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Overexpression of *Leptochloa fusca* H^+-pyrophosphatase (*LfVP1*) gene improves salinity and drought tolerance in tobacco

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ABSTRACT

Physiologically, salinity causes osmotic stress due to high solute concentration in soil and disturbs the metabolic and photosynthetic activity of the cells by increasing the toxicity of Na^+ in the cytoplasm. Plant adaptation to salt stress is characterized by cellular ion homeostasis and vacuolar sequestration of toxic ions from cytosol mediated by H^+-pyrophosphatase (*H^+-PPase*). The *LfVP1* gene was cloned under the control of the *Gal1* promoter for yeast transformation and the *CaMV35S* promoter for tobacco transformation. Yeast supplementation assay demonstrated that *ena1* and *ena1:nhx1* yeast mutants, transformed with *LfVP1* genes, could only be able to partially complement the effect of NaCl and hygromycin in the presence of a functional Na^+/H^+ antiporter gene. Transgenic tobacco plants transformed with the *LfVP1*gene had significantly higher photosynthetic levels, stomatal conductivity, relative water content, membrane stability index, and negative osmotic potential under osmotic stress compared to wild-type plants (WT). Seeds of the transgenic line *LfVP1*-PB4 (single gene insertion) and WT were germinated on the MS_0 medium supplemented with 200 and 250 mM NaCl. Transgenic plants showed better growth and tolerance to salinity stress than WT plants. Our findings indicate that the overexpressing *LfVP1* gene has a potential role in enhancing the abiotic stress tolerance in crops such as rice, wheat, and maize.

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H^+ proton pump; ion homeostasis; *Leptochloa fusca*; tonoplast function; transgenic stress response

Introduction

Climate change is significantly impacting global agricultural production.[1–4] Abiotic stresses such as drought and salinity are widely recognized as the two most significant factors reducing yields of major crops like wheat, maize, and rice.[5–7] According to the United Nations Convention to Combat Desertification (UNCCD), drought and desertification damaged 1.9 billion hectares of land and 1.5 billion people worldwide. The world loses 12 million hectares of land annually, reducing the potential to grow 20 million tons of grain. By 2050, over three-quarters of the world's population will face absolute water scarcity and live in water-stressed conditions.

Stress tolerance mechanisms in plants are categorized into two groups: osmotic-stress tolerance and ion-stress tolerance. Osmotic regulation is associated with the first group, while compartmentalization and exclusion of toxic ions belong to the second group. The ionic homeostasis response primarily focuses on the exclusion of Na^+ to prevent the accumulation of toxic concentrations in plant tissues.[8] Plants can mitigate the harmful effects of salinity by reducing ion accumulation in their shoots or sequestering excess Na^+ into vacuoles through the action of Na^+/H^+ antiporters.[9] The H^+ gradient generated by H ±pumping ATPases maintains the activity of these antiporters, facilitating the exchange of

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Na^+ for H^+ ions into the vacuole.[10] The energy-generating proton pumps are membrane proteins that aid the active transport of ions across the membrane by establishing an electrochemical gradient force.[11] The tonoplast contains two types of proton pumps: H^+-pyrophosphatase (*H^+-PPase*) and *H^+-ATPase*.[11–13] The expression of the *H^+-PPase* establishes a strong driving force for the activity of the Na^+/H^+ and other secondary antiporters by increasing the pH gradient between the vacuole and the cytoplasm.[14] Overexpression of the *H^+-PPase* gene has been shown to improve drought and salinity tolerance in both yeast and plants, highlighting the critical role of *H^+-PPase* in maintaining the electrochemical gradient necessary for various physiological processes, including stress responses. This proton pump utilizes energy from the hydrolysis of inorganic pyrophosphate (PPi) to transport H^+ ions across the plasma membrane, thereby enhancing the plant's ability to cope with osmotic stress conditions. The increased vacuolar sequestration of toxic ions, such as Na^+, effectively elevates osmotic pressure and mitigates the harmful effects of these cations.[15]

Heterologous expression of the *Arabidopsis thaliana* vacuolar *H^+-PPase* (*AVP1*) has been shown to confer salt tolerance to salt-sensitive *ena1* yeast mutants.[16] Additionally, the cloning of a wheat vacuolar *H^+-PPase* (*TVP1*) gene demonstrated that its expression reduced Na^+ hypersensitivity in the *Δena1* yeast mutant.[17] Further research involving the *TsVP1* gene from *Thellungiella halophila* and the *AVP1* gene from *Arabidopsis thaliana* revealed that overexpressing these genes in a yeast mutant (*Δena1*) resulted in improved Na^+ tolerance compared to wild-type yeast.[15]

Several studies have demonstrated that ectopic expression of *H^+ -PPase* gene homologs significantly enhances plant tolerance to drought and salinity stress. Notably, overexpression of the *AVP1* gene in transgenic *Arabidopsis thaliana* has led to improved performance under water-deficient and saline conditions compared to wild-type plants.[18] Similarly, transgenic alfalfa overexpressing *AVP1* exhibited superior growth under salt and drought stress.[19] The *AVP1* gene has become a focal point for improving abiotic stress resilience in various crops. Its expression in transgenic lines of cotton, rice, and barley has resulted in enhanced tolerance to drought, salinity, and heat, as well as increased biomass and yield.[20–22] These improvements are attributed to increased root growth, more effective sodium exclusion, and enhanced water use efficiency.[23–25]

Recent research has expanded to include cloning and functional characterization of *H^+ -PPase* genes from halophytic and stress-resilient species such as *Reaumuria trigyna*, *Zoysia matrella*, and *Salicornia europaea*. These genes, when introduced into model plants, have conferred robust tolerance to salt and drought stress.[26–28] In a more recent study, Wan et al.[29] reported that overexpression of the *NsVP1* gene from *Nitraria sibirica*, significantly enhanced salt tolerance in *Arabidopsis* by maintaining ion homeostasis and improving osmotic adjustment, further emphasizing the central role of vacuolar *H^+ -PPases* in stress adaptation.

Supporting evidence also comes from transgenic tobacco and cotton expressing *AVP1*, which demonstrated enhanced growth and water uptake under salinity and drought conditions compared to non-transgenic controls.[30,31] Similarly, peanut and tobacco plants overexpressing *AVP1* showed improved drought resistance, attributed to solute accumulation within vacuoles and resultant higher turgor pressure.[32,33] These studies consistently demonstrate that overexpression of *H^+ -PPase* genes promotes biomass accumulation, root system development, and grain yield under abiotic stress.[20–23] Moreover, *H^+ -PPases* from halophytic plants like *Zoysia matrella*, *Reaumuria trigyna*, and *Salicornia europaea* have been shown to enhance salt and drought tolerance through improved ion compartmentalization and vacuolar Na^+ sequestration when expressed in model crops.[26–28] These findings collectively support the hypothesis that *H^+ -PPase* overexpression enhances the proton electrochemical gradient across the vacuolar membrane, thereby facilitating Na^+ sequestration through vacuolar Na^+/H^+ antiporters.

