Isolation of Drought-Resistance Gene Encoded *GmNAC-D* in Dering-1 Variety of Soybean

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Abstract

*Drought is one of the most critical abiotic stresses limiting crop growth and productivity, causing substantial yield losses in major crops such as soybean (*Glycine max *L.). The No Apical Meristem (NAC) transcription factors (TFs) are plant-specific proteins that have been widely reported to play key roles in enhancing drought tolerance across various plant species. This study aimed to isolate and characterize a soybean NAC gene through a molecular approach. A NAC family gene was successfully isolated from drought-tolerant soybean cultivar Dering-1 and designated* GmNAC-D *(D = Dering-1). The gene was amplified by PCR-based cloning, sequenced and analyzed through bioinformatics, while tissue-specific expression was assessed using RT-PCR. The gene consists of a 762 bp open reading frame encoding 253 amino acids. Database analysis revealed that* GmNAC-D *shares high homology with* GmNAC02*, a known stress-responsive gene in soybean. Expression analysis across different tissues demonstrated that* GmNAC-D *is most abundantly expressed in stem tissue, followed by flowers and roots. Amino acid sequence analysis further confirmed its high similarity to* GmNAC02*, although with distinct expression profiles. The differential expression patterns of* GmNAC *genes suggest functional diversification during plant growth and development. Collectively, these findings expand the repertoire of soybean NAC transcription factors and provide a valuable genetic resource for the development of transgenic soybean with enhanced drought tolerance.*

Keywords: *Drought-resistant, GmNAC-D, soybean, transcription factor.*

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INTRODUCTION

Drought is one of the major environmental constraints that can suppress plant productivity through various physiological and biochemical processes, such as photosynthesis, respiration, nutrient metabolism, ion absorption and translocation (Oguz et al., 2022; El Haddad et al., 2022). The adverse effects of drought on soybean plants have become a major concern since this legume is considered one of the most important crops providing the largest sources of vegetable oil, protein, and macronutrient minerals for human consumption and livestock feed. The numerous benefits contained in soybeans as a high-quality food source have led to a steadily increasing demand for this crop from year to year (Bantacut, 2017).

Plants possess various defense mechanisms that enable them to cope with drought-induced stress. In response to such conditions, they activate biochemical, physiological, and morphological pathways (Pamungkas et al., 2022). At the biochemical level, plants enhance the production of antioxidant compounds, chlorophyll content, proline accumulation, as well as various hormones and other secondary metabolites. Physiologically, plants change stomatal activity, osmotic balance, photosynthesis, transpiration, water transport and leaf water content. In addition, morphological alterations such as

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reduced leaf area and number, accelerated leaf senescence, increased root length, and shifts in growth stages may also occur. All these changes are triggered by several molecular mechanisms, including the upregulation of gene expression (Conesa et al., 2016).

Great efforts have been made by researchers over the past decade to understand drought tolerance mechanisms in plants, including in maize (Hammad et al., 2017; Ghahfarokhi et al., 2015), mung bean (Jincy et al., 2021), wheat (Jin et al., 2018; Khan et al., 2019), sorghum (Sarshad et al., 2021; Sanjari et al., 2021), rice (Yang et al., 2019) and soybean (Dong et al., 2019). When plants are exposed to environmental stresses such as drought, their initial response is the activation of a set of genes involved in defense mechanisms that function to enhance tolerance against unfavorable conditions (Zhang et al., 2022). The defense mechanisms against stress are largely regulated by genes involved in physiological and metabolic processes, among which are Transcription Factors (TFs) and the cis-elements of target genes located in their promoters (Zuo et al., 2023). Transcription factors bind to specific DNA sequences and are involved in regulating the transfer of genetic information from DNA to RNA (Hao et al., 2021). Therefore, the identification of novel TFs holds great potential for overcoming key limitations in the development of transgenic soybean varieties with enhanced drought tolerance (Gahlaut et al., 2016).

