

Article

The Gshdz4-GsNAC019-GsEXPA8 Multi-Component Module Enhances Alkaline Stress Tolerance in *Lupinus angustifolius*

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Abstract

Lupinus angustifolius is an important ornamental plant; however, its poor tolerance to alkaline soils limits its cultivation and production. Based on the alkaline-tolerance-related *Gshdz4-GsNAC019-GsEXPA8* regulatory module previously screened and identified in soybean, we used *Agrobacterium rhizogenes*-mediated transformation to overexpress in lupine roots the combinations *Gshdz4-GsNAC019-GsEXPA8* (HNE), *Gshdz4-GsNAC019* (HN), and *GsNAC019-GsEXPA8* (NE) to investigate their effects on root development and alkaline tolerance. RT-PCR confirmed the successful generation of all overexpression lines. Under 100 mM NaHCO₃ stress, all overexpression lines exhibited less wilting and longer survival than the wild type (WT), with the HNE line showing the best phenotype. Physiological measurements showed that the overexpression lines had significantly higher proline content, antioxidant enzyme (SOD, CAT, POD) activities, and root activity, as well as lower malondialdehyde content. DAB and NBT staining of leaves indicated reduced accumulation of O₂^{•−} and H₂O₂, suggesting enhanced antioxidant capacity. Root architecture analysis revealed that root length, surface area, volume, tip number, and fork number were significantly increased in HNE, HN, and NE lines compared with WT, with the most pronounced effect observed in HNE. Bioinformatics analysis and qPCR confirmed that *Gshdz4* binds to and activates the promoter of the endogenous *LaNAC072* (the lupine homolog of *GsNAC019*), while *GsNAC019* binds to and activates the promoter of the endogenous *LaEXPA8* (the lupine homolog of *GsEXPA8*), thereby triggering the endogenous alkaline tolerance regulatory mechanism. Furthermore, the overexpression combinations significantly upregulated the expression of alkaline stress-responsive genes, including *LaSOS1*, *LaNHX6*, *LaP5CS*, *LaMYB39*, and *LaDnaJ1*. This study provides theoretical support for molecular breeding of alkaline-tolerant lupine.

Keywords: *Gshdz4*; *GsNAC019*; *GsEXPA8*; multi-combination overexpression; *Lupinus angustifolius*; alkaline tolerance



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1. Introduction

Soil salinization and alkalization affect agricultural productivity and soil ecological health, posing a serious challenge to global agriculture [1]. Exacerbated by climate change, irrational irrigation practices, and over-extraction of groundwater, it has become a major constraint to the sustainable development of global agriculture [2]. Such saline-alkali

damage is particularly severe in arid and semi-arid regions, where insufficient rainfall leads to continuous accumulation of soil salts. Worldwide, soil salinity results in the loss of 2000 hectares of farmland every day in more than 75 countries, causing annual agricultural economic losses of 27 billion US dollars. It is projected that one-third of global irrigated farmland will lose its agricultural productivity by 2100, seriously threatening global food security alongside population growth [2]. Saline-alkali stress inhibits plant growth and is mainly divided into salt stress and alkali stress [3]. Alkali stress, predominantly induced by NaHCO_3 and Na_2CO_3 , causes more severe damage than salt stress. Besides the universal Na^+ toxicity shared by both stresses, alkali stress also raises soil pH dramatically. A high pH environment severely disrupts intracellular pH homeostasis, impairs cell membrane integrity, reduces root activity, and inhibits leaf photosynthesis [4–6]. Furthermore, alkali stress disturbs cellular ion and osmotic homeostasis [7,8]. A large number of studies have demonstrated that alkali stress triggers a severe imbalance in nutrient ions, weakens the osmotic adjustment ability, suppresses antioxidant systems, and eventually causes pronounced growth inhibition [7–10]. Nevertheless, *Lupinus angustifolius* shows poor adaptability to alkaline environments and is highly susceptible to NaHCO_3 -induced alkaline stress at pH 8.5. Transcriptomic analysis has verified that alkaline stress with high bicarbonate concentration strongly restricts plant growth and nutrient uptake, which acts as the core factor limiting the cultivation and survival of *Lupinus angustifolius* in vast calcareous and alkaline soils [11]. Therefore, improving the alkaline tolerance of lupine to overcome cultivation bottlenecks can fully realize its landscape and ecological values, and this research has important theoretical significance and agricultural application prospects.

Among all cultivated species of the genus *Lupinus*, *Lupinus angustifolius* has the largest planting area across the globe [12]. As the cultivar with the longest cultivation history and the strongest adaptability, it is also the only cultivated lupine that can thrive in northern high-latitude regions. This crop is widely grown in Northern and Eastern Europe (including Germany, the Netherlands, Poland, and other countries), the United States, New Zealand, and Belarus [12]. It has multiple uses and has been traditionally planted as green manure and forage [12]. The Royal Horticultural Society (RHS), one of the world's most authoritative horticultural institutions, includes *Lupinus angustifolius* in its plant database and provides guidance on its cultivation and care: <https://www.rhs.org.uk/plants/24038/wd/details> (accessed on 10 June 2026). Australia ranks first worldwide in the planting and export of *Lupinus angustifolius*, with outstanding achievements in research on its genetic diversity and leading progress in variety breeding [13,14]. In 2019, the planting area of *Lupinus angustifolius* in the Russian Federation reached 78,971 hectares, making Russia one of the major producers of this crop worldwide [12]. At present, research on the hybrid breeding of lupine remains blank in China. Saline-alkali land in China accounts for approximately 10.1% of the global total. With the reduction in arable land and population growth, the country is confronted with mounting pressure on land resources [15]. The Songnen Plain of China is recognized as one of the three major concentrated areas of soda saline-alkali land across the world [16]. *Lupinus angustifolius* is highly sensitive to NaHCO_3 -induced alkaline stress. At soil pH ≥ 8.5 , its growth and nutrient uptake are severely inhibited, making it difficult to survive in calcareous soils and saline-alkali land [11]. Considering the great differences in application orientation of *Lupinus angustifolius* at home and abroad, as well as its weak tolerance to salt and alkali, improving its alkaline tolerance via molecular module technology can not only promote the industrial development of lupine in China, but also provide technical references for salt-alkali tolerance breeding of other leguminous crops. For the above reasons, *Lupinus angustifolius* was selected as the experimental material in this study.

The majority of field crops and horticultural plants suffer from severe damage under alkaline field conditions, generally manifested as suppressed growth, leaf chlorosis, and root damage [17]. Among major food crops, alkaline stress is one of the important abiotic stress factors limiting rice production, with its detrimental effects being particularly prominent at the seedling stage [18]. *Petunia*, a representative horticultural plant, also suffers severe damage under alkaline conditions at pH 8.3. Its plant biomass decreases by approximately 49–52%, and the total chlorophyll content declines by nearly 80% [19]. As a typical calcifuge plant, rhododendron fails to grow normally in calcareous alkaline soils. High soil pH markedly increases the incidence of leaf malformation and plant mortality [20]. Meanwhile, *Hordeum jubatum*, an ornamental grass, suffers the most severe inhibition in growth and photosynthetic performance under alkaline stress. The damage caused by alkalinity is even greater than that from salt stress and drought stress [21]. When the growing medium pH rises above 7.0, or alkaline irrigation water is used, the high pH environment significantly inhibits plant nutrient assimilation, reduces chlorophyll content, and leads to decreased photosynthetic rate and reduced biomass accumulation [22]. Studies have shown that iron deficiency under alkaline conditions is a key factor causing growth restriction, developmental disorders, and leaf chlorosis [23]. When soil pH exceeds 7.0, iron tends to form insoluble ferric hydroxide precipitates, significantly reducing its availability and thereby inducing iron-deficiency chlorosis in plants [24].

Lupine (*Lupinus angustifolius*) is an economically important legume crop with high ornamental value and high plant protein content. It is widely distributed around the world and holds potential for development and utilization [25–27]. It mainly grows in acidic or neutral soils (pH 5.0–7.0) [25] and has poor alkali tolerance. However, existing research on lupine has largely focused on abiotic stresses such as water deficit and high temperature, as well as on population genetics and evolution. For example, studies have examined the effects of water stress on protein, oil, total soluble sugar, and α -galactoside component contents in lupine seeds [28]; the impacts of high-temperature stress on photosynthetic physiology, yield, and grain quality in *Lupinus angustifolius* [29]; and the regulatory effects of gene flow on flowering and growth traits via assisted gene flow experiments in north–south populations, leading to the identification of key genetic loci [30]. Overall, research on the physiological mechanisms, germplasm improvement, and development of alkali-tolerant germplasm in lupine remains very limited, hindering its introduction and large-scale cultivation in saline-alkaline soil regions. Wild soybean (*Glycine soja*), the ancestor of cultivated soybean, possesses strong alkali tolerance and remarkable environmental adaptability, making it a valuable wild legume germplasm resource [31,32]. Wild soybean is rich in alkali-tolerance genes, which can be explored to uncover signal transduction pathways. Through genetic engineering, alkali-tolerance genes and regulatory modules can be heterologously transformed into lupine to enhance its alkali tolerance and thereby expand its cultivation range.

