



Full Length Article

Barley Vacuolar Pyrophosphatase (HVPI) Gene Confers Salinity Tolerance in Locally Adapted Wheat (*Triticum aestivum*)

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Abstract

Vacuolar H⁺-pyrophosphatase (VPI) gene is an efficient proton pump that develops a proton gradient to promote Na⁺/H⁺ antiporter activity. Thus, it plays vital role in conferring salinity tolerance in plants. This study was planned to evaluate the performance of *Hordium vulgare* vacuolar H⁺-pyrophosphatase (HVPI) gene in wheat (*Triticum aestivum*). Incorporation and expression of HVPI gene in wheat lines under maize ubiquitin (*UBI1*) promoter was confirmed through antibiotic selection, polymerase chain reaction, reverse-transcription polymerase chain reaction (RT-PCR) and Southern hybridization. Seeds of transgenic wheat exhibited better germination rate compared to non-transgenic when subjected to salt stress. Augmented HVPI expression in transgenic wheat plants improved their physiological performance compared to non-transgenic. Overexpression of HVPI in transgenic wheat plants also promoted the agronomic performance under saline field conditions (EC 16 dS m⁻¹; SAR 40.5; pH 7.82–8.92) compared to non-transgenic. Higher values of germination rate, plant height, spike length, number of spikelets per spike, 1000 grain weight, grain yield and harvest index were observed in transgenic wheat during 2017–2018 growing season at BSRI, Pakka Anna, Faisalabad. An increase of about 29% in grain yield of transgenic wheat compared to non-transgenic in saline field conditions highlights the performance of HVPI as an affective cation pump that promotes active transport of toxic salts in vacuoles. © 2019 Friends Science Publishers

Keywords: Genetic potential; Proton pump; Antiporter; Transgenic wheat; Osmotic potential; Vacuoles

Introduction

Agriculture plays a crucial role in the rural economy of Pakistan and shares 18.9 in GDP. A major proportion of population (64%) is engaged in one way or another to this sector (Pankaj and Ramyar, 2019). Wheat production has a significant positive relation to the agricultural GDP (Rehman *et al.*, 2015) as evident from its 1.7 percent contribution to GDP and 9.1 percent in the value added agriculture. The average yield of wheat is just 2.7 ton ha⁻¹ (Rahman, 2012), which is far below as compared to other wheat producing countries. Many factors contribute to low yield of wheat. Losses caused by abiotic stresses are immense. Among these abiotic stresses, salinity is the most important environmental anomaly as it can hamper productivity in both arid and semi-arid regions (Katerji *et al.*, 2009). Agricultural setbacks in the form of loss in production and decline in soil fertility cannot be accurately determined, however, there is no second opinion about the fact that these agro-ecological anomalies such as salinity affect a large land area and

contribute towards a significant loss to crop production (Cramer *et al.*, 2011). In future, ecological changes will have a significant impact on sea level, which has a direct role in increasing the salinity of surface and underground water sources. Therefore, it is evident that salinity will increase in subtropical areas (FAOSTAT, 2016). Unfortunately, in Pakistan, much of the climate is tropical or sub-tropical and it is already suffering from salinity (10% slightly saline, 4% moderately saline and 7% strongly saline soils (Qureshi, 2016). Only due to moderate saline soils, about 65% of wheat yield has been compromised (Shafi *et al.*, 2010). Therefore, it is imperative to develop high yielding stress tolerant crop varieties through new breeding strategies, genomics and genetic techniques of genome modifications.

Salinity stress either limits the entry of water into the plants or increases the accumulation of toxic ions in cytoplasm. It is critical for plants to maintain ion homeostasis in case of salt stress for normal physiological functioning. Plant cells respond to salinity stress by acidifying their vacuoles through proton pump H⁺-PPase

(H⁺-translocating inorganic pyrophosphatase) (Maeshima, 2000). This proton pump has characteristics comprising of single polypeptide (molecular mass of about 80 kDa), uses low cost substrate pyrophosphatase and efficient proton pump coexisting in vacuolar membrane with H⁺-ATPase (Maeshima, 2000). These characteristics of H⁺-PPase make it physiologically important for plants (Yu *et al.*, 2017). Among three classes of PPases, only H⁺-PPase has the ability to transport the protons across membrane (Maeshima, 2000).

