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Original Article

Assessment of *Solanum lycopersicum SlPI-II* gene under *OsRGLP2* promotor against salt and drought stress in transgenic wheat plants

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Abstract

Rapid change in global environmental conditions will increase the severity of salinity and drought stresses. Wheat (*Triticum aestivum* L.) is a major staple crop worldwide and its production is affected by these two major abiotic stresses. Use of breeding and transgenic techniques facilitated the development of tolerant varieties able to withstand in drought and saline environments. This work investigated the role of *Solanum lycopersicum* proteinase inhibitor II (PI-II), *SlPI-II*, gene for mounting tolerance under salinity and drought stress in transgenic wheat. Significant increase of 6.41-fold (TL1) was observed in transgenic plants at 1 day post 100m NaCl treatment. A gradual increase in the expression was observed at 200 mM NaCl treatment. At 300mM treatment, 3.78-fold (TL5) increase in the expression level was recorded. The maximum fold change of 5.01 and 3.99 was observed in 6 days post treatment at 100 mM and 200 mM in TL1, respectively. The extended saline treatment (14 days post treatment) in transgenic lines resulted the higher *SlPI-II* induction than control wheat plants which showed the efficacy of this gene in salinity tolerance. *SlPI-II* expression in 15% PEG treated plants showed transcript up to 7.31 fold increase during 1 day post treatment, while at 25% PEG transgene expression ranged from 3.45 to 2.27 fold increase. Conclusively, the role of *SlPI-II* gene in drought tolerance was due to sustained transcript increment during PEG treatment. We demonstrated that modern day transgenic approaches utilizing plant-based proteinase inhibitors will provide the new opportunities for the development of tolerant varieties in future.

Keywords: Wheat, NaCl, Proteinase inhibitor, Transgenic wheat, PEG6000

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Introduction

Defense related molecules play an important role in overcoming various biotic and abiotic factors. Such molecules are released in plant bodies in different scenarios in which one of the important aspects is the secretion of defense related material upon exposure of a particular stress. One of such defense related products includes plant proteinase inhibitors (PIs), which are known to play a significant role during salinity, drought and herbivory by interacting the proteases by targeting their active sites. The proteolytic activity of different plant-based proteinase inhibitors was reported in different researches (Fan et al., 2019; Benbow et al., 2019; Malefo et al., 2020; Xie et al., 2021; Li et al., 2022). Plant proteinase inhibitors which were identified from the *Arobidopsis, Fabaceaea* and *Nicotiana glutinosa* (NGP1-1) were found to be active during wounding and herbivory attack. The cysteine proteinase inhibitor from chestnut (*CsC*) was induced during heat, salinity and cold stress (Pernas et al., 2000). The plant proteinase inhibitors regulate various physiological process including developmental growth, macromolecular trafficking, program cell death and particular tissue accumulation in plant organs (Xu et al., 2001; He and Kermode, 2003; Fischer et al., 2008; Tamhane et al., 2012).

Wheat (*Triticum aestivum* L.) is a staple food crop fulfilling 25% of the global caloric requirements (Al-Otayk et al., 2019). The extremes in the climate change episodes recorded during the last three decades caused the 5.5% loss of global wheat production (Lobell et al., 2011). Continuous global environmental conditions will increase the severity of salinity and water deficit conditions (Tester and Langridge, 2011) which ultimately affect food availability (Tester and Langridge, 2010). It has been estimated that the required demand for wheat will increase by 60% by 2050. Drought stress is one of the important yield limiting factors which affect grain filling stage causing the reduction in kernel weight and grain yield (Zampieri et al., 2017). Use of breeding and transgenic methods have widen the horizons for development of crops with better yield and high tolerance against various biotic and abiotic stresses in wheat. The potential evidence of proteinase inhibitor genes in order to impart tolerance under abiotic stresses, was reported from various transgenic studies (Gaddour et al., 2001; Kidrič et al., 2014; Li et al., 2015).

