EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

Transformation and expression of *HvDRF1* drought tolerant gene from barley in two Alfalfa cultivars

Mohamed Hamed, Ibtisam Hammad, Abdelfattah Badr, Eman Tawfik

PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Transformation and expression of *HvDRF1* drought tolerant gene from barley in two Alfalfa cultivars

Mohamed Hamed, Ibtisam Hammad, Abdelfattah Badr, Eman Tawfik

Botany and Microbiology Department, Faculty of Science, Helwan University, Cairo, Egypt

Drought stress factors are the most dangerous issues for crop productivity and will be a more limiting factor in the future due to climate change. Genes for drought tolerance have been identified and isolated from barley to produce drought stress-tolerant cultivars in other plants with precise gene transfer. The main aim of the following study is to transfer the *HvDRF1* gene from barley, known for its drought tolerance properties, into alfalfa cultivars using chitosan nanoparticles as a gene vector. The successful transformation of the *HvDRF1* gene into the transformed alfalfa plants was confirmed using both PCR and RT-PCR techniques. The drought tolerance of the transformed alfalfa plants was assessed through various morphological and physiological measurements. These measurements demonstrated that the transformed alfalfa plants displayed a higher level of drought tolerance compared to non-transformed plants. The measurements of drought-responsive traits such as proline, plant biomass, photosynthetic pigments, phenolic compounds, and flavonoids as well as the RWC (relative water content), indicate that the transformed plants express more tolerance to drought compared to the control non-transformed plants. These results support the use of chitosan nanoparticles as a gene vector, proving to be an effective method for gene transfer to alfalfa and potentially other crop plants.

Keywords: *Medicago sativa*; *Hordium vulgare*; *DRF1*; Drought tolerance; Chitosan nanoparticles; Gene transformation

ARTICLE HISTORY
Submitted: December 11, 2025
Accepted: April 30, 2025

CORRESPONDENCE TO
Eman Tawfik,
Botany and Microbiology Department,
Faculty of Science, Helwan University,
Cairo, Egypt
Email: emantawfik@science.helwan.edu.eg
ORCID: 0000-0001-5574-7476
DOI: 10.21608/ejbo.2025.343781.3120

EDITED BY: R. Gaafar

INTRODUCTION

Alfalfa (*Medicago sativa* L.), a perennial herbaceous legume from the Fabaceae family, is globally recognized as an essential legume fodder crop due to its high nutritional value, abundant yield, and adaptability. It serves as a primary source of protein and is a key component in livestock diets (Latif et al., 2023). Additionally, alfalfa's ability to fix atmospheric nitrogen and synthesize protein makes it highly beneficial to farmers in semi-arid regions, helping to enhance soil fertility. Due to its perennial nature, Alfalfa offers high yield and carbon sequestration potential, making it a valuable crop. However, it can strain limited natural resources and warm climates in semi-arid regions, where water scarcity significantly constrains the productivity of alfalfa in numerous regions. Drought profoundly impacts its growth and output on a global scale (Wang et al., 2025). Therefore, enhancing the ability of alfalfa to withstand drought is a primary objective in breeding drought-tolerant cultivars. As a result, balancing water use with productivity is particularly vital for alfalfa cultivation, especially as climate change exacerbates fluctuations in water availability (Li et al. 2023). However, at the heart of alfalfa's high yield and carbon sinking potential lies the cost of water resources, which are scarce in warm regions that often have drier climates and depend on irrigation. Nevertheless, it remains sustainable to harness alfalfa's high yield despite its high-water consumption (Anthony et al., 2023).

Drought stress retards plants growth by interfering with several biochemical and physiological processes, such as chlorophyll synthesis, photosynthesis rate, nutrient metabolism, ion uptake and osmoregulation, translocation, and carbohydrates metabolism (Mohammed and Faisal, 2021; Pamungkas et al. 2022; Drwish et al., 2023). It therefore poses a significant challenge to agricultural output, exerting a notable adverse impact on plant growth, development, and overall productivity. In response to drought, plants display physiological adjustments such elongating their roots, closing their stomata, and slowing the aging of their leaves, which reduce water loss and improve water absorption (Ma et al. 2019). Plants can employ inorganic chemicals and soluble secondary metabolites, referred to as osmolytes, to uphold cell turgor and hydrostatic pressure during drought conditions (Lamaoui et al. 2018; Wahab et al. 2022). The relative water content (RWC) provides an accurate assessment of cell volume and serves as an indicator of the equilibrium between water absorption and water loss through transpiration. It is widely accepted as a suitable measure of plant water status, reflecting the physiological impact of cellular water deficiency (Badr and Brüggemann 2020). Maintaining plant cell water status through osmolyte production and increasing expression of ROS scavengers both enhance transformed alfalfa tolerance towards drought stress (Nadarajah 2020).

The DREB (dehydration responsive element binding) proteins are important transcription factors (TFs) in

regulating abiotic stress-related genes and play a crucial role in imparting environmental stress tolerance of plants. DREBs regulate the expression of many stress-inducible genes, mostly through ABA-independent mechanisms, which play a critical role in improving the abiotic stress tolerance in wheat by interacting with a DRE/CRT cis-element present in the promoter region of various abiotic stress-responsive genes (Lata and Prasad, 2011). The DREB genes also have a significant function in the stress-tolerance pathways that are not dependent on ABA and stimulate the activation of other stress-responsive genes in plants (Tawfik et al. 2016). The expression of OsDREB2A in transgenic tomatoes improved the drought tolerance in tomatoes (Hassan et al. 2021). Two DREB regulons, *DREB1* and *DREB2*, can thus be used to improve the tolerance of various kinds of agriculturally important crop plants to drought, high-salinity, and freezing stresses through gene transfer (Lata and Prasad, 2011). The evolutionary history of the C-repeat binding factor/dehydration-responsive element-binding 1 (CBF/DREB1) protein family was reconstructed in 43 plant species by Li et al. (2020), who also characterized the CBF/DREB1 proteins in *Solanum tuberosum* and proposed that the evolution of these proteins is useful for understanding their precise functions of the CBF/DREB1 proteins when plants are under abiotic stress.

The DREB protein is a plant-specific family of transcription factors that regulate the expression of specific genes in response to different abiotic and biotic stressors (Chen et al. 2022). An effective strategy for developing tolerance to diverse stress conditions involves the overexpression of transcription factors (TFs) that regulate multiple genes across several pathways. Transforming plants using different promoters to overexpress multiple DREB transcription factors has led to enhance plant tolerance to drought, salt, heat, and freezing stressors (Hrmova et al. 2021). Similarly, different forms of Os*DREB1* gene — 1C, 1E, and 1G — promote stress tolerance to chilling, heat, drought, and salt (Wang et al. 2022). The manipulation of a specific gene can be accomplished in two main ways: direct and indirect. The indirect approach entails utilizing a gene vector to transport the gene, whereas the direct approach involves employing diverse chemical and physical methods for gene transfer (Su et al., 2023). Conducting in vivo functional assessments is crucial for comprehending the molecular mechanisms underlying stress tolerance in plants and developing strategies to enhance crop output (Saharan et al. 2022).

The introduction of Nanoparticles (NPs) into crop research is an evolving field with diverse applications in agriculture and biotechnology. Their small size, high surface area, and tunable properties make them useful for enhancing the targeted delivery of these materials to plants (Sembada and Lenggoro, 2024). Results on NPs recent research shall play a vital role in improving agriculture productivity by increasing crop yield and enabling plants to cope with environmental stress and losses to pathogens and threats. Using NPs may help crop plants withstand drought and salinity stress by promoting sugar accumulation and reducing the expression of genes responsible for abiotic stress (Guerriero, Sutera et al., 2021). NPs also improved drought tolerance in strawberry plants by augmenting the activity of antioxidant enzymes, including ascorbate, peroxidase, catalase, peroxidase, and superoxide dismutase (SOD). In addition, biochemical parameters—viz., total phenolic compounds (TPCs), anthocyanin and vitamin C —were also enhanced in nanoparticle-treated strawberry plants (Zahedi et al. 2020). Magnetic NPs carrying exogenous DNA were delivered to pollen in the presence of a magnetic field.

NPs showed enhanced relative water content, membrane stability index, and water use efficiency. Such applications are enabled by real-time sensing and controlled delivery, designed for controlled and targeted release, especially if triggered on demand (Grillo et al., 2021). Meanwhile, Wu et al., (2023) described NPs assisted delivery methods for plant transformation into intact plant cells and mature plants through leaves and roots, with subsequent DNA transport through the vascular system or plant suspension cells and subsequent regeneration of transformed plants. This reference compiled a list of applications of NPs-mediated transformation in various plant species using different methods. Another advantage of NPs was proposed in genome editing applications for agricultural crops protection and yield improvement (Ahmar et al., 2021). An overview of the challenges and prospects focusing on the NP delivery methods and their detection of within plant tissues was published by Sembada and Lenggoro (2024). These authors recognized both passive and assisted delivery methods, including the use of roots and leaves as introduction sites. Osmani and Kulka (2025) discussed the factors influencing the efficacy of NPs for gene transfer to plants, exploring the challenges of NP cytotoxicity, transformation efficiency, regeneration capacity of the transformed

plants, as well as environmental impacts and regulatory considerations. Research on NPs-assisted gene transfer to plants is expected to make significant contributions to sustainable agriculture. However, it is important to consider their toxicity, long-term effects, and environmental impact, as well to optimize NP properties for specific delivery requirements.