H⁺-PPase is of two types. Type I and II are present on vacuolar membrane and golgi apparatus respectively (Belogurov and Lahti, 2002). Paez-Valencia *et al.* (2011) also confirmed its presence on plasma membrane. Cytosolic K⁺ influences the activity of type I H⁺-PPase (Maeshima, 2000; Belogurov and Lahti, 2002), while type II remains unaffected (Segami *et al.*, 2010). H⁺-PPase also known as V-PPase (Zhao *et al.*, 2016) acts as proton pump on tonoplast and plays crucial role in H⁺ electrochemical gradient. Numerous reports have been published highlighting increased expression levels of H⁺-PPase gene in tomato, tobacco, maize, sugarcane and cotton (Park *et al.*, 2005; Gao *et al.*, 2006; Li *et al.*, 2008; Kumar *et al.*, 2014; Zhao *et al.*, 2016) under stress conditions. In nature, wheat vacuolar pyrophosphatase does not over-express under salinity stress (Brini *et al.*, 2005). While, in barley, salt stress increases expression level of vacuolar pyrophosphatase gene in roots and leaves (Fukuda *et al.*, 2004) which helps it to cope salt stress efficiently. The relative salt tolerance of wheat crop is 5.5 dSm⁻¹ (Zörb *et al.*, 2019) while barley has threshold salinity level of 8 dSm⁻¹ (Vos *et al.*, 2016). Therefore, in nature, barley is relatively more tolerant to salinity stress and can be used for genetic improvement of other crops. The performance of barley *HVPI* gene has not been studied in any major crop including wheat.

The aim of this study was to clone barley *HVPI* gene under monocot specific *UBI1* promoter, transformation in wheat (cv. Ujala-2016) and expression under saline field conditions to explore the potential of the *HVPI* gene. These findings will open up a future horizon for utilizing the genetic potential of *HVPI* gene in other major crops to confer salinity tolerance to ensure food security under present climate change scenario.

Materials and Methods

Development of *HVPI* Plasmid Construct

Total RNA from tissues of *Hordeum vulgare* (Golden promise) was isolated by using RNase trizole reagent method (ThermoFisher Scientific cat # 15596026) as outlined by (Rio *et al.*, 2010). Full length cDNA of vacuolar pyrophosphatase gene (*HVPI*) (Accession number AB032839.1) was amplified by PCR using gene specific primers. The forward primer and the reverse primer were

designed from the 5' upstream and 3' downstream region of the gene respectively. The amplified product of PCR was purified using PCR purification kit (Thermo Fisher Scientific cat # K0701) and integrated into plant expression vector pTOOL37 downstream of the maize ubiquitin promoter (*UBI1*) and nopaline synthase (NOS) terminator. The plant expression vector containing *HVPI* gene cassette under constitutive *UBI1* promoter was confirmed by restriction analysis using *HindIII* and *SpeI* restriction endonucleases.

Wheat Transformation with *HVPI* Gene

The pTOOL37-*HVPI* binary vector was transferred into *Agrobacterium tumefaciens* strain *AGL1* and was used to transform wheat (*Triticum aestivum* cv. Ujala-2016). Successful transformation of *HVPI* gene in *Agrobacterium tumefaciens* was confirmed through PCR by using promoter specific (*UBI-F* 5'-CGACCTGTACGTCAGACACG-3'; *UBI-R* 5'-TGCATATGCCATCATCCAAG-3') and marker gene (Hyg) specific primers (Hyg-F 5'-GGCGACCTCGTATATTGGGAAT-3'; Hyg-R 5'-ACCGCAAGGAATCGGTCAAT-3'). Seeds of local wheat cultivar Ujala-2016 were sown under controlled conditions of temperature (25°C) and light (16 h light/8 h dark) in pots. After 2 weeks of anthesis initiation, immature seeds were collected and sterilized according to established protocol (Almeida *et al.*, 2004). Immature embryos were excised and cultured on MS callus induction media (Murashige and Skoog, 1962). After one week of culturing, explant embryos were shifted at MS₀ medium and half were inoculated with *Agrobacterium tumefaciens* harboring *HVPI* vector and half without vector supplemented with 400 mM Acetosyringon. After 2 h incubation period in dark at 28°C, excess *Agrobacterium* was blot dried. These embryos were sub-cultured at MS media + 400 mM Acetosyringon plates and incubated at 28°C. After 3 days, inoculated embryos were sub-cultured to MS medium containing 200 mg L⁻¹ timentin antibiotic to prevent *Agrobacterium* from further growth. Inoculated embryos were subsequently cultured after every 15 days till root elongation.

Antibiotic Selection Assay of Putative Transgenic Plants

Two weeks after *Agrobacterium* inoculation wheat calli were shifted to regeneration medium. For preliminary assessment of putative transformed calli, regeneration medium was supplied with 200 mg L⁻¹ hygromycin antibiotic. Plantlets with developed roots were shifted to soil in small pots. For further screening of transgenic plants antibiotic susceptibility assay was performed. Putative transgenic T₀ plants in pots were subjected to 200 mg L⁻¹ hygromycin antibiotic in two ways; first by using cotton swab the surface of attached leaves was painted with hygromycin antibiotic; second the detached leaves of

putative transgenic and non-transgenic (NS) wheat plants were cut and placed in 1.5 mL Eppendorf tubes (Rauf *et al.*, 2014) filled with MS liquid medium supplemented with 200 mg L⁻¹ hygromycin antibiotic. After two weeks, tissue necrosis in case of former and changes in green color of leaves in later method were visually observed and scored. Furthermore, seeds harvested from putative transgenic T₀ generation were harvested and grown on MS medium containing 200 mg L⁻¹ hygromycin antibiotic and 200 mM NaCl to check their germination potential under selection pressure.