Transgenic tobacco plants harboring the *NtPI* gene exhibited the tolerance against saline conditions (Srinivasan et al., 2009). A *PI* gene was characterized from rice which showed the role in drought tolerance (Huang et al., 2007). The tissues expression of *PI* was reported to be increased under abiotic stresses which showed it regulation via signaling networks in response to stress conditions (ABA, Jasmonic acid and ethylene) (Peña-Cortés et al., 1995; Fujita et al., 2006). Barley proteinase inhibitor (*HvPI*) also showed positive response under the drought stress (Gaddour et al., 2001).

The PIs belong to the super families which further include specific and non-specific classes, serine, cysteine and aspartic proteinase inhibitors, metalloproteinase inhibitors. Various PIs are sorted based on amino acids and classes of proteinases they target and inhibit (Rawlings at el., 2004). Serine and trypsin proteinases are inhibited by Kuntiz (KTIs; soybean) and Bowman-Brik families. The largest family of PIs includes serine PIs which are found in cereal, potato I, squash, and potato II (Haq et al., 2004). The serine PI-II endogenously regulates the developmental and stress responses in the *Solanaceae* (Sin et al., 2006).

Recently, the role of Germin-like proteins was studied in wheat for biotic stress resistance (Ilyas et al., 2020; Sun et al., 2021; Yuan et al., 2021. Recent research work based on rice showed the role of *OsRGLPs* (rice root germin like protein 2) against various biotic and abiotic stresses. The rice root germin like protein 2 promotor characterized by in-silico analysis in case of pathogenicity was tested in tobacco along with *Solanum tubeorsum* (Mahmood et al., 2007, 2013; Munir et al., 2016). In the current research work the expression of *Solanum lycopersicum* PI-II (*SlPI-II*) driven by a *OsRGLP2* promotor (wound inducible promotor) was observed in transgenic wheat plants under two major wheat affecting abiotic stress which are, salinity and drought.

Material and Methods

Plant material and vector for transformation

For transformation, the model spring cultivar Fielder was used as the recipient of target gene. Fielder was grown under controlled environmental conditions with 16 h light and 8 hours dark period at 20 ± 5 with 60 % humidity. Initially the plants were germinated in small 3L pots with various ration of peat moss, sand and green compost (2:1.5:2). The pots were checked for insects and fungal attack. After germination, the

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booting stage was observed, and all the spikes were tagged upon anthesis. After careful observation, the immature embryos were collected after 14 days post anthesis from the detached spikes of parent plant in the form of wheat kernels. The kernels were then excised for the isolation of immature embryos for the transfection process. Grains were surface sterilized (15% sodium hypochlorite followed by 70% ethanol) and extracted immature embryos were then preserved in 1.5ml microcentrifuge tubes containing liquid Murashige and Skoog (MS) medium along with 100 *μM Acetosyringone. The transforming vector* pCAMBIA1391Z comprising *Solanum lycopersicum* proteinase inhibitor-II *(SlPI-II)* gene fused with GUS driven by *OsRGLP2* promotor was used for the transfection procedure (Mahmood et al., 2013; Rehman et al., 2017) via Agrobacterium strain EHA101 (AC: AY007240).

Wheat transformation and confirmation of integration

The *Agrobacterium* mediated transformation was performed by the protocol followed by Ishida et al. (2015) with slight modifications for wheat cv. Fielder transformation via immature embryos. The transformed embryos were then shifted to series of shooting, rooting and regeneration medium to produce healthy plantlets. Transformed plants were used to confirm integration of target transgene by the PCR amplification with gene specific primers. For this purpose, the DNA was extracted using Richards et al. (1997) with slight optimized changes. Furthermore, the DNA was quantified using Colibri Spectrometer (Berthoid Detection systems, Germany). Gene specific primers were used for *SlPI-II* and hygromycin resistant gene to confirm the transgenic plants with sequence as follows, F: 5′-TATCCATCATGGCTGTCCAC-3′, R 5′-AACACACAACTTGATCCCCACA-3′ and F: 5′-GCTCCATACAAGCCAACCAC-3′, R: 5′-CGAAAAGTTCGACAGCGTCTC-3′. The amplification was performed at 95°C for 5 minutes which was followed by 30 cycles of denaturation for 1 minutes at 94°C then the annealing of primers was done at 64°C for of 1 minute and extension at 72°C prolonged for 1.5 minutes and then the extension at 72°C for the time interval of 10 minutes. The amplified product was confirmed by 1.5% agarose gel. Each confirmed generated plantlet was considered as the homozygous lines which were confirmed by PCR amplification of *SlPI-II* and hygromycin resistant gene. These positive plants (T_0) were then used to generate the T_1 progeny. The totals of eight transformed wheat lines (TL1 to TL8) were used to understand the drought and salinity responses.