The homeodomain–leucine zipper (HD-Zip) family is a plant-specific transcription factor family widely involved in plant growth, development, and abiotic stress responses [33]. This family is divided into four subfamilies, all of which contain a conserved DNA-binding homeodomain (HD) and a leucine zipper (LZ) domain responsible for protein dimerization [34]. Studies have shown that the HD-Zip II member *ZaHB15* induces flowering in *Zanthoxylum armatum* and negatively regulates plant growth and drought tolerance [35]; *JrHDZ28* in walnut participates in salt and drought stress responses and improves salt and drought tolerance [36]; and *ArHDZ22* in *Anoectochilus roxburghii* negatively regulates plant growth and salt tolerance, with overexpression significantly reducing plant height and salt tolerance [37]. Previous research has demonstrated that *Gshdz4*, a member of the HD-Zip I subfamily, is induced by alkali stress (NaHCO_3) in wild soybean, is expressed in both

leaves and roots, and its overexpression in *Arabidopsis* enhances tolerance to NaHCO_3 [38]. The NAC family (NAM, ATAF1/2, CUC1/2) is another plant-specific regulatory protein family widely distributed in plants, with nearly 100 members reported in most species studied to date [39]. These proteins are predominantly localized in the nucleus [40–42], although some are found outside the nucleus [43,44]. Expansins, discovered two decades ago, are cell wall proteins that mediate acid-induced cell wall extension and catalyze cell wall loosening without degrading cell wall polymers [45]. Plant expansins are classified into two major families based on sequence-based phylogenetic analysis: EXPA (α -expansin) and EXPB (β -expansin), with EXPA recognized as the mediator of acid-induced cell wall loosening [45].

Previous studies have verified a hierarchical regulatory relationship among three key genes, namely *Gshdz4*, *GsNAC019*, and *GsEXPA8*, which jointly mediate plant alkali stress tolerance. The transcription factor *Gshdz4* can specifically bind to the cis-acting elements CAATTA and CAATAA in the promoters of *AtNAC019/GsNAC019*, and heterologous overexpression of *Gshdz4* significantly upregulates *AtNAC019* expression in transgenic *Arabidopsis*, confirming that *AtNAC019/GsNAC019* acts as a downstream target gene of *Gshdz4* [46]. As a downstream transcription factor, *GsNAC019* specifically recognizes and binds to the CGTA sequence in the promoters of its target genes. The presence of typical CGTA binding sites in the *GsEXPA8* promoter further indicates that *GsEXPA8* is a downstream functional gene of *GsNAC019* [46] (Figure 1a). Under alkali stress, *Gshdz4* acts as an upstream regulatory gene to activate the transcription of *GsNAC019*, and the activated *GsNAC019* subsequently induces the expression of *GsEXPA8*. This regulatory pattern forms a unique three-tiered “nucleus-nucleus-membrane” signal transduction cascade of the *Gshdz4-GsNAC019-GsEXPA8* module, which coordinates multiple stress response pathways and ultimately enhances alkali tolerance in soybean [47] (Figure 1a). Functional verification of individual genes further supports the alkali-tolerant function of this regulatory module in lupine. Heterologous transformation assays have demonstrated that root-specific heterologous expression of the HD-Zip family gene *GsHZ4* (i.e., *Gshdz4*) in lupine improves alkali resistance by increasing the activities of antioxidant enzymes (CAT, POD, and SOD) and reducing malondialdehyde accumulation, accompanied by the upregulated expression of stress-responsive genes [48]. Consistent with this finding, heterologous expression of *GsEXPA8* in lupine roots also confers enhanced alkali tolerance. The improved stress adaptability is attributed to promoted root growth, enhanced antioxidant capacity and osmotic regulation ability, dynamic modulation of stress-related gene expression, and optimized rhizosphere microbial community structure [49].

To improve the alkali tolerance of lupine roots, this study employed an *Agrobacterium* rhizogenes (strain K599)-mediated transformation method [50] to perform multi-component transformations of *Gshdz4*, *GsNAC019*, and *GsEXPA8*. Specifically, we overexpressed the gene combinations *Gshdz4-GsNAC019-GsEXPA8* (HNE), *Gshdz4-GsNAC019* (HN), and *GsNAC019-GsEXPA8* (NE) in lupine roots. By systematically observing the growth phenotypes under alkali stress, measuring stress-related physiological indices, and determining the relative expression levels of alkali stress-responsive genes, we aimed to clarify the regulatory effects and functional differences in these multi-component overexpression constructs on alkali tolerance in lupine. This study goes beyond the limitations of single-gene functional studies by adopting a perspective of gene interaction and combinatorial regulation. It investigates the synergistic regulatory effects of different pairwise combinations of these three genes on lupine alkali tolerance and identifies the optimal gene combination pattern. The findings provide a strategy for improving lupine alkali tolerance using wild soybean as a related genetic resource, lay the foundation for lupine stress tol-

erance research and molecular breeding, and offer theoretical and technical support for expanding the cultivation range of lupine.

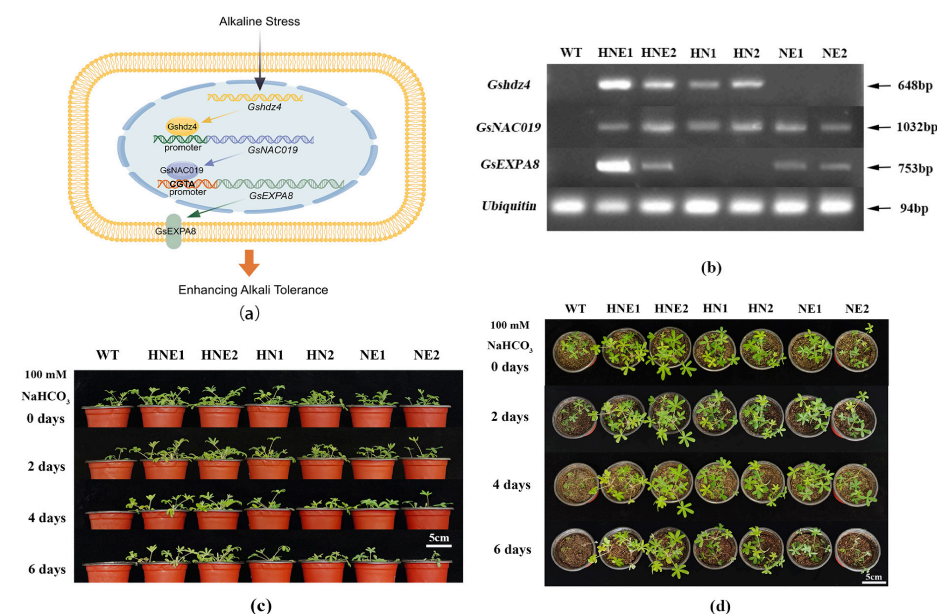


Figure 1. Regulatory mechanism of the target genes, their expression patterns in lupine, and phenotypic characterization. (a) Hierarchical regulatory model of the *Glycine soja* *Gshdz4*-*GsNAC019*-*GsEXPA8* module under alkali stress, showing protein localization in the “nucleus-nucleus-cell membrane” cascade. The diagram was created using BioGDP (<https://BioGDP.com>). (b) RT-PCR analysis of *Gshdz4*, *GsNAC019*, and *GsEXPA8* expression in WT, HNE, HN, and NE plants. (c,d) Phenotypes of 60-day-old wild-type (WT) lupine and overexpression lines (HNE, HN, NE) treated with 100 mM NaHCO₃ for 0, 2, 4, and 6 days. Scale bars = 5 cm.

2. Materials and Methods

2.1. Plant Growth Conditions

This experiment was carried out at the Horticultural Experiment Center of Northeast Agricultural University (44°45′28″ N, 126°43′16″ E). *Lupinus angustifolius* seeds were purchased from Huayouxixu commercial seed supplier (Suqian, China) [48]. To generate overexpression constructs, the coding sequence (CDS) of *Gshdz4* was cloned into the pCAMBIA1302 vector using the NcoI restriction site [47]; the CDS of *GsNAC019* was inserted into the pBI121 vector using BamHI and EcoRI sites [47]; and the full-length cDNA of *GsEXPA8* was cloned into the pBI121 vector using BamHI and EcoRI sites [49]. All target genes were driven by the CaMV 35S constitutive promoter in the recombinant vectors described above. The recombinant vectors were amplified in *Escherichia coli* DH5α cells (DL1001; Weidi Biology, Shanghai, China). Purified recombinant expression vectors were introduced into *Agrobacterium rhizogenes* strain K599 (AC1080; Weidi Biology, Shanghai, China) by the freeze–thaw method. The three engineered strains were individually cultured in LB liquid medium containing 50 mg/L kanamycin at 28 °C with shaking at 200 rpm until the late-logarithmic phase (OD₆₀₀ of approximately 1.0). The bacterial cells were collected by centrifugation (5000 × g, 10 min, 4 °C), resuspended in liquid LB medium, and adjusted to an OD₆₀₀ of 1.0. According to the three different combinations (*Gshdz4*-*GsNAC019*-*GsEXPA8*, *Gshdz4*-*GsNAC019*, and *GsNAC019*-*GsEXPA8*), the corresponding bacterial suspensions were mixed in equal volume ratios (1:1:1 or 1:1). Then, 100 μL of each mixed suspension was evenly spread onto solid LB medium containing 50 mg/L kanamycin and incubated in an inverted position at 28 °C for 48 h to form a bacterial lawn. Transgenic lupine plants were generated using a conventional *Agrobacterium rhizogenes*-mediated root transformation system [49,50]. Hair roots were induced by inoculating

wounds on cotyledons of sterile seedlings with the bacterial lawn of *Agrobacterium rhizogenes* K599 [50]. For infection, a sterile inoculation loop was used to scrape a fresh bacterial lawn, which was then evenly smeared onto the wounds of sterile lupine seedlings. This method has been reported to achieve a high hairy root induction rate in soybean and has also been shown to be effective for genetic transformation of *Lupinus angustifolius* [49,50].