NAC TFs are plant-specific transcription factors and are proteins involved in various processes, including flowering, senescence, seed development, secondary cell wall thickening, and responses to stresses caused by both biotic and abiotic factors (Li et al., 2023). One of the important roles of NAC TFs that has been investigated over the past decades is their contribution to enhancing resistance against both biotic and abiotic stresses. Stress-responsive NAC TFs are largely encoded by members of the ATAF subfamily, including StNAC262 in potato plants (Zhang et al., 2018), *Brassica napus*, *BnNAC485* (Ying et al., 2014), and Arabidopsis, NAC transcription factors from the ATAF subfamily such as ATAF1, ATAF2, ANAC019, ANAC055, and ANAC072, also known as ERD11 (Responsive to Dehydration Stress) play crucial roles in regulating plant responses to environmental stresses (Jensen et al., 2010), and ANAC032 is involved in regulating tolerance to drought and salinity stress, as well as in the signaling pathways of jasmonic acid and abscisic acid (ABA). Overexpression of the ANAC032 protein in Arabidopsis not only enhances plant tolerance to drought and salinity stress but is also associated with sugar biosynthesis, which is likely closely linked to the photosynthetic process (Zhang et al., 2022). According to Zuo et al. (2023), NAC proteins, particularly members of the ATAF and ANAC subfamilies, have been shown to activate genes involved in osmotic adjustment, reactive oxygen species (ROS) detoxification, and abscisic acid (ABA)-mediated stress signaling.

The identification and cloning of cDNA for NAC proteins in soybean revealed the presence of 32 different NACs, seven of which encode stress-inducible genes (Melo et al., 2018). In this study, one NAC gene was successfully isolated from a drought-tolerant soybean variety (var. Dering-1), which shows high homology with Arabidopsis NAC genes that have been proven to confer drought-tolerant phenotypes (Ermawati et al., 2024). The existence of this gene represents a novel source of genetic diversity that can be utilized for the development of superior drought-tolerant soybean varieties through genetic engineering.

MATERIALS AND METHODS

Experiment Site

The research was conducted at the Biosains Laboratory and Greenhouse, Politeknik Negeri Jember, from June to November 2024.

Materials and Tools

The materials used in this study are soybean seeds (Dering-1 variety), pots (Ø 25 cm), soil-compost mixture, RNeasy Plant Mini Kit (Qiagen), Transcriptor First Strand cDNA Synthesis Kit (Roche, USA), Core Kit Taq DNA Polymerase (Roche, USA), NPK Mutiara 16-16-16, Liquid Nitrogen. The tools are UV-Vis spectrophotometer (Amersham Biosciences), Biometra PCR Thermal Cycler.

Planting and Stress Treatment

The soybean variety used in this study was Dering-1, which is known for its drought tolerance (BRMP, Malang). Planting was carried out in the greenhouse of Politeknik Negeri Jember under day/night temperatures of approximately 31°C/25°C, using pots (Ø 25 cm) filled with a soil-compost mixture at a 1:1 ratio. One plant was grown in each pot. Drought stress treatment was applied when the plants were about

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three weeks old after planting, by withholding irrigation for 7 consecutive days, or until the soil moisture content had decreased to around 30% of field capacity.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from 100 mg of various plant tissues, such as roots, stems, leaves, and flowers, using the RNeasy Plant Mini Kit (Qiagen). The RNA concentration was determined using a UV-Vis spectrophotometer (Amersham Biosciences). The first-strand cDNA was prepared from 1 µg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions.

PCR and Cloning of *GmNAC-D*

The *GmNAC-D* gene was amplified from cDNA derived from stem, leaf, root, pod, and flower tissues subjected to drought stress using the Core Kit Taq DNA Polymerase (Roche, USA). Primers were designed based on the Arabidopsis NAC gene (*At1g77450*) with the following sequences: Forward: 5'-GCGGATCCATGATGAAATCTGGGGCTGATT-3', Reverse: 5'-GCTCTAGATCAGAAAGTTCCCTGCCTA ACC-3'. The PCR conditions were 94°C for 3 min, followed by 30 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel and visualized under a UV illuminator (Biometra, Germany). The amplified fragments were subsequently cloned into the pGEM-T vector (Promega, USA) as previously described byPrasetyo et al., (2018) and sequenced using Sanger dideoxy sequencing technology (The 1st BASE, Malaysia).

Phylogenetic Analysis

The amino acid sequences of six *GmNAC* genes obtained from the NCBI GenBank database were analyzed for sequence homology using ClustalX (Thompson et al., 1997), with the following parameters: gap open penalty = 10 and gap extension penalty = 0.2. A phylogenetic tree was then constructed using the neighbor-joining method with the online EMBL-EBI software (www.ebi ac.uk/).

