighbor-joining method with

the following parameters to evaluate the significance of the nodes: JTTmodel, pairwise gap deletion, and 1,000 bootstraps. MEME (<http://meme.nbcr.net/meme/cgi-bin/meme.cgi>) online software was used to analyze conserved structures of TF proteins of yellowhorn (Bailey et al. 2009). The length of a motif was set at 6-100, and the number of motif sites was 2-120; the maximum number of motifs was 10.

Go Annotation

To further understand the functional diversity of identified TFs, QuickGO v2020-12-11 (<https://www.ebi.ac.uk/QuickGO/>) was used. This online tool executes GO annotations. GO numbers of TF genes were input for InterPro annotation, providing information on molecular functions, biological processes and cell compositions for each TF.

Protein Interaction Network

To determine the interactions between TF proteins, the functional Protein Interaction Network String v11.0 (<http://string-db.org>) database was used in COG mode. Considering Arabidopsis as a biological reference, the optimal allocation cogs based on the most significant E-value were used to construct the interaction network. Based on the co-expression and experimental data, interactions with confidence scores ≥ 0.7 were used to construct the network.

Statistical analysis

Statistical analysis in basic descriptive was performed using Microsoft Office Excel 2007; $p < 0.05$ was considered statistically significant.

Results

Effects of low-nitrogen stress on yellowhorn growth and development

The growth status of yellowhorn under different N conditions was evaluated to assess its phenotypic change under low-nitrogen stress. After 15 days of treatment, compared to those in CK, plant heights of yellowhorn decreased by 5.7% and 14.66% in the LN and NN treatment groups (Table 1), respectively, and fresh weights also decreased by 4.38% and 14.93%, respectively. Contrastingly, yellowhorn root growth and development under N deficiency conditions were stronger than those in CK (Table 1) (Fig. 1). Root lengths were 21.86% and 38.89% higher than that in CK under LN and NN treatments, respectively, and the number of lateral roots also significantly increased by 52.36% and 50.07%, respectively, compared to that of CK. N is the main component of proteins and nucleotides, and is related to plant nutrition accumulation and metabolism, so that N deficiency will hinder plant growth and development (Liu et al. 2020). Soluble proteins are important osmotic regulatory substances and nutrients, whose content

increase and accumulation can improve the cell water retention ability and play a protective role on cell life material and membrane. In this experiment, the content of soluble protein was increased in yellowhorn after low-nitrogen treatment, indicating that plant cells had been destroyed, which affected the osmotic regulation of yellowhorn (Fig. 2).

Sequencing Quality

Illumina HiSeq™ 2000 was used to sequence the yellowhorn leaves. More than 95% of the reads were obtained in the nine libraries, reaching 5.90 Gb in each sample, which indicated that sequencing data were suitable for gene expression analysis.

Identification Of Tfs

To identify all the TFs in yellowhorn that respond to low-N stress, we performed RNA-seq analysis of the leaf tissue after low-N treatment. In total, 178,376 CDS sequences were uploaded to Plant TFDB v4.0 for comparison and 1,873 TFs were obtained, composed of 56 families harboring 1–334 TFs, are shown in Table 2 (Table S1). For convenience, the identified TFs were named Xs followed by the transcript number and the family name (e.g., Xs_72802_c4_g3_WRKY) (Nath et al. 2019). The families marked with * are the next eight families to be deeply excavated. Nitrogen deficiency is known to affect various biological and metabolic pathways, in which the activation and inhibition of transcription are complex multi-dimensional processes (Deng et al. 2019; Ueda et al. 2020). The expression profile of yellowhorn TFs could provide important clues for further gene function research so that we examined the differentially expressed TF genes in yellowhorn under low-N treatments using the threshold criteria based on P-values and the TPM ratio ($P \leq 0.05$ and $|\log_2 \text{ratio}| \geq 1$). A total of 871 TFs are differentially expressed, comprising 53 families. However, the number of DEGs in each TF family is induced to different degrees (Fig. 3). For up-regulated TFs in NN vs. CK, the largest members of TFs belonged to the C2H2 and ERF families (33 members were up-regulated in each family), followed by NAC (29), and GRAS (25). Meanwhile, for down-regulated TFs, members of the C2H2 family were most prevalent (171), followed by GRAS (38), and C3H (33). For up-regulated TFs in LN vs. CK, ERF (32), NAC (28), and C2H2 (25) family members were differentially regulated. For those down-regulated, C2H2 (205), C3H (42), GRF, and bHLH (41) families were identified. Subsequently, eight families were selected for further analysis, including bHLH, Dof, ERF, LBD, MYB, MYB-related, NAC and WRKY, with a total of 593 members. These families were selected based on previous studies reporting their association with low-N stress (Lea et al. 2007; Zebarth et al. 2012; Pal et al. 2017; Wang et al. 2017; Xu et al. 2018; Joshi 2020). Using the clustering normalization method, a heat map of the expression of each family member was drawn, as showed in Fig. 4. Among up-regulated TFs, Xs_73870_c1_g1_MYB was significant with both N treatments compared to the CK group (FC values in NN, 4.523046804; LN, 4.351006543, $P \leq 0.05$), in addition to Xs_73319_c0_g1_ERF and Xs_69533_c0_g2_ERF in the NN treatment. Among down-regulated TFs, two TFs were strongly regulated, namely Xs_71944_c0_g4_MYB-related (NN) and Xs_64172_c0_g1_bHLH (LN), both of which were changed more than 10-fold ($P \leq 0.05$). Based on these analyses, although

differentially expressed TFs in yellowhorn were strongly differentially expressed in NN and LN, there were no significant differences in the number or expression values between the two treatments.

Phylogenetic Analysis

In order to investigate the phylogenetic relationships, we used phylogenetic analysis to analyze the evolutionary relationships among TF families related to the low-N response between yellowhorn and those of *A. thaliana* (downloaded from PlantTFDB v4.0). As Fig. 5 shows, the TFs of yellowhorn were evenly distributed in each phylogenetic lineage. The bHLH family was divided into six representative classes. bHLH of yellowhorn is divided into 17 members of classes 1, 2, and 3, and 28, 14, and 33 members of classes 4, 5, and 6, respectively. The Dof family was divided into four representative classes, and the number of members in each class was 3, 7, 3, and 10, respectively. The ERF family was divided into five representative classes, with 20, 39, 21, 32, and 18 members, respectively. LBD family had four categories, with 2, 9, 14, and 8 members. MYB family comprised six categories, with 12, 10, 8, 6, 4, and 8 members, respectively. MYB-related family was divided into 72 members, which were divided into four categories, with 5, 6, 27, and 34 members, respectively. The 87 members of the NAC family were divided into six categories, one category had only one member, whereas the numbers of members of the 2nd, 3rd, and 4th categories were 11, 8, and 31 respectively; the number of members in the 5th and 6th categories were 18. The 62 members of the WRKY family were divided into five categories, comprising 4, 6, 18, 13, and 21 TFs, respectively. These results indicate that all the yellowhorn TF proteins may be efficiently grouped into their respective families with subgroups based on phylogenetic affinity.