Transgenic lupine seedlings were transplanted into pots filled with a growth substrate (nutrient soil–vermiculite = 1:1). The plants were grown under controlled conditions at 22–25 °C with a 16 h light/8 h dark photoperiod until the third pair of palmate compound leaves had fully developed [49]. The resulting plants with root-specific overexpression of different gene combinations were designated as *Gshdz4-GsNAC019-GsEXPA8* (abbreviated HNE), *Gshdz4-GsNAC019* (HN), and *GsNAC019-GsEXPA8* (NE).

2.2. RT-PCR Identification of Transgenic Roots

Roots of wild-type (WT) and HNE, HN, and NE plants were collected, immediately frozen in liquid nitrogen, and stored at −80 °C for total RNA extraction. Total RNA was extracted using the TransZol Up reagent kit (ET111-01-V2; TransGen Biotech, Beijing, China). First-strand cDNA was synthesized from 1 µg of total RNA using the SPARKSCRIPT II RT Plus kit (with gDNA Eraser; AG0304-B; Sparkjade Biotechnology, Jinan, China). PCR was performed using the cDNA as template, and the products were analyzed by electrophoresis to verify the integration of *Gshdz4*, *GsNAC019*, and *GsEXPA8* into the lupine plants. The primers used for RT-PCR are listed in Table 1.

Table 1. Primers for RT-PCR analysis.

Primer Name	Sequence (5′ → 3′)
<i>Gshdz4</i> -F	ATGAATCATCGACCACCTTTCC
<i>Gshdz4</i> -R	CAGATTAAATCCATTCCATGCCG
<i>GsNAC019</i> -F	ATGGGAGTTCCAGAGAAAGACCC
<i>GsNAC019</i> -R	TCAATTTCTGAACCCGAACCCGA
<i>GsEXPA8</i> -F	ATGCCCATTTGTAGCAACC
<i>GsEXPA8</i> -R	CTAGAACTGTGCCCTTCA
<i>Ubiquitin</i> -F	GGCAAGACCATCACTCTCGA
<i>Ubiquitin</i> -R	ACCTCAAGGGTGATGGTCT

2.3. Plant Phenotyping Under Stress Treatment

Healthy 45-day-old WT and root-transgenic lupine plants were photographed to record initial phenotypes, which served as the day 0 baseline before NaHCO₃ treatment. The plants were then irrigated with 0 mM or 100 mM NaHCO₃ solutions, and subsequent phenotypic changes were continuously observed and recorded.

For physiological measurements, healthy and uniformly grown plants at 45 days after growth were selected. Their roots were treated with water or 100 mM NaHCO₃ for 6 h [49]. This study evaluated the wild-type (WT) along with six independent transgenic lines (designated HNE1, HNE2, HN1, HN2, NE1, and NE2). For each treatment (0 or 100 mM NaHCO₃), three biological replicates were used per line. Each biological replicate consisted of equal amounts of root tissue pooled from 15 individual plants. The alkali-stressed (treated with 100 mM NaHCO₃) WT and overexpression plants constituted the experimental groups, while 0 mM NaHCO₃-treated (water-treated) WT and overexpression plants served as the controls. Roots were used as experimental materials for physiological assays. Proline (PRO) content, malondialdehyde (MDA) content, superoxide dismutase (SOD) activity, catalase (CAT) activity, peroxidase (POD) activity, and root activity were determined using commercial kits: PRO kit (No. G0111W; Geruisi Biotechnology, Suzhou, China), MDA kit (No. G0109W; Geruisi Biotechnology, Suzhou, China),

SOD kit (No. G0101W; Geruisi Biotechnology, Suzhou, China), CAT kit (No. G0105W; Geruisi Biotechnology, Suzhou, China), POD kit (No. BN0051-W96; Bainian Chuangxing Biotechnology, Chongqing, China), and root activity kit (No. G0124W; Geruisi Biotechnology, Suzhou, China). All measurements were performed according to the manufacturers' instructions [48,49].

2.4. Diaminobenzidine (DAB) Staining

DAB staining was used to visualize hydrogen peroxide (H_2O_2) levels in plant tissues. Healthy 45-day-old WT and overexpression plants were treated with water or 100 mM NaHCO_3 for 6 h [49], and then leaves were collected. DAB powder (Biotopped, Beijing, China) was prepared as follows: 0.1 g DAB powder was dissolved in 45 mL of distilled water (prepared in the dark) in a brown bottle. The pH was adjusted to 5.7, and the volume was brought to 50 mL with distilled water. Leaves from treated WT and overexpression plants were immersed in DAB staining solution and subjected to vacuum infiltration, followed by staining for 18 h. The stained leaves were placed in a beaker containing absolute ethanol, and the beaker was heated in boiling water. Absolute ethanol was replenished as needed to prevent drying. After complete decolorization, the leaves were removed and photographed [51]. The percentage of stained leaf area was calculated using ImageJ 1.54p (National Institutes of Health, Bethesda, MD, USA).

2.5. Nitroblue Tetrazolium (NBT) Staining

NBT staining was used to visualize superoxide anion (O_2^-) levels in plant tissues. Healthy 45-day-old WT and overexpression plants were treated with water or 100 mM NaHCO_3 for 6 h [49], and then the leaves were harvested. NBT powder (Biotopped, Beijing, China) was prepared by mixing 1.6 mL of NaH_2PO_4 buffer and 8.4 mL of Na_2HPO_4 buffer, adjusting the volume to 200 mL with distilled water (pH 7.5). Then, 0.1 g of NBT powder was dissolved in 50 mL of this buffer in a brown bottle (prepared in the dark). Leaves from treated WT and overexpression plants were immersed in the NBT solution, vacuum-infiltrated, and stained for 18 h in the dark. The stained leaves were decolorized with absolute ethanol as described for DAB staining, then photographed [51]. The percentage of stained leaf area was calculated using ImageJ 1.54p (National Institutes of Health, Bethesda, MD, USA).

2.6. Root Scanning

At 60 days after cultivation, WT and overexpression lupine plants exhibited similar above-ground growth. Plants at this non-stressed growth stage were sampled. Plant height was measured with a digital vernier caliper (DWKC-2013; Delixi Electric, Wenzhou, China) for statistical analysis, and whole-plant phenotypes were recorded with a digital camera (two pots per line, three plants per pot). To investigate the effects of multi-component overexpression on root growth, we performed root scanning on plants of the same age under non-stressed conditions. Roots were thoroughly cleaned and scanned using an LA-S root analyzer (Wanshen, Hangzhou, China). Total root length, surface area, root volume, number of root tips, and number of root forks were automatically calculated by the system [52].

2.7. Stress Treatment, RNA Extraction, and qPCR

For alkali stress treatment, lupine roots were treated with water or 100 mM NaHCO_3 for 0, 3, 6, or 12 h [49]. Root samples of WT, HNE, HN, and NE plants were collected, frozen in liquid nitrogen, and stored at -80°C for RNA extraction. Total RNA was extracted using the TransZol Up kit (ET111-01-V2; TransGen Biotech, Beijing, China). First-strand cDNA was synthesized from 1 μg of total RNA using the SPARKSCRIPT II RT Plus kit (with

gDNA Eraser; AG0304-B; Sparkjade Biotechnology, Jinan, China). Specific primers for the endogenous lupine genes *LaNAC072* and *LaEXPA8*, as well as for alkali stress-responsive genes, were designed. Quantitative real-time PCR (qPCR) was performed on a CFX384 Real-Time System (Bio-Rad, Hercules, CA, USA) using 2× Universal SYBR Green qPCR Mix (AH0105-B; Sparkjade Biotechnology, Jinan, China) [47]. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method [53], with the lupine Ubiquitin gene as an internal control. All qPCR primers are listed in Table 2.

Table 2. Primers for qPCR analysis of marker genes.

Primer Name	Sequence (5' → 3')
qLaNAC072-F	CAGTTCTTCACGCTTCCACG
qLaNAC072-R	CACCGAGTCTAAACCGGACG
qLaEXPA8-F	GTGACTATGGTGGAGGATGGC
qLaEXPA8-R	CATAACCACAAGCTCCTCCCAT
qLaP5CS-F	TGTTCTAGACGGCTTCAGGC
qLaP5CS-R	AACCCAGCCTAGCAACCAAG
qLaSOS1-F	TCTTCACTCTGGCAGGTTCC
qLaSOS1-R	CTGTGGGCACGAAGAAATGC
qLaNHX6-F	AGGAGCTTGGCACTGATGTC
qLaNHX6-R	CAACACCTGCCGACATTGAC
qLaMYB39-F	TGTCATGGGAAACAAGTGGGC
qLaMYB39-R	CGGGGTCAATCCCCATACG
qLaDnaJ1-F	GGAAATCCATTTGGTGGCGG
qLaDnaJ1-R	CCAAGCTGACCTTGAGAGGG
qUbiquitin-F	GGCAAGACCATCACTCTCGA
qUbiquitin-R	ACCTCAAGGGTGATGGTCT

2.8. Promoter Analysis of Endogenous *LaNAC072* and *LaEXPA8*

Using BLAST on the NCBI website (<https://www.ncbi.nlm.nih.gov/>, accessed on 10 May 2026), we identified the lupine homologs of *GsNAC019* and *GsEXPA8*, designated *LaNAC072* and *LaEXPA8*, respectively. The 2000 bp promoter sequence upstream of the transcription start site (TSS) of *LaNAC072* and the 2000 bp reverse-complement promoter sequence downstream of the TSS of *LaEXPA8* (located on the negative strand) were downloaded from NCBI. Putative cis-regulatory motifs were identified using MEGA 12.0.14 (Mega Software, LLC, North Salt Lake, UT, USA).