The present study aims to further explore the potential of *H^+ -PPase* genes in conferring abiotic stress tolerance by characterizing a novel gene variant from *Leptochloa fusca*, a salt-tolerant forage grass species. Known for its ability to grow in saline environments where other forage crops fail, *L. fusca* exhibits key physiological traits including salt accumulation and excretion, a well-developed root system, and the ability to acidify the

rhizosphere, thereby improving soil permeability.[34] This research focuses on functionally analyzing the vacuolar H^+ -*PPase* gene from *L. fusca* in model systems to evaluate its effects on growth, ion regulation, and physiological responses under drought and salinity stress.

Materials and Methods

Cloning and Construction of Yeast and Plant Expression Vectors

The full-length *LfVP1* gene[35] was amplified from cDNA *Leptochloa fusca* using LfVPFwd1: 5′-CACC ATGGCGATCCTCTCGG-3′; LfVPRev1: 5′-TTAAAACAGCTTGAAGAGCAGGCCAC-3′) primer pair and sub-cloned into pENTR/SD TOPO® cloning vector (Invitrogen®). The successful cloning of the *LfVP1* gene was confirmed by performing colony PCR and sequencing of the plasmid. Then, LR reaction was performed to clone the full-length *LfVP1* gene into Gateway® compatible vector YES-DEST52 (Invitrogen® US A) and pB7WG2D,1 (VIB, Belgium) for yeast (*Saccharomyces cerevisiae)* and plant transformation, respectively.

Yeast Transformation

Yeast expression vectors harboring *LfVP1* under the control of GAL promoter and a control plasmid pYES2/CT (Invitrogen, Cat # V8251–20) were transformed into *Saccharomyces cerevisiae* Wild-type and *ena1* mutant and *ena1; nhx1* double-mutant strains. The transformation was done using the EZ-Yeast Transformation Kit, following the protocol described by the manufacturer (Qbiogene; Irvine, CA, USA). A 10 mL of Yeast potato dextrose (YPD) medium was inoculated with a colony of yeast strains (*ena1, ena1*; nhx1 mutants and RG9) and left on a shaker overnight at 30°C. The OD of the overnight culture was determined, and the culture was diluted to an OD_{600} of 0.4 in 50 mL of YPD medium and grown for an additional 2–4 hours. The culture was centrifuged at 2500 rpm to pellet the cells, and the pellet was resuspended in 40 mL 1X TE. The culture was centrifuged again at 2500 rpm to pellet the cells and resuspended in 2 mL of 1X LiAc/0.5X TE, followed by incubation at room temperature for 10 minutes. For each transformation, 1 µg of plasmid DNA and 100 µg of denatured sheared Salmon sperm DNA were mixed with 100 µL of the yeast suspension. After that, 700 µL of 1X LiAc/40% PEG-3350/1X TE was added and mixed well. Subsequently, solutions were incubated at 30°C for 30 minutes. Then 88 µl of DMSO was added, mixed well, and heat shock was given at 42°C for 7 minutes. Each of the samples was centrifuged for 10 seconds, and the supernatant was removed. The cell pellet was resuspended in 1 mL of TE buffer and re-pelleted by centrifuging for 10 seconds. The cell pellet was again resuspended in 50–100 µL of 1X TE buffer and plated on a selective SC-U plate.

Functional Complementation Assays of LfVp1 Gene

A single colony of each transformed strain, including WT (Rg-9, *LfVP1-ena1*, *LfVP1-ena1*; *nhx1*, and control (pYES2/CT) constructs (CT-*ena1* and CT-*ena1;nhx1*), was grown in 15 mL of SC-U media (containing 2% glucose) overnight at 30°C with shaking. The OD_{600} of overnight-grown culture was determined and placed in 10–15 mL of induction medium (Galactose + Raffinose). The amount of overnight culture was calculated to get OD_{600} ~1.0 of the induction media (SC medium containing 2% galactose). The cells were pelleted at 1500 × g for 5 minutes at 4°C, and the supernatant was discarded from each culture. Subsequently, cells were re-suspended in 1–2 mL of ddH_2O (sterilized autoclaved) and centrifuged at 1500 × g for 5 minutes at 4°C, and the supernatant was again discarded from each sample. Cells were washed three times with autoclaved ddH_2O, and the final OD_{600} of 1.0 was also adjusted with autoclaved distilled water. Serial, dilution of OD_{600} of 1.0 was set as; 0, 1/10, 1/25, 1/50, and 1/100. A serial dilution of 0 corresponds to OD_{600} of 1.0. A 5 µL of serial dilutions was dropped out on solid YPD plates with different concentrations of hygromycin and NaCl using a multi-channel pipette.

Agrobacterium-Mediated Plant Transformation

A full-length coding sequence of the *LfVP1* gene under the control of the *CaMV35S* promoter was

transformed into *Nicotiana tabacum* by *Agrobacterium*-mediated *AGL1* strain. A total of 160 explants were used. After inoculation with *Agrobacterium*, the explants were placed on callus induction and regeneration medium containing 0.1mgL^{-1} NAA and 1 mg L^{-1} BAP supplemented with 2mgL^{-1} Basta® as a selectable marker. After 6–7 days, non-transformed leaf discs started dying, and transformed cells started callusing and regeneration. After 7–10 days, callus induction started from the inoculated leaf discs. Out of 160 inoculated explants, 78 explants survived and grew on the selection medium. After 10–12 days, regenerated plantlets were carefully excised from callus and shifted to jars of MS media containing 0.1mgL^{-1} NAA and 1 mg L^{-1} BAP supplemented with 3mgL^{-1} Basta®. After primary root formation, the plantlets were shifted to the root induction medium in jars without selection. After the formation of enough secondary roots, plants were shifted to pots containing peat moss and covered with polythene bags to avoid rapid desiccation.

DNA Isolation and PCR Confirmation of Transgenic Plants

Genomic DNA was isolated from the fresh leaves of the putative transgenic and wild-type (WT) plants. The integrity of the DNA was ensured by running 2 µL of DNA on 1% agarose gel, and the quantity was determined with the help of a spectrophotometer. The PCR was performed to confirm the putative *LfVP1* transgenic plants using a *bar* gene-specific primer pair (5′-GTACCGGCAGGCTGAAGTC-3′ and 5′-GAAGTCCAGCTGCCAGAAAC-3′). The PCR program was set as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 2 min; followed by a final extension at 72°C for 10 min.