Conserved Domain Analysis

To study the structural diversity in the TFs of yellowhorn, a total of 10 conserved motifs in each family were captured using MEME software. Results showed that all the TF genes have highly conserved core domain characteristics (Fig. 6). For example, in the WRKY family, motif 1 (WRKYGQK domain) is confirmed to be a highly conserved region (Xu et al. 2018), which is widely distributed among members of the family. Some TF members of the same family have highly similar motifs, indicating that they play a key role in the specific functions of the protein group and reflecting the similarity in function. On the other hand, there were large structural differences within the same family. It is worth noting that although families had a highly conserved motif or DNA-binding domain characteristics. The sequence similarity of other regions was different in most TF genes.

Go Annotation

The yellowhorn GO annotations were divided into three main functional biological categories (Fig. S1): biological processes, cellular components, and molecular functions. In the context of biological processes, a major proportion of TFs was involved in cellular, metabolic, and biosynthetic processes, and in hormone-mediated signaling pathways. TF activity and DNA binding accounted for the highest

proportion of molecular functions. The analysis of the cellular components showed that the yellowhorn proteins were almost all nucleus-localized and the rest individually localized in the endoplasmic reticulum, cytoplasm, ribosome, and mitochondrion. TF nuclear localization and nucleoplasm transport are important checkpoints for the fine regulation of gene expression. The signals from the ribosome, mitochondrion, and endoplasmic reticulum play key roles in regulating the defense response against stress. In addition to basic transcriptional pathways, such as transcriptional regulation, the annotated biological pathways were mainly enriched in hormone conduction and metabolism, defense responses, and stress responses. The detailed GO annotations are provided in Table S2.

Protein Interaction Network

Protein interaction networks provide a comprehensive understanding of their role in different biological processes. Interactive networks show the interconnection of different TF families in response to biological stress. Using *A. thaliana* as the reference, key proteins performed hormone-mediated and defense responses (Fig. 7). In addition, different families had different functions with respect to the key genes in their interaction networks. For example, the interacting proteins of the bHLH family also included photoreceptors, flavonoid pathway and cell differentiation regulatory proteins (Table S3). The WRKY family included abiotic stress signals, oxidative stress responses and other family interaction networks, while key genes also included genes that regulate cell differentiation and rhythm. The key genes of the interaction network are detail in Table S3.

Discussion

The ability to perceive and respond quickly to stress is essential for the survival of plants. When N is deficient, damage to the plant structure is inevitable, such as to glutamine, chlorophyll, proteins, and other N compounds. Photosynthetic activity is significantly reduced, carbohydrate synthesis is hindered, and plant aging is accelerated (Paul et al. 1997; Scheible et al. 2004; Lin et al. 2016; Fataftah et al. 2018). Quan et al. (2016) reported, based on transcriptome analysis, that under low N conditions, plants respond to stress via hormone conduction, flavonoids, phenylpropane metabolism, C and N metabolism, and antioxidant stress pathways. Based on previous research and the results of our analyses, yellowhorn TFs may help resist low-N stress.

Hormone Response

Abscissic acid (ABA) and jasmonic acid (JA) mediate plant responses to various biological and abiotic stresses to help resist damage. MYBR1 (MYB44) can suppress many ABA-induced stress genes and is a negative regulator of ABA, stress, injury response and anti-aging activity (Jaradat et al. 2013). In addition, AtMYB44 can modulate antagonism by activating SA-mediated defense and directly controlling WRKY70 to inhibit JA-mediated defense in Arabidopsis (Shim et al. 2013). In yellowhorn, the MYB44-like TF Xs_58368_c1_g1_MYB and WRKY70 TF Xs_72802_c4_g3_WRKY continued to be up-regulated under NN

and LN ($P \leq 0.05$). In addition, using KEGG analysis, we found that the SA and JA pathways of yellowhorn were up-regulated, which implies that they play a role in defense against low-N stress. However, in JA-stimulated plants, the JA signaling molecule activates the

SCFCO11 protein complex to bind to JAZ (compete with MYC2), and the release of MYC2 bound to the JA response gene may eventually regulate plant physiology (Que et al. 2014). However, Xs_57480_c0_g1_bHLH (MYC2-like) in yellowhorn continued to be up-regulated under N deficiency, suggesting that this TF could also be involved in related pathways.

In addition, cytokinin (CTK) plays a vital role as a signaling molecule in coordinating N utilization in plants (Gu et al. 2018), while CRF is responsible for regulating CTK. CRF is known as cytokinin response factor (Cutcliffe 2011). Interestingly, although both N treatments induced CRF, the induced expression was not significant and some conditions even resulted in down-regulation. This implies that yellowhorn might not respond to N-deficiency stress through CTK signaling.

Oxidative Stress Response

In the absence of N, plants exhibit reduced photosynthesis and are more susceptible to oxidative damage caused by excess energy (Foyer et al. 1994; Diaz et al. 2006). In addition, recent studies have found that plants accumulate reactive oxygen species (ROS) under N deficiency (Shin et al. 2005; Kong et al. 2012), which act as toxic agents stimulating signals for the plant cell death program (Overmyer et al. 2003). Excessive ROS accumulation causes considerable plant cell death. To alleviate the damage, plants have developed a powerful antioxidant system in which TFs play a pivotal role (Overmyer et al. 2003; Bahieldin et al. 2018; Zhao et al. 2019). In our transcriptome data, we found that Xs_69533_c0_g2_ERF (ERF109) was strongly induced in NN ($P \leq 0.05$). Bahieldin et al. (2016) reported that ERF109 can delay cell death by regulating PCD-related genes, and its over-expression also improves the salt tolerance of Arabidopsis. In addition, the authors found that this TF might be a “main switch” mediator for the anabolism of multiple amino acids and hormone regulation, helping plants to promote cell growth under adverse conditions (Bahieldin et al. 2018).

Based on this, we can speculate that ERF109 also plays a crucial role in the low-N stress response of yellowhorn. In addition, some highly expressed TFs ($P \leq 0.05$) were reported. They affect the scavenging of ROS, helping plants resist oxidation. For example, PagWRKY75 (Zhao et al. 2019) can reduce the scavenging capacity for ROS and the accumulation of proline under stress, while also playing a positive role in regulating water loss from leaves. In this study, Xs_98156_c0_g1_WRKY with LN under different conditions was significantly up-regulated. Moreover, bHLH18 helps plants to regulate active oxygen homeostasis by regulating antioxidant genes (Geng et al. 2018), while Xs_69716_c0_g10_bHLH (bHLH18-like) showed continuous up-regulated expression ($P \leq 0.05$). In addition, we also found some strongly induced TFs (FC value ≥ 2 , $P \leq 0.05$) in the leaves of yellowhorn. Among them, CBF/DREB-like 1 protein, a typical cold-induced TF, can also be induced by drought, ABA treatment and high salt to improve plant resistance (Wang et al. 2014). Under N deficiency conditions, this TF showed high differential expression

in yellowhorn, which has not been reported previously. Further, we found that many of them have antioxidative-related functions ($P \leq 0.05$). Therefore, yellowhorn may mobilize this pathway to resist stress injury in case of N deficiency.

Biological Pathway Of Flavonoids

Plants accumulate flavonoids under low N conditions (Lea et al. 2007). In addition, flavonoids, as important secondary metabolites in plants, also provide plants with a “protective umbrella” against oxidative damage (Stewart et al. 2001; Diaz et al. 2006). Among them, bHLH and R2R3-MYB are the main TFs regulating flavonoids (Zhao et al. 2013; Liu et al. 2018), which are widely induced in the leaves of yellowhorn. In the transcriptome data, GO annotation yielded 13 TFs related to flavonoid and anthocyanin metabolism, namely bHLH and NAC. However, although they might be hypothetical proteins, they were not differentially expressed. According to the transcriptome data, we found that the key genes of the flavonoid KEGG pathway were generally up-regulated. Consequently, yellowhorn may mobilize the flavonoid pathway to help resist the damage caused by N deficiency. Upstream TFs and their associated mechanism will therefore be an interesting topic for future research.