Genomic DNA Isolation and PCR Confirmation of Transgenic Plants

Genomic DNA of putative transgenic and NT wheat plants was extracted using modified CTAB method (Zhou and Lin, 2002) and analyzed for DNA concentration and purity on NanoDrop (ND-2000, Thermo Scientific). To check the presence or absence of *HVPI* gene in each plant, a PCR reaction of 100 ng genomic DNA using *HVPI* gene specific primers was conducted at following conditions; Initial denaturation at 95°C for 2 min; 34 cycles of denaturation at 94°C for 20 s; annealing at 60°C for 30 s and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. PCR reaction mixture contained 10X Standard Taq reaction buffer (Fermentas), 1.5 mM MgCl₂, 2.5 mM dNTPs, 0.2 μM forward and reverse primers and 1.25 unit of hot start Taq DNA polymerase. Gel electrophoresis of PCR product was carried out with 1% agarose gel documentation and imaging system.

RNA Isolation and Semi-quantitative Reverse-transcription Polymerase Chain Reaction (RT-PCR)

To check expression of *HVPI* gene under the constitutive expression of *UBI1* promoter, total RNA was isolated from fresh leaves of transgenic plants of T₁ generation by using Trizole reagent method (Invitrogen, Cat # 15596-026) followed by DNase treatment to remove any DNA contamination. STRATGENE AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Catalog #200820) was used for cDNA synthesis. 1 μL of cDNA was used for semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) using *HVPI* gene specific primers (mentioned above).

Southern Blot Hybridization

Copy number of *HVPI* insert in positive transgenic wheat plants was assessed through Southern hybridization following the standard procedure (Sambrook *et al.*, 2001). Approximately 10 μg of genomic DNA was digested by using *HindIII* and *SpeI* fractioned on 1% agarose gel and transferred to nylon membrane. The blot was then hybridized with DIG labelled probe produced by integration

of DIG-11-dUTP into *UBI1* template through PCR DIG probe synthesis. The remaining hybridization steps were performed with DIG high prime DNA Labelling and Detection Starter Kit I (Roche Applied Sciences, Germany).

Physiological Performance of Transgenic Plants

For characterization of *HVPI* transgenic lines, salinity stress was applied to T₃ transgenic and NT plants. Non-porous plastic pots were filled with naturally saline soil (EC 16 dS m⁻¹; pH 7.82–8.92) from Biosaline Research Station (BSRS) Pakka Anna. Three replicates of 5 independent transgenic events alongside non-transgenic control having five seeds per pot were sown. To maintain high level of salinity, plants were subjected to 200 mM NaCl. A 50 mM NaCl was added to pots after three days interval until salt concentration reached to 200 mM NaCl, plants were irrigated afterwards with distilled water. At anthesis stage transgenic and non-transgenic wheat plants were phenotypically and physiologically examined. Data regarding leaf temperature, relative humidity, quantum energy absorbed, durable resistance of leaf, temporary resistance of leaf, (not shown), stomatal conductance, transpiration rate, photosynthesis, and water use efficiency (shown) was recorded by using AP4 Leaf Porometer.

Biochemical Analysis of Salt Tolerant Transgenic Wheat

High level of Na⁺ accumulation in vacuole is a key factor in conferring salinity tolerance to plants. So, in order to investigate the difference of Na⁺ accumulation in transgenic wheat and non-transgenic wheat plants, 5 cm of leaf samples from transgenic and non-transgenic wheat grown in saline field conditions were collected before reproductive stage, air dried, cleaned and then placed in an oven for 24 h. Then leaves were ground and 1 g fine powder was put in a flask. 5 mL of concentrated H₂SO₄ was added to flask for digestion of leaf sample. Further, 1 mL of H₂O₂ was added to flask at 240°C until sample was turned transparent. Discolored sample was kept at room temperature for a period of two hours followed by adding the distilled water to make total volume 50 mL. Concentrations of sodium were determined by using standard protocol of flame atomic emission spectrometry (Ghanem *et al.*, 2010).

Agronomic Performance of Transgenic Wheat under Saline Conditions

To evaluate the performance of transgenic wheat lines expressing *HVPI* in saline field conditions, an open field study of homozygous T₃ transgenic lines (TW1, TW2, TW3, TW4) and non-transgenic (NT) was conducted during the session 2017-2018 at Biosaline Research Station (BSRS) Pakka Anna, Faisalabad. Soil having EC 12–16 dS m⁻¹; pH 7.82–8.92) and irrigation water with EC 4.97 dS m⁻¹, SAR 40.5, pH 8.2 were chosen for characterization study. The

field trial was planted in completely randomized block design (RCBD) with three repeats having 45 cm row spacing, 9 cm seed spacing and 0.75 cm seed depth. Two lines of each transgenic wheat line with twenty seeds alternating with two lines of NT wheat plants were sown for comparative study. Standard agronomics practices were carried out for weed control along with spray of weedicides. Fertilizer was applied @ 100-90-75 NPK kg ha⁻¹ using urea, DAP and sulphate of potash as nutrient sources. Whole P and K and half dose of N was applied as basal dose while remaining N was applied in two splits. Performance of transgenic lines in agronomic traits such as germination percentage (%), plant height (cm), spike length (cm), number of spikelets per spike, grain weight (g), grain yield (ton ha⁻¹) and harvest index (%) were compared with NT.