RESULTS AND DISCUSSION

Isolation of *GmNAC-D*

Drought stress remains one of the most damaging abiotic factors affecting global crop yields (Oguz et al., 2022). In soybeans, drought during the flowering and pod-filling stages can lead to significant yield losses due to impaired photosynthesis, altered carbohydrate partitioning, and reduced reproductive success (Dong et al., 2019). Plants mitigate these effects by activating complex stress-response networks, many of which are regulated by transcription factors. NAC proteins, particularly members of the ATAF and ANAC subfamilies, have been shown to activate genes involved in osmotic adjustment, reactive oxygen species (ROS) detoxification, and abscisic acid (ABA)-mediated stress signaling (Zhang et al., 2022; Zuo et al., 2023).

To investigate the involvement of genes from the NAC transcription factor (TF) family in regulating drought stress, gene isolation was performed using the drought-tolerant cultivar Dering-1 as the genetic source. The gene isolation was not performed through conventional cDNA library screening, but instead utilized literature studies and bioinformatics databases, including GenBank (www.ncbi.nml.nih.gov/) and the Plant Transcription Factor Database (http://planttfdb.cbi.pku.edu.cn/). Previous studies highlighting the role of NAC TFs in Arabidopsis, particularly *ANAC032*, were also taken into consideration. Notably, overexpression of *ANAC032* has been reported to confer enhanced tolerance to drought and salinity stress, providing a strong rationale for its use as a reference in soybean gene isolation (Ermawati et al., 2024). Based on database information, the NAC family in soybean has been identified to comprise 180 members, of which 32 full-length open reading frames (ORFs) have been characterized (Melo et al., 2018). Building upon this knowledge, primers were designed from the Arabidopsis NAC gene sequences as templates to amplify and synthesize NAC genes from drought-stressed soybean plants.

The gene isolation process revealed the presence of a putative 762-bp fragment, designated as *GmNAC-D* (D = Dering-1) (Figure 1). Sequencing and bioinformatic analysis using BLAST (www.ncbi.nml.nih.gov/) revealed that *GmNAC-D* contains an open reading frame (ORF) of 762 bp, encoding 253 amino acids (Figure 2). Two nucleotide differences were detected when comparing the GmNAC-D sequence with that of Arabidopsis NAC32; however, these substitutions were synonymous and did not alter the amino acid composition, indicating that *GmNAC-D* and Arabidopsis *NAC32* share an identical amino acid sequence (data not shown).

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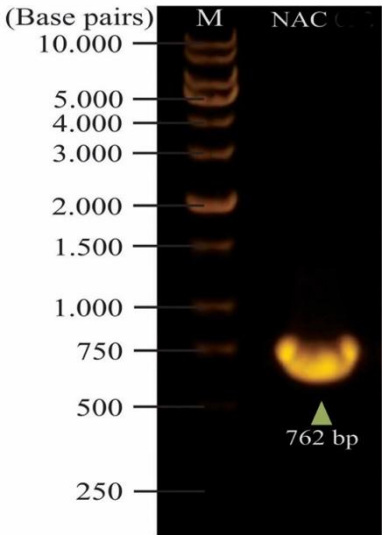

Figure 1. The *GmNAC-D* gene, With a Fragment Size of 762 bp, was Confirmed by Electrophoresis of a 20 µl PCR Product on a 1% Agarose Gel.