Chitosan (CTS) is a polymer derived from the partial deacetylation and hydrolysis of chitin, which is found in the exoskeleton of mollusks and crustaceans, as well as cuticles of fungi and insects. Both chitin and CTS are composed of randomly distributed *β*-(1-4)-linked d-glucosamine and *N*-acetyl glucosamine units (Jiménez-Gómez et al., 2020). On a commercial scale, CTS is mainly obtained from the crustacean shells and is therefore considered as a natural biopolymer, generated by performing N-deacetylation on chitin extracted from the exoskeleton of crustaceans. However, several types of mushrooms, green algae, and yeasts can produce CTS through biosynthesis (Kaur and Dhillon, 2014). CTS exhibits low solubility in most solvents but demonstrates limited solubility in weak organic acids when diluted. Its degree of deacetylation (DDA) quantifies the concentration of glucosamine residue in the polymer chain, indicating that the molecule carries a positive charge when diluted in an acidic solution. This is apparent from the ratio of unbound amino groups in the chitosan biopolymer (Kołodziejska et al., 2021). Meanwhile, Hidangmayum et al. (2019) addressed the role of CTS in mitigating the adverse effect of abiotic stress through the physiological response and transduction pathway via secondary messengers. They concluded that CTS treatment stimulates the photosynthetic rate, promotes stomatal closure through ABA synthesis, enhances antioxidant enzymes activity via nitric oxide and hydrogen peroxide signaling pathways, and stimulates the production of organic acids, sugars, amino acids and other metabolites required for osmotic adjustment, stress signaling, and energy metabolism in plants under abiotic stresses.

Li et al. (2015) proposed the use of CTS-based nanoparticles (CTS-NPs) as non-viral vectors in gene delivery due to their biocompatibility, efficiency, biodegradability, and ease of modification. CTS-NPs possess inherent features, including cationic charge, biocompatibility, high loading capacity, and effective penetration potential, making them suitable as nano-carriers. In addition, these NPs exhibit favorable release kinetics (Abyadeh et al., 2017). Chitosan, the main component of the chitosan nanoparticles, demonstrates inherent biocompatibility with plants and a high affinity for binding with DNA. It acts as a protective carrier during DNA delivery, ensuring high efficiency and successful gene transformation. Furthermore, CTS-NPs best application as nano-carrier is supported by their intrinsic properties such as their cationic nature, biocompatibility, high loading capacity, and good penetration potential, and good release kinetics (Akashpriya et al., 2022). Meanwhile, Zongyou et al. (2020) reviewed the use of nanoparticle-mediated gene transformation strategies for plant genetic engineering, while Karayianni et al., (2023) examined the technological aspects, applications, and future perspectives of chitosan-based nanoparticles in packages of tissue engineering and nucleic acid delivery.

Gene delivery by CTS-NPs for introducing new traits in plants, such as disease and pest resistance, is much better than conventional methods, with a higher success rate (Riseh et al., 2024). However, major concern in the application of chitosan nanoparticles arises when they are complexed with metals, genes and other compounds, as this can result in toxicity, posing a threat to the environment and causing health issues. Greater attention from the scientific community is required to understand the application and mechanism of action of these nanoparticles to effectively implement nanotechnology for sustainable agricultural development. Barley (*Hordeum vulgare*. L.) is one of the oldest cultivated crops in the world with a high adaptive capacity to drought stress. It is proposed as a cereal model for investigating the mechanisms of adaptation to abiotic stress, as it naturally tolerates drought stress better than other cereals. Barley ranks fourth in terms of harvested area among cereal crops, as stated in reports by the Food and Agriculture Organization (Dawson et al., 2015; Collin et al. 2020). There is growing interest in identifying drought stress-responsive genes in barley through various methods, such as small/large-scale omics research, comparative genomics, and genetic transformation to overexpress certain genes (Gürel et al., 2016).

A study by Shen (2013) on the characterization of drought stress regulator *CBF/DREB* genes in *Hordeum vulgare* identified ten barley *CBF/DREB* gene transcripts (*CBF1, CBF2, CBF3, CBF4, CBF6, CBF11, DREB1, DRF1.1, DRF1.3* and *DRF2*) that were isolated from different barley cultivars using gene-specific primers. Phylogenetic analysis showed that all candidate *CBF/DREB* genes could be grouped into three phylogenetic subgroups, designated *HvCBF1, HvCBF4 and HvDREB1*. A strong constitutive

expression of the three genes *HvDREB1, HvDRF1.1 and HvDRF1*.3 in response to drought were contrary to data in the literature, possibly due to differences in the age and physiological state of the plants. By analyzing *CBF* expression patterns in response to drought in leaf tissues of plants grown in the field, researchers observed that barley cultivars respond to drought at different time points. One of these genes, *HvDRF1*, was selected for isolation from barley for introduction into alfalfa cultivars to increase their drought stress tolerance. Kavipriya et al. (2019) analyzed genetic diversity across many species to discover the specific *HvDRF1* gene that could be used to develop a drought stress-tolerant alfalfa genotype.

The objectives of this study were to design a new clone of recombinant vectors carrying the *HvDRF1* gene isolated from barley to transform alfalfa plants for improved drought tolerance using CTS-NPs as gene delivery vehicles. Both drought-responsive traits and biomass productivity of two alfalfa cultivars were monitored after drought stress application in transformed and non-transformed alfalfa plants of both cultivars.

MATERIALS AND METHODS

The research work was carried out at the "Genetics and Molecular Biology Lab of Botany and Microbiology Department - Faculty of Science - Helwan University - Egypt", from 2021 to 2023. The reagents and chemicals used were of high purity, either analytical or molecular grade. Plant material: Barley (*Hordeum vulgare* L.) CV. Giza 126, a tolerant cultivar of drought stress as highlighted by Mariey and Khedr (2017); was provided by "The Central Laboratory for Research and Development of Barley", and two alfalfa cultivars (*Medicago sativa* L.) (CV. Helali, and CV. Serow 1) were brought from "The Central Laboratory for Research and Development of alfalfa"; Agricultural Research Centre, Giza, Egypt.

Barley germination and genomic DNA extraction

Barley grains were planted in 1 L pots having 600 g of sandy clay soil, fifteen seeds in each pot, and grown at room temperature for 21 days before DNA extraction. Total genomic DNA of barley leaves was isolated using the CTAB method following Edwards et al. (1991) protocol, as follows: 0.3 g of barley leaves were ground in 700 µl of 2% CTAB buffer then incubated for 30 min at 65°C with vortex every 10 min. The supernatant was transferred into new Eppendorf tubes after centrifugation at 12,000 rpm for 10 min. Each tube was filled with an equal volume of chloroform: isoamyl alcohol (24:1) and allowed to sit for 2 min at room temperature before being centrifuged at 12,000 rpm for 10 min at 4°C.The upper aqueous layer was transferred to new Eppendorf tubes and 800 µl of absolute ice-cold ethanol was added before being left at - 20°C for about 2 h. After that, tubes were centrifuged at 12,000 rpm for 10 min at 4°C to extract DNA pellets, then pellets were washed in 70% ice-cold ethanol and allowed for air drying at room temperature. Finally, the pellets were resuspended in 50 µl of Tris/ EDTA buffer (TE buffer) then kept at -20 °C till applying the PCR reaction". Genomic DNA extraction from barley leaves was confirmed by running through 1.2 % agarose gel.

Primer design, Polymerase chain reaction, and cloning

The primers for the *HvDRF1* gene were designed based on the NCBI GenBank accession number AY223807 using SnapGene® (version 2.3.3) software. The primers were selected to specifically amplify the target region. The T_m for each primer was calculated according to Ahsen *et al*. (2001) using the following equation:

$$\mathrm{Tm} = 4\mathrm{oC}\ (\mathrm{No.\ of\ G + C}) + 2\mathrm{oC}\ (\mathrm{No.\ of\ A + T})$$

The primers had the following forward and reverse sequences: HvDRF1F:5'GGGTTTCCGACTTTTCTTTC3', HvDRF1R:5'ATTCCACTTCCAAAGATCGT3'.

PCR reactions were performed in a Biometra thermocycler as follows: For each 25 µl PCR reaction mixture composed of Taq master mix (12.5 µl) (COSMO PCR RED M. Mix, W1020300x), 2 µl genomic DNA (50 ng of template DNA was used), 1µl (50nmole/base) of each forward and reverse primer (Willowfort), and ddH_2O (8.5 µl). PCR amplification was cycled as: initial denaturing of 5 min at 95°C, followed by 40 cycles of 1 min at 95◦C, 1 min at 53°C, 2 min at 72°C followed by one step of final extension at 72°C for 10 min then cooling at 4°C. The amplified PCR product was electrophoresed on a 1.7% agarose gel compared to 1kb DNA ladder (New England Biolab, N3232S). The amplified PCR product was purified using Gene JETTM PCR Purification Kit (Thermo K0701). For cloning, the purified HvDRF1 PCR product was ligated into the pLUG-Prime TA-Cloning Vector Kit II (Intron Biotechnology®) (25 ng/µl), which utilizes TA cloning for efficient insert integration following the manual's instructions. The vector contains a T7 promoter, multiple cloning site (MCS), lacZα for blue-white screening, ampicillin resistance gene (AmpR), and a T7 terminator. Both the *HvDRF1* gene map and the cloned pLUG vector map were designed using SnapGene® (2.3.3) software,

clearly demonstrating the specific location where the *HvDRF1* gene was introduced.