To evaluate the performance of transgenic *HVP1* wheat in field, significant difference and comparison between individual data regarding physiological and agronomic traits was determined by ANOVA (Statistics 8.1) at LSD, $P < 0.05$.

Results

Construction of Plant Expression Vector Containing *HVP1* Gene

The *HVP1* gene was cloned in binary vector pTOOL37. The *HindIII* and *SacI* restriction sites in maize ubiquitin promoter (*UBI1*) and NOS terminator were added to the *HVP1* gene. A 1146 bp fragment of *HVP1* cDNA was successfully cloned into pTOOL37 vector under *UBI1* and NOS terminator (Fig. 1A). The integration of gene cassette was validated by restriction digestion with *HindIII* and *SacI* endonucleases. This restriction digestion analysis of pTOOL37-*HVP1* pDNA liberated ~ 2.8 kb and 9.2 kb fragments (Fig. 1B). This vector comprising *HVP1* was transformed into *AGL1* strain of *Agrobacterium tumefaciens* followed by validation through PCR by promoter specific and marker gene specific primers Fig. 1 (C and D).

Selection of Transformed Tissues on Antibiotic Medium

After transformation of wheat callus with *Agrobacterium* having *HVP1* gene, tissues were cultured on the MS medium containing 200 mg L⁻¹ hygromycin along with untransformed callus as negative control. About 100 plantlets originated from independent inoculated calli. Among those, about 30 were phenotypically similar to parental variety and established roots in rooting medium. Further, 14 lines were screened for their ability to survive on selection medium. No regeneration was observed in untransformed callus on antibiotic selection medium that was used as negative control.

Antibiotic Susceptibility Assessment by Leaf Paint Method

Transgenic (TW) and non-transgenic (NT) wheat plants

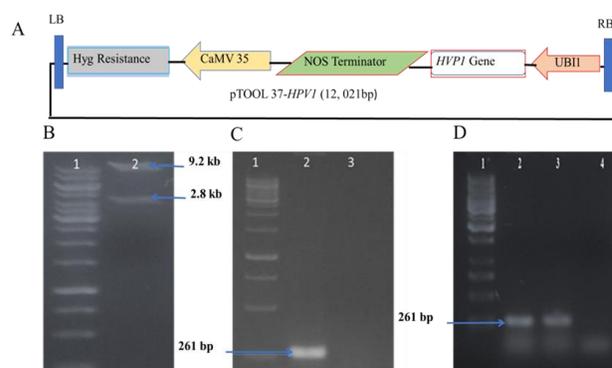


Fig. 1: A: Strategy for cloning of *HVP1* gene under *UBI1* promoter and NOS terminator in binary vector for transformation of wheat. B: Confirmation of successful cloning of *HVP1* gene under maize ubiquitin promoter (*UBI1*) and NOS terminator in the pTOOL37 vector. Restriction digestion with *HindIII* and *SpeI* gave two fragments of 2.8 kbp gene and 9.8 kb vector backbone in comparison with 1 kb DNA Ladder (Fermentas). Lanes 1 is 1kb Ladder and Lane 2 is digested clone that liberated two fragments. C: PCR confirmation of transformed *Agrobacterium tumefaciens* strain using maize ubiquitin (*UBI1*) promoter specific primers that gave band of 261 bp. Lane 1 is 1kb ladder; Lane 2 is transformed culture of *Agrobacterium tumefaciens*; Lane 3 is negative control (water). D: PCR confirmation of transformed *Agrobacterium tumefaciens* strain using Hyg gene specific primers and gave product size of 260 bp. Lane 1 is 1kb ladder; Lane 2 and 3 is transformed culture of *Agrobacterium tumefaciens*; Lane 4 is negative control (water)

exhibited distinct response when subjected to antibiotic application at T₁ generation. Leaves of 14 transgenic plants remained green two weeks of post leaf painting with 200 mg L⁻¹ hygromycin whereas non-transgenic leaves showed necrosis. Marked lesions and brownish color was evident in NT (Fig. 2A). In case of second method of leaf antibiotic susceptibility assay leaves of transgenic and NT plants were immersed in MS medium supplied with 200 mg L⁻¹ hygromycin. Non-transgenic leaf segments could not survive in antibiotic solution and showed necrosis while leaves of four transgenic wheat plants remained unaffected (Fig. 2B). Additionally, only putative transgenic wheat plants germinated when T₁ seeds were grown on MS medium containing 200 mg L⁻¹ hygromycin and 200 Mm NaCl. No germination was observed in seeds of NT Ujala-2016 (Fig. 2C).