Plant preparation for abiotic stress

The seeds of wheat plant were surface sterilized using 10% NaClO followed by washing with distilled water. Initially the seeds of transgenic wheat plant and control were placed in petri dishes for the germination (The Whatman filter paper). The petri plates were kept under controlled environmental conditions, which are 55±5% humidity, 18°C and darkness. After the seeds germination the plates were exposed to light of 57–65 $\mu E \cdot m^{-2} \cdot s^{-1}$ to develop chlorophyll. Each petri plate was added with Hoagland solution which prevented the germinating seedling from desiccation. The plants at 3-5 leaf stage were used for the stress treatment.

Salinity and drought stress treatment

The Hoagland solution with half strength was used for the application of salinity and drought stress in transgenic and control wheat plants. The composition of the solution included 0.5 mM KH_2PO_4, 2.5 mM KNO_3, 1 mM $MgSO_4$, 2.5 mM $Ca(NO_3)_2.4H_2O$, Fe-EDTA solution. The micronutrients were also added, 0.9 µM $ZnSO_4.7H_2O$, 0.5 µM $CuSO_4.5H_2O$, 7.1 µM H_3BO_3, 0.0016 µM $(NH4)_6Mo_7O_{24}.H_2O$, 7.3 µM $MnCl_2.4H_2O$, and 0.5 µM $Ni(NO_3)_2.6H_2O$. The hydroponic application required continuous aerated system which was adjusted and renewed for keeping the nutrient content of hydroponic solution. To understand the expression regulation of *SlPI-II* gene under salinity and drought, the transgenic plants were treated with different strengths of salt including 100 mM, 200mM, 300Mm within Hoagland solution. For the drought stress 6000PEG was added within the nutrient media with 0%, 15% and 25% concentrations. Leaves were collected from the stressed applied plants after 1, 6 and 14 days post treatment for RNA extraction. Five replicates were used for each treatment.

RNA extraction and cDNA synthesis for expression analysis

The expression analysis of target gene was accessed by qRT-PCR. For this purpose, total RNA was extracted from the independent transgenic wheat lines. The RNA extraction was performed with TRIZOL reagent (Invitrogen, CA, USA) according to

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manufacturer protocol along with slight modifications (Chomczynski and sacchi, 2006). Furthermore, NanoDrop NDLite was used for RNA quantification. TRUE cDNA synthesis Kit was used to synthesize cDNA (ZOKEYO, Wuhan, China**.** The qPCR was performed by using ten times diluted cDNA via CFX96 real-time PCR detection system (BIO-RAD, California, US). The total reaction mixture of 5 µl was prepared along with Genious 2.5 µl of 2x SYBER Green Fast qPCR Mix (ABclonal, CAT# RM21204), 0.1 µl of each primer, prepped cDNA, ROX water about 1 µl and 1.3 µl of water (PCR). Actin primers forward- (5/ GCTGGAAGGTGCTGAGGGA -3/) and reverse- (5/ GCATCGCCGACAGGATGAG -3/) was used control. The qPCR program comprised of a total of 40 cycles under 95°C for the time interval of 30 secs , 95°C for 10 secs, and 60 °C for about 30 secs and 72 °C was given for 10 seconds. The analysis of variance was calculated on $2^{-\Delta\Delta CT}$ values using Statistix 8.1. The data means within specific response were measured with LSD (least significant difference test) with threshold probability LSD ($p<0.05$).