Nitrogen Transport And Assimilation

As the main N form absorbed by plants, nitrate is also one of the compounds that regulate the metabolism and growth of plants to adapt to changes in N availability (Scheible et al. 1997). Nitrogen deficiency enhances the expression of high affinity nitrate transport systems (Crawford et al. 1998). LBD37, LBD38 and LBD39 were identified as a negative regulator of N availability signaling and Arabidopsis anthocyanin biosynthesis (Rubin et al. 2009). LBD TFs inhibit various other known N response genes, including the key genes for NO⁻ uptake and assimilation (Rubin et al. 2009), whereas NaCl is the central regulator of low-N responses in poplar roots (Wei et al. 2013). Moreover, LBD38 and NaCl are up-regulated but not strongly expressed in yellowhorn leaves. Many studies have reported that the Dof family plays an important role in low-N conditions (Kurai et al. 2011; Xu et al. 2018), including an active role in plant N metabolism. Although the expression of 23 members of the Dof family was induced under low-N stress, there were no significant differences among them. In addition, this was not related to N transport and metabolism based on the GO annotation. In conclusion, yellowhorn may prefer N transport and assimilation in the absence of N, but this might not be the main mechanism used to resist damage due to low-N stress.

Conclusion

At present, our description of the plant low-N stress transcription network is very fragmented, mainly relying on individual cases of selective signaling pathways affected by TFs and the expression of a limited number of single target genes. This study provides a comprehensive view of yellowhorn TF genes and expression mapping at two low-N treatment concentrations. Our results emphasize the complex

interactions between the activation and inhibition of TFs from multiple families, which may play an important role in the adaptation of yellowhorn to N-deficiency stress. Some TFs can have assigned putative defensive functions based on their typical model plant homologous genes. In particular, the strong induction of TFs in hormone conduction and oxidative stress responses implies that this mechanism plays a central role in helping yellowhorn adapt and alleviate the damage caused by low-N stress. These findings provide a reference for further research on the molecular mechanisms of yellowhorn to adapt to low-N stress.

Declarations

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Conflicts of interest/Competing interests

Not applicable.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are not publicly available as the data are being used for the next part of the research, but are available from the corresponding author upon reasonable request.

Code availability

The links for software used in this study are as follows: Plant transcription factor database, Plant TFDB v4.0 (<http://planttfdb.gao-lab.org>); BMK Cloud Online Tool v1.0 (<https://international.biocloud.net/zh/software/tools/detail/small/305>); MEME (<http://meme.nbcr.net/meme/cgi-bin/meme.cgi>); Protein Interaction Network String v11.0 (<http://string-db.org>); and QuickGO v2020-12-11 (<https://www.ebi.ac.uk/QuickGO/>).

Authors' contributions

CYJ, JXZ, XLL and HJ conceived the study. JLS, CYJ and CCY carried out the lab work. JLS, CYJ, JXZ and LLL performed data analysis. JLS and CYJ wrote the manuscript. HJ and MW performed the final revision of the manuscript. All authors read and approved the final manuscript.

References

1. A. Razal RA, Ellis S, Singh S, G. Lewis N, Towers GHN (1996) Nitrogen recycling in phenylpropanoid metabolism. *Phytochemistry* 41: 31-35.
2. Ao Y (2016) Comparison of floral ontogeny between wild-type *Xanthoceras sorbifolia bunge* and its double-flowered mutant. *Bangl J Bot* 45: 367-375.
3. Bahieldin A, Atef A, Edris S, O. Gadalla N, M. Ramadan A, M. Hassan S, G. Al Attas S, A. Al-Kordy M, S. M. Al-Hajar A, S. M. Sabir J, E. Nasr M, H. Osman G, M. El-Domyati F (2018) Multifunctional activities of ERF109 as affected by salt stress in *Arabidopsis*. *Sci Rep* 8: 6403.
4. Bahieldin A, Atef A, Edris S, O. GadallaN, M. Ali H, M. Hassan S, A. Al-Kordy M, M. Ramadan A, M. Makki R, Abdulrahman S. M. Al-Hajar A, M. El-Domyati F (2016) Ethylene responsive transcription factor ERF109 retards PCD and improves salt tolerance in plant. *BMC Plant Biol* 16: 216.
5. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res* 37(W): 202-208.
6. Becker RA, Chambers JM, Wilks AR. *The New S Language*. Chapman & Hall, 1988.
7. Crawford N, Glass A (1998) Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci* 3: 389-395.
8. Cutcliffe JW, Hellmann E, Heyl A, M. Rashotte A (2011) CRFs form protein-protein interactions with each other and with members of the cytokinin signalling pathway in *Arabidopsis* via the CRF domain. *J. Exp Bot* 68: 4995-5002.
9. Deng B, Li Y, Lei G, Liu G (2019) Nitrogen availability alters flavonoid accumulation in *Cyclocarya paliurus* via the effects on the internal carbon/nitrogen balance. *Sci Rep* 9: 2370.
10. Diaz C, Saliba-Colombani V, Loudet O, Belluomo P, Moreau L, Daniel-Vedele F, Morot-Gaudry JF, Masclaux-Daubresse C (2006) Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in *Arabidopsis thaliana*. *Plant Cell Physiol* 47: 74-83.
11. Ding L, Wang KJ, Jian GM, Biswas DK, Xu H, Li LF, Li YH (2005) Effects of nitrogen deficiency on photosynthetic traits of maize hybrids released in different years. *Ann Bot* 96: 925-930.
12. Du X, Wang G, Ji J, Shi L, Guan C, Jin C (2017) Comparative transcriptome analysis of transcription factors in different maize varieties under salt stress conditions. *Plant Growth Regul* 81: 183-195.
13. Duffy J E, Canuel E A, Adey W, Swaddle J P (2009). *Biofuels: algae*. *Science* 326:1345.