Confirmation of Putative Transgenic Wheat Plants

Wheat (*Triticum aestivum* cv. Ujala- 2016) was used for the *Agrobacterium tumefaciens* mediated transformation with pTOOL37-*HVP1* vector regulated by constitutive *UBI1* promoter and NOS terminator. Molecular confirmation of transgenic T₁ lines surviving on antibiotic selection medium was done through PCR of genomic DNA and semi-quantitative reverse transcriptase polymerase chain reaction through *UBI1* in primers. PCR confirmed the expression of

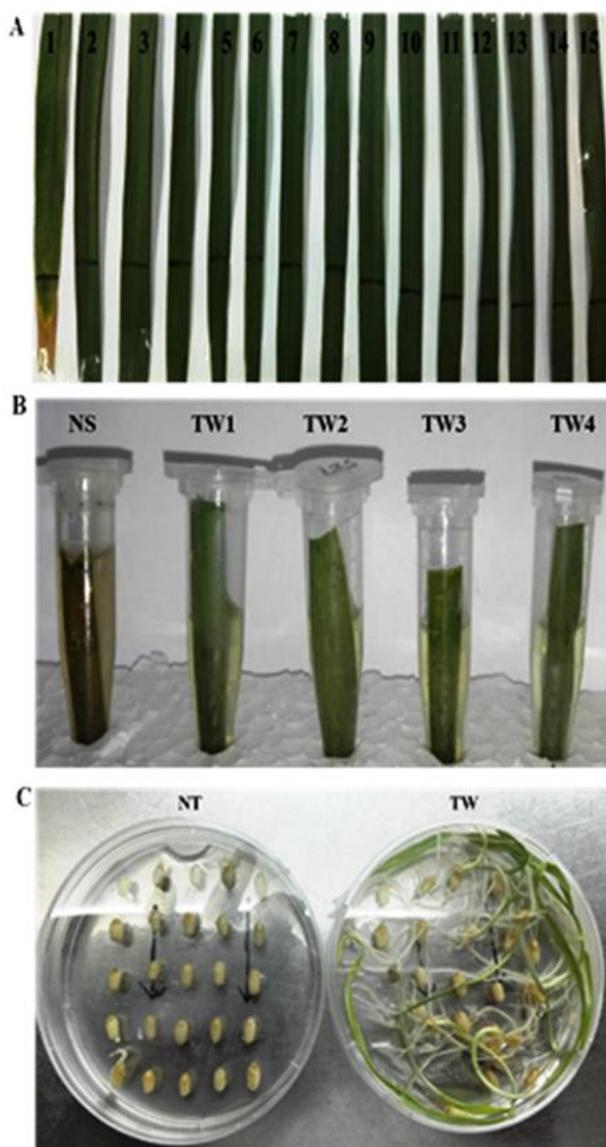


Fig. 2: Antibiotic susceptibility assessment of transgenic wheat plants expressing *HVP1* gene and non-transgenic wheat plants. **A:** Leaf paint assay using 200 mg L⁻¹ hygromycin; 1 is Non transgenic (NT) while lanes 2-15 are putative transgenic (TW) plants. **B:** Detached leaf dip assay of non-transgenic and putative transgenic wheat plants using 200 mg L⁻¹ hygromycin. NT is non-transgenic; lane TW1-TW4 are transgenic wheat lines showing tolerance to hygromycin. **C:** Antibiotic and salt stress assay of T1 seeds grown on MS medium containing 200 mg L⁻¹ hygromycin and 200 mM NaCl; Non transgenic (NT); Putative transgenic (TW) seeds transformed with *HVP1* gene

HVP1 gene in 11 out of 14 wheat lines that survived on antibiotic selection medium (Fig. 3A). For additional validation at posttranscriptional level, a RT-PCR was performed that confirmed expression of *HVP1* in four transgenic lines (Fig. 3B). Southern analysis of transgenic lines expressing *HVP1* and NS (cv. Ujala- 2016) exhibited successful integration of gene in four transgenic lines while