Results

Induction of *SlPI-II* gene under salinity stress

The regulation of *SlPI-II* gene in response to salinity was monitored during 1, 6 and 14 days of post treatment via qRT-PCR at the 100mM, 200mM and 300mM NaCl treatments (Figure 1a, b, and c). For the purpose of expression analysis, the RNA was extracted from transgenic and control plant with specific stress treatment at 1-, 6- and 14-days post treatment. The recorded fold change values resulted in positive regulation of target gene. At 1 day of 100mM, fold change values were 4.21 for TL4 and 6.41 for TL1. At 200 mM, the gradual increase in the expression was observed, the folds change values showed by TL3 was 3.44 and TL1 was 3.99 compared to the control was 0.36. At 300 mM, TL5 had 3.78-fold higher expression levels while the least values were observed for TL6 as 1.23 which indicated decline in tolerance against salinity stress.

The transgenic lines TL5 and TL8 initially showed 5.82 and 5.41-fold increase, but declined to 3.78 and 2.21-fold increase at 300 mM after one day post saline treatment. At 6 days post stress, fold change values for TL1 were 5.01 and 3.99 at 100 mM and 200mM, respectively, while 1.71-fold increase was observed at 300 mM. The transgenic line TL4 showed the least fold change values at 100 mM and 200 mM as 3.04 and 2.0, respectively. The transgenic line TL7 showed higher expression with 4.0 and 3.92-fold increase at 100 mM and 200 mM NaCl, respectively. The gene expression in wild-type was recorded as 0.1 and 0.07 at 100 mM and 200 mM NaCl, respectively. The prolonged exposure of NaCl for 14 days resulted in induction of *SlPI-II* with fold change values ranging from 3.6 to 2.8 at100 mM NaCl. At 200 mM, fold increase was 2.32 and 1.8 at14 days post stress in TL3 and TL5, respectively.

Effect of drought stress treatment

In the current study, the *SlPI-II* gene expression under osmotic stress in transgenic and wild-type wheat plants was studied with treatment of PEG6000 at 1-, 6- and 14-days (Figure 2a, b, and c). The *SlPI-II* gene expression showed the significant fold increase values ranging from 7.31 for TL1 and 5.44 for TL3. The increase in *SlPI-II* transcript during stress indicated the positive gene regulation in wheat plants. The wheat plants including TL1, TL3 and TL5 showed the significant increase in expression at 15% PEG6000 application. At the 25%PEG treatment, the expression level ranged from 3.45 to 2.27. The 3.45-fold increase was observed in TL3 as compared to wild-type which was observed as 0.27. The fold increase values were significant at 15% PEG treated transgenic lines as compared to 25% PEG at 1 day post treatment. In case of 6 days post treatment, the TL1 showed 4.41-fold increase while TL6 showed 3.76-fold increase. At 25% PEG, the fold increase was recorded from 3.61 to 1.71 as in TL4 and TL6, while control exhibited 0.28-fold increase. The extended treatment for 14 days showed varied expression among wheat plants, the transcript increment was relatively lower compared to 1- and 6-days post treatment. The expression was recorded in TL6 as 3.56 and in TL4 was 3.24 and TL2 showed 2.15-fold change values as compared to control with 0.19 levels. TL1 showed 2.92 level and TL8 with 2.47 during 25% PEG at 14 days post stress for mounting defense against drought conditions.