Southern Blot Hybridization

For Southern hybridization, 20 µg of the genomic DNA was digested with *BamH*I and *EcoR*I. The digested DNA was separated on 1% agarose gel by electrophoresis and then transferred to a nylon membrane. A 600 bp PCR product of the *bar* gene was used as a probe. The membrane was hybridized with the *bar* gene probe.[36]

RNA Extraction and Semiquantitative rt-Pcr

Semi-quantitative RT-PCR (Reverse transcriptase PCR) was performed to confirm the expression of the transgene in putative transgenic plants. The total RNA was extracted from 100 mg of young leaves of transgenic and WT tobacco plants using the Trizol® reagent. The first strand of cDNA was synthesized using a revert Aid H^{-} cDNA synthesis kit (Fermentas®, USA). For RT-PCR, 1 µl of the first strand cDNA template and a pair of gene-specific primers (*LfVP1*F: 5′-TTGTCAAGCACCTTTC TTGG-3′ and *LfVP1*R: 5′-GCAGAAGAACAC GGTGAGAA-3′) were used. The *NtActin* gene-specific forward (5′-CAGGAATGGTTAAGGC TGGA-3′) and reverse primers (5′-CCATATC GTCCCAGTTGCTT-3′) were used as a control to confirm the expression of *Nicotiana tabacum Actin1* gene. The PCR reaction was carried out on the following conditions: 1 cycle for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 30s with a final extension at 72°C for 10 min. The PCR product was analyzed by 1% agarose gel electrophoresis.

Basta® Leaf Paint Assay

Transgenic plants were screened using the Basta® leaf paint assay. Two types of leaf assay were performed: attached leaf assay and detached leaf assay. For the attached leaf assay, 0.025% Basta® solution was prepared in MS_0 and painted on the attached leaf surface with the help of a cotton swab. In the case of the detached leaf assay, a small portion of transgenic as well as WT plants' leaves were dipped in Eppendorf® tubes containing 700 µl of 0.025% Basta® solution. All tubes were placed at 25±℃ under the light.

Physiological Analysis

For the characterization of the *LfVP1* transgenic plants, osmotic stress was applied, and different physiological characters were measured. Transpiration rate (E), stomatal conductance (g_s), and net photosynthetic rate (Pn) were determined using an open

system LCA-4 ADC portable infrared gas analyzer (Analytical Development Co., Hoddesdon, England).

Measurement of Osmotic Potential (Ψs)

Fully expanded youngest leaves were excised from transgenic and WT plants. Leaf material was shifted into a 1.5 mL Eppendorf® tube and stored at −80°C for two weeks. Samples were thawed, and the frozen sap was extracted by crushing the material with a micro mortar. The leaf sap was collected, and 10 µL of sap was used for the determination of osmotic potential in an osmometer (Vapro 5520). The value obtained in mmol/kg was converted into – Mpa using the following formula.

$$\Psi\ s(-MPa) = -2.5 \times (mmol/kg)/1000$$

Measurement of Relative Water Contents

The relative water content (RWC) of the leaf was measured according to the method described by.[37] Fresh weight from the youngest fully expanded and equal-sized leaf discs was determined after excision. Turgid weight was measured after soaking the discs in distilled water for 22–24 hours. All discs were quickly and carefully dried on tissue paper before measuring turgid weight. Dry weight was also measured after drying the leaf disc sample for 72 h at 80°C in the incubator. The RWC was calculated using the following equation.

$$RWC = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100$$

Leaf Membrane Stability Index

The membrane stability index (MSI) of the leaf was determined according to the method described by[38] and modified by.[39] Leaf discs of uniform size and weight (0.2 g) were taken in 15 mL Falcon® tubes containing 10 mL of double-distilled water and kept at 40°C in a water bath for 30 min, and the electrical conductivity (C_1) of the water containing the sample was measured using an electrical conductivity meter. Another sample set was taken in test tubes containing the same amount of water and incubated at 100°C in the boiling water bath for 15 min, and their electrical conductivity was also measured (C_2) as given above. MSI was calculated using the formulae given below:

$$MSI = [1 - C1/C2] \times 100$$

Germination Analysis of Transgenic and WT Control Plants

To analyze herbicide and salinity tolerance of *LfVP1* overexpressing tobacco plants, seeds from T_0 transgenic plants were germinated on an MS_0 medium supplemented with Basta® ($2mgL^{-1}$) and different concentrations of NaCl (100, 200, and 250 mM). After 10 days, Basta® resistant T_1 and WT seedlings were again transferred to MS medium supplemented with 200 and 250 mM NaCl.

Statistical Analysis

Data were statistically analyzed by performing an analysis of variance (ANOVA) technique using the Statistix 8.1 software.[40] Treatment means were compared using the least significant difference (LSD) test at a 0.05% probability level.[41]

Results

Cloning and Functional Characterization of LfVp1 Genes in Yeast

Kallar grass grown in the salinity stress research station at Pakka Ana was used to isolate the *LfVP1* gene. After sequence confirmation, the full-length gene was cloned and transformed along with a control plasmid in WT and different mutant yeast strains (Figure 1). WT, *ena1* mutant, and *ena1;nhx1* double-mutant strains were used for functional complementation analysis at 0, 10, 25, and 50 µg hygromycin. Furthermore, Na^+ ions were supplemented in YPD media (as NaCl salt at 0.5 and 0.7 M) serially diluted to 1:10 and 1:100 (Figure 2). Visible differences were observed in wild type, ena1, and ena1; nhx1 empty control vector at $1:100^{th}$ dilution on YPD media without any supplemented Na^+ or hygromycin. These differences became prominent when YPD media was supplemented with 0.5 M NaCl, where the empty vector was unable to grow in both *ena1* and *ena1; nhx1* double-mutant at all dilutions. Furthermore,

