RESEARCH ARTICLE

Identification and characterization of a novel multi-stress responsive gene in Arabidopsis

Faiza Tawab1, Iqbal Munir1*, Zeeshan Nasim1, Mohammad Sayyar Khan2, Saleha Tawab3, Adnan Nasim3, Aqib Iqbal1, Mian Afqah Ahmad1, Waqar Ali4, Raheel Munir1, Maria Munir1, Noreen Asim2

1 Division of Biochemistry, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan, 2 Genomics and Bioinformatics Division, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan, 3 Agriculture Research System, Peshawar, Khyber Pakhtunkhwa, Pakistan, 4 Department of Biotechnology, University of Malakand, Chakdara, Lower Dir, Khyber Pakhtunkhwa, Pakistan

* iqmunir@aup.edu.pk

Abstract

Abiotic stresses especially salinity, drought and high temperature result in considerable reduction of crop productivity. In this study, we identified AT4G18280 annotated as a glycine-rich cell wall protein-like (hereafter refer to as GRPL1) protein as a potential multi-stress-responsive gene. Analysis of public transcriptome data and GUS assay of pGRPL1::GUS showed a strong induction of GRPL1 under drought, salinity and heat stresses. Transgenic plants overexpressing GRPL1-3HA showed significantly higher germination, root elongation and survival rate under salt stress. Moreover, the 35S::GRPL1-3HA transgenic lines also showed higher survival rates under drought and heat stresses. GRPL1 showed similar expression patterns with Abscisic acid (ABA)-pathway genes under different growth and stress conditions, suggesting a possibility that GRPL1 might act in the ABA pathway that is further supported by the inability of ABA-deficient mutant (aba2-1) to induce GRPL1 under drought stress. Taken together, our data presents GRPL1 as a potential multi-stress responsive gene working downstream of ABA.

Introduction

Being sessile in nature the plants are continuously exposed to different kinds of environmental stresses, including elevated level of salinity, drought and intense temperature [1] that causes reduction in crop productivity and pose a major threat to global food security [2]. The adverse effects of these abiotic stresses are further worsening by climate change, persistent reduction in the arable land and limiting water resources [3,4]. Extensive research in model plants and crops has aimed to understand the responses of plants to various biotic and abiotic stresses [5]. To meet the demands of growing population and varying climatic conditions of the globe there is a great urge to increase the global food production, consequently the demand of stress-tolerant crop varieties has been increased than in the past [6,7].
To cope with stress condition, plants have developed numerous adoptive strategies which include physiological, morphological, molecular and biochemical responses [8]. Plants have evolved several molecular mechanisms for adaptation and subsequent survival under stress conditions as revealed by stress induced transcriptomics. Hundreds of plant genes are differentially regulated in response to abiotic stresses, as demonstrated by RNA-seq analyses. A variety of stresses has been extensively studied in Arabidopsis thaliana [9]. RNA-seq has been proven as a powerful method for understanding the complex regulation networks and gene expression in many plant species responding to several kinds of biotic and abiotic stresses, such as Chinese cabbage [10], chickpea [11], maize [12], potato [13], Ammopiptanthus mongolicus [14] and soybean [15].

To mitigate the negative effects of these stresses, plants have evolved a sophisticated system of stress sensors, signaling transduction pathways and transcription factors (TFs) [16,17]. Over the past several years, the functional roles of glycine-rich proteins (GRPs) in the stress response have been investigated, and some members of this family in Arabidopsis have been shown to enhance seed germination and seedling tolerance to cold stress [18]. A large number of glycine rich proteins (GRPs) are also modulated by biotic and abiotic factors [19]. GRPs are also involved in responses to water stress, including drought and water logging. The grain yield of rice (Oryza sativa) during drought stress was improved following the incorporation of AtGRP2 and AtGRP7 into the rice genome [20].

In this study, we identified a novel gene GRPL1 (AT4G18280) that is annotated as a glycine-rich cell wall protein-like protein in Arabidopsis thaliana by using publicly available transcriptomic data of plants exposed to different abiotic stresses. The co-regulation of AT4G18280 with high-affinity K+ 5 (HAK5) under K+ deficiency and salt stress has been reported [21]; however, no attempts were made to functionally characterize this gene. In this study, we characterized GRPL1 by generating overexpression lines and its performance analyses under different abiotic stresses.Overpopulation of GRPL1 results in increased tolerance to salt, drought and heat stresses in Arabidopsis thaliana. Manipulating the orthologues of this gene in economical important crops can be helpful to ensure the global food security under changing environmental conditions.