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                                                        TTCTTTTGCGGATTCCC
ATGATGAAATCTGGGGCTGATTTGCAATTTCCACCAGGATTTAGATTTCATCCTACGGAT    60
 M  M  K  S  G  A  D  L  Q  F  P  P  G  F  R  F  H  P  T  D     20
GAGGAGCTAGTCCTCATGTATCTCTGTCGTAAATGCGCGTCGCAGCCGATCCCTGCTCCG   120
 E  E  L  V  L  M  Y  L  C  R  K  C  A  S  Q  P  I  P  A  P     40
ATTATCACCGAACTCGATTTGTACCGATATGATCCTTGGGACCTTCCCGACATGGCTTTG   180
 I  I  T  E  L  D  L  Y  R  Y  D  P  W  D  L  P  D  M  A  L     60
TACGGTGAAAAGGAGTGGTATTTTTTCTCACCAAGAGATCGAAAGTATCCAAACGGTTCA   240
 Y  G  E  K  E  W  Y  F  F  S  P  R  D  R  K  Y  P  N  G  S     80
AGACCCAACCGTGCAGCTGGTACTGGATATTGGAAAGCTACCGGAGCTGATAAACCAATA   300
 R  P  N  R  A  A  G  T  G  Y  W  K  A  T  G  A  D  K  P  I    100
GGTCGTCCTAAACCGGTTGGTATTAAGAAGGCTCTAGTGTTTTACTCGGGAAAACCTCCA   360
 G  R  P  K  P  V  G  I  K  K  A  L  V  F  Y  S  G  K  P  P    120
AATGGAGAGAAAACCAATTGGATTATGCACGAATACCGGCTCGCTGACGTTGACCGGTCG   420
 N  G  E  K  T  N  W  I  M  H  E  Y  R  L  A  D  V  D  R  S    140
GTTCGTAAGAAAAACAGTCTAAGATTGGACGATTGGGTATTGTGTCGTATATATAACAAG   480
 V  R  K  K  N  S  L  R  L  D  D  W  V  L  C  R  I  Y  N  K    160
AAAGGTGTCATCGAGAAGCGACGAAGCGATATCGAGGACGGGTTAAAGCCTGTGACTGAC   540
 K  G  V  I  E  K  R  R  S  D  I  E  D  G  L  K  P  V  T  D    180
ACGTGTCCACCGGAATCTGTGGCGAGATTGATCTCCGGCTCGGAGCAAGCGGTGTCACCG   600
 T  C  P  P  E  S  V  A  R  L  I  S  G  S  E  Q  A  V  S  P    200
GAATTCACGTGTAGCAACGGTCGGTTGAGTAATGCCCTTGATTTTCCGTTTAATTACGTA   660
 E  F  T  C  S  N  G  R  L  S  N  A  L  D  F  P  F  N  Y  V    220
GATGCCATCGCCGATAACGAGATTGTGTCACGGCTATTGGGCGGGAATCAGATGTGGTCG   720
 D  A  I  A  D  N  E  I  V  S  R  L  L  G  G  N  Q  M  W  S    240
ACGACGCTTGATCCACTTGTGGTTAGGCAGGGAACTTTCTAATCTAGAGCAAAA        762
 T  T  L  D  P  L  V  V  R  Q  G  T  F  *                     253
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Figure 2. The Predicted Nucleotide and Amino Acid Sequences of *GmNAC-D* are Shown, with the Putative Nuclear Localization Signal (NLS) Highlighted within the Boxed Region.

NAC transcription factors (TFs) represent one of the largest plant-specific TF families (Melo et al., 2018). They are characterized by a conserved N-terminal DNA-binding NAC domain and a variable C-terminal region, which functions as a transcriptional activation or repression domain. The NAC family has been implicated in diverse biological processes including cell wall thickening, senescence, hormone signaling, and abiotic stress responses (Li et al., 2021; Mohanta et al., 2020). Database analysis revealed that *GmNAC-D* contains a conserved NAC domain, which is a characteristic feature of NAC transcription factors, and shares high similarity with other members of the NAC family. However, the C-terminal region