Preparation of competent DH5α

The preparation of chemically competent *E. coli* (DH5α) was prepared according to Maniatis *et al*. (1989). The culture of DH5α was grown on LB agar plate media with no antibiotics and incubated at 37°C for 24h. One colony was placed in 10mL LB broth media and grew overnight at 37°C then 1ml of this culture was transferred into 100 mL LB media in 0.5 L flask. Cells were allowed to grow at 37°C (250rpm), until OD600= 0.4 (after 3 hours), 3ml volume of LB media were used as a blank for spectrophotometer measurements. Then, cells were transferred to 2 centrifuge bottles (50mL), which were placed on ice for 20 min and then centrifuged at 4°C for 10 min at 3,000g. Media poured off and cells were re-suspended in 10 mL of cold 0.1 M $CaCl_2$. The suspended cells were transferred into 50 mL polypropylene falcon tubes then tubes were incubated on ice for 30 min. Cells were centrifuged at 4°C for 10 min at 3,000g (2500rpm). Finally, supernatant poured, and cells were re-suspended (by pipetting) in 1.6 mL cold 0.1 M $CaCl_2$ containing 15% glycerol. Aliquot 100µl into 1.5mL Eppendorf tubes placed on ice. Subsequently, the recombinant plasmids were introduced into *E. coli* (DH5α) competent cells by transformation.

Transformation into competent DH5α

pLUG vector carrying *HvDRF1* gene was transformed into the competent *E. coli* cells following Kavas (2011) as follows: 100µl of competent cells were allowed to thaw. 5µl plasmid DNA was added and incubated on ice for 30 min. Samples were subjected to 42°C for 1min and they were immediately transferred onto ice and left for 3 min. 900µl of SOC medium was added onto bacterial culture. The samples were incubated at 37°C for 50 min with shaking at 200 rpm. An inoculum of *E. coli* (DH5α) contains cloned vector was inoculated into a surface of LB agar plates containing appropriate antibiotics (100µg/L ampicillin). The plates were incubated for 24h at 37°C. One single colony was transferred into 10mL LB broth media with 10µl ampicillin (100µg/L) in falcon tube, then media was incubated at 37°C with 200 rpm overnight. After that, the amplified plasmid was isolated with gene of interest.

Plasmid mini prep

Plasmid DNA isolation was carried out using alkaline lysis method following the protocol of Maniatis et al., (1989). Bacteria were harvested from 10 mL overnight culture by centrifugation at 14000 rpm for 20 sec in 1.5 mL Eppendorf tube. The supernatant was poured off and the bacterial pellet was drained at room temperature by inverting the tube over paper towels for 10 min. The pellet was re-suspended in 100µl of ice-cold GTE buffer (freshly prepared), mixed thoroughly and left in room temperature for 5 min. Then, 200µl freshly prepared solution II was added. The suspension was mixed by inversion several times with gentle vortexing and kept on ice for 5min followed by the addition of 150µl cold 3M potassium acetate (Sol III) and mixed thoroughly by inversion and the tube was incubated on ice for 5 min and then centrifuged at room temperature for 5min at 14000 rpm. The supernatant was transferred into a new 1.5 mL Eppendorf tube then 800µl of isopropanol was added and mixed thoroughly by inverting. The pDNA was then recovered by centrifuging at 14000 rpm for 2 min at 4°C. The supernatant was poured off and the pellet was washed by slowly adding 0.5 mL 70% (v/v) ethanol. The ethanol was slowly discarded, and the pellet was dried at room temperature by inverting over paper towels for 15 min. The pDNA was re-suspended in 100µl 1x TE buffer.

Confirmation of bacterial transformation

To select the successful transformants colony PCR was carried out with *HvDRF1* gene specific cloning primers. Separate colonies obtained from bacterial transformation were used as a template for PCR reaction. Colony PCR was employed to distinguish recombinant and non-recombinant colonies by selecting colonies instead of the DNA template in the PCR components. The recombinant bacteria were cultivated on LB agar plates supplemented with 100 µg/L ampicillin. PCR reactions were conducted using the same *HvDRF1* primers and the same conditions of the PCR program, with single colonies serving as templates. Subsequently, electrophoresis was performed on a 1.7% agarose gel to confirm the presence of *HvDRF1* transgene in transformed bacteria. The *HvDRF1* gene was located at a position of approximately 3495 base pairs.

Chitosan nanoparticles characterization

Chitosan nanoparticles were purchased from "VitroLab" the Nano-chitosan manufacturer in Egypt. Chitosan nanoparticles were characterized by imaging via Transmission electron microscopy (TEM) following the protocol of (Kiang et al. 2004). Carbon coated 400 mesh copper grids were put over one drop of the complex (CTS/pDNA) and left to stand for 1.5 min. The grid was stained with one drop of filtered solution

containing 2% uranyl acetate for 1.5 min, then any excess of uranyl acetate was removed with filter paper. The grids were dried for 10 min then photographed with a transmission electron microscope (TEM) in the Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo., Egypt.

Preparation of chitosan-DNA nanoparticles complex (CTS/pDNA)

Mansouri et al. (2006) designed an essential protocol for CTS/DNA nanoparticle complex formation by dissolving chitosan nanoparticles (CTSNPs) at a concentration of 0.08% in 25 mM acetic acid, which was then adjusted to pH 5.5. Both recombinant pLUG and CTSNPs were incubated for 15 min at 55°C in a water bath and then added equally to each other followed by immediately intense stirring on a vortex mixer for 1min.

Transformation of chitosan/pDNA into alfalfa

The protocol of chitosan nanoparticles transformation into alfalfa plant tissue was performed according to Tawfik et al. (2022). Alfalfa seeds were cultivated in 1-liter pots containing 600 grams of sandy clay soil. After seven days, the top three leaves were injected with a syringe containing 100µl of CTS/pDNA complex. The injury in the seedlings enabled chitosan nanoparticles carrying the recombinant plasmid to be transferred into plant tissues. The injected seedlings were grown, alongside non-transformed alfalfa seedlings for 8 weeks (experiment period) at room temperature.

Drought treatment

Both transformed and non-transformed alfalfa plants (first generation) were initially watered with 150 mL of tap water per pot twice a week for a period of 4 weeks, followed by a 2-week drought period. After the drought phase, both transformed and non-transformed plants received regular irrigation of 150 mL per pot for another 2 weeks as part of the drought recovery phase, following the method of Alwutayd et al. (2023). Samples from both transformed and non-transformed alfalfa leaves were collected for subsequent analysis and measurements. Additionally, seeds of the two transgenic alfalfa cultivars were collected for further studies."

Molecular analysis of transformed alfalfa plants
Conventional PCR

This study utilized PCR to identify the presence of the *HvDRF1* gene in transformed alfalfa plants. Total Genomic DNA was isolated from leaves of non-transformed and transformed alfalfa plants by using cetyl-trimethyl ammonium bromide (CTAB) method following Edwards et al. (1991) protocol as described before. DNA fragments of *HvDRF1* gene were amplified by PCR with the same *HvDRF1* primers and the same conditions of the PCR program (as described previously) to test the amplification of the *HvDRF1* gene (3495 bp). The PCR product was then electrophoresed through 1.7 % agarose gel electrophoresis to confirm the presence of *HvDRF1* transgene.

Gene expression analysis using qRT-PCR

Gene expression analysis of *HvDRF1* gene was performed on eight weeks old alfalfa cultivars (Helali and Serow 1). This reaction was performed in three steps: RNA extraction, cDNA synthesis and real time-PCR reaction.

RNA extraction and cDNA synthesis

For RT-PCR, total RNA was extracted from leaf tissues of transgenic and non-transgenic alfalfa plants using TRIzol protocol following Ahmad et al. (2021), 250mg plant leaves were frozen with liquid nitrogen and ground to a fine powder in a pre-chilled mortar. Plant material was then homogenized for about 30 sec in 500µl of pre-warmed (80^{o}C) mixture of RNA extraction buffer (The composition of RNA extraction buffer was 100 mM LiCl, 100 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% (w/v) SDS, then autoclaved and kept at bench (RT)) and phenol in a 1:1 ratio. This mixture was re-suspended in 250µl Chloroform: Isoamyl alcohol (24:1) for 30 sec. Then samples were kept on ice and other samples were homogenized in the same way. When all samples were homogenized, centrifugation was done at room temperature for 5min at 14000rpm. Supernatant was transferred into new Eppendorf tubes and equal volume of 4M LiCl was added and kept overnight on ice box at 4^{o}C. Centrifugation was performed for 20 min at 4^{o}C and 14000rpm in a pre-chilled centrifuge.

The supernatant was carefully discarded and the pellet dissolved in 250µl sterile milliQ water. At this step, 0.1 volumes (25µl) of 3M sodium acetate pH 5.2 and 2 volumes (550µl) of absolute ethanol were added then this mixture was kept at -70^{o}C for 2 hours. Centrifugation was performed at 4^{o}C and 14000rpm for 10 min. The supernatant was carefully discarded, and the pellet was washed twice with 70% ethanol. For each washing, 1 mL 70% ethanol was added without dissolving the pellet. The supernatant was carefully discarded after a centrifugation at 4^{o}C and

14000rpm for 2 min. The pellet was air dried for 10 min at room temperature and re-suspended in 25µl RNase free water then stored at -70°C. Total RNA was DNase-treated using RNase-free DNase (Promega kit RQ1) according to the manufacturer's instructions. The cDNA was synthesized from 1 µg of total RNA using SuperScript® III RT-PCR System with Platinum® Taq DNA Polymerase (Thermo scientific) according to the manufacturer's instructions using the Semi-quantitative RT-PCR method. The Semi-quantitative RT-PCR method involves amplifying cDNA using PCR with a limited number of cycles, ensuring that the amplification remains within the exponential phase. The relative expression levels of the target gene are then analyzed by comparing band intensities on an agarose gel, normalized to a housekeeping gene. This approach provides a qualitative and comparative measure of gene expression.

Quantitative real-time PCR (qPCR)

Real-Time PCR reactions were carried to quantify selected genes transcripts using TaqMan™ Gene Expression Cells-to-CT™ Kit, and SYBR Green Master Mix (Thermo Fisher, USA) following the instructions provided by the manufacturer as follows: each reaction (15µl total volume) contained 7.5µl SYBER green mix, 0.5µM of primer pair, 2µl of cDNA. The 16SrDNA gene expression was determined as an internal control (housekeeping gene). The expression level of *HvDRF1* gene was studied in the two different alfalfa cultivars using quantitative reverse transcriptase PCR (qRT-PCR).