2.9. Data Visualization and Statistical Analysis

Data visualization was performed using GraphPad Prism 10.6.1 (GraphPad Software, San Diego, CA, USA), and schematic diagrams were created using BioGDP (<https://BioGDP.com>). All data are presented as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 10.6.1. One-way ANOVA was used for plant height and root scanning data. Two-way ANOVA followed by Tukey's post hoc test was used for physiological indices, stained area percentages, and qPCR data. Different lowercase letters indicate significant differences between groups ($p < 0.05$). Asterisks denote levels of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3.1. Identification of Positive Transgenic Lupine Roots

RT-PCR analysis was performed to detect the expression of *Gshdz4*, *GsNAC019*, and *GsEXPA8* in wild-type (WT) and transgenic lupine lines (HNE, HN, NE) (Figure 1b). The internal control Ubiquitin band was present in all samples. The HNE line showed bands corresponding to *Gshdz4*, *GsNAC019*, and *GsEXPA8*; the HN line showed bands for *Gshdz4*

and *GsNAC019*; and the NE line showed bands for *GsNAC019* and *GsEXPA8* (Figure 1b). These results confirmed that the target genes were successfully transformed into lupine plants and that root-specific overexpression lines were successfully established.

WT and overexpression plants (45-day-old) were treated with 100 mM NaHCO_3 . Two days after treatment, the WT plants were the first to show wilting symptoms (Figure 1c,d). After four days, WT plants were completely wilted, at which time the NE line began to show wilting (Figure 1c,d). After six days, WT plants had completely died, while NE plants exhibited pronounced wilting, and HNE and HN plants displayed only mild wilting (Figure 1c,d). Compared with WT, the HNE, HN, and NE lines all showed enhanced tolerance to alkali stress, with the HNE line exhibiting the strongest tolerance (Figure 1c,d). Taken together, these phenotypic observations indicate that multi-component overexpression of *Gshdz4*, *GsNAC019*, and *GsEXPA8* improves the alkali tolerance of lupine plants, and among the tested combinations, the HNE line conferred the highest level of tolerance.

3.2. Root-Specific Multi-Component Overexpression of *Gshdz4*/*GsNAC019*/*GsEXPA8* Enhances Alkali Tolerance in Lupine

The proline (PRO) content, malondialdehyde (MDA) content, antioxidant enzyme activities, and root activity of WT plants and overexpression plants under water (untreated) and NaHCO_3 treatment reflect differences in alkali tolerance (Figure 2).

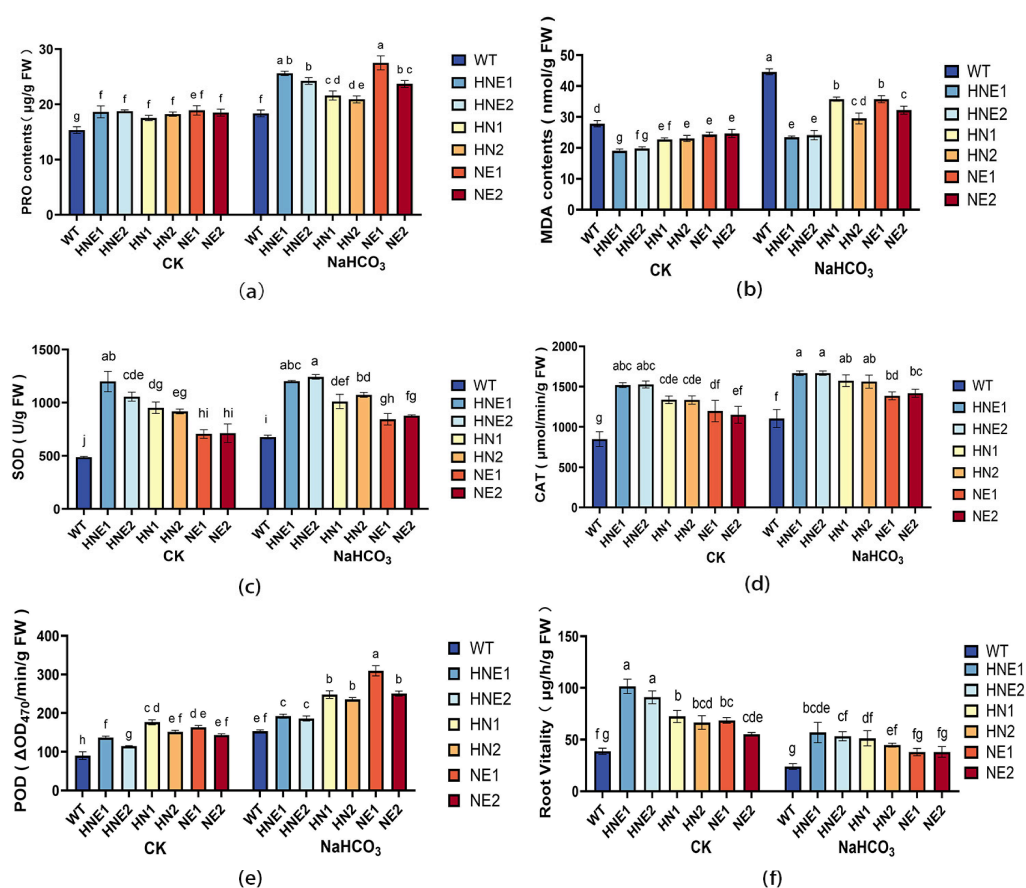


Figure 2. Multi-component overexpression of the *Gshdz4*-*GsNAC019*-*GsEXPA8* module in lupine roots enhances lupine alkaline tolerance. (a) Proline (PRO) content; (b) malondialdehyde (MDA) content; (c) superoxide dismutase (SOD) activity; (d) catalase (CAT) activity; (e) peroxidase (POD) activity; (f) root activity. Data are presented as mean $\pm$ SD ($n = 3$ biological replicates). Statistical significance was determined by two-way ANOVA. Different lowercase letters denote significant differences at $p < 0.05$ among treatments.

Compared with the untreated control group, the proline content and peroxidase (POD) activity of multi-component overexpression lines increased significantly under NaHCO_3 stress (Figure 2a,e). Meanwhile, under NaHCO_3 stress, the activities of superoxide dismutase (SOD) and catalase (CAT) in multi-component overexpression lines were higher than those in the untreated control group (Figure 2c,d). Under both untreated and NaHCO_3 -treated conditions, all overexpression lines exhibited significantly higher PRO content, SOD activity, catalase (CAT) activity, and peroxidase (POD) activity than WT (Figure 2a,c,d,e), whereas MDA content was significantly lower in the overexpression lines than WT (Figure 2b). For root activity, all overexpression lines had significantly higher values than WT under normal conditions. Under NaHCO_3 stress, the HNE and HN lines showed significantly higher root activity than WT, and the NE line also possessed higher root activity than WT (Figure 2f). These results demonstrate that multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* in lupine roots significantly increases PRO accumulation, antioxidant enzyme activities, and root activity under both normal and alkali-stress conditions, while reducing lipid peroxidation levels, thereby enhancing stress tolerance.

Specifically, under untreated conditions, all overexpression lines had high proline (PRO) levels, which were significantly higher than those of WT (Figure 2a). The HNE line had the lowest malondialdehyde (MDA) content, while the HN and NE lines showed similar MDA levels, all of which were significantly lower than WT (Figure 2b). Under NaHCO_3 treatment, the NE line showed the highest PRO content (approximately 1.3–1.5-fold that of WT) and the highest POD activity (approximately 1.6–2-fold that of WT). The HNE line consistently exhibited the lowest MDA content under both untreated and NaHCO_3 -treated conditions (approximately 50% reduction compared with WT under NaHCO_3 treatment), and the highest SOD activity (approximately 1.8-fold that of WT under NaHCO_3), CAT activity (approximately 1.5-fold that of WT under NaHCO_3), and root activity (approximately 2.2-fold that of WT under NaHCO_3). Alkali stress caused a significant reduction in root activity. However, all overexpression lines still exhibited higher root activity than WT under alkaline conditions, and the HNE and HN lines showed significantly higher values than WT. Taken together, these results confirm that multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* effectively enhances alkali tolerance in lupine, with the HNE line exhibiting the best overall performance (Figure 2a–f).