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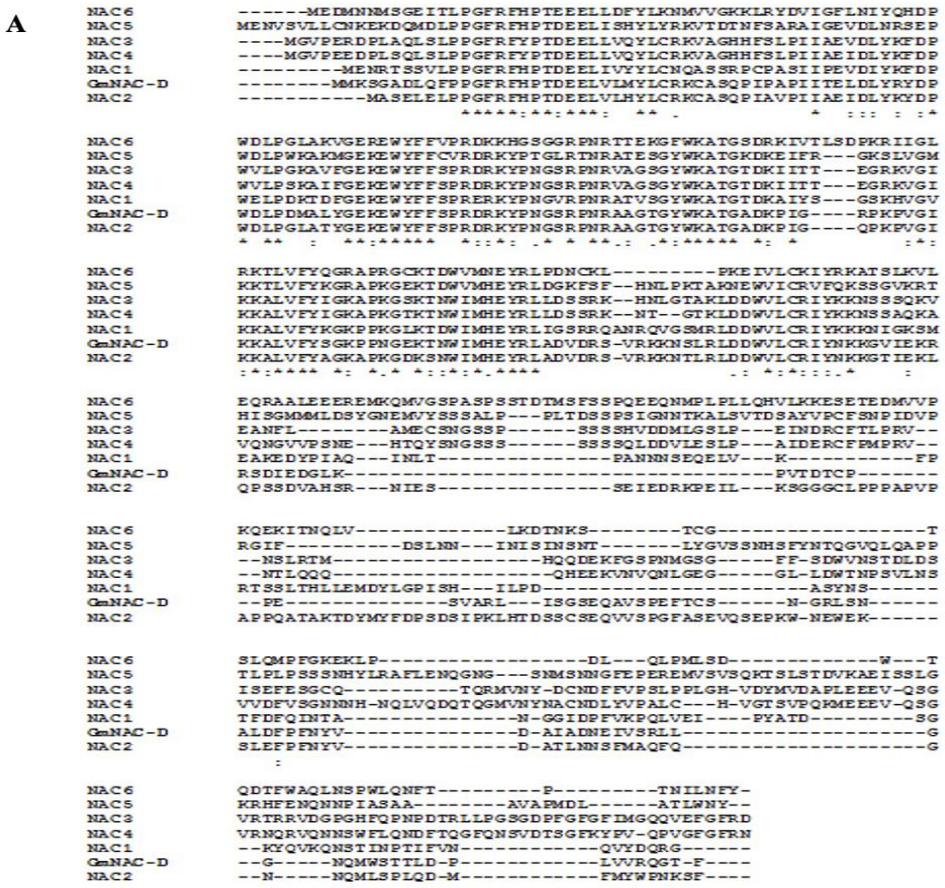
exhibited a high degree of variation within the NAC family (Figures 2 and 3). According to Li et al. (2021), in addition to exhibiting high variation in the C-terminal region, a large number of repetitive amino acids, particularly serine, proline, and threonine, were also identified. Sequence prediction further revealed a putative nuclear localization signal (NLS) with the motif PRDRKYPNGS (Mohanta et al., 2020), located in the N-terminal region of *GmNAC-D* (Figure 2). The structural features of GmNAC-D, including the nuclear localization signal and conserved NAC DNA-binding domain, suggest its potential function as a regulator of downstream drought-responsive genes. As reported by Bian et al. (2020), the NAC domain contains an NLS whose primary function is to facilitate the translocation of proteins from the cytoplasmic matrix into the nucleus, where they can regulate gene expression. The NAC-domain **has** dimerization ability, which is crucial for the proper functioning of NAC transcription factors to enhance their DNA-binding capacity, leading to more efficient and targeted gene regulation.

Bioinformatics Analysis of the *GmNAC-D* Protein

From the soybean database, 32 genes encoding NAC proteins have been independently identified in full length (Melo et al., 2018). However, only six NAC genes (*GmNAC1–GmNAC6*) are listed and accessible in the GenBank database. The relationship between these six *GmNAC* genes and *GmNAC-D*, based on the high degree of domain conservation, is shown in Figure 3A. Although NAC proteins share conserved domains, they often exhibit divergent functions depending on tissue expression patterns and stress conditions. In soybean, at least 32 NAC genes have been reported as full-length, with only a subset characterized in detail (Melo et al., 2018). The N-terminal region of *GmNAC* proteins exhibits a very high level of homology among members, whereas the C-terminal region, known as the Transcriptional Activation Region (TAR), consists of simple repeated amino acids and is typically enriched in serine/threonine, glutamine and proline residues (Li et al., 2021). According to Bian et al. (2020), NAC proteins generally possess two relatively independent domains: the N-terminal domain is highly conserved and typically consists of about 151–159 amino acids**.** This domain contains the NAC domain itself, which is responsible for its primary function as a DNA-binding domain. The NAC domain enables the protein to interact with specific sequences of DNA, particularly in the promoter regions of target genes. The C-terminal domain**,** referred to as the TAR is more diverse and shows significant variability across different NAC proteins, and it is involved in activating gene transcription**.**