14. Fataftah N, Mohr C, Hajirezaei MR, Nicolaus von Wirén NV, Klaus Humbeck K (2018) Changes in nitrogen availability lead to a reprogramming of pyruvate metabolism. *BMC Plant Biol* 18: 77.
15. Foyer CH, Lelandais M, Kunert KJ (1994) Photooxidative stress in plants. *Physiol Plantarum* 92: 696-717.
16. Frink CR, Waggoner PE, Ausubel JH (1999) Nitrogen fertilizer: retrospect and prospect. *Proc Natl Acad Sci USA* 96: 1175-1180.
17. Geng JJ, Liu JH (2018) The transcription factor CsbHLH18 of sweet orange functions in modulation of cold tolerance and homeostasis of reactive oxygen species by regulating the antioxidant gene. *J Exp Bot* 69: 2677-2692.
18. Gu JF, Li ZK, Mao YQ, C. Struik P, Zhang H, Liu L, Yang J (2018) Roles of nitrogen and cytokinin signals in root and shoot communications in maximizing of plant productivity and their agronomic applications. *Plant Sci* 274: 320-331.
19. Gu L, Zhang G, Du L, Du J, Qi K, Zhu X, Zhang X, Jiang Z (2019) Comparative study on the extraction of *Xanthoceras sorbifolia* Bunge (yellowhorn) seed oil using subcritical n-butane, supercritical CO₂, and the Soxhlet method. *LWT* 111: 548-554.
20. Guo Y, Zhang W, He J, Zhou J, Yu H (2012) Effects of water stress and seed mass on germination and antioxidative enzymes of *Xanthoceras sorbifolia*. *Afr J Biotechnol* 11: 4187-4195.
21. Halim R, Gladman B, Danquah M K, Webley P A (2011). Oil extraction from microalgae for biodiesel production. *Bioresour. Technol.* 102: 178-185.
22. Jaradat MR, Feurtado JA, Huang D, Lu Y, Cutler AJ (2013) Multiple roles of the transcription factor AtMYBR1/AtMYB44 in ABA signaling, stress responses, and leaf senescence. *BMC Plant Biol* 13: 192.
23. Jin H, Zou JX, Li L, Bai X, Zhu T, Li J, Xu B, Wang Z (2020) Physiological responses of yellow-horn seedlings to high temperatures under drought condition. *Plant Biotechnol Rep* 14: 111-120.
24. Jin J, Tian F, Yang DC, Meng YQ, Kong L, Luo JC, Gao G (2017) Plant TFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. *Nucleic Acids Res* 45: D1040-D1045.
25. Joshi V, Joshi M, Penalosa A (2020) Comparative analysis of tissue-specific transcriptomic responses to nitrogen stress in spinach (*Spinacia oleracea*). *Plos ONE* 15: 5.
26. Kong L, Wang F, Si J, Feng B, Zhang B, Li S, Wang Z (2012) Increasing in ROS levels and callose deposition in peduncle vascular bundles of wheat (*Triticumaestivum* L.) grown under nitrogen deficiency. *J Plant Interact* 8: 109- 116.
27. Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol Biol Evol* 33: 1870-1874.
28. Kurai T, Wakayama M, Abiko T, Yanagisawa S, Aoki N, Ohsugi R (2011) Introduction of the ZmDof1 gene into rice enhance carbon and nitrogen assimilation under low-nitrogen condition. *Plant Biotechnol. J* 9: 826-837.

29. Lang X, Dalai A K, Bakhshi N N, Reaney M J, Hertz P B (2001). Preparation and characterization of bio-diesels from various bio-oils. *Bioresour. Technol.* 80: 53-62.
30. Lea US, Slimestad R, Smedvig P, Lillo C (2007) Nitrogen deficiency enhances expression of specific MYB and bHLH transcription factors and accumulation of end products in the flavonoid pathway. *Planta Med* 225: 1245-1253.
31. Li J, Fu Y J, Qu X J, Wang W, Zu Y G (2012). Biodiesel production from yellow horn (*xanthoceras sorbifolia bunge.*) seed oil using ion exchange resin as heterogeneous catalyst. *Bioresource Technology*, 108: 112-118.
32. Li J, Zu Y G, Fu Y J, Yang Y C, Li S M, Li Z N, Michael W (2010) Optimization of microwave-assisted extraction of triterpene saponins from defatted residue of yellow horn (*Xanthoceras sorbifolia Bunge.*) kernel and evaluation of its antioxidant activity. *Innov. Food Sci. Emerg. Technol.* 11: 637-643.
33. Li L S, Zhang H, Chai X H, Lv J, Hu L L, Wang J, Li Z Z, Yu J H, Liu Z C (2021) Genome-wide identification and expression analysis of the MYC transcription factor family and its response to sulfur stress in cabbage (*Brassica oleracea L.*). *Gene* 814: 146116.
34. Lin Z, Zhong Q, Chen C, Ruan Q, Chen Z, You X (2016) Carbon dioxide assimilation and photosynthetic electron transport of tea leaves under nitrogen deficiency. *Carbon dioxide assimilation and photosynthetic electron transport of tea leaves under nitrogen deficiency. Bot Stud* 57: 37.
35. Liu Z W, Wu Z J, Li X H, Huang Y, Li H, Wang Y X, Zhuang J (2015) Identification, classification, and expression profiles of heat shock transcription factors in tea plant (*Camellia sinensis*) under temperature stress. *Gene* 576: 52-59.
36. Liu Y, Hou H, Jiang X, Wang P, Dai X, Chen W, Xia T (2018) A WD40 Repeat Protein from *Camellia sinensis* Regulates Anthocyanin and Proanthocyanidin Accumulation through the Formation of MYB-bHLH-WD40 Ternary Complexes. *Int J Mol Sci* 19: 1686.
37. Luo J, Zhou JJ, Masclaux-Daubresse C, Wang N, Wang H, Zheng B (2019) Morphological and physiological responses to contrasting nitrogen regimes in *Populus cathayana* is linked to resources allocation and carbon/nitrogen partition. *Environ Exp Bot* 162: 247-255.
38. Miyake K, Ito T, Senda M, Ishikawa R, Harada T, Niizeki M, Akada S (2003) Isolation of a subfamily of genes for R2R3-MYB transcription factors showing up-regulated expression under nitrogen nutrient-limited conditions. *Plant Mol Bio* 53: 237-245.
39. Nath VS, Koyyappurath S, Alex TE, Geetha KA, Augustine L, Nasser A, Thomas G (2019) Transcriptome-based mining and expression profiling of *Pythium* responsive transcription factors in *Zingiber sp.* *Funct Integr Genomic* 19: 249-264.
40. Overmyer K, Brosché M, Kangasjärvi J (2003) Reactive oxygen species and hormonal control of cell death. *Trends Plant Sci* 8: 335-342.
41. Pal S, Yadav AK, Singh AK, Rastogi S, Gupta MM, Verma RK, A. Nagegowda D, Pal A, Shasany AK (2017) Nitrogen treatment enhances sterols and withaferin A through transcriptional activation of