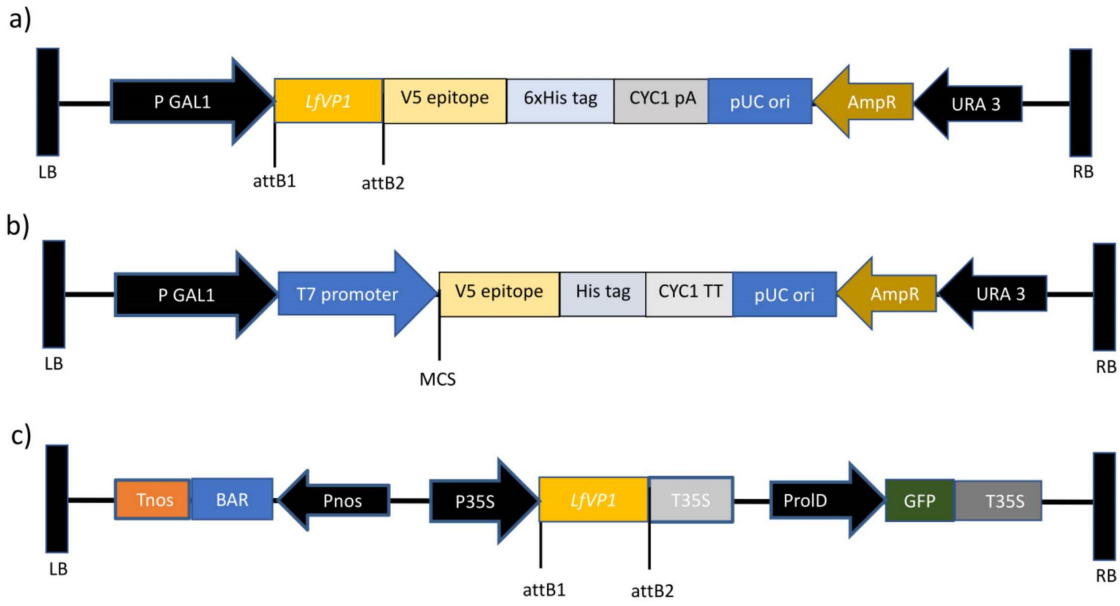

Figure 1. Diagrammatic representation of the LfVP1 constructs used in this study a) construct for yeast transformation, b) control vector for yeast transformation c) construct for tobacco transformation.

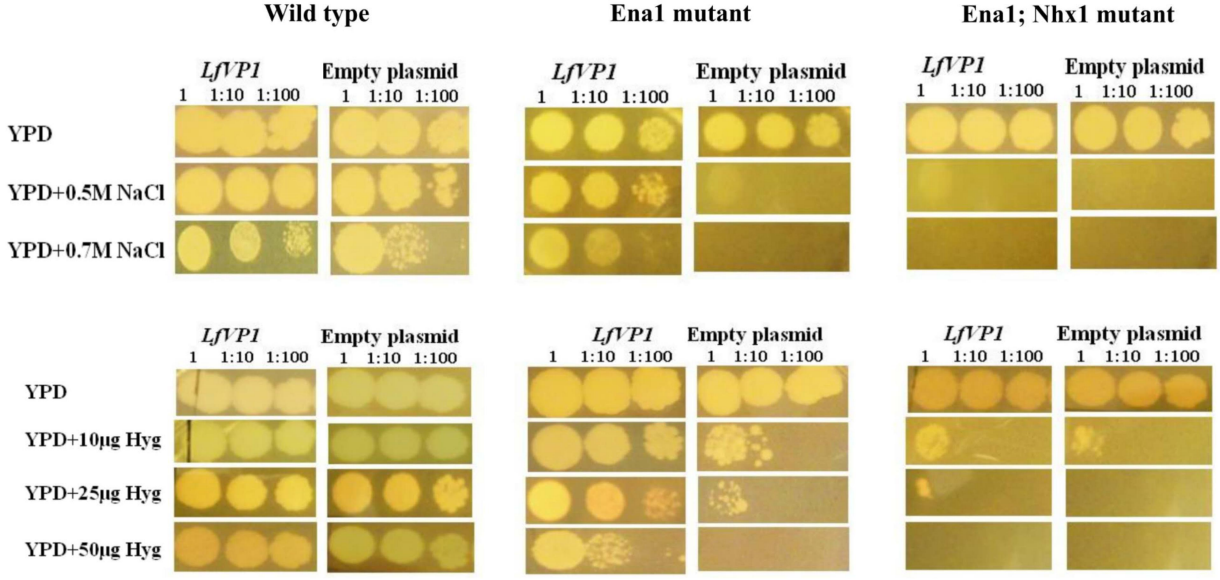

Figure 2. Growth response of wild-type, mutant, and LfVP1-transformed yeast strains at different concentrations of NaCl and hygromycin. Molecular analysis of transgenic tobacco plants.

LfVP1 expressed in *ena1* mutant showed growth retardation at 1:100 dilution, whereas ena1; nhx1 mutant expressing LfVP1 showed no growth. An *Ena1* mutant deficient in functional sodium efflux pump showed only sensitivity to NaCl due to the presence of the functional *NHX1* gene. This functional *NHX1* gene detoxifies the effect of toxic cations such as Hygromycin, which is absorbed by the cell through an electrochemical proton gradient. *Ena1;nhx1* double-mutant strain, deficient in functional sodium efflux pump and vacuolar cation/H^+ antiporter (*NHX1*) gene, showed sensitivity to NaCl and hygromycin. WT yeast strains transformed with *LfVP1*, and an empty plasmid could suppress the hygromycin phenotype at 10, 25, and 50 µg/L hygromycin concentrations due to the presence of all functional genes involved in cation detoxification (Figure 2). However, relatively less growth was observed with an empty plasmid WT strain compared to WT strains

transformed with *LfVP1* constructs, which indicated the overexpression of the *LfVP1* gene. Yeast mutant ena1 also showed suppression of the hygromycin phenotype in all strains transformed with *LfVP1* and an empty plasmid in the presence of the functional *NHX1* gene. In the case of *ena1;nhx1* yeast mutant increased the net hygromycin uptake into the cell was increased by disturbing the sequestration into the vacuole in the absence of the functional *nhx1* gene. Therefore, no growth was observed in *ena1;nhx1* mutant strain transformed with *LfVP1* and an empty plasmid. The overexpression of H^+-*PPase* depends upon the functional chloride channel (Geg1) and *NHX1*. Results of complementation analysis on various concentrations of NaCl also showed differences in the growth of yeast mutants. In the case of WT yeast strains transformed with *LfVP1* and an empty plasmid, normal growth was observed at 0, 0.5, and 0.7 M NaCl. Overexpression of the *LfVP1* gene in *ena1* mutant strains showed tolerance to 0.5 and 0.7 M NaCl as compared to strains transformed with an empty plasmid. The *ena1;nhx1* mutant, deficient in both functional efflux pump and nhx1 gene, showed intolerance to different concentrations of NaCl when transformed with either *LfVP1* or empty plasmid due to deficiency of functional cation/H^+ antiporter gene (Figure 2).

To verify transgenic plants, the primers of the plant selectable marker bar gene were used. The Basta® leaf assay was also used to confirm the transgenic plants. In both attached and detached leaf assays (Figure 3(a)), transgenic plant leaves remained unaffected at 0.025% Basta® while WT plant leaves lost their chlorophyll. Amplification of the required 600 bp fragment showed that the putative transgenic crops were positive (Figure 3(b), lines 2–6) and no amplification was observed in WT (Figure 3(b), lane 7). In transgenic plants, RT-PCR showed amplification and gene expression. A 184 bp fragment from cDNA was amplified for *LfVP1* transgenes, but no amplification was observed in the WT plant (Figure 3(c)). The *actin* gene of the *Nicotiana tabacum* was used as an internal control. A 200 bp fragment of the *NtActin* gene was amplified from the cDNA of all transgenes as well as WT control tobacco plants. In *LfVP1*-PB1 and *LfVP1*-PB6 transgenes, 2 copies of the *LfVP1* gene were found in the southern hybridization of the *LfVP1*