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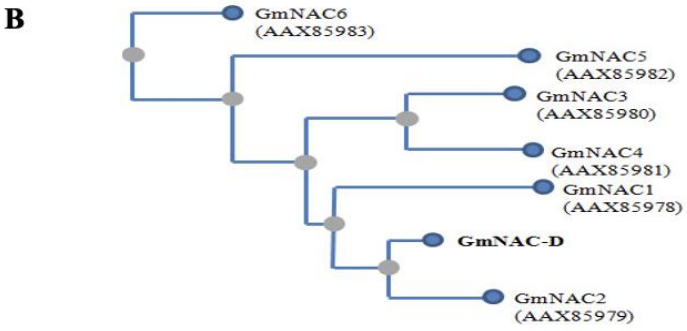

Figure 3. Comparison of Amino Acid Sequences and Phylogenetic Tree of GmNAC-D and other NAC Proteins. (A) Amino acid sequence comparison of GmNAC-D with GmNAC1 (AAX85978.1), GmNAC2 (AAX85979.1), GmNAC3 (AAX85980.1), *GmNAC4* (AAX85981.1), *GmNAC5* (AAX85982.1), and *GmNAC6* (AAX85983.1). Identical amino acids are indicated with an asterisk (*). (B) Phylogenetic tree showing the relationship of *GmNAC-D* with the six other *GmNAC* proteins.

Expression of *GmNAC-D*

Previous studies have reported that tissue-specific expression of NAC transcription factors (TFs) plays a critical role in plant growth and development (Li et al., 2018). Root development, in particular, is strongly correlated with mechanisms of stress tolerance, and organ-specific gene expression profiling provides valuable information for identifying candidate genes with potential applications in improving drought resistance, especially when exploited in transgenic crop development systems (Amin et al., 2024). To support this evidence, expression pattern analysis of *GmNAC-D* was carried out across several soybean tissues. Repeated PCR-based expression assays revealed that *GmNAC-D* exhibited the highest expression in stems, followed in descending order by flowers, pods, roots, and leaves (Figure 4B). Interestingly, this expression profile differs from that of *GmNAC02*, a closely related homolog with high sequence similarity to *GmNAC-D*, which was previously reported to show the highest expression in roots (Tran et al., 2009). This indicates possible functional diversification among soybean NAC transcription factors, supporting earlier findings that NAC TFs display tissue- and stress-dependent variation in expression (Li et al., 2018; Amin et al., 2024).

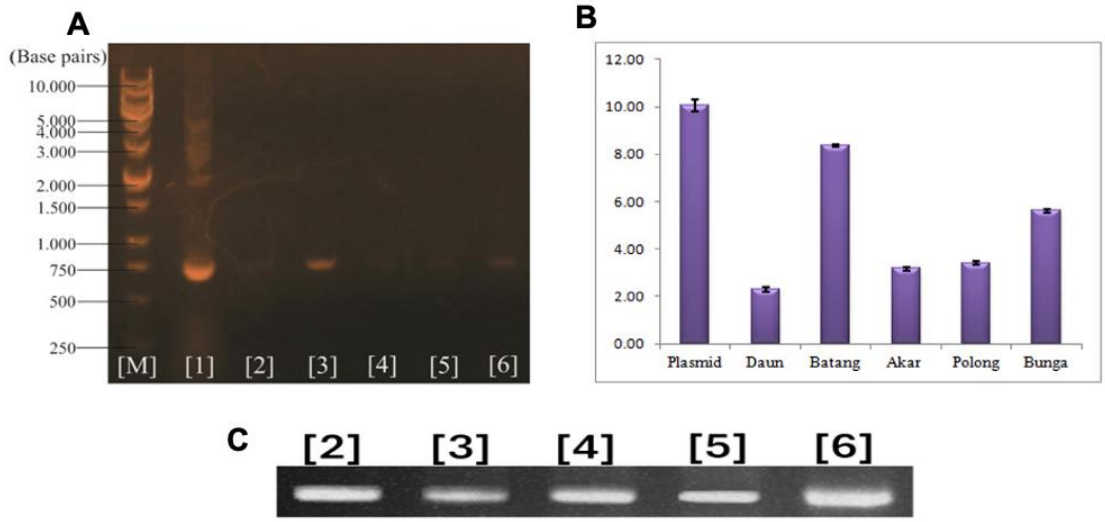

Figure 4. Expression of GmNAC-D in various plant tissues. Plasmid: *GmNAC-D* (1), leaf (2), stem (3), root (4), pod (5), and flower (6). (A) 10 µl aliquot of the PCR product was subjected to electrophoresis on a 1% agarose gel. (B) The PCR gel electrophoresis signal was analyzed with ImageJ software from digital images of the gel. (C) *Actin* gene as a control