Biological samples were tested in triplicates, along with negative controls containing no template DNA. The NTC (negative control) was used as a control to confirm the absence of contamination and non-specific amplification. Specifically designed primers and an internal probe were used to amplify a section of the target gene. The product was 210 bp in size. Real-time PCR was performed using the following cycling conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The reaction mixture included template DNA, forward and reverse primers (200nM each), probe (100nM), and reporter dye. The primers for *HvDRF* 1 gene and TaqMan probe were designed using Snap gene® software 2.3.2. (Table 1).

Data analysis and fold change calculation

CT values of the *HvDRF1* gene for the two alfalfa cultivars were subtracted from the CT values of the *16SrDNA* gene to determine the ΔCT values. Ct values were analyzed using the 2^−ΔΔCt method, as described by Livak and Schmittgen (2001) to determine the relative expression of the *HvDRF1* gene in transgenic plants. This method is widely used for analyzing relative changes in gene expression in quantitative PCR experiments.

The formula used was:
ΔCt = Ct (target gene) - Ct (reference gene)
ΔΔCt = ΔCt (transgenic) - ΔCt (nontransgenic)
Fold change = 2^ (-ΔΔCt)

Determination of drought responsive traits
Morphological parameters measurements

Measurements of seven morphological traits were conducted on genetically modified and non-genetically modified alfalfa plants from two different alfalfa cultivars. Specifically in the first generation. The measurements were evaluated to assess the impact of *HvDRF1* gene transformation and expression on the morphological characteristics of the transgenic alfalfa plants. The analyzed parameters were plantlet fresh weight (g), total dry weight (g), main shoot length (cm), main tap root length (cm), number of leaves, number of lateral roots, and number of branches. Ten replicates for each parameter were estimated for accurate average results.

Physiological parameters measurements

Several physiological parameters were measured in both transformed and non-transformed alfalfa plants to assess the effects of gene transformation and expression. The evaluated parameters included photosynthetic pigments (mg/g fresh weight), total phenolic compounds (mg/g dry weight), total flavonoids (mg/g dry weight), proline content (mg/g dry weight), and relative water content (%). Five replicates were analyzed for each parameter to ensure accurate average results. The photosynthetic pigments were measured following the method of Metzener et al. (1965). Proline, the stress-responsive amino acid, was estimated according to the method described by Bates et al. (1973). Total phenolics were quantified using the procedure outlined by Kujala et al. (2000), while total flavonoids were determined based on the method of Piyanete et al. (2009). All methods were adapted with slight modifications as described by Hussain et al. (2021).

Table 1. RT-PCR primers, sequences, and probe characteristics.

Name	Type	Min. length of amplicon	Max. length of amplicon	Primer Sequence Direction	Length	GC %	Tm
236 F	forward	236	255	5'AGGAAAAAGCGACCUCGGAG3'	20	55	60
445 R	reverse	426	445	5'UUGCCUUACCCCACGGAAUC3'	20	55	60
probe	probe	260	279	5'CGUGAUGGCCCUAAUUCCGU3'	20	55	62.1

Relative water content measurements

Leaves of transformed and non-transformed alfalfa cultivars were cut and immediately weighed fresh mass (FM), then saturated to turgidity by immersing in cold water overnight to ensure complete hydration of the leaves to measure the turgid mass (DM) of the leaves, Subsequently, the leaves were placed in an oven at a temperature of 80°C for 48 hours to measure the dry mass (DM) of the leaves following Barrs and Weatherley (1962). A collection of 9 fully developed leaves were used as a replicate for accurate average results. The calculation of relative water content (RWC) was performed using the given equation.

$$RWC = \frac{FM - DM}{TM - DM} * 100$$

Where FM, TM, and DM, are the fresh, turgid, and dry masses, respectively.

Calculation of drought tolerance indices and performance for the measured traits

Expressing the improvement in drought tolerance for the transformed alfalfa plants of the two cultivars, a drought tolerance index for each of the 14 measured traits was calculated according to the following equation:

$$Drought\ Tolerance\ Index\ (\%) = \frac{(\text{Transformed} - \text{Nontransformed})}{\text{Nontransformed (Control)}} \text{x}100$$

This formula calculates the percentage change in a trait due to transformation, relative to the non-transformed control. Performance of different morphological and physiological traits measurements for transgenic alfalfa cultivars was calculated by the following equation:

$$\text{Trait Performance } (\%) = \left(\frac{\text{Transgenic Value for Each Trait}}{\text{Control Value for the Same Trait}}\right) \text{x}100$$

This formula is commonly used to assess the relative performance of transgenic plants compared to their non-transgenic counterparts as described by Xu et al. (2023). This method expresses the transgenic trait value as a percentage of the control, facilitating a straightforward comparison.

Statistical analysis

The data obtained were analyzed using one-way ANOVA - SPSS 21 software according to Wohlmann and Kater (2022) to calculate means and determine significance. The gel data was analyzed using BioRAD Quantity One software (version 4.6.2). The data in SPSS 21 (IPM, USA) underwent variance test analysis. Calculations were performed to determine the standard deviations and mean averages.

RESULTS

The total genomic DNA was successfully extracted from barley leaves, and the *HvDRF1* gene was amplified using PCR with gene-specific primers. The resulting PCR product was analyzed by electrophoresis on a 1.7% agarose gel, alongside a 1 kb DNA ladder (New England Biolab, N3232S) for size comparison, as illustrated in Figure 1. The restriction map of the *HvDRF1* gene (Figure 2) and the cloning map of the pLUG vector (Figure 3) were generated using SnapGene® software (version 2.3.3). These maps clearly illustrate the precise insertion site of the *HvDRF1* gene within the pLUG vector.

Transformation and Confirmation of Chitosan/pDNA in Alfalfa

After eight weeks of growth, the integration of the *HvDRF1* gene in transformed alfalfa plants was confirmed through conventional PCR. Non-transformed plants were used as a negative control to

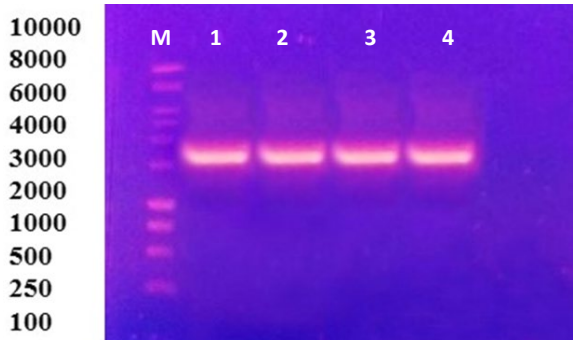

Figure 1. Agarose gel electrophoresis of the PCR-amplified *HvDRF1* gene. A distinct band corresponding to the expected size of 3495 bp is observed, as compared to the 1 kb DNA ladder (New England Biolab, N3232S).

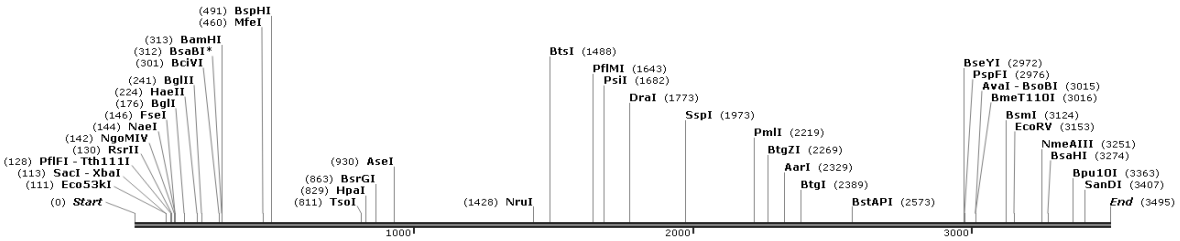

Figure 2. Restriction map of the *HvDRF1* gene. The map was generated using SnapGene® software (version 2.3.3).

verify the absence of the *HvDRF1* gene. Gel electrophoresis analysis revealed a distinct band corresponding to the expected size of 3495 base pairs, as shown in Figure 4.

Expression analysis of the transferred *HvDRF1* gene

The expression of the transferred *HvDRF1* gene was evaluated using both semi-quantitative (Figure 5a) and quantitative (Figure 5b) methods. Semi-quantitative analysis was performed using agarose gel electrophoresis, comparing *HvDRF1* expression to that of the housekeeping gene *16SrDNA* in transformed and non-transformed alfalfa cultivars (Helali and Serow 1). Quantitative real-time PCR (qRT–PCR) was used to assess the gene expression profiles of *HvDRF1* in response to drought stress in both transformed and non-transformed alfalfa cultivars. The results revealed that the *HvDRF1* gene was significantly upregulated in transformed alfalfa plants under drought stress, with higher transcript levels observed in the Helali cultivar compared to Serow1. In contrast, no expression was detected in the non-transformed control plants.

The relative expression levels of *HvDRF1*, normalized to the housekeeping gene *16SrDNA*, were calculated from three biological replicates and are presented graphically as expression ratios. For the real-time PCR assay, two positive samples (S1 and S2), representing the Helali and Serow1 cultivars, respectively, were tested with three replicates for each. A negative control (n) was also included, with three replicates, to ensure the absence of contamination. No amplification was observed in the negative control samples, confirming the reliability of the assay. Efficient amplification was detected in both positive samples, with the Helali cultivar (S1) exhibiting lower quantification cycle (Cq) values compared to the Serow1 cultivar (S2). This indicates a higher initial concentration of target DNA in the Helali cultivar compared to the Serow1 cultivar.