3.3. Root-Specific Multi-Component Overexpression of *Gshdz4/GsNAC019/GsEXPA8* Improves Antioxidant Capacity in Lupine Leaves Under Alkali Stress

DAB and NBT staining revealed that, compared with the untreated groups, the stained leaf area increased in plants treated with NaHCO_3 (Figure 3). Under both untreated and NaHCO_3 -treated conditions, the DAB-stained area in leaves of HNE, HN, and NE lines was significantly smaller than that in WT leaves, with the HNE line showing the smallest stained area (Figure 3a,b). Under NaHCO_3 treatment, the NBT-stained area in leaves of HNE, HN, and NE lines was significantly smaller than that in WT leaves, and the HNE line again exhibited the smallest stained area (Figure 3c,d). Collectively, these results indicate that the overexpression lines HNE, HN, and NE significantly reduce the accumulation of O_2^- and H_2O_2 in leaves after alkali stress, with the HNE line showing the lowest levels of both reactive oxygen species. Therefore, multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* in lupine roots enhances root alkali tolerance, which in turn effectively reduces the accumulation of reactive oxygen species (ROS) in lupine leaves under alkali stress.

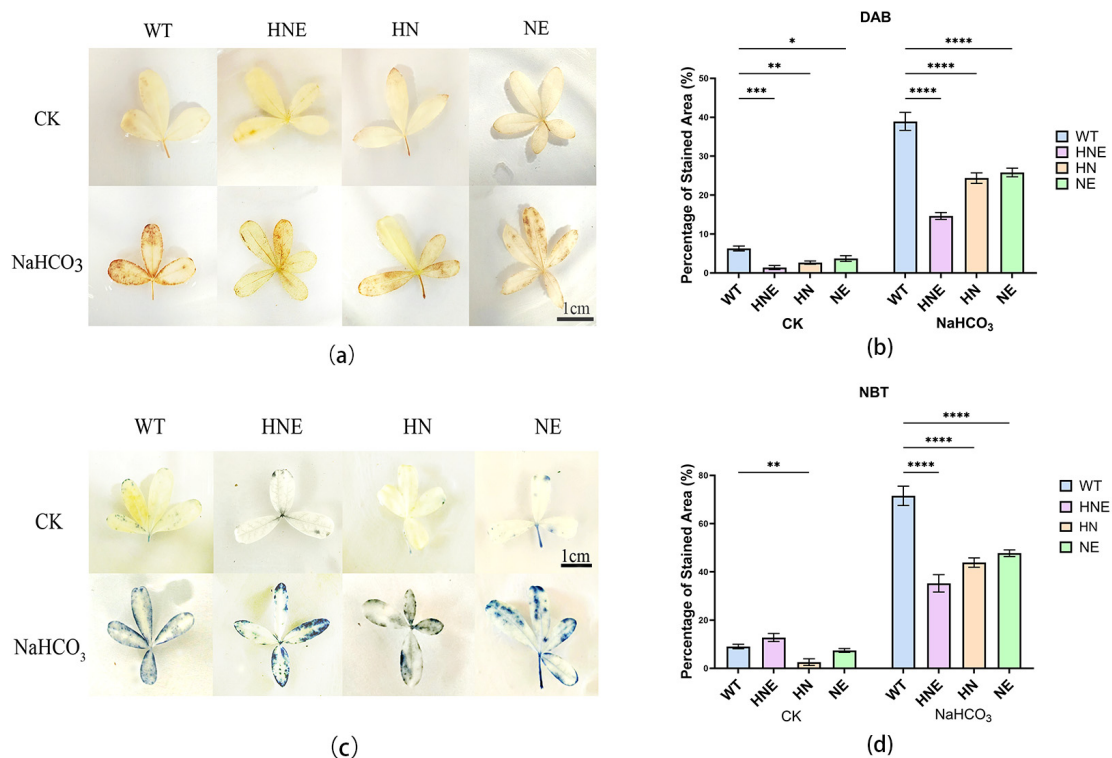


Figure 3. Multi-component overexpression of the *Gshdz4-GsNAC019-GsEXPA8* module in lupine roots improves shoot antioxidant capacity. (a) DAB staining of leaves from WT, HNE, HN, and NE lines treated with H₂O (CK) or 100 mM NaHCO₃; (b) quantitative analysis of DAB-stained area percentage; (c) NBT staining of leaves from WT, HNE, HN, and NE lines treated with H₂O (CK) or 100 mM NaHCO₃; (d) quantitative analysis of NBT-stained area percentage. Data in (b,d) are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined by two-way ANOVA. Asterisks indicate significant differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Root-Specific Multi-Component Overexpression of *Gshdz4/GsNAC019/GsEXPA8* Promotes Root Growth in Lupine

When WT and overexpression plants were grown for 60 days, no significant difference in plant height was observed (Figure 4h), and the aerial parts showed similar growth patterns (Figure 4a). Root architecture analysis of 60-day-old plants revealed that the roots of HNE, HN, and NE lines were more robust than those of WT, with the HNE line exhibiting the best root growth (Figure 4b). Compared with WT, the HNE, HN, and NE lines showed significant increases in root length, surface area, root volume, number of root tips, and number of root forks (Figure 4c–g). These results demonstrate that overexpression of *Gshdz4-GsNAC019-GsEXPA8* (HNE), *Gshdz4-GsNAC019* (HN), and *GsNAC019-GsEXPA8* (NE) all significantly promote root growth in lupine plants, thereby enhancing nutrient uptake. Among these, overexpression of *Gshdz4-GsNAC019-GsEXPA8* (HNE) had the most pronounced effect on promoting root growth.

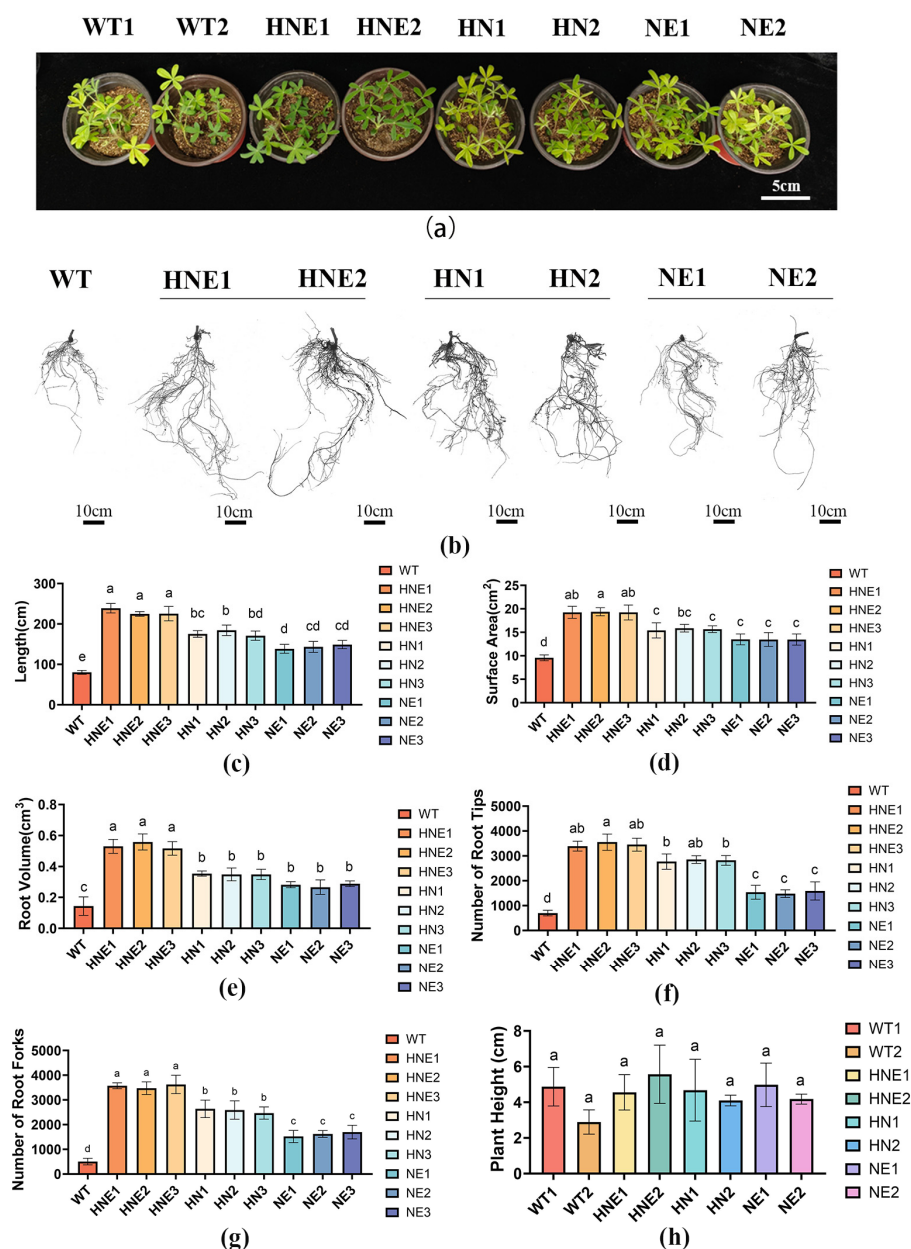


Figure 4. Multi-component overexpression of the *Gshdz4-GsNAC019-GsEXPA8* module in lupine roots promotes root growth. Wild-type (WT), HNE, HN, and NE lupine plants were grown for 60 days. (a) Whole-plant phenotypes, scale bar = 5 cm; (b) root phenotypes, scale bar = 10 cm; (c) root length; (d) root surface area; (e) root volume; (f) number of root tips; (g) number of root forks; (h) plant height. Data in (c–h) are presented as mean ± SD (n = 3). One-way ANOVA was performed, and different lowercase letters indicate significant differences between groups (p < 0.05).