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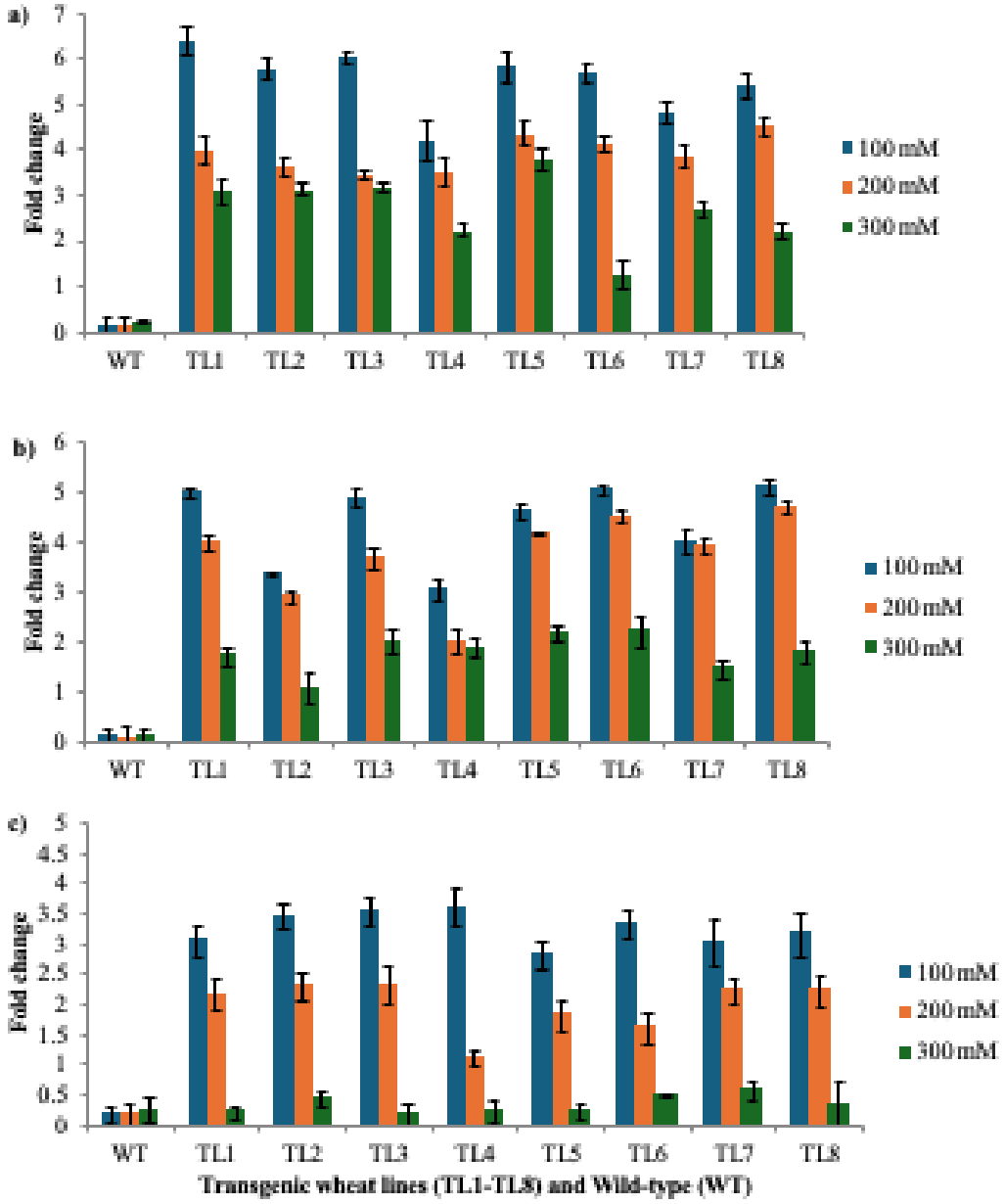

Figure-1. *SlPI-II* gene expression under salinity stress. Each graph represent the transcript level of *SlPI-II* gene under 100mM, 200mM and 300mM NaCl. a) Graphs show the fold change in gene expression for 1 day post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8), b) Graphs show the fold change in gene expression for 6 days post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8), and c) Graphs show the fold change in gene expression for 14 days post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8).