- jasmonate pathway, WRKY transcription factors, and biosynthesis genes in *Withania somnifera* (L.) Dunal. *Protoplasma* 254: 389-399.
42. Paul MJ, Driscoll SP (1997) Sugar repression of photosynthesis: the role of carbohydrates in signaling nitrogen deficiency through source:sink imbalance. *Plant Cell Environ* 20: 110-116.
 43. Paul MJ, Driscoll SP (2008) Sugar repression of photosynthesis: the role of carbohydrates in signaling nitrogen deficiency through source:sink imbalance. *Plant Cell Environ* 20: 110-116.
 44. Quan X, Zeng J, Ye L, Chen G, Han Z, Shah JM, Zhang G (2016) Transcriptome profiling analysis for two Tibetan wild barley genotypes in responses to low nitrogen. *BMC Plant Biol* 16: 30.
 45. Que Y, Su Y, Guo J, Wu Q, Xu L (2014) A global view of transcriptome dynamics during *Sporisorium scitamineum* challenge in sugarcane by RNA-Seq. *Plos ONE* 9: e106476.
 46. Ruan CJ, Yan R, Wang BX, Mopper S, Guan WK, Zhang J (2017) The importance of yellow horn (*Xanthoceras sorbifolia*) for restoration of arid habitats and production of bioactive seed oils. *Ecol Eng* 99: 504-512.
 47. Rubin G, Tohge T, Matsuda F, Saito K, Scheible W-R (2009) Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. *Plant Cell* 21: 3567-3584.
 48. Scheible WR, Morcuende R, Czechowski T, Fritz C, Osuna D, Palacios-Rojas N, Schindelasch D, Thimm O, K. Udvardi M, Stitt M (2004) Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. *Plant Physiol* 136: 2483-2499.
 49. Scheible WR, Gonzale-Zfontes A, Lauerer M, Muller-Rober B, Caboche M, Stitt M (1997) Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. *Plant Cell* 9: 783.
 50. Sendzikiene E, Makareviciene V, Janulis P (2006). Influence of fuel oxygen content on diesel engine exhaust emissions. *Renew. Energ.* 31: 2505-2512.
 51. Shamimuzzaman M, Vodkin L (2013) Genome-wide identification of binding sites for NAC and YABBY transcription factors and co-regulated genes during soybean seedling development by ChIP-Seq and RNA-Seq. *BMC Genom* 14: 477.
 52. Shao H, Chu L (2008). Resource evaluation of typical energy plants and possible functional zone planning in china. *Biomass and Bioenergy*, 32: 283-288.
 53. Shim JS, Jung C, Lee S, Min K, Lee YW, Choi Y, Lee JT, Kim JK, Choi YD (2013) AtMYB44 regulates WRKY70 expression and modulates antagonistic interaction between salicylic acid and jasmonic acid signaling. *Plant J* 73: 483-495.
 54. Shin R, Berg RH, Schachtman DP (2005) Reactive oxygen species and root hairs in *Arabidopsis* root response to nitrogen, phosphorus and potassium deficiency. *Plant Cell Physiol* 46: 1350-7.
 55. Stewart AJ, Chapman W, Jenkins GI, Graham I, Martin T, Crozier A (2002) The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. *Plant Cell Environ* 24:1189-1198.

56. Supriadi, Duangkhet M, Thepsukhon A, Widyastuti R, Santosa DA, Tajima S, Nomuta M (2016) A MYB-related transcription factor affects nodule formation in *Lotus japonicus*. *Plant Biotechnol* 33: 187-194.
57. Ueda Y, Ohtsuki N, Kadota K, Tezuka A, Nagano AJ, Kadowaki T, Kim Y, Miyao M, Yanagisawa S (2020) Gene regulatory network and its constituent transcription factors that control nitrogen-deficiency responses in rice. *New phytol* 227: 1434-1452.
58. Wagner GP, Kin K, Lynch V J (2013) A model based criterion for gene expression calls using RNA-seq data. *Theor Biosci* 132: 159-164.
59. Wan G S, Wang X B, Wu L J, Gao H Y (2013). Advances in Studies on Chemical Constituents of *Xanthoceras Sorbifolia* and Their Pharmacological Activities. *Chin. Tradit. Herbal. Drugs* 13: 1842-1851.
60. Wang D, Su D, Yu B, Chen C, Cheng L, Li X, Xi R, Gao H, Wang X (2017) Novel anti-tumour barringenol-like triterpenoids from the husks of *Xanthoceras sorbifolia* Bunge and their three dimensional quantitative structure activity relationships analysis. *Fitoterapia* 116: 51-60.
61. Wang L, Ruan C, Liu L, Du W, Bao A (2018) Comparative RNA-Seq Analysis of High- and Low-Oil Yellow Horn During Embryonic Development. *Int J Mol Sci* 19: 3071.
62. Wang X, Li D, Jiang J, Dong J, Ma Y (2017) Soybean NAC gene family: sequence analysis and expression under low nitrogen supply. *Biol Plantarum* 61: 473-482.
63. Wang Z, Liu J, Guo H, He X, Wu W, Du J, Zhang Z, An X (2014) Characterization of two highly similar CBF/DREB1-like genes, PhCBF4a and PhCBF4b, in *Populus hopeiensis*. *Plant Physiol Bioch* 83: 107-116.
64. Wei H, Yordanov YS, Georgieva T, Li X, Busov V (2013) Nitrogen deprivation promotes *Populus* root growth through global transcriptome reprogramming and activation of hierarchical genetic networks. *New Phytol* 200: 483-497.
65. Xu X, Li F, Wang Y, Tang S, Dai Q, Zhu S, Liu T (2018) Identification of Dof transcription factors in ramie (*Boehmeria nivea* L. Gaud) and their expression in response to different nitrogen treatments. *3 Biotech* 8: 496.
66. Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. *Proc Natl Acad Sci U S A* 101: 7833-7838.
67. Yang Y, Gao S, Su Y, Lin Z, Guo J, Li M, Wang Z, Que Y, Xu L (2019) Transcripts and low nitrogen tolerance: Regulatory and metabolic pathways in sugarcane under low nitrogen stress. *Environ Exp Bot* 163: 97-111.
68. Yao ZY, Qi JH, Yin LM (2013) Biodiesel production from *Xanthoceras sorbifolia* in China: Opportunities and challenges. *Renew Sust Energ Rev* 24: 57-65.
69. Zang E, Qiu B, Chen N, Li C, Li M (2021). *Xanthoceras sorbifolium bunge*: a review on botany, phytochemistry, pharmacology, and applications. *Frontiers in Pharmacology*, 12: 708549.

70. Zebarth B, Tai H, Luo S, Millard P, Koeyer DD, Li XQ, Xiong X (2012) Effect of Nitrogen Form on Gene Expression in Leaf Tissue of Greenhouse Grown Potatoes During Three Stages of Growth. *Am J Potato Res* 89: 315-327.
71. Zhang F, Liu Y, Wang L Y, Bai P X, Ruan L, Zhang C C, Wei K, Cheng H (2018) Molecular cloning and expression analysis of ammonium transporters in tea plants (*Camellia sinensis* (L.) O. Kuntze) under different nitrogen treatments. *Gene* 658: 136-145.
72. Zhao D, Reddy KR, Kakani VG, Reddy VR (2005) Nitrogen deficiency effects on plant growth, leaf photosynthesis, and hyperspectral reflectance properties of sorghum. *Eur J Agron* 22: 391-403.
73. Zhao K, Zhang D, Lv K, Zhang X, Cheng Z, Li R, Zhou B, Jiang T (2019) Functional characterization of poplar WRKY75 in salt and osmotic tolerance. *Plant Sci* 289: 110259.
74. Zhao L, Gao L, Wang H, Chen X, Wang Y, Yang H, Wei C, Wan X, Xia T (2013) The R2R3-MYB, bHLH, WD40, and related transcription factors in flavonoid biosynthesis. *Funct Integr Genomic* 13: 75-98.
75. Zhao N, Zhang Y, Li Q, Li R, Xia X, Qin X, Guo H (2015) Identification and expression of a stearyl-ACP desaturase gene responsible for oleic acid accumulation in *Xanthoceras sorbifolia* seeds. *Plant Physiol Bioch* 87: 9-16.
76. Zhong C, Cao Y X, Li B Z, Yuan Y J (2010). Biofuels in china: past, present and future. *Biofuels Bioproducts and Biorefining*, 4: 326-342.

Tables

Tables 1 & 2 are available in the Supplementary Files section.

Figures



Figure 1

Root of yellowhorn under low nitrogen stress. The root of control (CK) and stressed (LN, NN) yellowhorn seedlings were observed after 15 days low-N treatment.