3.5. *Gshdz4* and *GsNAC019* Bind to Promoters of Endogenous Lupine Genes to Activate the Endogenous Alkali Tolerance Regulatory Mechanism

Since the lupine homolog of *GsNAC019* is *LaNAC072*, and the lupine homolog of *GsEXPA8* is *LaEXPA8*, we analyzed the promoter sequences of *LaNAC072* and *LaEXPA8*. The analysis revealed the presence of the *Gshdz4*-binding motif CAATAA at positions −76 bp, −95 bp, and −753 bp in the *LaNAC072* promoter, and the *GsNAC019*-binding motif CGTA at positions −186 bp, −728 bp, and −811 bp in the *LaEXPA8* promoter (Figure 5a). Previous studies have demonstrated that *Gshdz4* directly binds the CAATAA/CAATTA motif via yeast one-hybrid and dual-luciferase assays [46], and that *GsNAC019* recognizes the CGTA motif [46]. Furthermore, under alkaline stress, *Gshdz4* directly activates the *GsNAC019*

promoter as validated by dual-luciferase assays and CUT&Tag-seq [47]. Based on these findings, we hypothesize that under alkali stress, Gshdz4 may bind to the promoter of the endogenous lupine gene *LaNAC072* and activate its expression, and that GsNAC019 may bind to the promoter of the endogenous lupine gene *LaEXPA8* and activate its expression.

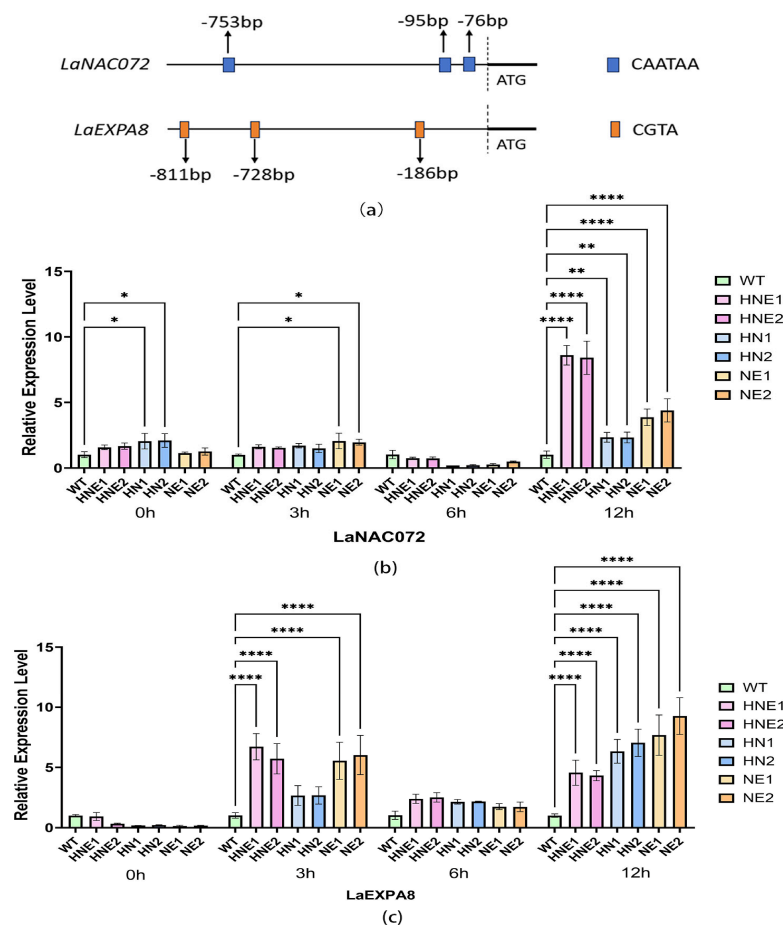


Figure 5. Promoter analysis and relative expression levels of the endogenous lupine genes *LaNAC072* and *LaEXPA8*. (a) Schematic diagrams of the promoter regions of *LaNAC072* and *LaEXPA8*: the upper diagram shows the positions of the CAATAA motif in the *LaNAC072* promoter; the lower diagram shows the positions of the CGTA motif in the *LaEXPA8* promoter. (b) Relative expression levels of *LaNAC072* in plants treated with 100 mM NaHCO₃ for 0, 3, 6, and 12 h. (c) Relative expression levels of *LaEXPA8* in plants treated with 100 mM NaHCO₃ for 0, 3, 6, and 12 h. Data in (b,c) are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined by two-way ANOVA. Asterisks indicate significant differences between groups: * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

To validate the above hypothesis, we determined the expression levels of the endogenous lupine genes *LaNAC072* and *LaEXPA8* by qPCR in WT and overexpression lines (HNE, HN, NE) treated with 100 mM NaHCO₃ for 0, 3, 6, and 12 h. The results showed that at 12 h of NaHCO₃ treatment, the expression levels of *LaNAC072* in all overexpression lines (HNE, HN, NE) were significantly higher than those in WT (Figure 5b). For *LaEXPA8*, expression levels in the overexpression lines were higher than in WT at 3, 6, and 12 h, and the differences became significant at 12 h for all overexpression lines (Figure 5c). In summary, the qPCR results are consistent with the hypothesis that, under long-term NaHCO₃-alkali stress, Gshdz4 may bind to the promoter of the endogenous target gene *LaNAC072* and activate its expression, while GsNAC019 may bind to the promoter of the endogenous target gene *LaEXPA8* and activate its expression, thereby potentially triggering the endogenous alkali tolerance regulatory mechanism and enhancing lupine tolerance to

alkali stress. Thus, exogenous genes and regulatory mechanisms can effectively modulate the expression levels of endogenous genes, thereby influencing stress-related traits.

To investigate the effects of multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* on the expression of endogenous alkali stress-responsive genes in lupine, we performed qPCR to measure the expression dynamics of these genes at different time points (Figure 6). Based on previous studies, we selected *LaSOS1*, *LaNHX6*, *LaP5CS*, *LaMYB39*, and *LaDnaJ1* for analysis, all of which are known to respond to alkaline salt stress and play important roles in plant adaptation to alkali stress.

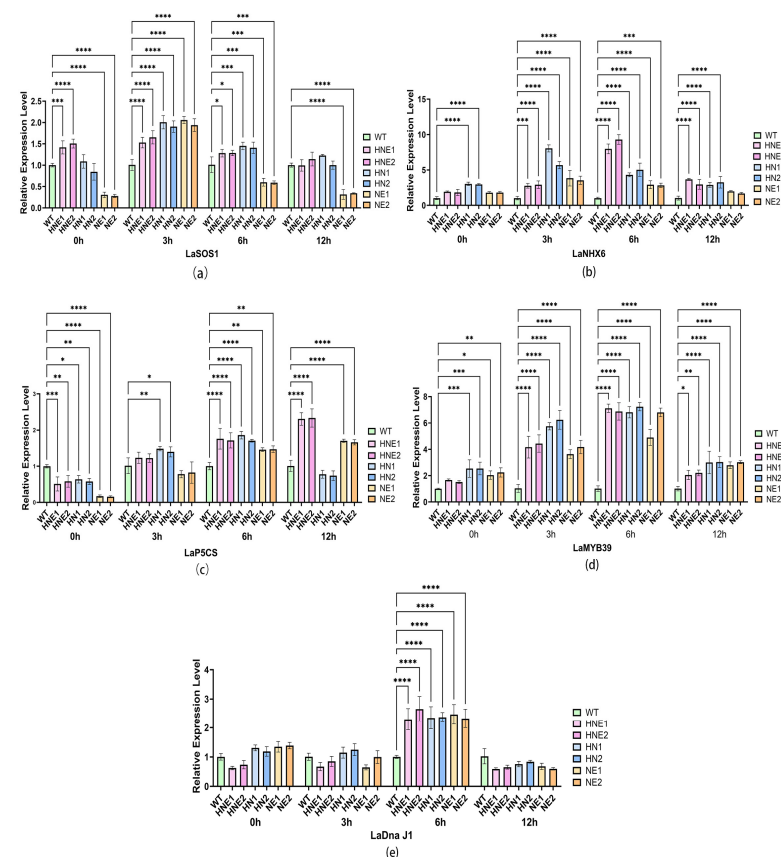


Figure 6. Relative expression levels of alkali stress-responsive genes in wild-type and overexpression lupine plants treated with NaHCO₃ for 0, 3, 6, and 12 h. (a) *LaSOS1*; (b) *LaNHX6*; (c) *LaP5CS*; (d) *LaMYB39*; (e) *LaDnaJ1*. Data in (a–e) are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined by two-way ANOVA. Asterisks indicate significant differences between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The expression of *LaSOS1* in HNE, HN, and NE lines initially increased and then decreased over the 0–12 h alkali stress period, peaking at 3 h. At this time point, *LaSOS1* expression was significantly higher in all overexpression lines than in WT (Figure 6a). Specifically, at 3 h, the relative expression level of *LaSOS1* in HNE was approximately 1.4-fold that of WT, while in HN and NE it was approximately 1.7-fold that of WT (Figure 6a).

The expression of *LaNHX6* in HNE, HN, and NE also followed an initial increase, followed by a decline. In HNE plants, *LaNHX6* expression reached its highest level at 6 h of stress, approximately 8–9-fold that of WT; in HN and NE plants, the peak occurred at 3 h, with expression in HN being approximately 5–8-fold that of WT and in NE approximately 4-fold that of WT (Figure 6b). At 3 h and 6 h of alkali stress, *LaNHX6* expression was significantly higher in HNE, HN, and NE than in WT; at 12 h, expression remained higher in all overexpression lines, with HNE and HN showing significant differences compared to WT (Figure 6b).