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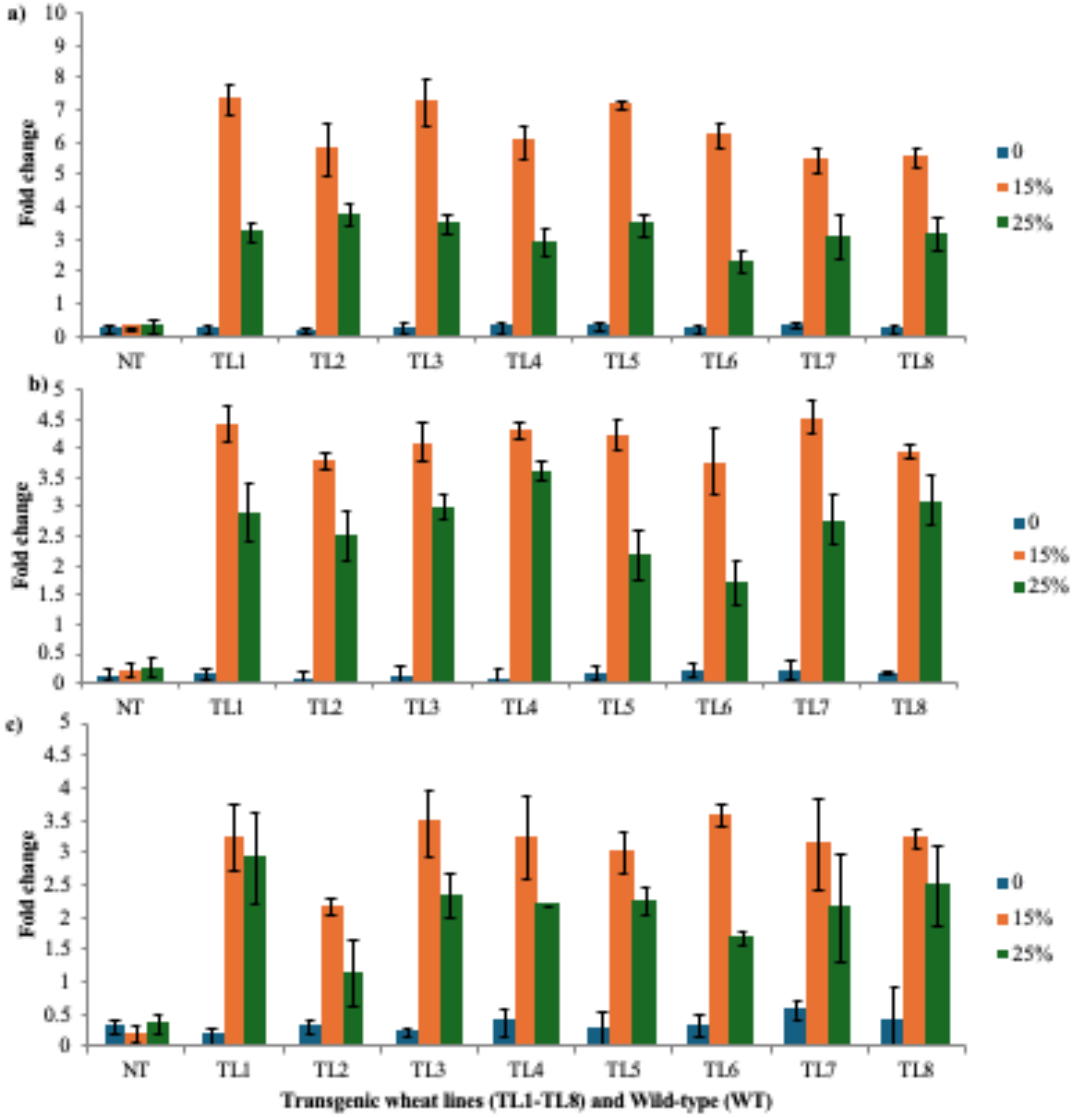

Figure-2. *SlPI-II* gene expression under salinity stress. Each graph represent the transcript level of *SlPI-II* gene under 0%, 15 % and 25% PEG 6000. a) Graphs show the fold change in gene expression for 1 day post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8), b) Graphs show the fold change in gene expression for 6 days post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8), and c) Graphs show the fold change in gene expression for 14 days post treatment in wild-type (WT) and 8 transgenic lines (TL1-TL8).