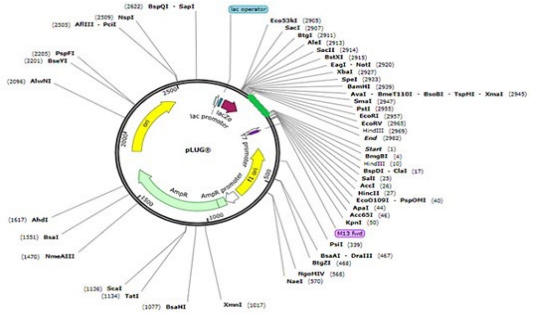

Figure 3. Circular restriction map of the recombinant pLUG vector. The inserted *HvDRF1* gene is highlighted in green. The map was designed using SnapGene® software (version 2.3.3).

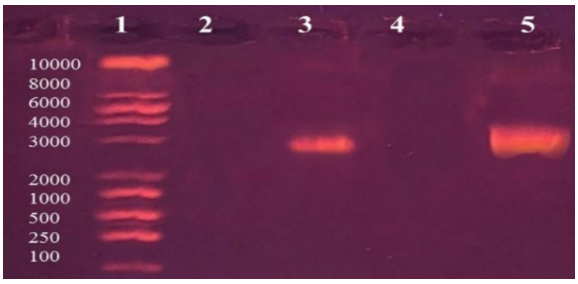

Figure 4. Agarose gel electrophoresis of conventional PCR products confirming the transformation of the *HvDRF1* gene in alfalfa plants. Lanes: 1 – DNA ladder; 2 – non-transformed Serow1 plants; 3 – transformed Serow1 plants; 4 – non-transformed Helali plants; 5 – transformed Helali plants.

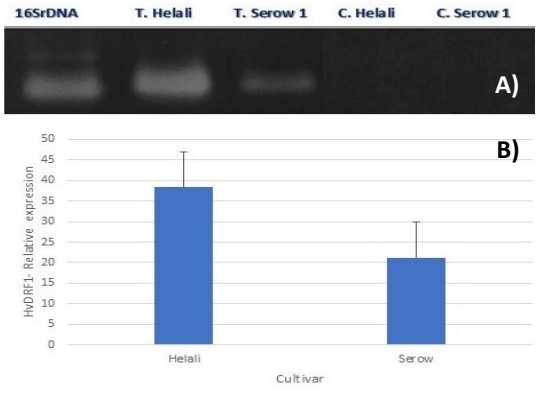

Figure 5. Expression analysis of the *HvDRF1* gene in two alfalfa cultivars, Helali and Serow1. (A) Semi-quantitative RT-PCR results visualized on an agarose gel. (B) Relative expression levels of the *HvDRF1* gene determined by qRT-PCR.

Effect of drought on morphological growth parameters

The impact of drought stress on morphological growth parameters was evaluated in both transformed and non-transformed alfalfa cultivars after a two-week drought period. As shown in Tables 2 and 3, all measured growth parameters were significantly higher in transformed plants compared to non-transformed controls. Drought stress significantly notably affected key growth traits, including shoot length, main taproot length, fresh weight, dry weight, number of leaves, number of branches, and number of lateral roots. Among the transformed cultivars, Helali exhibited superior growth performance, displaying higher values for all measured morphological traits compared to the transformed Serow1 cultivar. Both transformed cultivars, however, outperformed their corresponding non-transformed controls under drought conditions.

Effect of drought on leaf relative water content (RWC)

The relative water content (RWC) of leaves was measured in both transformed and non-transformed alfalfa cultivars under drought conditions. The results indicated that the transformed plants of both cultivars maintained significantly higher RWC values compared to their non-transformed controls, as illustrated in Figure 6.

Effect of drought on photosynthetic pigments, secondary metabolites, and proline content

The levels of chlorophyll *a*, chlorophyll *b*, carotenoids, phenols, flavonoids, and proline were measured in both transformed and non-transformed alfalfa cultivars under drought conditions. As illustrated in Figure 7, the transformed plants exhibited significantly higher levels of physiological parameters compared to their non-transformed controls. These traits are well-established indicators of drought tolerance, and the results demonstrate that the transformed plants were more resilient to drought stress than their non-transformed counterparts.

Drought tolerance indices of the measured traits

Tolerance indices for 14 measured traits were calculated to evaluate the response of transformed and non-transformed alfalfa cultivars to drought stress. The results, illustrated in Figure 8, show the percentage change in each trait for the transformed Helali and Serow1 cultivars compared to their non-transformed controls. The transformed Serow1 plants exhibited significant improvements across multiple traits under drought conditions.

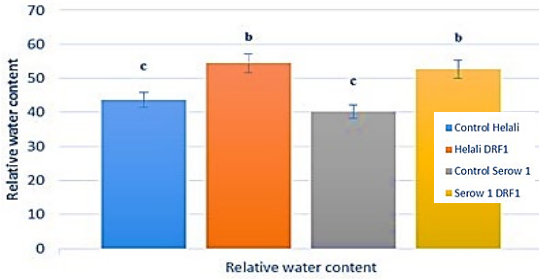

Figure 6. Histogram shows the relative water content (RWC) values for control and transformed plants of two alfalfa cultivars (Helali and Serow1) following drought treatment.

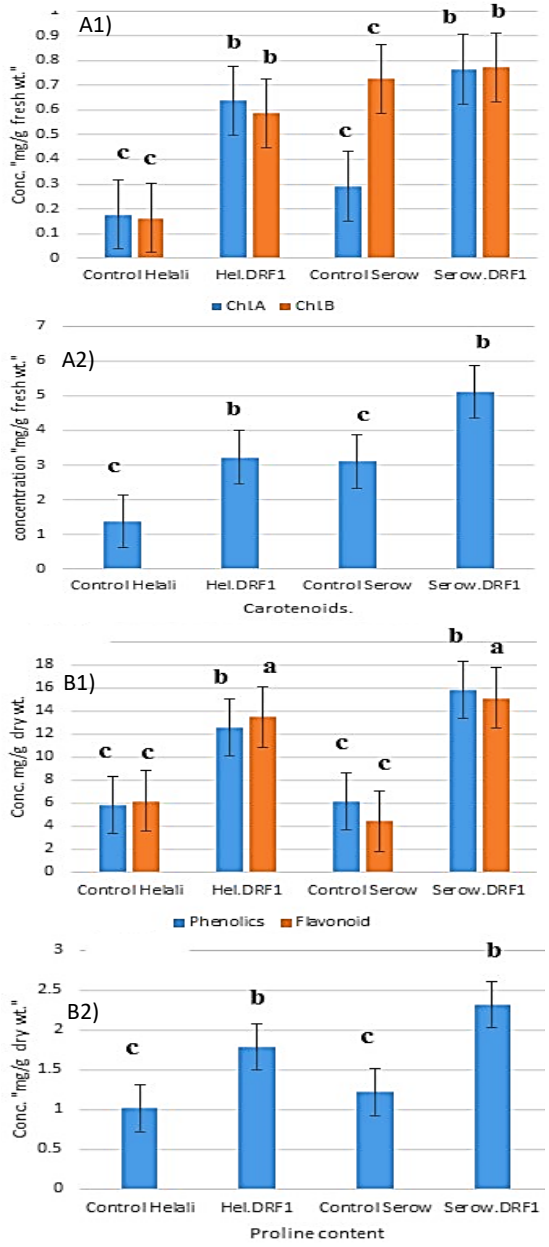

Figure 7. Histograms showing changes in photosynthetic pigments, secondary metabolites, and proline content in transformed and non-transformed alfalfa cultivars. (A1) Chlorophyll *a* and *b*; (A2) Carotenoids; (B1) Phenolics and flavonoids; (B2) Proline content.

Table 2. Mean values of morphological parameters for the Helali alfalfa cultivar (transformed and non-transformed plants) under drought conditions, measured after a two-week drought period.

Cultivar	Helali			
Parameter	Non-Transformed	Transformed	F-value	P-value
Fresh weight in gram	0.48± 0.092^{b}	1.22± 0.410^{a}	5.04*	0.050
dry weight in gram	0.050± 0.010^{a}	0.166± 0.094^{a}	3.22	0.112
Shoot length in cm	23.23± 2.25^{a}	27.40± 8.66^{a}	3.62	0.093
Main tap root length in cm	6.60± 1.31^{a}	8.73± 0.47^{a}	2.14	0.199
No. of Leaves	5.667± 0.577^{b}	7.667± 1.528^{ab}	6.91**	0.028
No. of Lateral Roots	15.00± 3.00^{a}	23.33± 4.26^{a}	1.24	0.353
No. of Branches	5.667± 0.577^{b}	7.667± 1.528^{ab}	6.91**	0.028

*Significant, ** highly significant, *** super highly significant

Table 3. Mean values of morphological parameters for the Serow1 alfalfa cultivar (transformed and non-transformed plants) under drought conditions, measured after a two-week drought period.