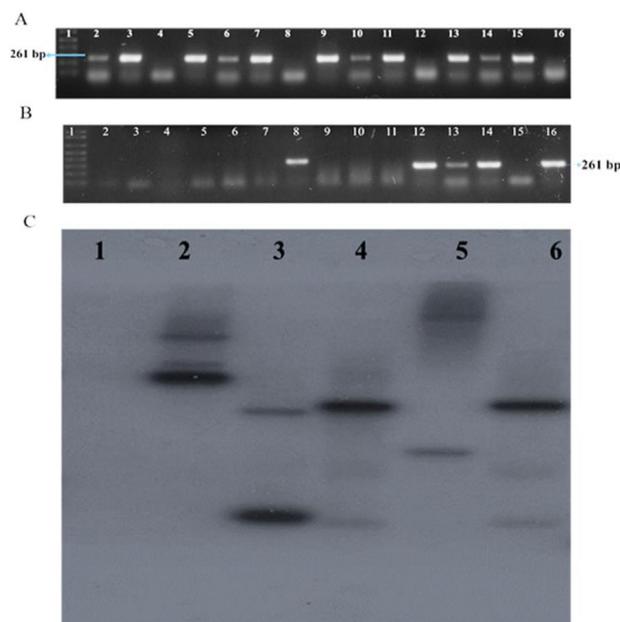


Fig. 3: **A:** PCR confirmation of T1 transgenic wheat lines by using maize ubiquitin (*UBI1*) primers. Lane 1 is DNA ladder (Fermentas); lanes 2, 3, 5, 6, 7, 9, 10, 11, 13, 14, 15 are transgenic wheat plants; Lane 4, 8, 12 are segregating nulls; Lane 15 is positive control (plasmid); Lane 16 is non-transgenic wheat plants. A 261 bp fragment was amplified in transgenic wheat lines while no amplification was observed in segregating nulls and wild type. **B:** Expression analysis of DNA fragment of 261 bp using reverse transcriptase PCR (RT-PCR) with maize ubiquitin (*UBI1*) primers. Lane 1 is DNA ladder. Lane 2, 3, 4, 4, 5, 6, 7, 9, 10, 11 are segregating nulls. Lane 8, 12, 13, 14 are transgenic wheat expressing *HVP1*. Lane 15 was Non-transgenic wheat plant and lane 16 was positive control (plasmid). **C:** Southern blot analysis of transgenic wheat plants expressing *HVP1* gene and non-transgenic wheat. Lane 1 is non-transgenic wheat plants; lane 2 is positive control; lane 3–6 are transgenic wheat plants

no insert copy was detected in NT (Fig. 3C). Molecular validation of four transgenic lines showed successful integration of *Hordium vulgare* vacuolar pyrophosphatase (*HVP1*) gene in wheat genome. These four transgenic lines were used in all later studies for assessment of their performance in field conditions.

Physiological Analysis of *HVP1* Transgenic Plants

Transgenic lines (designated as TW1, TW2, TW3, TW4) and non-transgenic (NT) plants at T₂ generation showed marked difference in their growth pattern when subjected to salinity stress in sealed plastic pots containing saline soil. Transgenic plants were phenotypically better in plant height as compared to NT plants (Fig. 4A). Significant differences ($P < 0.05$) regarding all measured physiological parameters were observed between transgenic wheat and NT wheat plants. Transgenic wheat plants expressing *HVP1* gene maintained higher rate of photosynthesis when compared to NT plants (Fig. 4B). The *HVP1* expressing wheat plants

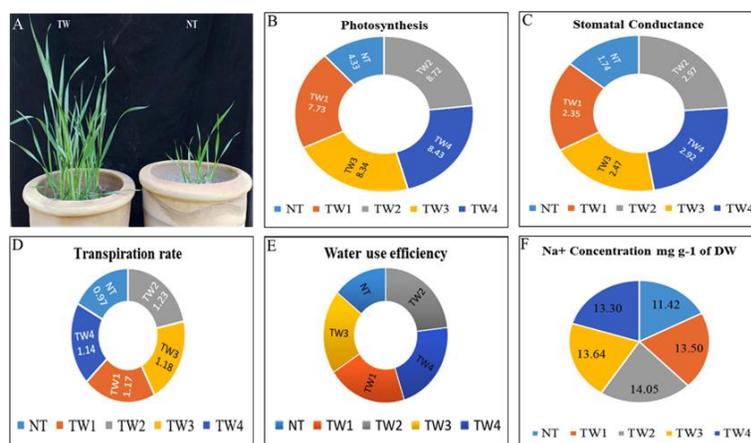


Fig. 4: A: Plants expressing *HVP1* gene (TW) showed better performance in pots filled with saline soil and irrigated with 200 mM NaCl water as compared non-transgenic (NT) wheat. Physiological analysis of T2 transgenic plants (TW1, TW2, TW3, TW4) expressing *HVP1* gene and non-transgenic (NT) wheat subjected to salinity in pots. The response was measured in terms of, B: photosynthesis ($\mu\text{mol m}^{-2} \text{s}^{-1}$), C: stomatal conductance ($\text{mmol m}^{-2} \text{s}^{-1}$), D: transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$), E: water use efficiency ($\mu\text{mol m}^{-2} \text{s}^{-1}$), F: Na^+ concentration (mg g^{-1} of DW)

also possessed significantly higher stomatal conductance, transpiration rate and water use efficiency as compared to NT plants Fig. 4 (C, D and E). Overall, our study in pot experiment showed that *HVP1* expression in transgenic wheat plants render them more tolerant to salinity stress that help them to ameliorate the negative effects of salinity on their physiology and consequently all yield related parameters.

Sodium Accumulation under High Salinity

The cellular sodium concentration of the transgenic wheat plants was significantly higher than non-transgenic plants under saline field conditions. 23% more Na^+ accumulation was observed in TW2 compared to non-transgenic wheat plants (Fig. 4F). This result indicates that *HVP1* expression confers salt tolerance to transgenic wheat plants *via* ion homeostasis through enhanced Na^+ accumulation capacity.