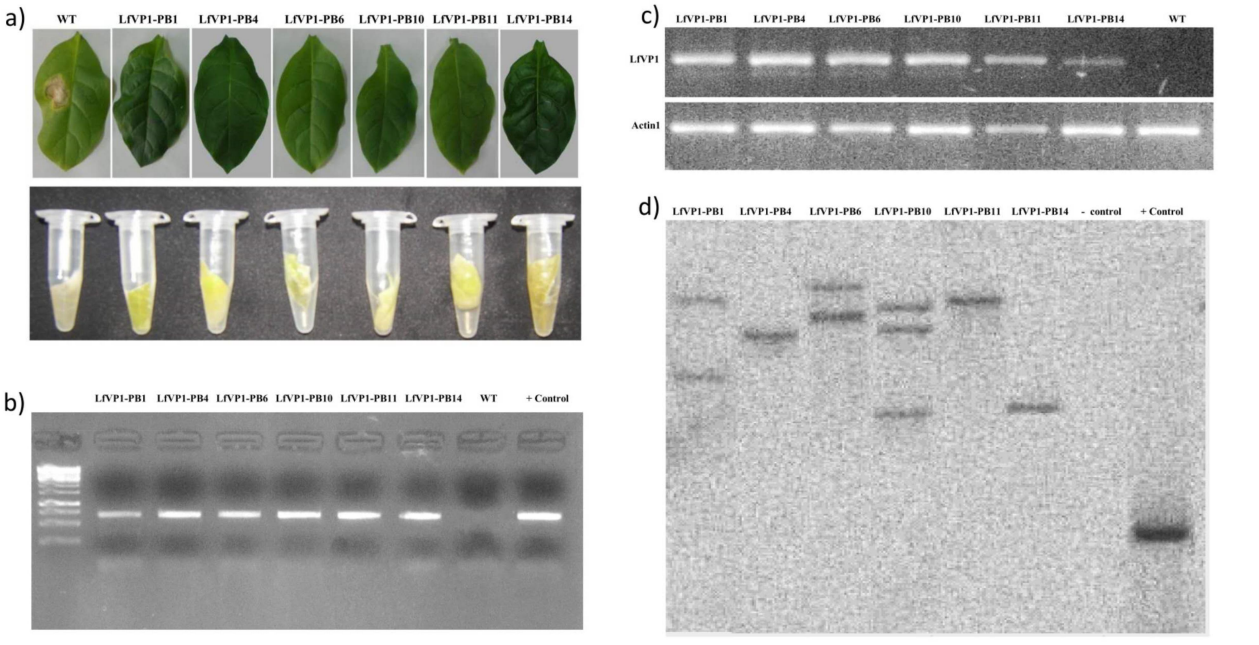

Figure 3. Confirmation of the putative LfVP1 transgenic tobacco plants through a) Basta® leaf paint assays. b) PCR using bar primers c) semi-quantitative RT-PCR d) Southern blot analysis.

transgenes (Figure 3(d)). In *LfVP1*-PB4, *LfVP1*-PB11, and *LfVP1*-PB14 transgenic lines, a single copy number was noted. Furthermore, the 3-copy integration was noticed in *LfVP1*-PB10, whereas integration of the *LfVP1* gene was not observed in WT (Figure 3(d)).

Effect of Osmotic Stress on the Growth of Transgenic Tobacco

To access the effect of osmotic stress on the growth of transgenic tobacco plants, drought stress for 7 days was applied to transgenic plants (*LfVP1*-PB4 and *LfVP1*-PB11) and WT plants (Figure 4(a)).

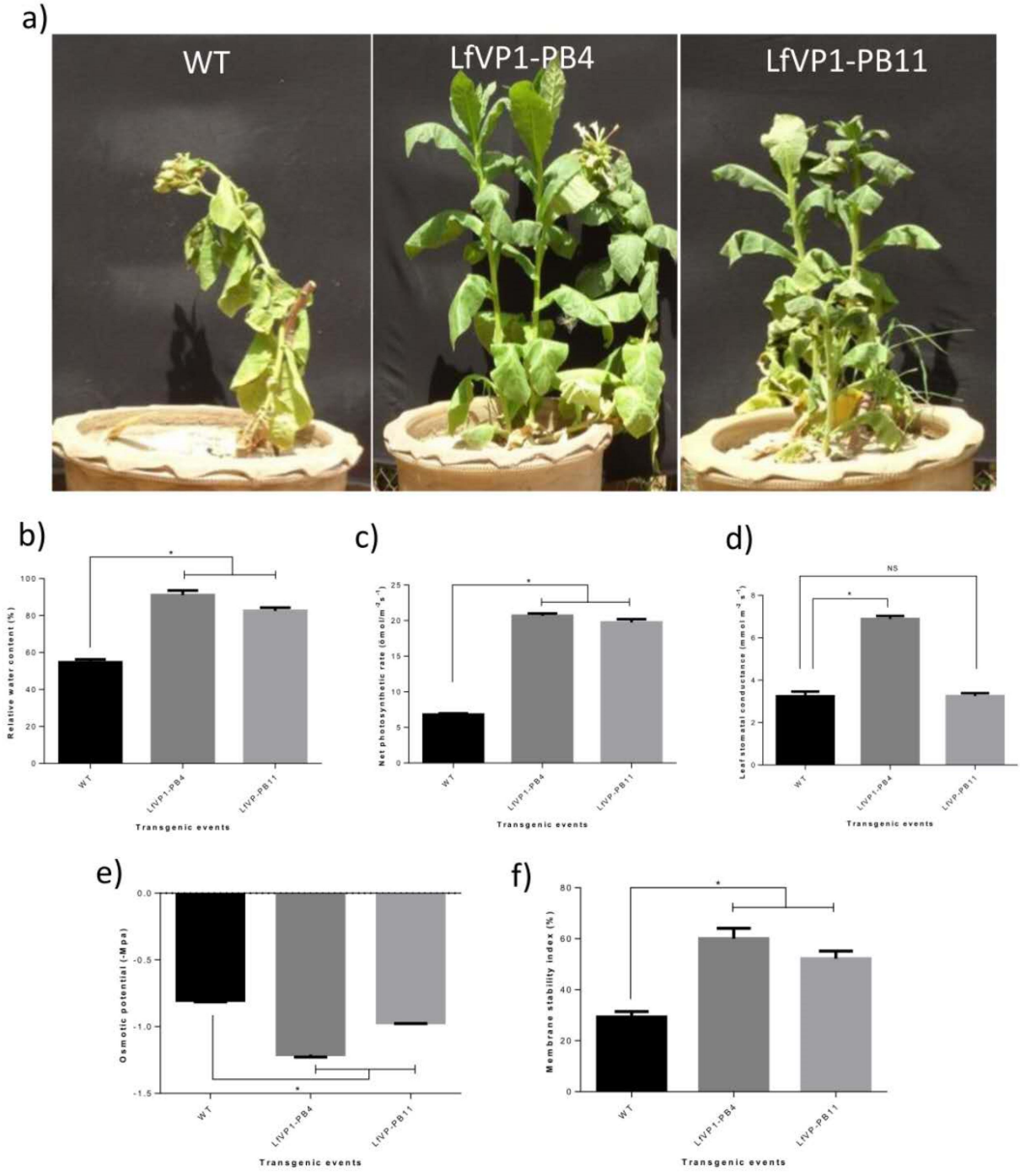

Figure 4. a) Growth of wild-type (WT) and transgenic plants (*LfVP1*-PB4 and *LfVP1*-PB11) in response to osmotic stress for 7 days. b) Relative water content RWC, b) photosynthetic rate, c) stomatal conductance, d) osmotic potential, and e) cell membrane stability index of WT and transgenic plants in response to osmotic stress. Bars represent the mean ± standard deviation.