Cultivar	Serow 1			
Parameter	Non-Transformed	Transformed	F-value	P-value
Fresh weight in gram	0.230±0.026^{c}	0.630±0.108^{b}	36.61***	0.000
Total dry weight in gram	0.025±0.005^{b}	0.083±0.020^{a}	36.46***	0.000
Shoot length in cm	14.70±2.46^{b}	26.43±3.49^{a}	26.94**	0.001
Main tap root length in cm	3.633±0.15^{b}	8.60±1.80^{a}	6.66*	0.030
No. of Leaves	4.667±0.577^{a}	6.333±0.577^{a}	4.20	0.072
No. of Lateral Roots	6.333±0.57^{b}	18.67±3.79^{a}	18.60**	0.003
No. of Branches	4.667±0.577^{b}	6.333±0.577^{a}	10.33**	0.011

*Significant, ** highly significant, *** super highly significant

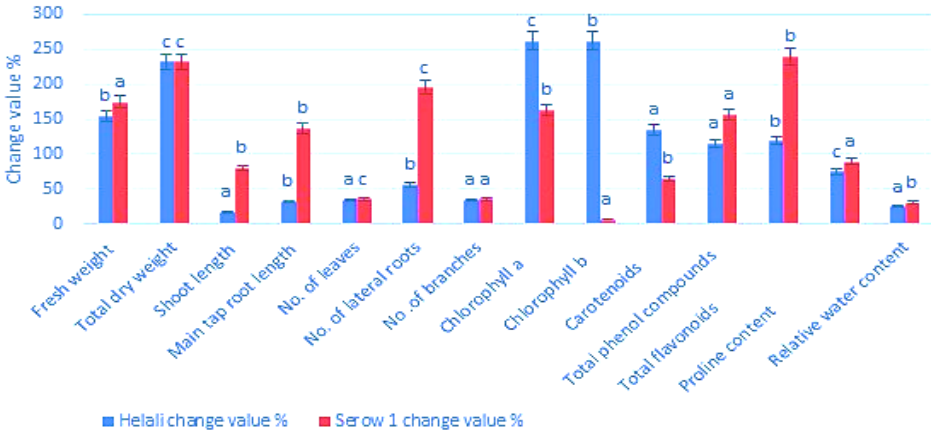

Figure 8. Histogram showing the drought tolerance indices for the measured traits expressed as the percentage change in response to drought in transformed alfalfa cultivars (Helali and Serow1) compared to non-transformed control plants.

Key enhancements included a 174% increase in fresh weight, a 232% increase in total dry weight, an 80% increase in shoot length, a 137% increase in main taproot length, and a 36% increase in the number of leaves and branches. Photosynthetic pigments also showed notable improvements, with chlorophyll *a* increasing by 163%, chlorophyll *b* by 7%, and carotenoids by 65%. Additionally, protective compounds such as total phenols and flavonoids increased by 157% and 240%, respectively, while proline content, an osmo-protectant, rose by 90%. The transformed Helali plants demonstrated similar trends but with generally lower values compared to the transformed Serow1 plants. However, the Helali cultivar outperformed Serow1 in three specific traits: chlorophyll *a* and *b* levels increased by 262%, and carotenoid levels increased by 135%.

Performance of morphological and physiological traits

The performance of various morphological and physiological traits was evaluated for the two transgenic alfalfa cultivars. The results demonstrated that the transgenic plants outperformed their non-transgenic control counterparts across all measured traits, as illustrated in Figure 9.

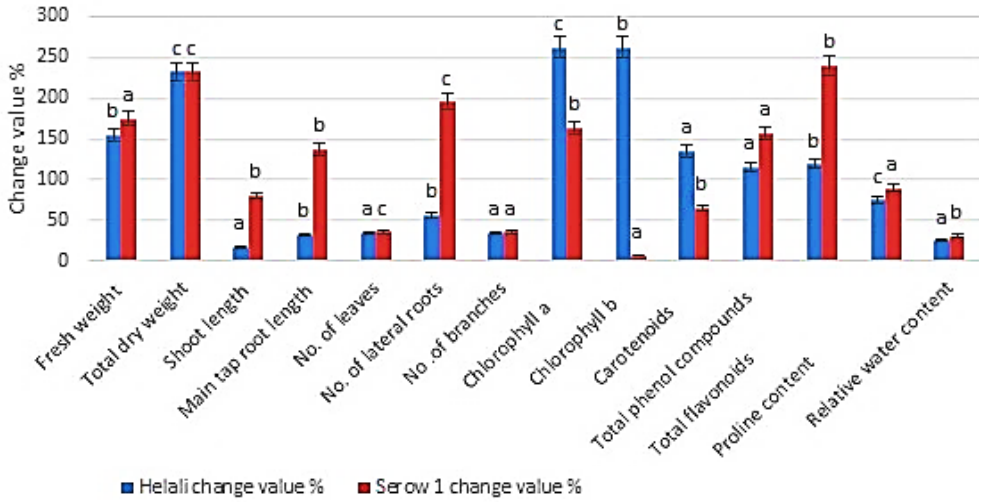

Figure 9. Performance of morphological and physiological traits in two transgenic alfalfa cultivars (Helali and Serow1) following drought stress.

DISCUSSION

The main objective of this study was to transfer the *HvDRF1* gene from barley to alfalfa cultivars using chitosan nanoparticles as a means of direct gene transformation into plant tissues. The expression of this gene in alfalfa plants plays a vital role in improving their tolerance to drought-induced stress, as demonstrated by the noticeable alterations in their morphological and physiological traits. These findings may make alfalfa cultivation suitable for areas with semi-arid and arid climates.

The improvement in the drought-responsive traits of the transformed plants confirms the successful application of chitosan nanoparticles in delivering the *HvDRF1* gene from barley to alfalfa plants. This is supported by reviews from Zongyou et al. (2020) and Karayianni et al. (2023) on the effectiveness of nanoparticles, including nano-chitosan and carbon nanotubes, in plant gene transformation, as well as the future perspectives of chitosan-based nanoparticles in gene delivery and tissue engineering.

The measurements of the morphological traits expressing plant growth were consistently higher in the transformed alfalfa plants compared to the non-transformed plants for both the CV. Helali and CV. Serow 1 cultivars. Additionally, the control and transformed Helali cultivars exhibited higher measurements than the control and transformed Serow 1 cultivars, respectively. These results suggest that the CV Helali cultivar is well-suited for cultivation in arid and semi-arid regions under both normal and water stress conditions.

The transformed alfalfa plants exhibited higher levels of all measured physiological parameters compared to the non-transformed plants (control) in both alfalfa cultivars. Additionally, the physiological parameters were higher in both control and transformed Serow 1 cultivar compared to CV Helali, except for relative water content. These findings demonstrate that the transformed alfalfa plants exhibit higher levels of drought-responsive traits, such as free proline, photosynthetic pigments, phenols, and flavonoids, compared to wild-type plants.

The increased accumulation of these compounds contributes to improved cellular relative water content, ultimately enhancing drought tolerance of the transformed alfalfa plants. Under abiotic stresses, plants accumulate compatible osmolytes like proline, soluble sugars, and other metabolites (Ghosh et al., 2021). These osmolytes act as protective agents, enabling plants to withstand drought stress. Meanwhile, RWC (Relative Water Content) provides an accurate representation of the volume of cells and reveals the equilibrium between water absorption and water loss through transpiration. It also serves as an indicator of the plant water status, namely the physiological effects of cellular water deficiency (Smart and Bingham, 1974).

During periods of environmental stress, the level of reactive oxygen species (ROS) in plants increases. While ROS can serve as signaling molecules at low concentrations, they become toxic at higher levels. To counteract the harmful effects of ROS, plants activate various enzymatic and non-enzymatic antioxidant systems that work together to scavenge and detoxify

ROS molecules. Non-enzymatic antioxidants, including phenolic acids, alkaloids, flavonoids, carotenoids, and other secondary metabolites, play a crucial role in combating oxidative stress in plant cells (Hasanuzzaman et al., 2020). The regulation of antioxidant genes during abiotic stress is controlled through signaling pathways and transcription factor regulation (Bhalani et al., 2019).

According to Dehghanian et al. (2022), plants may increase their levels of phenolic compounds during oxidative stress as a protective mechanism against oxidative damage. These phenols can directly or indirectly activate other defense mechanisms that safeguard plants from extensive oxidative stress. The oxidative bursts resulting from this stress can harm various biomolecules, including proteins, nucleic acids, and lipids. When reactive oxygen species (ROS) interact with membrane lipids, they form lipid peroxides (LOOH) and generate different carbonyl compounds like aldehydes and ketones. Some of these compounds contain carbonyl-conjugated C-C bonds, referred to as reactive carbonyl species (RCS). It is worth noting that RCS, due to their toxic effects on organisms, typically feature one or more carbonyl groups (Zhang et al., 2021).

In this study, the transformed alfalfa plants showed increased levels of carotenoids, flavonoids, and phenolic compounds. This increased accumulation of these compounds may contribute to enhanced protection against oxidative damage caused by ROS-induced oxidative stress. Additionally, the transformed alfalfa plants showed higher levels of chlorophyll A and B compared to the non-transformed control plants for both alfalfa cultivars, indicating increased protection. Most of the physiological parameters measured also showed elevated content in the transformed alfalfa cultivars, resulting in improved tolerance to drought stress. As a result, the transformed alfalfa plants also showed improvements in morphological parameters and biomass production, which was the main target of this study.

The real-time PCR assay successfully identified samples containing the *HvDRF1* gene, while the negative control showed no amplification, indicating the absence of contamination. The positive samples were amplified effectively, with samples for transforming Helali having a higher initial amount of the target DNA. The real-time PCR assay was effective in quickly and accurately analyzing biological samples to detect and measure the *HvDRF1* gene.

The drought tolerance indices calculations reveal that, except for chlorophyll a, chlorophyll b, and carotenoids, all indices are higher for the Serow 1 cultivar compared to the Helali cultivar. This suggests that the Serow 1 cultivar is more drought-tolerant than the Helali cultivar. However, for the control and transformed alfalfa plants, most parameters of the Helali cultivar are higher than those of the Serow 1 cultivar. Therefore, it can be concluded that the Helali cultivar is more suitable for cultivation under both normal and water-stressed environments, while the transformed Helali cultivar is the best choice for water-stressed conditions in arid and semi-arid regions. The production of transformed alfalfa cultivars can help increase alfalfa biomass and productivity.