Agronomic Traits Assessment of Transgenic Wheat Plants in Saline Field Conditions

Transgenic T₃ wheat plant (TW1, TW2, TW3, TW4) expressing *HVP1* gene and NT wheat (cv. Ujala-2016) plants were grown in a saline field (BSRS, Pakka Anna). All four transgenic wheat plants expressing *HVP1* gene showed almost double germination rate as compared to NT wheat plants. Highest germination was recorded in TW2 with germination percentage of 91.3% followed by 89% in TW4 while NT wheat only showed 48.7 percent germination rate. Transgenic wheat TW1 and TW3 showed 80.7 and 83.3 percent germination respectively (Fig. 5A). Data regarding plant height showed approximately 24% increase in TW2 as compared to NT wheat plants. TW1, TW3 and TW4 exhibited plant height of 80.2 cm, 79.1 cm and 80.7 cm as compared to 66.2 cm in case of NT (Fig. 5B). The results

showed that spike length and number of spikelets per spike demonstrated 9–14% and 28–32% relative increase respectively in plants expressing *HVP1* gene as compared to NT Fig. 5 (C and D). About 20 and 15% respective increase in 1000 grain weight and harvest index was recorded in transgenic plants as compared to NT Fig. 5 (E and F). Grain yield of transgenic wheat was also found to be about 29% higher than non-transgenic wheat plants (Fig. 5G). These results emphasize the fact that transgenic plants expressing *HVP1* gene showed better performance through better ion homeostasis resulting in reduction of stress induced injury.

Discussion

Among major environmental stresses soil salinity is one of the most crucial constraint contributing major reductions in cultivated land area and hence crop productivity (Yamaguchi and Blumwald, 2005; Shahbaz and Ashraf, 2013; Al-Khaishany *et al.*, 2018). Use of stress tolerance enhancing genes from distinct genetic backgrounds into major crops has promising prospect in future. Transgenic cotton expressing *AVPI* gene showed significantly improved tolerance to salt stress (Pasapula *et al.*, 2011). To check whether use of vacuolar pyrophosphatase gene improves the salt stress tolerance in wheat under saline field conditions, transgenic wheat plants expressing *HVP1* gene were generated. As barley is considered a salt tolerant crop with threshold salinity level of 8 ds m^{-1} (Vos *et al.*, 2016), vacuolar pyrophosphatase gene was isolated from barley and transformed into wheat through *Agrobacterium tumefaciens* method. Among three different vacuolar pyrophosphatase genes reported in barley (Shavrukov, 2014), *HVP1* gene was selected for wheat transformation as it encodes amino acids sequence site DVGADLVGKVE, a potential site for H^+ -PPase activity and EYYTS motif

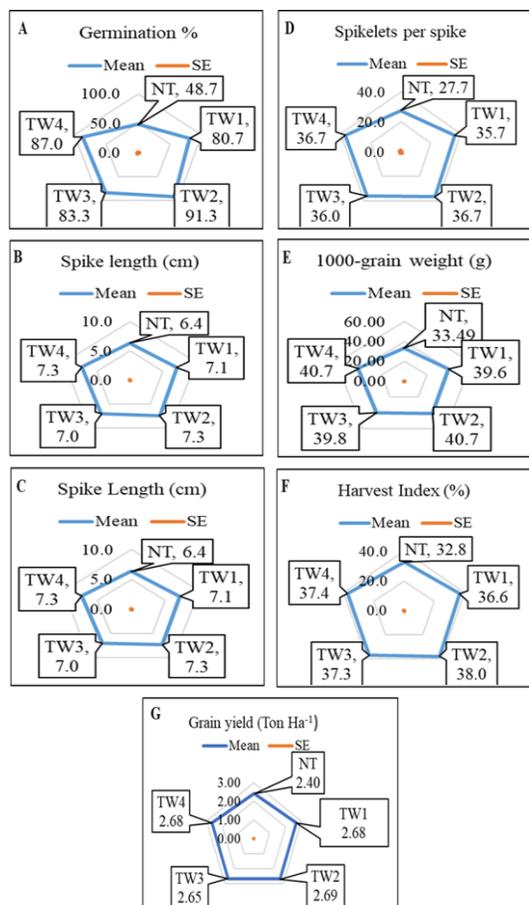


Fig. 5: Agronomic performance of T3 transgenic plants (TW1, TW2, TW3, TW4) expressing *HVPI* gene and non-transgenic (NT) wheat plants under saline field conditions at BSRS in year 2017-1018. **A:** Difference in germination rate (%), **B:** plant height (cm), **C:** spike length (cm), **D:** spikelet per spike, **E:** 1000 grain weight (g), **F:** harvest index (%), **G:** Grain yield (Ton Ha⁻¹)

involved in proton pumping (Brini *et al.*, 2005). Further *HVPI* was cloned under maize *UBI1* promoter who significantly enhances gene expression (Zhang *et al.*, 2016).