Transgenic plants showed a very minor effect of osmotic stress, while the WT plants showed wilting. Furthermore, we measured different physiological parameters related to drought stress. The *LfVP1* transgenes showed significantly higher values of the difference between transgenic lines and WT plants for relative water contents (RWC). The *LfVP1*-PB4 and *LfVP1*-PB11 transgenic lines showed maximum RWC of 90% and 83%, respectively, compared to WT plants (53%) (Figure 4(b)). Similarly, statistically significant differences ($p < .05$) in the net photosynthetic rate were observed between transgenic lines and WT. The maximum photosynthetic rate was observed in the transgenic line *LfVP*-PB4, followed by *LfVP*-PB11 (Figure 4(c)). Due to the dedication, the minimum photosynthetic rate was observed in the WT plant. Leaf stomatal conductance was also observed with significant differences between transgenic and WT plants. The *LfVP1*-PB4 followed by the *LfVP1*-PB11 transgenic line showed the highest stomatal conductance compared to *LfVP1*-PB10 and WT plants (Figure 4(d)). Drought-tolerant plants can maintain their turgor at low water potential. Significant differences ($p < .05$) were observed among transgenic and WT plants. Most of the *LfVP1* transgenic lines (*LfVP1*-PB4 and *LfVP1*-PB6) showed a more negative value of osmotic potential compared to the WT plants (Figure 4(e)). Membrane stability index (MSI) for *LfVP1*-PB4 and *LfVP1*-PB11 lines was also significantly higher than WT plants (Figure 4(f)).

Characterization of Transgenic Plants for Herbicide and Salinity Tolerance

Transgenic seeds of the *LfVP1*-PB4 (Figure 5(a)), and control plants (Figure 5(b)) were germinated on MS_0 media supplemented with 2mgL^{-1} Basta® for herbicide resistance. Seeds of the WT plant were unable to survive and lost their chlorophyll; however, seeds of the transgenic line survived with normal root and shoot growth. Moreover, transgenic seeds of the *LfVP1*-PB4 and WT plant were also germinated on MS_0 supplemented with 200 mM NaCl. Transgenic plants showed better growth at 200 mM than WT plants (Figure 5(c)). The WT plants turned yellowish and de-shaped after a few days of germination (Figure 5(d)). Furthermore, we shifted seedlings of transgenic and WT plants to jars containing MS_0 with 250 mM NaCl. Transgenic plants maintained their normal growth (Figure 5(e)), while WT plants were unable to maintain their growth (Figure 5(f)).

In this study, the functional characterization of the *LfVP1* gene in yeast has significant implications in understanding cation detoxification mechanisms and enhancing stress tolerance in plants. The complementation analysis demonstrated that both *LfVP1* can suppress hygromycin susceptibility in yeast mutants, indicating their roles in cation transport and detoxification processes. These findings suggest that the overexpression of *LfVP1* enhances the ability of yeast strains to withstand osmotic stress caused by high NaCl concentrations. Moreover, the successful transformation of these genes in tobacco plants, along with the observed drought and herbicide tolerance, highlights the potential for using the *LfVP1* gene in developing transgenic crops that can thrive in challenging environments.

Discussion

Salinity and drought are major abiotic stresses that adversely affect plant growth and productivity. High salinity results in an increase in the concentration of sodium ions (Na^+) in the soil, which can lead to ion toxicity and osmotic stress. Drought, on the other hand, results in water deficit, which can lead to dehydration and osmotic stress. Plants have developed various mechanisms to cope with these stresses, including the regulation of ion homeostasis, osmotic adjustment, and stress signaling pathways. The overexpression of the H^+-pyrophosphatase gene (*H^+-PPase*) has been shown to confer drought and salinity tolerance in yeast and plants. This finding has important implications for improving the ability of plants to grow and thrive in harsh environmental conditions. Salinity and drought are major abiotic stresses that adversely affect plant growth and productivity. High salinity results in an increase in the concentration of sodium ions (Na^+) in the soil, which can lead to ion toxicity and osmotic stress. Drought, on the other hand, results in water deficit, which can lead to dehydration and osmotic stress. Plants