Discussion

This study identified the importance of *SlPI-II* gene transformed in wheat under *OsRGLP2* promotor. The eight transgenic lines were evaluated under different salinity and osmotic stress treatments to reveal the role of this gene to induce resilience to abiotic stresses. All the transgenic lines showed significant increase in the expression of transgene compared to the wild-type and the change in expression was observed until 14 days post treatment. In many transgenic plants studies the plant PIs were reported for the induction of tolerance in plants against several abiotic stresses (Kidrič et al., 2014; Li et al., 2015). Kim et al. (2001) reported the significant increase in the expression of serine proteinase inhibitors (*CaPI-2*) for mounting defense against salinity. It has been observed that jasmonic acid in *PI* gene induction played a significant role to mitigate various abiotic stress conditions including UV exposure, water deficit condition and salinity (Dombrowski, 2003; Fujita et al., 2006; Huang et al., 2007). Similarly, the transgenic tobacco with

proteinase inhibitor, *NtPI,* exhibited tolerance against salinity (Srinivasan et al., 2009). Some of the transgenic lines like TL1 showed higher expression, however some lines showed higher expression at 6- and 14-days post treatment like TL4 and TL6, respectively. Recently, Chen et al. (2021) revealed that *ZmCIPK42* was expressed in diverse tissues and was induced by salinity stress. Then yeast two-hybrid screen identified that a *ZmPI* as well as calcineurin B-like protein 1 and 4 were responsible as interaction partner of *ZmCIPK42*.

It was observed that expression of transgene successively decreased with the increase in time after stress was imposed, and maximum expression was observed at 1 day post treatment. The proteinase inhibitor gene in chestnuts originating from barely showed tolerance under drought stress via positive regulation (Gaddour et al., 2001). Fujita et al. (2006) reported the regulation of various defense related molecules upon *PI* gene expression. The constitutive expression of *PIs* has the direct advantage of control over the activity of proteases and the enhanced protease inhibitory activity is reported in PI transgenics (Huang et al., 2007). *PIs* may also be involved in response to several biotic and abiotic stresses, such as drought and salinity, and regulate physiological and developmental processes in plants (Huang et al., 2007). In plants, *PIs* family has few genes and their proteins regulate many abiotic stresses especially salt-stress. Proteases and their inhibitors participate in ABA-signaling events and the up-regulation of ABA-responsive genes is essential for drought adaptation (Sebastián et al., 2020). As a result, transgenic plants overexpressing either protease or protease inhibitor genes exhibit enhanced performance, plant growth and yield, survival rate, and resilience to water deficits (Malefo et al., 2020).

Conclusion

The efficacy of tomato *SlPI-II* gene in salinity and drought tolerance was observed by change in gene expression level during drought and salinity stress exposure. The significant change in expression was observed at early hours of post stress application at100mM, 200mM and 300mM NaCl stress. For osmotic stress, the significant fold change values were observed at 1 day post stress of 15% PEG application, while sustained expression was observed during 6- and 14-days post stress. *SlPI-II* gene provided tolerance until 14 days post salinity stress. Different aspects related to abiotic stress for *SlPI-II* gene are still needed to be explored which are ultimately linked to wheat growth and survival against salinity and drought. Modern day transgenic approaches utilizing plant-based proteinase inhibitors will provide the new opportunities for the development of tolerant varieties in future.

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Contribution of Authors

Sarfraz W: Performed the experiment and wrote first draft of manuscript, read and approved the final draft.
Khalid M: Performed the experiment and analyzed the data, read and approved the final draft.
Rasheed A: Designed the experiment and drafted the manuscript, read and approved the final draft.
Mahmood T: Conceived idea and designed the experiment, read and approved the final draft.

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Supplementary Data

Table-S1. Table represents the values as mean± SE for control and transgenic wheat plants (TL1-8). The values were obtained for fold change resulted from data analysis of qRT-PCR. Various letters showed significant difference for wheat plants using one tailed ANOVA with LSD test ($p<0.05$)