Under NaHCO_3 stress, *LaP5CS* expression increased continuously with stress duration in HNE and HN, whereas in NE it first increased and then decreased over 0–12 h (Figure 6c). At 0 h, *LaP5CS* expression in all overexpression lines was significantly lower than in WT. At 3 h, expression in HN was significantly higher than in WT, approximately 1.4-fold. At 6 h, expression in HNE, HN, and NE was significantly higher than in WT, approximately 1.7-fold, 1.8-fold, and 1.4-fold, respectively. At 12 h, expression in HNE and NE remained significantly higher than in WT, approximately 2.3-fold and 1.7-fold, respectively (Figure 6c).

The expression of *LaMYB39* in HNE, HN, and NE first increased and then decreased over 0–12 h of alkali stress, peaking at 6 h (Figure 6d). At 3, 6, and 12 h of NaHCO_3 treatment, *LaMYB39* expression was significantly higher in all overexpression lines than in WT (Figure 6d). At 6 h, the expression levels in HNE and HN were approximately 7-fold that of WT, and in NE approximately 5–7-fold that of WT.

Finally, *LaDnaJ1* expression peaked at 6 h of NaHCO_3 treatment in HNE, HN, and NE, and was significantly higher than in WT at that time point, with all three overexpression lines showing expression levels approximately 2.5-fold that of WT (Figure 6e).

Taken together, these results demonstrate that multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* activates the endogenous alkali tolerance regulatory mechanism in lupine under alkali stress, upregulates the expression of endogenous alkali stress-responsive genes, and thereby enhances lupine tolerance to alkali stress. Among the tested combinations, simultaneous expression of all three genes conferred the strongest regulatory capacity and the most pronounced improvement in alkali tolerance.

4. Discussion

4.1. Relationship Between Alkali Stress and the Reactive Oxygen Species Scavenging Mechanism

Under NaHCO_3 stress, all overexpression lines (HNE, HN, NE) in this study exhibited significantly enhanced antioxidant capacity, elevated proline (PRO) content and root activity, as well as reduced malondialdehyde (MDA) accumulation compared with the wild-type (WT) plants. Meanwhile, multi-gene overexpression effectively reduced alkali stress-induced accumulation of reactive oxygen species (ROS) in lupine leaves. These results suggest that multi-component module overexpression may enhance the adaptability of plants to alkali stress by modulating physiological and metabolic processes. The osmoprotective function of proline was first discovered in bacteria, and subsequent studies have confirmed a clear causal relationship between proline accumulation and salt tolerance in plants, establishing proline as a key osmolyte that plays a central role in plant responses to abiotic stress [54,55]. Furthermore, the ROS-scavenging capability of proline was first documented in the pioneering work of Smirnoff and Cumbes [56]. As early as 1989, they demonstrated that proline can effectively scavenge hydroxyl radicals ($\cdot\text{OH}$) and interact with various reactive oxygen species, laying the theoretical foundation for subsequent in-depth investigations into the antioxidant properties of proline [57]. Based on these findings and our results, the significantly increased proline content in the overexpression lines is speculated to help maintain cellular osmotic balance and stabilize membrane integrity, thereby alleviating NaHCO_3 -induced cell damage and enhancing plant alkali tolerance. Notably, the antioxidant capacity of the transgenic lines was also significantly higher than that of WT under normal growth conditions without alkaline stress. This can be explained by the CaMV 35S constitutive promoter used in the overexpression vectors, which drives continuous and stable expression of individual exogenous genes independent of external stress signals. Consistent with the hierarchical regulatory relationships among *Gshdz4*, *GsNAC019*, and *GsEXPA8* described above, these persistently expressed genes can autonomously activate downstream antioxidant signaling pathways under non-stressed

conditions, thereby elevating basal antioxidant levels and improving the intrinsic stress tolerance of transgenic lupine plants.

Meanwhile, environmental stress often induces oxidative stress in plant cells, leading to ROS accumulation. MDA, as a marker of membrane lipid peroxidation, directly reflects the degree of membrane damage [58]. In this study, after alkali stress treatment, the activities of three key antioxidant enzymes—catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD)—were significantly higher in the overexpression lines than in WT, indicating a marked enhancement of antioxidant capacity. We speculate that there may be a direct causal relationship between the improved antioxidant capacity and the significantly reduced MDA accumulation in the overexpression lines. This phenomenon suggests that multi-component overexpression of *Gshdz4/GsNAC019/GsEXPA8* strengthens the ROS scavenging system, effectively reduces oxidative damage caused by ROS accumulation, alleviates membrane lipid peroxidation under alkali stress, and ultimately improves plant alkali tolerance. However, the precise regulatory connections among these physiological and metabolic processes remain to be fully elucidated and require further experimental validation.

4.2. Association Between Promoted Root Growth and Alkali Tolerance Phenotype by Multi-Component Module Overexpression

Our results showed that multi-component overexpression of *Gshdz4*, *GsNAC019*, and *GsEXPA8* in lupine roots significantly promoted root growth, with the HNE line (*Gshdz4-GsNAC019-GsEXPA8*) exhibiting the best root growth. Roots are crucial organs for plant growth and development, providing mechanical support and absorbing water and essential nutrients from the soil to meet the needs of normal physiological metabolism [59]. Meanwhile, in alkali stress phenotyping experiments with NaHCO_3 , the HNE line displayed the strongest alkali tolerance, exhibiting significantly better growth under alkali stress than the other lines and the longest survival time. It is worth noting whether a direct link exists between superior root growth and alkali tolerance—that is, whether a well-developed root system enhances water uptake efficiency, optimizes nutrient transport, or accelerates stress signal transduction, thereby improving lupine adaptation to NaHCO_3 stress. This scientific question requires further experimental verification and in-depth investigation.

4.3. Activation Mechanism of Endogenous Alkali Tolerance Regulatory Pathways by Exogenous Genes

The *Gshdz4-GsNAC019-GsEXPA8* module constitutes a “nucleus–nucleus–membrane” three-tiered signaling cascade that integrates multiple stress-response pathways and participates in the comprehensive regulation of abiotic stress tolerance [47]. Within this module, *Gshdz4* acts as a master regulator, not only directly binding and regulating a series of different stress-responsive genes but also modulating core defense mechanisms against multiple stresses. Under alkaline stress, *Gshdz4* differentially regulates *GmSLX8*, *GmSF3*, and *GmPP4*; under cadmium stress, it specifically regulates *GmGMFL01* and *GmUNC* expression [47]. Previous studies have demonstrated that the transcription factor *Gshdz4* specifically binds to the cis-acting elements (CAATTA or CAATAA sequences) in the promoter region of *AtNAC019/GsNAC019*, and overexpression of *Gshdz4* positively regulates *AtNAC019* expression in transgenic Arabidopsis, suggesting that *AtNAC019/GsNAC019* is a downstream target of *Gshdz4* [46]. In this study, the lupine homolog of *GsNAC019*, *LaNAC072*, was identified. Bioinformatics analysis of its promoter sequence revealed the presence of a specific *Gshdz4*-binding motif in the promoter region, leading us to hypothesize that *Gshdz4* directly binds the *LaNAC072* promoter and activates its transcription. To test this hypothesis, we measured the expression levels of *LaNAC072* in WT and overexpression lines under alkali stress. The results showed that under prolonged alkali stress,

LaNAC072 expression was significantly higher in the overexpression lines than in WT. This finding raised the question of whether the introduction of *GsNAC019* might indirectly regulate *LaNAC072* expression. However, given the presence of the *Gshdz4* binding motif in the *LaNAC072* promoter and the marked increase in its expression under alkali stress, these lines of evidence indirectly suggest that *Gshdz4* may directly bind to specific sequences in the *LaNAC072* promoter and activate its transcription under alkali stress. Similarly, these findings also indirectly indicate that, under alkali stress, *GsNAC019* may directly bind to the corresponding binding motif in the *LaEXPA8* promoter and initiate its transcription.

4.4. Rationale for Selection of Physiological Indices and Endogenous Alkali Stress-Responsive Genes

We chose to measure the expression levels of the endogenous lupine genes *LaSOS1*, *LaNHX6*, *LaP5CS*, *LaMYB39*, and *LaDnaJ1* to assess differences in the regulation of endogenous alkali stress-responsive genes between WT and overexpression lines. Plant stress responses are closely associated with the expression regulation of a series of endogenous stress-responsive genes. *SOS1* encodes a Na^+/H^+ exchanger, and plants can export Na^+ out of the cytoplasm via the plasma membrane Na^+/H^+ antiporter *SOS1* to counteract salt stress; this transport activity is critical for Na^+ efflux in Arabidopsis cells [60–62]. *NHX*-type $\text{Na}^+(\text{K}^+)/\text{H}^+$ exchangers are important for regulating intracellular pH and ion homeostasis and are also involved in various physiological processes such as osmotic adjustment and stress responses [63]. For example, *GmNHX6* encodes a Golgi-localized Na^+/H^+ exchanger whose transcript abundance is upregulated in alkaline-tolerant soybean varieties in response to NaHCO_3 stress, and overexpression of *GmNHX6* improves tolerance to alkaline salt stress in soybean [64]. Proline accumulation is a common physiological response of higher plants to drought and salt stress [65,66]. It is synthesized from glutamate and ornithine [65], and the synthesis of proline from glutamate is catalyzed by delta-1-pyrroline-5-carboxylate synthase (*P5CS*) [67]. Furthermore, previous studies have shown that MYB transcription factors, one of the largest families of plant transcription factors, play important regulatory roles in plant secondary metabolism, hormonal and environmental factor responses, and organ morphogenesis. They bind to cis-acting elements upstream of stress-responsive genes and specifically regulate their expression, playing crucial roles in breeding for abiotic stress tolerance, such as drought, salt, and temperature [68,69]. One study found that the *SHR-MYB36-MYB39-FAR5* pathway coordinately regulates root suberin formation in plants, and the suberin lamellae form a hydrophobic protective barrier against biotic and abiotic stresses [70]. For lupine itself, previous transcriptome sequencing and qPCR validation confirmed that *LaDnaJ1* is a gene responsive to alkaline stress, and transformation of *GsHZ4* (i.e., *Gshdz4*) into lupine may improve alkali tolerance by modulating the expression of *LaDnaJ1* under alkaline stress [48].