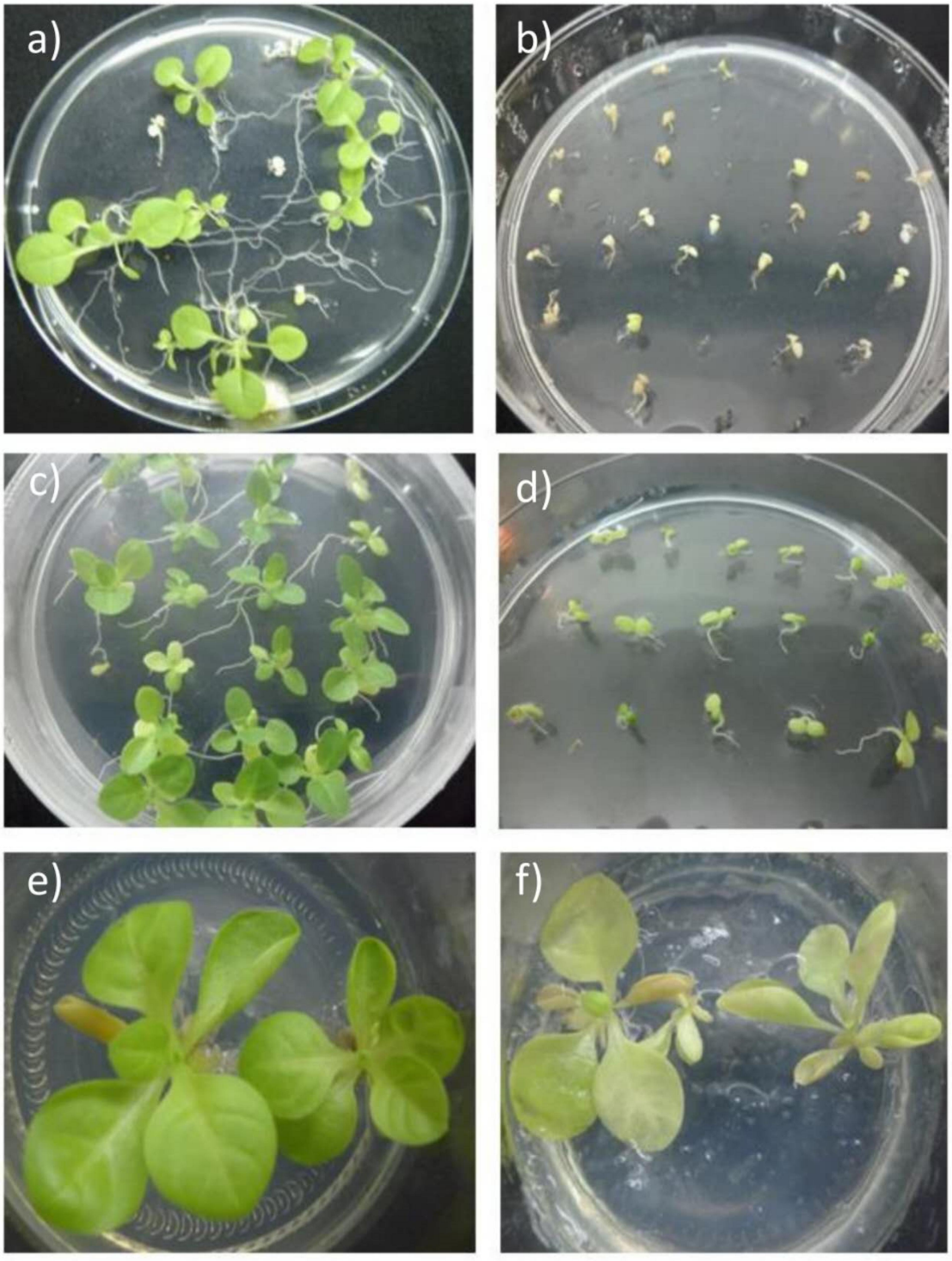

Figure 5. Germination response of transgenic and WT plants for herbicide and salinity tolerance. a-b) Germination of LfVP1-PB4 transgene and WT at 2mgL-1 Basta® for herbicide tolerance, c-d) germination of the transgenic and WT plant seeds at 200 mM NaCl, e-f) germination of transgenic and WT plantlets at 250 mM NaCl.

have developed various mechanisms to cope with these stresses, including the regulation of ion homeostasis, osmotic adjustment, and stress signaling pathways.

The wild-type (WT) yeast strain, equipped with all functional genes, exhibits a remarkable ability to survive across a range of concentrations of both hygromycin and NaCl. This resilience is largely due

to the coordinated action of various transporters and detoxification mechanisms present in the WT strain. In contrast, the *ena1* mutant, which lacks a functional sodium efflux pump, demonstrates a pronounced sensitivity specifically to NaCl. This vulnerability stems from the mutant's inability to effectively extrude sodium ions, resulting in toxic accumulation within the cell. However, the presence of a functional *NHX1* gene in *ena1* mutant allows for some level of detoxification of toxic cations, including hygromycin. The *NHX1* gene encodes a vacuolar cation/H+ antiporter that plays a critical role in maintaining ion homeostasis by facilitating the sequestration of toxic cations into the vacuole. This process is driven by the electrochemical proton gradient, which enables the cell to absorb hygromycin while simultaneously mitigating its toxic effects. Consequently, the contrasting responses observed in the WT and ena1 mutants highlight the significance of these transport mechanisms in cellular detoxification and ion regulation, offering valuable insights into the adaptive strategies employed by yeast under stress conditions.

Several studies have reported that overexpression of the *H^+-PPase* gene can increase salt tolerance in plants. For example, overexpression of the *AVP1* gene, which encodes the H^+-pyrophosphatase pump in *Arabidopsis thaliana*, significantly increased salt tolerance by reducing Na^+ accumulation in the shoot tissues.[42] In another study, the knockdown of the *HVP1* gene inhibited growth and increased Na^+ accumulation in barley, while its overexpression enhanced salt tolerance by improving Na^+ sequestration and K^+ homeostasis.[43] These studies suggest that the *H^+-PPase* pump plays an important role in regulating ion homeostasis, particularly Na^+ exclusion, which is crucial for plant adaptation to saline environments.

The H^+-PPase gene has also been shown to enhance drought tolerance in plants. For instance, overexpression of the maize H^+-pyrophosphatase gene in *Arabidopsis thaliana* and tobacco plants improved drought tolerance by increasing water use efficiency and reducing stomatal conductance. Similarly, overexpression of the *H^+-PPase* gene from the halophyte *Salicornia europaea* in *Arabidopsis thaliana* improved drought tolerance by increasing photosynthetic efficiency and reducing oxidative stress. These studies suggest that the *H^+-PPase* pump plays a key role in regulating water balance and stress responses under drought conditions. Moreover, in yeast, overexpression of the *H^+-PPase* gene has been shown to increase tolerance to high salt and osmotic stress, indicating that the *H^+-H^+-PPase* pump is conserved across eukaryotic cells and plays a similar role in regulating ion homeostasis and pH balance. Collectively, these findings highlight the multifunctional role of *H^+-PPase* in enhancing plant resilience to both drought and salinity stress, thereby contributing to improved agricultural productivity in challenging environmental conditions.

The primary pathway for Na^+ extrusion in yeast is mediated by *ena1*, which is a plasma membrane *Na^+ ATPase*. The yeast mutant strain *ena1* lacks the plasma membrane sodium efflux pump, relying on an internal detoxification system to overcome sodium toxicity. The phenotype of the yeast mutant strain *ena1*, which lacks the plasma membrane sodium efflux pump and relies on an internal detoxification system to overcome sodium toxicity. The *ena1* mutant shows increased sensitivity to sodium and impaired growth in high-salt conditions, and these phenotypes can be rescued by overexpression of the *ena1* gene or by supplementing the growth medium with potassium. The growth of the *ena1* strain is sensitive to a low concentration of sodium, whereas the growth of the wild-type yeast strain is not inhibited by such low NaCl concentrations. Hygromycin also acts as a toxic cation that enters the cell in response to the electrochemical proton gradient.[44] Mutant strains lacking a functional *NHX1* gene are sensitive to even lower concentrations of NaCl and Hygromycin. Based on a previous report,[17] to use the *ena1* yeast mutant strain to determine the function of the plant vacuolar *H^+-PPase*, we employed wild-type, *ena1*, and *ena1;nhx1* mutants to study functional complementation analysis by transforming the *Gal1-LfVP1* gene construct. Our results showed that the wild-type yeast strain had normal growth under different concentrations of hygromycin and NaCl. However, *ena1*, with overexpression of the *LfVP1* gene, conferred partial tolerance to hygromycin and NaCl compared to an empty plasmid *ena1* mutant (Figure 2). These