Wheat	100 mM	200 mM	300 mM
	1day post stress treatment		
NT	0.14±0.15a	0.16±0.17a	0.21±0.03a
TL1	6.41±0.31b	3.99±0.32a	3.09±0.28a
TL2	5.77±0.25b	3.62±0.22a	3.11±0.13b
TL3	6.02±0.12b	3.44±0.13b	3.17±0.11ab
TL4	4.21±0.44a	3.51±0.32b	2.24±0.15a
TL5	5.82±0.34ab	4.34±0.27ab	3.78±0.22a
TL6	5.70±0.20b	4.12±0.18ab	1.23±0.32a
TL7	4.82±0.24b	3.83±0.25b	2.70±0.18a
TL8	5.41±0.29b	4.51±0.21b	2.21±0.17a
Wheat	6 days post stress treatment		
NT	0.10±0.13a	0.07±0.27a	0.12±0.14a
TL1	5.01±0.10a	3.99±0.16a	1.71±0.19a
TL2	3.38±0.04a	2.90±0.13b	1.06±0.31b
TL3	4.89±0.16ab	3.66±0.20b	2.00±0.24ab
TL4	3.04±0.21b	2.00±0.24a	1.88±0.21b
TL5	4.60±0.15b	4.20±0.03ab	2.17±0.15b
TL6	5.05±0.12a	4.50±0.12ab	2.21±0.30b
TL7	4.00±0.26ab	3.92±0.15b	1.47±0.19a
TL8	5.10±0.14a	4.70±0.14a	1.8±0.23a
Wheat	14 days post stress treatment		
NT	0.18±0.14a	0.15±0.18a	0.22±0.21a
TL1	3.04±0.27a	2.15±0.24b	0.20±0.11a
TL2	3.43±0.21b	2.29±0.22b	0.43±0.12b
TL3	3.52±0.22b	2.31±0.29a	0.16±0.17a
TL4	3.60±0.30ab	1.10±0.11a	0.22±0.16b
TL5	2.80±0.21a	1.80±0.24a	0.21±0.13ab
TL6	3.34±0.23b	1.59±0.27ab	0.49±0.01ab
TL7	3.01±0.37b	2.23±0.21b	0.56±0.17a
TL8	3.14±0.34b	2.22±0.25b	0.30±0.40ab

Table-S2. Expression analysis values are represented as mean± SE. Following results were conducted from qRT-PCR for Control and transgenic wheat lines (TL1-8). Different letters representing the significant difference in transgenic wheat plants and control calculated via one tailed ANOVA [LSD test ($p<0.05$)].

Wheat	0	15%	25%
	1 day post stress treatment		
NT	0.18±0.17a	0.23±0.03a	0.27±0.19a
TL1	0.17±0.10b	7.31±0.44ab	3.18±0.31ab
TL2	0.13±0.11b	5.77±0.81b	3.71±0.35a
TL3	0.02±0.17ab	7.22±0.71a	3.45±0.31a
TL4	0.24±0.15a	5.98±0.48ab	2.87±0.42b
TL5	0.27±0.10b	7.10±0.13b	3.18±0.34b
TL6	0.19±0.12a	6.18±0.36b	2.27±0.34b
TL7	0.31±0.11ab	5.41±0.41b	3.04±0.71ab
TL8	0.20±0.14a	5.51±0.28ab	3.12±0.52ab
Wheat	6 days post stress treatment		
NT	0.13±0.10a	0.21±0.12a	0.28±0.17a
TL1	0.15±0.39a	4.41±0.29a	2.89±0.50b
TL2	0.09±0.11ab	3.78±0.16ab	2.51±0.42b
TL3	0.30±0.17b	4.10±0.33ab	3.03±0.21a
TL4	0.10±0.14b	4.30±0.14ab	3.61±0.15a
TL5	0.17±0.10ab	4.23±0.27b	2.18±0.41ab
TL6	0.21±0.13a	3.76±0.56b	1.71±0.37a
TL7	0.23±0.17ab	4.51±0.28b	2.77±0.43ab
TL8	0.16±0.03a	3.95±0.13a	3.11±0.44b
Wheat	14 days post stress treatment		
NT	0.29±0.10a	0.19±0.13a	0.35±0.15a
TL1	0.15±0.13ab	3.22±0.51ab	2.92±0.71b
TL2	0.30±0.12ab	2.15±0.14b	1.12±0.51ab
TL3	0.21±0.08b	3.45±0.51b	2.32±0.33b
TL4	0.37±0.22a	3.21±0.64ab	2.17±0.41b
TL5	0.25±0.27ab	2.99±0.31b	2.23±0.21b
TL6	0.31±0.16a	3.56±0.18b	1.67±0.12a
TL7	0.56±0.44a	3.12±0.71b	2.12±0.83a
TL8	0.39±0.51b	3.21±0.14a	2.47±0.61b