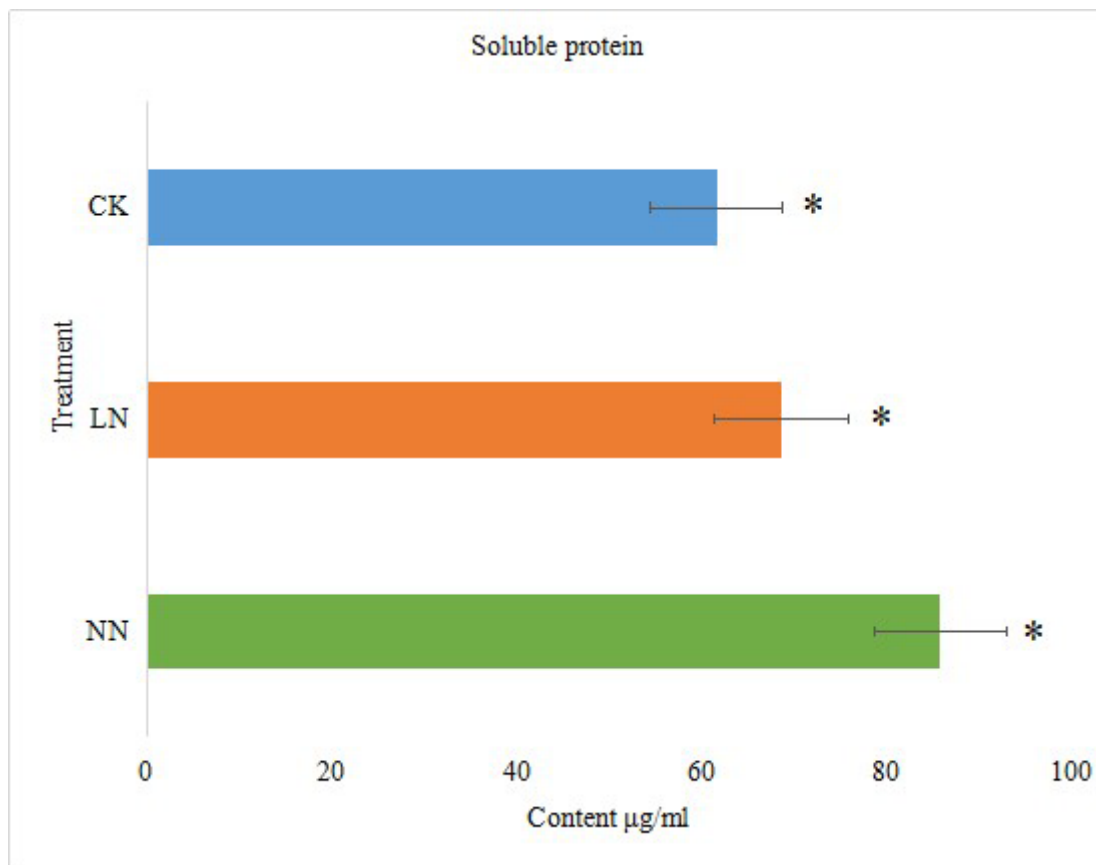


Figure 2

Soluble protein content. The amount of soluble protein content of seedlings treated under normal condition and low-N-stress were measured by Bradford (1976) using bovine serum albumin as the standard.

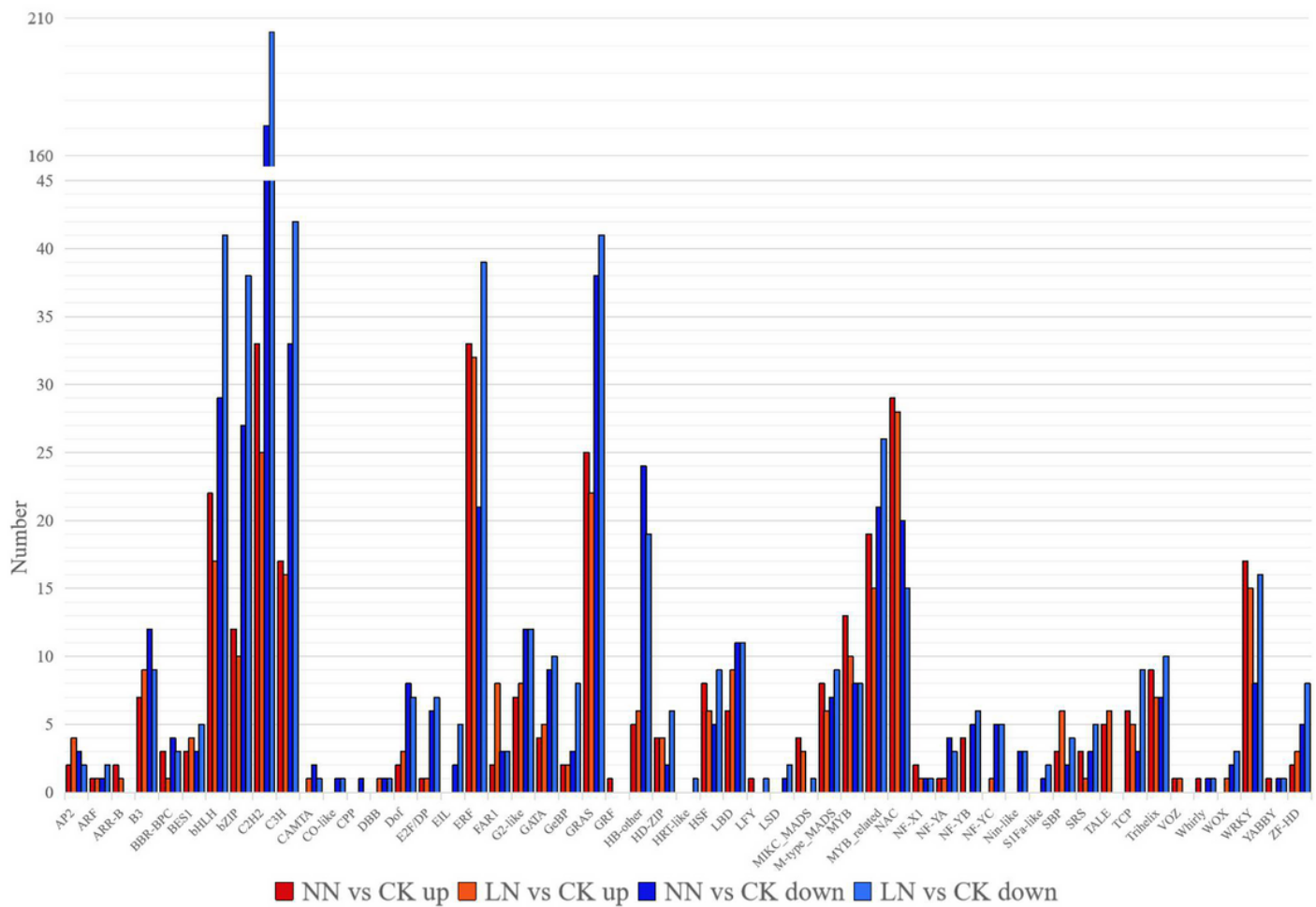


Figure 3

Expression profile of yellowhorn transcription factors (TFs) when comparing control (CK) and two nitrogen deficiency conditions. The X-axis indicates various TF families; the Y-axis represents the number of differentially expressed TFs. Red indicates up-regulated in no-N (NN vs CK); orange indicates up-regulated in low-N (LN vs. CK); blue indicates down-regulated in NN vs. CK; sky blue indicates down-regulated in NN vs CK.