Potential Off-Target Effects

Despite the promising results, it is important to consider potential off-target effects associated with the gene transformation process. The unintended integration of the *HvDRF1* gene into non-target genomic regions could lead to unintended phenotypic changes, including growth abnormalities or altered metabolic pathways. Additionally, chitosan nanoparticles, while effective as gene delivery vehicles, may interact with cellular components in unforeseen ways, potentially affecting gene expression regulation (Zhang et al., 2022). Future studies should include whole-genome sequencing or transcriptomic analysis to assess any unintended genetic modifications and evaluate their impact on plant performance and stability. Moreover, long-term field trials are necessary to investigate whether any off-target effects persist across multiple generations. Addressing these concerns will be crucial for ensuring the safe and sustainable use of genetic transformation technologies in crop improvement.

CONCLUSION

The *HvDRF1* gene, associated with drought tolerance, was transformed from barley to alfalfa plants using chitosan nanoparticles as a gene vector. Gene transformation was confirmed using PCR and RT-PCR. The transformed alfalfa plants exhibited higher drought tolerance compared to non-transformed plants, as demonstrated by measurements of drought-responsive traits and trait performance. These results provide valuable insights for developing drought-tolerant alfalfa cultivars - a major fodder crop - for cultivation in the newly reclaimed and arid lands in Egypt, and other lands across the Middle East.

ACKNOWLEDGMENT

The Practical research for this article was conducted at the Genetics and Molecular Biology Laboratory in the Botany and Microbiology Department, Faculty of Science, Helwan University. The laboratory has been updated by a "Capacity Building Project funded by a budget from the Helwan University's scientific research fund.

AUTHOR CONTRIBUTION

All authors contributed to the study's conception and design.

FUNDING

No funding was received for this work.

CONFLICT OF INTEREST

The authors declare any conflict of interest.

REFERENCEB

Abyadeh M, Sadroddiny E, and Ebrahimi A (2017) Electrosprayed chitosan nanoparticles: facile and efficient approach for bacterial transformation. Int Nano Lett 7:291–295. https://doi.org/10.1007/s40089-017-0224-0.

Ahmad H, Maher M, Abdel-Salam EM, Li Y, Yang C, ElSafty N, Ewas M, Nishawy E, and Luo J (2021) Integrated de novo analysis of transcriptional and metabolic variations in salt-treated Solenostemma argel desert plants. Front Plant Sci. 12:744699. Doi: 10.3389/fpls.2021.744699.

Ahmar S, Mahmood T, Fiaz S, Mora-Poblete F, Shafique MS, Chattha MS, Jung KH. (2021). Advantage of nanotechnology-based genome editing system and its application in crop improvement. Front Plant Sci. 2021 May 28; 12:663849. Doi: 10.3389/fpls.2021.663849. PMID: 34122485; PMCID: PMC8194497.

Akashpriya C, Gopishankar T, Praveen N, and Vasantha VL (2022) Chapter 18 - impact of chitosan and chitosan-based nanoparticles on genetic transformation: an overview. Nanomaterial-Plant Interactions 387-400.

Alwutayd, K. M., Rawat, A. A., Sheikh, A. H., Almeida-Trapp, M., Veluchamy, A., Jalal, R., Karampelias, M., Froehlich, K., Alzaed, W., Tabassum, N., Schley, T. R., Schäffner, A. R., Daur, I., Saad, M. M., & Hirt, H. (2023). Microbe-induced drought tolerance by ABA-mediated root architecture and epigenetic reprogramming. EMBO reports, 24(8), e56754. https://doi.org/10.15252/embr.202256754

Anthony, T.L., Szutu, D.J., Verfaillie, J.G. et al. (2023.) Carbon-sink potential of continuous alfalfa agriculture lowered by short-term nitrous oxide emission events. Nat Common 14, 1926 (2023). https://doi.org/10.1038/s41467-023-37391-2

Badr, A., & Brüggemann, W. (2020). Comparative analysis of drought stress response of maize genotypes using chlorophyll fluorescence measurements and leaf relative water content. Photosynthetica, 58(2), 638–645. https://doi.org/10.32615/ps.2020.014

Barrs, H. D., & Weatherley, P. E. (1962). A re-examination of the relative turgidity technique for estimating water deficits in leaves. Australian Journal of Biological Sciences, 15(3), 413–428. https://doi.org/10.1071/BI9620413

Bates L.S, and Waldern R.P (1973) Teare rapid determination of free proline for water stress studies. Plant Soil 39:pp. 205-207.

Bhalani H, Thankappan R, Mishra G. P, Sarkar T, Bosamia T.C, and Dobaria J.R (2019) Regulation of antioxidant mechanisms by AtDREB1A improves soil-moisture deficit stress tolerance in transformed peanut (Arachis hypogaea L.). PloS one 14(5):e0216706. https://doi.org/10.1371/journal.pone.0216706.

Chen Y, Li Z, Sun T, Wang D, Wang Z, Zhang C, Que Y, Guo J, Xu L, and Su Y (2022) Sugarcane ScDREB2B-1 confers drought stress tolerance in transformed Nicotiana benthamiana by regulating the ABA signal, ROS level and stress-related gene expression. International journal of molecular sciences 23(17):9557. https://doi.org/10.3390/ijms23179557.

Collin A, Daszkowska-Golec A, Kurowska M, and Szarejko I (2020) Barley ABI5 (abscisic Acid INSENSITIVE 5) is involved in abscisic acid-dependent drought response. Front. Plant Sci 11:1138.doi: 10.3389/fpls.2020.01138.

Dawson I.K, Russell J, Powell W, Steffenson B, Thomas W.T, and Waugh R (2015) Barley: a translational model for adaptation to climate change. New Phytol 206:913–931.doi: 10.1111/nph.13266.

Dehghanian Z, Habibi K, Dehghanian M, Aliyar S, Lajayer B.A, Astatkie T, Minkina T, and Keswani C (2022) Reinforcing the bulwark: unravelling the efficient applications of plant phenolics and tannins against environmental stresses. Heliyon e09094.

Drwish, A., Fergani, M., Hamoda, S., El-temsah, M. (2023). Effect of Drought Tolerance Inducers on Growth, Productivity and some Chemical Properties of Cotton under Prolonging Irrigation Intervals. Egyptian Journal of Botany, 63(1), 113-127. doi: 10.21608/ejbo.2022.150010.2041

Edwards K, Johnstone C, and Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acid Research 19:1349.

Ghosh U.K, Islam M.N, Siddiqui M.N, and Khan M.A. R (2021) Understanding the roles of osmolytes for acclimatizing pants to changing environment: a review of potential mechanism. Plant signaling & behavior 16(8):1913306. https://doi.org/10.1080/15592324.2021.1913306.

Grillo, R., Mattos, B. D., Antunes, D. R., Forini, M. M., Monikh, F. A., & Rojas, O. J. (2021). Foliage adhesion and interactions with particulate delivery systems for plant nano bionics and intelligent agriculture. Nano Today, 37, 101078.

Guerriero, G., Hausman, J. F., Legay, S., & Siddiqui, K. S. (2021). Phyto-Courier, a silicon particle-based nano-biostimulant. ACS Nano, 15(3), 4783–4797. https://doi.org/10.1021/acsnano.0c09488

Gürel F, Öztürk ZN, Uçarlı C, and Rosellini D (2016) Barley genes as tools to confer abiotic stress tolerance in crops. Front. Plant Sci 7:1137.doi: 10.3389/fpls.2016.01137.

Hasanuzzaman M, Bhuyan M.H.M.B, Zulfiqar F, Raza A, Mohsin S.M, Mahmud J.A, Fujita M, and Fotopoulos V (2020) Reactive oxygen species and antioxidant defense in plants under abiotic stress: revisiting the crucial role of a universal defense regulator. Antioxidants 9:681. https://doi.org/10.3390/antiox9080681.

Hassan N, El-Shafey NM, Khodary SEA, El-Shabrawi H, and Badr A (2021) Expression of OsDREB2A in transgenic tomatoes

improves drought tolerance. Rom Biotechnol Lett 26(6): 3145-3154. https://doi:10.25083/rbl/26.6/3145-3154.

Hidangmayum A, Dwivedi P, Katiyar D, Hemantaranjan A. (2019). Application of chitosan on plant responses with special reference to abiotic stress. Physiol Mol Biol Plants. 2019 Mar; 25(2):313-326. Doi: 10.1007/s12298-018-0633-1.

Hrmova M, and Hussain SS (2021) Plant transcription factors involved in drought and associated stresses. Int J Mol Sci 22(11):5662.doi: 10.3390/ijms22115662. PMID: 34073446; PMCID: PMC8199153.

Hussain, A., Kausar, T., Din, A., Murtaza, M. A., Jamil, M. A., Noreen, S., Rafique, A., Iftikhar, K., & Quddoos, M. Y. (2021). Determination of total phenolic, flavonoid, carotenoid and mineral contents in peel, flesh and seeds of pumpkin (Cucurbita maxima). Journal of Food Processing and Preservation, 45(1), e15542. https://doi.org/10.1111/jfpp.15542

Jiménez-Gómez CP, Cecilia JA. (2020). Chitosan: A natural biopolymer with a wide and varied range of applications. Molecules. 2020, 25(17):3981. Doi: 10.3390/molecules25173981.

Karayianni M, Sentoukas T, Skandalis A, Pippa N, and Pispas S (2023) Chitosan-based nanoparticles for nucleic acid delivery: technological aspects, applications, and future perspectives. Pharmaceutics 15(7):1849. https://doi.org/10.3390/pharmaceutics15071849.