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The differential expression profiles of *GmNAC* genes indicate that their products are involved in diverse functional roles during plant growth and development. According to Li et al. (2018), this differential expression pattern highlights the likelihood of sub-functionalization following gene duplication events in soybean. Genome duplication has provided leguminous crops with an expanded repertoire of stress-related genes, enabling functional diversification and fine-tuned responses to environmental challenges. In this context, GmNAC-D may have evolved to regulate stress responses in tissues where hydraulic conductivity and reproductive integrity are crucial. Stem-specific expression of NAC TFs has been linked to enhanced water transport efficiency, xylem differentiation, and lignin biosynthesis under drought conditions (Bian et al., 2020). In addition, flower expression suggests a role in safeguarding reproductive organs during water deficit, which is critical since drought-induced yield losses are most pronounced when stress coincides with flowering (Yang et al., 2019). The stem-dominant expression of GmNAC-D suggests its involvement in regulating vascular tissue development and secondary cell wall deposition. Such functions are consistent with reports of NAC TFs like *VND* and *NST* genes, which regulate xylem differentiation and secondary wall thickening (Li et al., 2021). By reinforcing stem vascular tissues, GmNAC-D may help mitigate the detrimental effects of drought on long-distance water transport. Furthermore, flowers exhibited the second-highest expression of GmNAC-D. This observation aligns with evidence that NAC TFs contribute to the maintenance of reproductive structures under stress by modulating hormonal and sugar signaling pathways (Dong et al., 2019). Thus, GmNAC-D may contribute to soybean resilience by reinforcing water transport pathways and protecting reproductive development.

More detailed functional characterization of *GmNAC-D* is required, particularly in light of the preliminary findings from this study, which suggest its potential involvement in abiotic stress responses. These results imply that *GmNAC-D* could serve as a promising candidate gene for genetic manipulation aimed at enhancing tolerance to environmental stresses. The integration of molecular, bioinformatic, and functional genomics approaches will be crucial to fully elucidate the role of *GmNAC-D*. Such efforts not only advance fundamental understanding of NAC transcription factors but also hold promise for developing climate-resilient crops through targeted genetic improvement.

Overexpression studies of NAC genes in Arabidopsis, rice, and wheat have demonstrated substantial improvements in stress tolerance (Ermawati et al., 2024; Khan et al., 2019). Similarly, CRISPR/Cas9-mediated editing has been successfully applied to dissect the role of NACs in stress signaling (Li et al., 2023). Introducing or modifying GmNAC-D in elite soybean varieties could therefore represent a promising strategy to improve drought resilience. Furthermore, the stem- and flower-specific expression of GmNAC-D may help minimize pleiotropic effects often associated with constitutive overexpression of stress-responsive genes. By targeting tissues most vulnerable to drought-induced damage, GmNAC-D could enhance stress tolerance while maintaining overall plant growth and yield stability.

CONCLUSIONS

This study successfully isolated the *GmNAC-D* gene from drought-tolerant soybean (*Glycine max*) var. Dering-1. The gene comprises a 762 bp open reading frame (ORF) encoding 253 amino acids, with strong sequence homology to *GmNAC02*, a stress-responsive NAC transcription factor. Phylogenetic analysis further confirmed its close relationship with members of the stress-responsive gene cluster, while bioinformatics analysis revealed conserved features typical of NAC proteins, including a nuclear localization signal and the presence of a characteristic NAC domain. Expression profiling indicated that *GmNAC-D* is expressed in multiple tissues, with the highest expression detected in the stem. However, overall, the findings from this preliminary study are not yet sufficient to confirm the direct involvement of *GmNAC-D* in drought tolerance. Further detailed functional analyses are required to elucidate the precise regulatory role of the *GmNAC-D* protein in plant drought stress responses.

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