These five genes were selected because they have been established as downstream targets of the *Gshdz4-GsNAC019-GsEXPA8* regulatory module. Specifically, heterologous overexpression of *GsEXPA8* in narrow-leaved lupine (*Lupinus angustifolius*) significantly upregulated the expression of *LaSOS1*, *LaNHX6*, *LaP5CS*, and *LaMYB39* in a time-dependent manner under alkaline stress conditions [49]. For *LaDnaJ1*, independent studies have confirmed that transformation of *Gshdz4* into narrow-leaved lupine upregulates *LaDnaJ1* expression under alkaline stress conditions. After treatment with 50 mM and 100 mM NaHCO_3 , *LaDnaJ1* transcript levels were significantly higher in *Gshdz4*-overexpressing hairy root lines than in wild-type controls, indicating that *LaDnaJ1* is a downstream target of *Gshdz4* under alkaline conditions [48]. From a physiological perspective, these downstream effector genes function synergistically: *LaSOS1* and *LaNHX6* maintain ion homeostasis; *LaP5CS* drives osmotic adjustment; *LaMYB39* orchestrates transcriptional defense responses; and *LaDnaJ1* responds to alkaline stress as a downstream gene of *Gshdz4*. The cooperative

activation of these genes contributes to the enhanced alkaline tolerance of transgenic lines overexpressing the *Gshdz4-GsNAC019-GsEXPA8* module.

4.5. Limitations of This Study

Several limitations of this study should be acknowledged. This study focused solely on a single stress condition of 100 mM NaHCO₃ to compare alkali tolerance differences between WT and overexpression lupine plants. Whether the alkali tolerance of the overexpression lines remains stable across different concentrations of NaHCO₃, other types of alkali stress (e.g., Na₂CO₃ stress), or at different growth stages (especially with regard to ornamental value) remains unclear. In addition, the specific regulatory mechanisms of endogenous lupine alkali stress-responsive genes (such as *LaSOS1* and *LaP5CS*) have not yet been elucidated, and the upstream/downstream regulatory relationships between these genes and the target genes identified in this study (*LaNAC072* and *LaEXPA8*) still lack in-depth analysis. Further studies are needed to validate and refine these aspects.

4.6. Research Value and Application Potential

Agrobacterium rhizogenes K599 is widely utilized for hairy root transformation in diverse plant species, largely attributed to its broad host adaptability and stable transformation efficiency. This technique is highly portable across different crops. Previous studies conducted in vivo inoculation experiments using wild-type K599 on soybean (Fabaceae), cucumber (Cucurbitaceae), and garden balsam (Balsaminaceae) and demonstrated that K599 induced adventitious root formation from wounded cotyledons with frequencies of 100%, 65%, and 91%, respectively, and from unwounded cucumber axillary buds with a frequency of 10% [50]. PCR amplification of the *rolC* gene confirmed the presence of K599 T-DNA sequences in the regenerated hairy roots, confirming the universality and stability of this transformation system across different plant families. The transgenic roots obtained in that study provided valuable materials for further research on root-knot nematode pathology in soybean and cucumber, as well as dwarf breeding in garden balsam [50]. Among leguminous crops beyond soybean, K599 achieved a transformation efficiency of 92% in fenugreek (*Trigonella foenum-graecum*), with the transgenic roots retaining full symbiotic competence with both arbuscular mycorrhizal fungi (*Rhizophagus irregularis*) and rhizobia (*Sinorhizobium meliloti*) [71]. In Rosaceae species, stable K599-mediated hairy root protocols have been developed for rose, with the latter achieving a transformation efficiency of 74.1% and enabling CRISPR/Cas9-mediated gene editing for the first time in rose (with an efficiency of 62%) [72]. In woody plants, K599 achieved a transformation efficiency of up to 60.38% in *Liriodendron* hybrids, also enabling gene editing applications [73]. Collectively, these case studies demonstrate that the K599 hairy root transformation system has reliable cross-species adaptability. When integrated with the multi-gene alkali-tolerance module characterized in the present study, this feasible transformation strategy can be applied to genetic improvement and multi-gene overexpression in various crop species, providing broad application prospects for crop stress tolerance breeding.

5. Conclusions

In summary, we overexpressed three gene combinations—*Gshdz4-GsNAC019-GsEXPA8* (HNE), *Gshdz4-GsNAC019* (HN), and *GsNAC019-GsEXPA8* (NE)—in lupine roots via *Agrobacterium rhizogenes*-mediated root transformation. Under 100 mM NaHCO₃ stress, all overexpression lines (HNE, HN, NE) showed milder wilting and longer survival compared to the wild type (WT). Physiologically, they exhibited significantly enhanced antioxidant capacity, increased proline content, reduced MDA accumulation, and improved root activity (Figure 7). The overexpression lines also showed markedly reduced accumulation

of reactive oxygen species (ROS) in leaves under alkali stress (Figure 7). Furthermore, the HNE, HN, and NE lines displayed better root growth than WT (Figure 7). This study revealed that *Gshdz4* likely activates the expression of the endogenous target gene *LaNAC072*, and *GsNAC019* activates the expression of *LaEXPA8*, thereby regulating the endogenous alkali tolerance mechanism and upregulating the alkali stress-responsive genes *LaSOS1*, *LaNHX6*, *LaP5CS*, *LaMYB39*, and *LaDnaJ1* (Figure 7), ultimately enhancing lupine tolerance to alkali stress. Collectively, the line overexpressing all three genes in roots exhibited the strongest alkali tolerance.

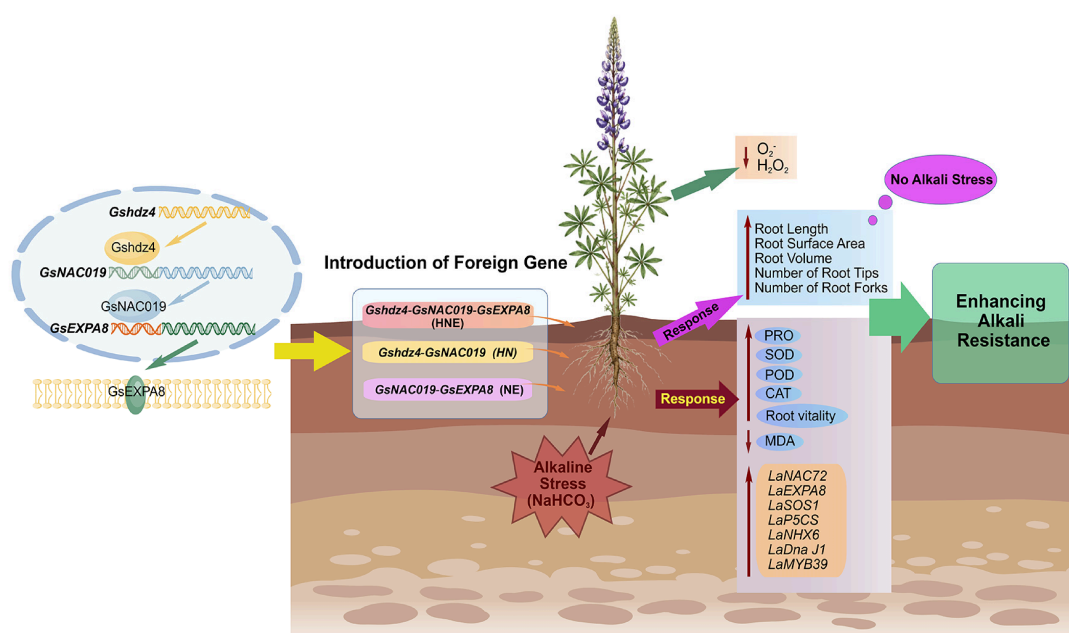


Figure 7. Schematic diagram illustrating the enhanced alkali stress tolerance conferred by multi-component overexpression of the *Gshdz4*–*GsNAC019*–*GsEXPA8* module in lupine roots. The diagram was created using BioGDP (<https://BioGDP.com>).

Author Contributions: H.W.: writing—original draft, investigation, visualization, formal analysis. Y.R.: investigation. M.Z.: investigation. Y.L.: investigation. X.W.: investigation. X.D.: validation. Y.F.: validation. T.Z.: validation. J.Z. and L.C.: project administration, conceptualization, writing—review and editing, resources, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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