Kaur, S., Dhillon, G. S. (2014). The versatile biopolymer chitosan: Potential sources, evaluation of extraction methods and applications. Critical Reviews in Microbiology, 40(2), 155–175. https://doi.org/10.3109/1040841X.2013.770385

Kavas, M. (2011). Development of salt-resistant transgenic plants by using TANHX1 and TASTR genes (Doctoral dissertation). The Graduate School of Natural and Applied Sciences, Middle East Technical University.

Kavipriya C, Yuvaraja A, Senthil K, and Menaka C (2019) Genetic transformation methods for crop improvement: A brief review. Agric Rev 40(4):281-288.

Kiang T, Wen J, Lim HW, and Leong KW (2004). The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. Biomaterials 25:5293–5301.

Kołodziejska M, Jankowska K, Klak M, and Wszoła M (2021) Chitosan as an underrated polymer in modern tissue engineering. Nanomaterials (Basel, Switzerland) 11(11):3019. https://doi.org/10.3390/nano11113019.

Kujala TS, Loponen JM, KlikaKD, and Pihlaja K (2000) Phenolics and betacyanins in red beet root (Beta vulgaris) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. J. Agric. Food Chem 48:5338–5342.

Lamaoui M, Jemo M, Datla R, and Bekkaoui F (2018) Heat and drought stresses in crops and approaches for their mitigation. Front. Chem 6:26.

Lata, C., Prasad, M. (2011). Role of DREBs in regulation of abiotic stress responses in plants, J. Experital Bot 62, 2011, Pages 4731–4748, https://doi.org/10.1093/jxb/err210

Latif, S., Ijaz, M., & Bukhari, S. A. H. (2023). Herbaceous alfalfa plant as a multipurpose crop and predominant choice for dairy farming. Frontiers in Sustainable Food Systems, 7, 1126151. https://doi.org/10.3389/fsufs.2023.1126151

Li W, Chen Y, Ye M, Lu H, Wang D, Chen Q. (2020). Evolutionary history of the C-repeat binding factor/dehydration-responsive element-binding 1 (CBF/DREB1) protein family in 43 plant species and characterization of CBF/DREB1 proteins in Solanum tuberosum. BMC Evol Biol. 2020 Nov 3; 20(1):142. Doi: 10.1186/s12862-020-01710-8. 1.

Li X, An J, and Hou X (2023) Effects of six consecutive years of irrigation and phosphorus fertilization on alfalfa yield. Plants 12: 2227.

Li, G., Wang, J., Feng, X., Liu, Z., Jiang, C., Yang, J., 2015. Preparation and testing of quaternized chitosan nanoparticles as gene delivery vehicles. Appl Biochem Biotech. 175, 3244–3257. https://doi.org/10.1007/s12010....

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^−ΔΔCt method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Ma Y, Vosátka M, and Freitas H (2019) Editorial: beneficial microbes alleviate climatic stresses in plants. Front. Plant Sci 10:595.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1989). Molecular Cloning: A Laboratory Manual (2nd ed.). Cold Spring Harbor Laboratory Press.

Mansouri S, Cuie Y, Winnik F, Shi Q, Lavigne P, Benderdour M, and Beaumont E (2006) Characterization of folate-chitosan-DNA nanoparticles for gene therapy. Biomaterials 27:2060–2065.

Mariey, S. A., & Khedr, R. A. (2017). Evaluation of some Egyptian barley cultivars under water stress conditions using drought tolerance indices and multivariate analysis. Journal of Sustainable Agricultural Sciences, 43(2), 105-114. https://doi.org/10.21608/jsas.2017.1061.1005

Metzener H, Rau H, and Senger H (1965) Studies on synchronization of some pigment-deficient chlorella mutants. Planta 65:186–194.

Mohammed, K., Faisal, M. (2021). Effect of Various Environmental Factors and Treatment with Ascorbic Acid on Several Physiological Attributes in Wheat (Triticum aestivum L.). Egyptian Journal of Botany, 61(3), 891-899. doi: 10.21608/ejbo.2021.82711.1715

Nadarajah K.K (2020) ROS homeostasis in abiotic stress tolerance in plants. International journal of molecular sciences 21(15): 5208. https://doi.org/10.3390/ijms21155208.

Osmani, Z., & Kulka, M. (2025). Form and Function: The factors that influence the efficacy of nanomaterials for gene transfer to plants. Molecules, 30(3), 446. https://doi.org/10.3390/molecules30030446

Pamungkas, S. S. T., Suwarto, Suprayogi, & Farid, N. (2022). Drought Stress: Responses and Mechanism in Plants. Reviews in Agricultural Science, 10, 168–185. https://doi.org/10.7831/ras.10.0_168

Piyanete C, Meechai P, and Nakbanpotecc W (2009) Antioxidant activities and phenolic contents of extracts from Salvinia molesta and Eichornia crassipes. Research Journal of Biological Science 4:1113-1117.

Riseh, RS, Vatankhah, M, Hassanisaadi, M, Varma, R. S (2024). A review of chitosan nanoparticles: Nature's gift for transforming agriculture through smart and effective delivery mechanisms, International Journal of Biological Macromolecules 260(2), 2024,129522,

Saharan BS, Brar B, Duhan JS, Kumar R, Marwaha S, Rajput VD, and Minkina T (2022) Molecular and physiological

mechanisms to mitigate abiotic stress conditions in plants. Life (Basel) 12(10):1634.doi: 10.3390/life12101634. PMID: 36295069; PMCID: PMC9605384.

Sembada, A. A., & Lenggoro, I. W. (2024). Transport of nanoparticles into plants and their detection methods. Nanomaterials, 14(2), 131. https://doi.org/10.3390/nano14020131

Shen, J. (2013). Characterization of drought stress regulator CBF/DREB genes in *Hordeum vulgare*: Expression analysis in ten different barley cultivars. - Bonn, 2013. - Dissertation, Rheinische Friedrich-Wilhelms-Universität Bonn. Online-Ausgabe in bonndoc: https://nbn-resolving.org/urn:nbn:de:hbz:5n-32746

Smart R, and Bingham GE (1974) Rapid estimates of relative water content. – Plant Physiol. 53:258-260.

Su W, Xu M, Radani Y, and Yang L (2023) Technological development and application of plant genetic transformation. International journal of molecular sciences 24(13):10646. https://doi.org/10.3390/ijms241310646.

Tawfik E, Hammad I, and Bakry A (2022) Production of transformed Allium cepa by nanoparticles to resist *Aspergillus niger* infection. Molecular Biology Reports 49(3):1783-1790. https://doi.org/10.1007/s11033-021-06988-5.

Tawfik RS, Badr A, Sammour R, Ibrahim U, Matter M, and Sakr M (2016) Improvement of flax drought tolerance using gene transfer. Plant Tissue Cult. & Biotech 26(2): 197-207.

Von Ahsen, N., Wittwer, C. T., & Schütz, E. (2001). Oligonucleotide melting temperatures under PCR conditions: Nearest-neighbor corrections for Mg^{2+}, deoxynucleotide triphosphate, and dimethyl sulfoxide concentrations with comparison to alternative empirical formulas. Clinical Chemistry, 47(11), 1956–1961. https://www.gene-quantification.de/ahsen-hrm-2004.pdf

Wahab A, Abdi G, Saleem MH, Ali B, Ullah S, Shah W, Mumtaz S, Yasin G, Muresan CC, and Marc RA (2022) Plants' physio-biochemical and phyto-hormonal responses to alleviate the adverse effects of drought stress: A comprehensive review. Plants 11(13):1620. https://doi.org/10.3390/plants11131620.

Wang H, Lu S, Guan X, Jiang Y, Wang B, Hua J, and Zou B (2022) Dehydration-responsive element binding protein 1C, 1E, and 1G promote stress tolerance to chilling, heat, drought, and salt in rice. Front. Plant Sci. 13:851731.doi: 10.3389/fpls.2022.851731.

Wang, T, Mallick, K., Verfaille, J., Szutu, D., Baldocchi, D. (2025). Water scarcity in semi-arid California compromises perennial alfalfa's high yield and carbon sinking potentials. Agricultural Water Management 308, 2025,109284.

Wohlmann, E., & Kater, M. M. (2022). Analysis of BRCT5 domain-containing proteins reveals a new role of DNA damage repair components in root development. Frontiers in Plant Science, 13, 1023358.

Wu K, Xu C, Li T, Ma H, Gong J, Li X, Sun X, Hu X. (2023). Application of nanotechnology in plant genetic engineering. Int J Mol Sci. 2023 Oct 2; 24(19):14836. Doi: 10.3390/ijms241914836. PMID: 37834283; PMCID: PMC10573821.

Xu, Z., Heuschele, D. J., Lamb, J. F. S., Jung, H.-J. G., & Samac, D. A. (2023). Improved Forage Quality in Alfalfa (Medicago sativa L.) via Selection for Increased Stem Fiber Digestibility. Agronomy, 13(3), 770. https://doi.org/10.3390/agronomy13030770

Zahedi S.M. Zahedi, F. Maharani, S. Sarikhani, M. PadervandSelenium and silica nanostructure-based recovery of strawberry plants subjected to drought stress. Sci Rep. 10 (2020) p 17672.

Zhang H, Troise A.D, Qi Y, Wu G, Zhang H, and Fogliano V (2021) Insoluble dietary fibre scavenges reactive carbonyl species under simulated physiological conditions: The key role of fibre-bound polyphenols. Food Chem 349:129018.

Zhang, X., Zhang, H., Liang, X., Zhang, J., Tao, W., & Zhu, X. (2022). Chitosan nanoparticles as gene delivery vehicles: Potential impacts on cellular components and gene expression regulation. *Journal of Nanobiotechnology, 20*(1), 123. https://doi.org/10.1186/s12951-022-01345-5

Zongyou Lv, Jiang R, Chen J, Chen W (2020) Nanoparticle-mediated gene transformation strategies for plant genetic engineering. The Plant Journal 104:880–891.