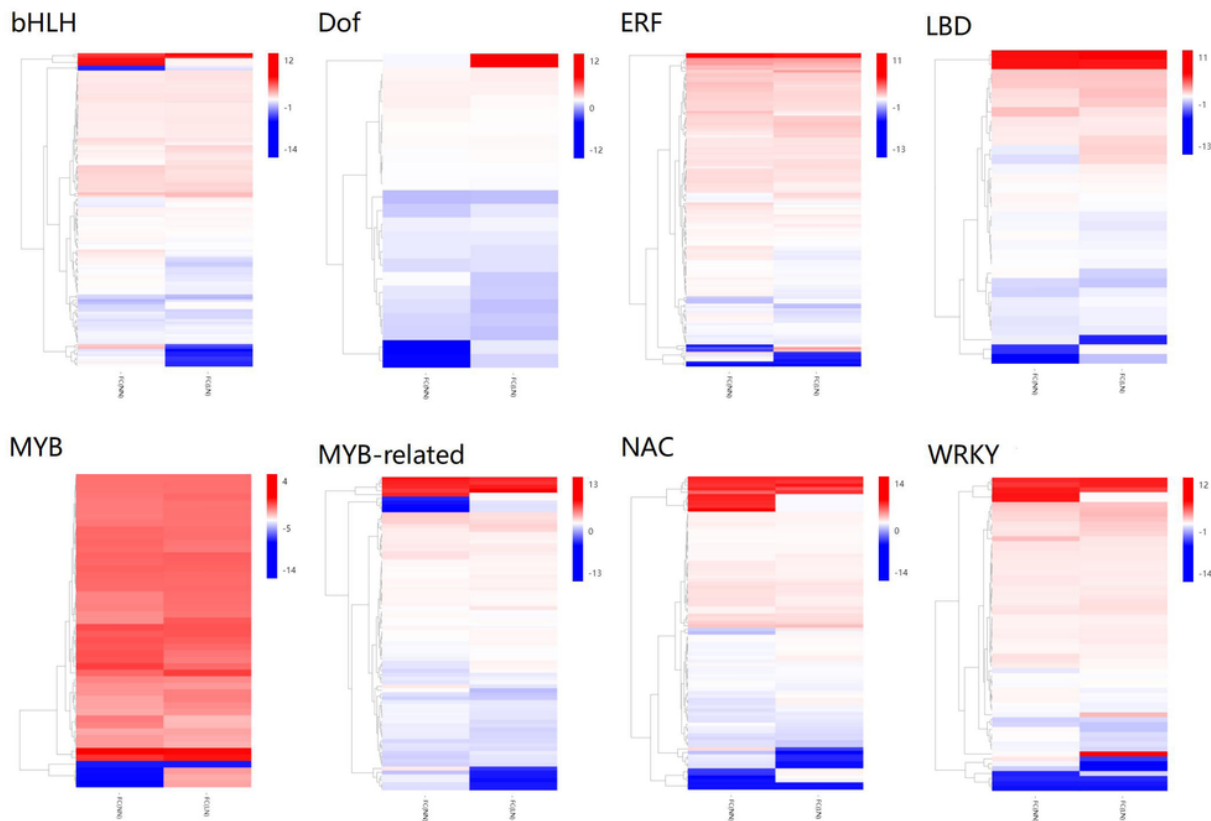


Figure 4

Heat map representing expression profiles of yellowhorn TFs under two N deficiency conditions compared with those of CK. The left and right columns in each family represent NN and LN compared with CK, respectively.

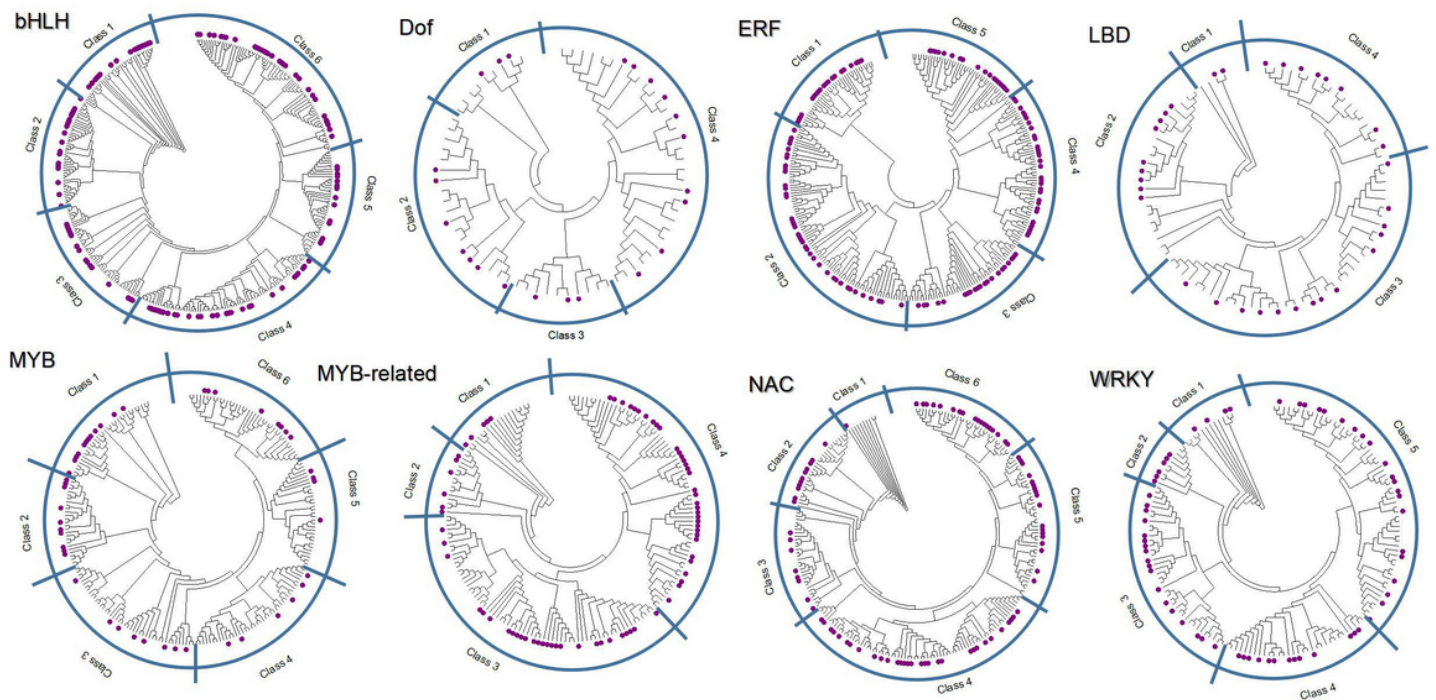


Figure 5

Phylogenetic relationships and subfamily designations between yellowhorn and the model species *Arabidopsis* TF families. Purple marks represent the proteins of *Arabidopsis*, whereas unmarked branches are the proteins of yellowhorn.