As expected, *HVPI* expressing transgenic wheat plants grown under salt stress performed much better than NT wheat plants (Fig. 4A). This result was consistent with previous reports in which vacuolar pyrophosphatase gene rendered better performance in plants under salt stress. Rice plants expressing *AVPI* gene grown under paddy field conditions have shown greater biomass production and grain yield (Kim *et al.*, 2014). Additionally, transgenic barley expressing *AVPI* gene resulted in higher biomass production (Schilling *et al.*, 2014). However, to our knowledge there is no previous study investigating the role of vacuolar pyrophosphatase gene from barley (*HVPI*) in wheat.

In this study, *HVPI* transgenic wheat subjected to salinity stress in green house conditions performed better in physiological attributes as compared to NT wheat plants Fig. 4 (B, C, D and E). The better performance of transgenic

wheat plants in pot experiment complimented the results of field study presented in this study. Higher rate of germination in transgenic wheat plants expressing *HVPI* as compared to NT wheat plants laid the foundation for better yield attributes (Fig. 5A). Highest germination rate was observed in TW2 followed closely by other three transgenic wheat lines however, NT wheat plants with germination rate of only 48.7% could not germinate well under salt stress. The higher germination potential of transgenic plants expressing *HVPI* gene under maize ubiquitin promoter (*UBI1*) is attributed to its role as proton pump that provides force required to prevent K⁺ loss and Na⁺ exclusion through enhanced SOS1 activity (Bose *et al.*, 2013). However, transgenic wheat plants under salt stress showed greater Na⁺ concentration in cellular tissues compared to non-transgenic plants. Elevated levels of Na⁺ in leaf tissues is potentially harmful if retained in cytosol, but plants can mitigate its detrimental effects by promoting its transport to vacuole. This was perhaps the possible reason that enabled the transgenic wheat plants to perform better under saline field conditions compared to non-transgenic plants. Previously, higher accumulation of Na⁺ in transgenic rice expressing *AVPI* (Kim *et al.*, 2014) and in *Arabidopsis thaliana* plants expressing *TVPI* (Brini *et al.*, 2007) has been reported. These reports also laid emphasis on that vacuolar pyrophosphatase gene present on tonoplast acts as an electro-chemical pump that promotes the uptake of Na⁺ in the vacuole.

Transgenic lines expressing *HVPI* gene showed edge in important yield related parameters when grown in saline field conditions Fig. 5 (B, C and D). This was in agreement with previous study in barley, where expression of *AVPI* showed significant higher shoot area as compared to NT and segregating nulls (Schilling *et al.*, 2014). The better performance of transgenic plants in above mentioned parameters laid the foundation for improvement in other yield attributes like 1000 grain weight grain yield and harvest index. The previous reports also augmented the fact that vacuolar pyrophosphatase activity enhances production in cotton and rice (Pasapula *et al.*, 2011; Kim *et al.*, 2014).

The difference in growth attributes between transgenic and NT plants is likely the outcome of the difference in their photosynthesis. Transgenic plants expressing *HVPI* gene maintained higher photosynthesis rate than NT under 200 mM NaCl stress (Fig. 4B) which explains the difference in yield and harvest index. Efficient sequestration of Na⁺ in vacuole could be the likely reason for enhanced photosynthesis in transgenic plants expressing *HVPI* gene. Salt stress affects stomatal conductance through reduction in turgor pressure of guard cells (Dionisio-Sese and Tobita, 2000) that ultimately leads to reduction in photosynthetic rate of non-transgenic wheat. This difference in photosynthesis could be the potential reason of higher grain yield in transgenic wheat plants as compared to non-transgenic wheat (Wang *et al.*, 2016).

Moreover, salt stress reduces the transpiration rate at

critical stages of salt sensitive rice cultivars (Moradi and Ismail, 2007) Therefore, higher stomatal conductance and transpiration rate in transgenic wheat plants could be the reason of their better performance in saline field conditions as compared to NT plants Fig. 4 (C and D). The better performance of transgenic plants in physiological and agronomic traits as compared to NT could be attributed to the expression of *HVPI* gene in them. Fukuda *et al.* (2004) reported increased transcript levels of *HVPI* under salt stress that enhances pyrophosphatase activity and hence confer salinity tolerance in plants. Failure of NT to cope salt stress could be best explained by taking into account the low transcript level of vacuolar pyrophosphatase gene under salt stress in roots and leaves (Brini *et al.*, 2005).

Conclusion

The expression of *HVPI* gene can confer the salinity tolerance to otherwise salt susceptible wheat. Interestingly, there is no prior report suggesting the use of *HVPI* gene in conferring salinity tolerance to cereal crops other than barley itself. This is the first time that the role of *HVPI* has been explored in wheat for salinity tolerance and it additionally revealed that water use efficiency of transgenic wheat expressing *HVPI* gene was higher than NT under salinity stress. This laid the road for maximizing the potential of *HVPI* gene for improving tolerance against other abiotic stresses such as drought and terminal heat shock.

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