results are in accordance with Gaxiola et al.,[16] who reported hetero-expressions of vacuolar *H*$^+$*-PPase* gene in yeast mutant *ena1*, which could partly suppress its hypersensitivity to NaCl. Similarly, the overexpression of *TsVP1* restores the salt tolerance of the salt-sensitive *ena1* mutant in a similar way to *AVP1*. In their study, the *ena1* mutants carrying the pYES2-TsVP or pYES2-AVP1 plasmids showed much better growth than the *ena1* mutants with empty pYES2 plasmids.[15,45] In the present work, overexpression of *LfVP1* could restore the salt tolerance of the salt-sensitive *ena1* mutant in a similar way to *AVP1*. We initially characterized the function of *AmVP1 and TsVP1*, which partially restored the NaCl tolerance of ena1. Moreover, in the case of *ena1;nhx1* mutant, overexpression of *LfVP1* did not confer resistance even at a low concentration of hygromycin and NaCl. It indicates that the wild-type yeast strain, with all functional genes, can detoxify the toxic effects of hygromycin and Na^+. The *ena1* is involved in the movement of toxic Na^+ out of the cell. Therefore, the *ena1* yeast mutant is sensitive to even a low concentration of NaCl. In the absence of the *ena1* gene, it must rely on the internal detoxification system for the exclusion of Na^+ from the cytoplasm into the vacuole by the action of the Na^+/H^+ antiporter gene. Therefore, overexpression of the *LfVP1* gene in *ena1;nhx1* mutant could not complement even at low concentrations of NaCl and hygromycin, mainly due to the absence of a functional Na^+/H^+ antiporter gene.

Hygromycin, a cationic antibiotic, is toxic to cells upon accumulation in the cytosol. The basis for *hyg* hypersensitivity of the yeast nhx1 mutant is linked with a reduction in NHX-mediated sequestration of the toxic cations in the yeast vacuole.[46] Previously, Gaxiola et al.[16] reported that heterologous expressions of vacuolar H^+-pyrophosphatase genes in yeast mutant *ena1* could partly suppress its hypersensitivity to NaCl. A similar method was employed in this study to characterize the function of the LfVP1 gene, which partially restores the NaCl tolerance of ena1 (Figure 2). In this study, the *LfVP1* gene was found to be involved in suppressing the Na+ hypersensitivity of *ena1*. All *ena1* mutants, along with the WT yeast, grew on YPGAL medium with no added NaCl; however, only *ena1* mutants with *LfVP1* cDNA grew in the presence of NaCl and Hygromycin (Figure 2). Our results with *LfVP1* are consistent with prior studies of *AVP1* expression in yeast,[17] suggesting that *LfVP1* is also an *H*$^+$*-PPase* pump localized to the vacuolar and/or pre-vacuolar tonoplast membrane. Moreover, our leaf bioassay results showed that putative transgenic plants were tolerant to Basta® compared to WT (control) plants (Figure 4). The Basta® leaf paint assay was used to select transgenic plants containing the *bar* gene as a plant selectable marker, as has been utilized in previous studies for transgenic plant production.[47–49]

To study the role of the *LfVP1* gene for drought tolerance, *LfVP1* transgenic and WT tobacco plants were subjected to osmotic stress. The WT control plant showed rapid leaf desiccation and growth inhibition compared to transgenic lines (overexpressing *LfVP1)*. Transgenic lines showed higher net photosynthetic rate, relative water contents, stomatal conductance, a more negative value of leaf osmotic potential, and higher cell membrane stability index compared to control plants. These results are per the previous studies on the *AVP1* expressing transgenic Arabidopsis plants.[50,51] Low or negative leaf osmotic potential values in this study indicate that plants have adapted to osmotic stress due to effective osmoregulation under drought stress. Moreover, tolerant plants maintain higher stomatal conductance and increased cell growth at low osmotic potential.[45] Furthermore, we collected the T1 seeds of the *LfVP1*-PB4 transgenic plants and performed a germination test at MS_0 supplemented with $2mgL^{-1}$ Basta®. Transgenic tobacco plants exhibited better growth on Basta® while WT plants did not maintain their survival after germination (Figure 5). Transgenic plants were also segregated for wilting after osmotic stress. Seeds of the *LfVP1*-PB4 transgenic plants were also able to grow and maintain their growth under salinity stress of various concentrations (100, 200, and 250 mM NaCl). The reason for salt stress tolerance in these transgenic plants could be due to the increased activity of *H*$^+$*-PPase* under salinity stress.[52] Furthermore, longer root and shoot growth were observed in the transgenic plants (*LfVP1)* compared to WT plants. A larger and longer root system would make it easier for plants to absorb nutrients and, in turn, promote plant growth under different conditions. Similarly,

transgenic plants also performed better under drought and stress compared to WT plants in terms of higher leaf area and leaf water potential, which could be due to an enhanced osmotic adjustment in the transgenic plants.[45]

Conclusion

The overexpression of the H^+-pyrophosphatase (*H^+-PPase*) gene has been shown to enhance drought and salinity tolerance in both yeast and plants, indicating its important role in ion homeostasis and pH regulation in plant cells. The *H^+-PPase* encodes for a proton pump that utilizes the energy derived from the hydrolysis of inorganic pyrophosphate (PPi) to transport H^+ ions across the plasma membrane. This pump plays a crucial role in the maintenance of the electrochemical gradient across the membrane, which is essential for various physiological processes, including stress responses. This research could pave the way for agricultural advancements, particularly in areas facing salinity and drought stress, ultimately contributing to food security and sustainable farming practices. Further studies could explore the application of these findings in other crop species, enhancing their resilience to environmental factors under changing climatic conditions.

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Author contributions

CRediT: **Muhammad Rauf:** Conceptualization, Data curation, Writing – original draft; **Khurram Shahzad:** Formal analysis, Methodology, Writing – original draft; **Imran Habib:** Data curation, Formal analysis, Writing – original draft; **Badr Alharthi:** Funding acquisition, Methodology, Writing – review & editing; **Sajid Fiaz:** Methodology, Writing – review & editing; **Mehwish Kanwal:** Data curation, Formal analysis, Writing – original draft; **Moddassir Ahmed:** Data curation, Formal analysis, Resources; **Rashid Ali:** Formal analysis, Methodology, Writing – original draft; **Gerald A Berkowitz:** Methodology, Writing – original draft; **Asjad Ali:** Methodology, Writing – original draft; **Seung Hwan Yang:** Funding acquisition, Writing – review & editing; **Nasir A. Saeed:** Methodology, Writing – original draft.

Disclosure Statement

Authors declare absence of any potential conflict of interest.

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Data Availability Statement

Data can be made available on reasonable request to the first/corresponding author of the study.

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