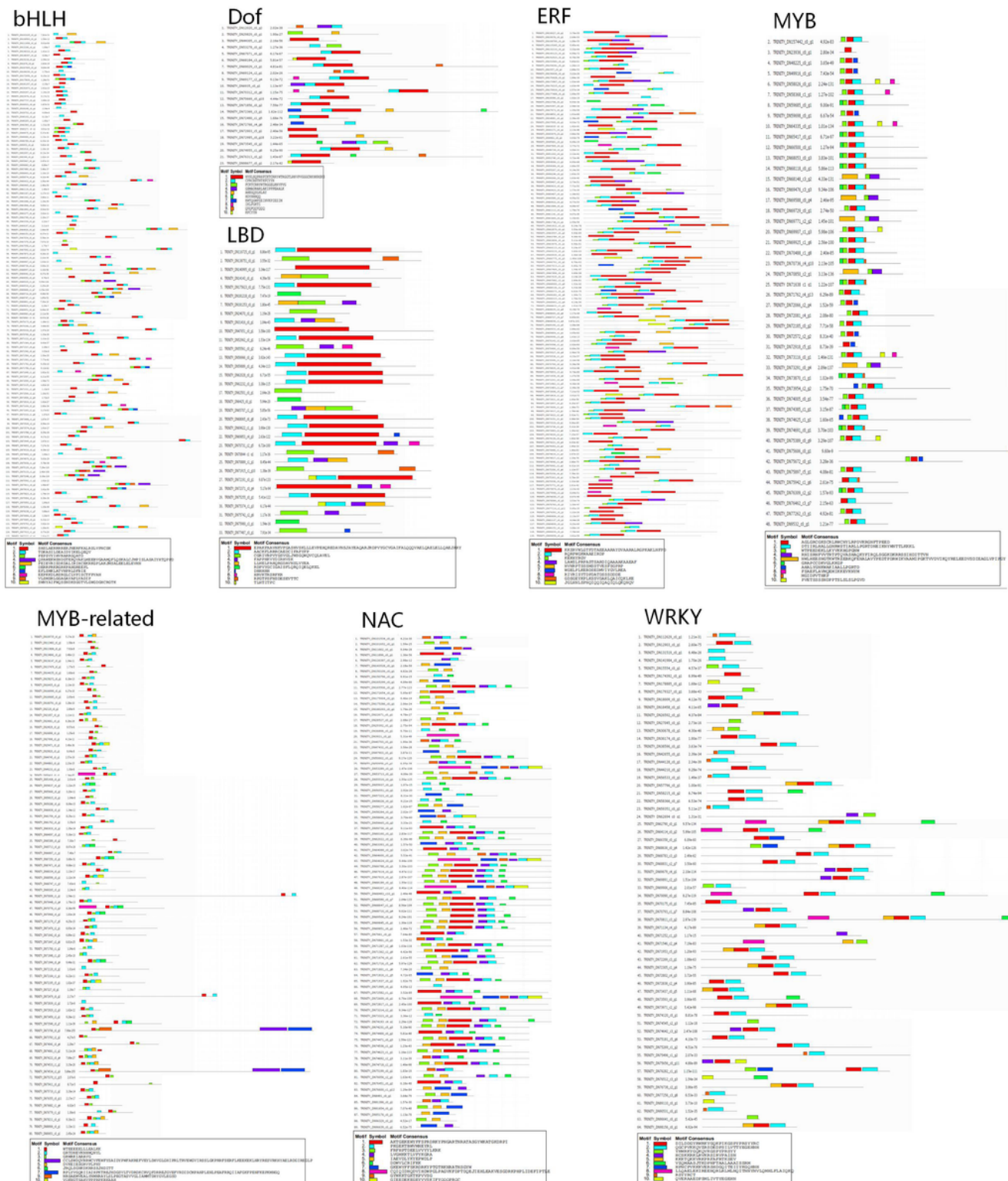


Figure 6

Conservative domain analysis of yellowhorn TFs. Different motifs are highlighted with different colored boxes with numbers 1-10.

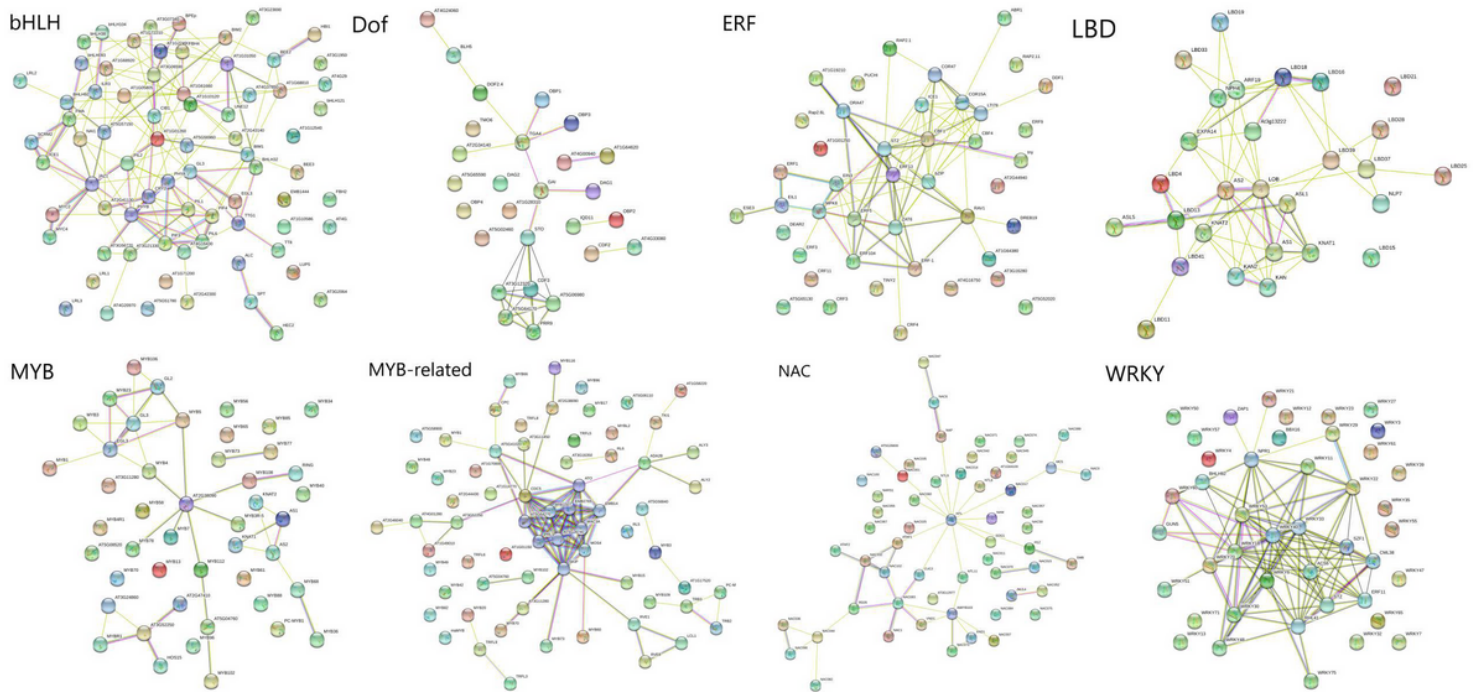


Figure 7

Network analysis of yellowhorn TFs in COG mode using the STRING 11.0 database. The connection colors represent the types of evidence for inferring association: recurring neighborhood in different genomes (green line), events of gene fusion (red), co-occurrence of those genes in the same organisms (dark blue), co-expression (black), experimental protein–protein interaction data (pink), pathway described by other data-bases (light blue), literature text-mining (yellow), and homology (purple lines). Showing all TF genes connections with a confidence score >0.4.

Supplementary Files

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