Materials and methods

RNA-seq public datasets retrieval and analysis using Tuxedo protocol

To gain insights of transcriptome wide genes expression, we selected publicly available transcriptome datasets of Arabidopsis thaliana plants based on the criteria that the samples used should be of similar developmental stage and ideally the level and duration of the specific abiotic stress should be similar. Based on this criterion, SRA009031 [22], GSE72806 [23], and SRP035234 [24] of Arabidopsis thaliana (Col-0) were downloaded and analyzed with the Tuxedo protocol [25]. Briefly, the raw reads were quality assessed, adopter trimmed and then aligned with TOPHAT2 followed by the transcript assembly with Cufflinks and differential gene expression analysis with Cuffdiff2. Downstream analysis and visualization of differentially expressed genes (DEGs) were done with custom R and Python scripts. Common target genes of different stresses were determined using Venny2 (available at: bioinfogp.cnb.csic.es/tools/venny).

Plant material, growth conditions and stress response analyses

For all experiments Columbia accession (Col-0) of Arabidopsis thaliana was used. Two-week-old plants grown under controlled conditions (16:8h day/night) were used for stress response experiments.
Expression analysis. For transcript level expression of genes and validation of RNA-seq data, RNA was extracted from Arabidopsis seedlings using TRI Reagent (Sigma), and cDNA was synthesized from total RNA (2 μg) using Superscript-II reverse transcriptase (Invitrogen). Quantification was performed using LightCycler® 480 instrument. qPCR experiments were performed in three technical and biological replicates each. The average Ct values obtained from the qPCR reaction were used to calculate the relative quantity (RQ) for reference and gene of interest using the equation given below:

Relative Quantity (RQ) = E[(Ct[control]–Ct[treatment])

Where: E = Primer efficiency
T = Target gene

The relative quantities were then used to estimate the relative expression of the target gene using the equation:

Normalized Expression = RQsample(GOI)/RQsample(REF)

Where: GOI = Gene of interest
REF = Reference gene

Gene expression was normalized to Ubiquitin. Whereas, the protein gel blot was used for detection of HA-epitope-tagged proteins in the overexpression lines. For protein gel blot, anti-HA monoclonal antibody (Agrisera) was used. Ponceau staining was used as loading control.

PGRPL1::GUS cloning and GUS assay. To check the promoter activity of GRPL1 promoter, we cloned 1,758bp upstream region of GRPL1 translation start site using Infusion cloning system. Briefly, the pBI101 vector was linearized using SmaI restriction enzyme whereas the promoter region was amplified using a high fidelity pfu DNA polymerase using the primers mentioned in (S1 Table). The GUS assay was performed on the stable T3 transgenic lines as described earlier [26]. Briefly, the samples were fixed with 90% acetone for 20min, washed and treated with staining buffer (containing X-Gluc) and incubated at 37˚C for 2h. After the removal of staining buffer and washing with ethanol the samples were observed under light microscope.

Generation of over expression lines. The potential multi-stress tolerance AT4G18280 gene was amplified with gene specific primers (S1 Table) and cloned into a pUC19 background based binary vector containing a 35S Cauliflower Mosaic fused Virus (CaMV) promoter. Overexpression of GRPL1 was confirmed at transcript level via qPCR (S1 Table) and at protein level by western blot of the stable transgenic lines.

Stress assays. To access the functionality of AT4G18280 as a potential multi stress tolerance candidate, performance of the overexpression lines of T4 generation under salt, drought and heat stress was determined in comparison with wild-type plants. Salt stress was induced by addition of 100 and 200mM NaCl to the MS plates. Wild type and over expression lines were evaluated for fresh weight, germination percentage, relative root elongation, chlorophyll contents, proline content, melondialdehyde content (MDA) and survival rate (%). After completion of stress period, seedlings (10 plants/genotype) were taken from control and stressed pots and their fresh weights (mg/plant) were determined using a digital balance. For germination test, differences in the seeds germination under salt stress was recorded on the third day of transfer to growth chamber (22˚C). Relative root elongation (%) was measured as the percentage of root elongation under salt stress normalized to the control condition. Chlorophyll contents from leaves of the plants treated with 100 and 200 mM NaCl were quantified using a spectrophotometer (U-2810, Hitachi). After extraction of the supernatant from samples
homogenized in acetone the chlorophyll a and b were determined in totality in mg/g FW using the methodology described earlier [27]. Proline contents (µg/g FW) were quantified using the method reported earlier with minor modifications [28]. Melondialdehyde (MDA) content was measured by obtaining the supernatant from leaf samples of control and stressed plants through series of steps and then the obtained supernatant was used to measure the OD at 532, 600, and 450 nm and the MDA content was calculated using the method described by Zhang et al. [29]. Survival rate (%) was determined by subjecting two week-old seedlings for NaCl (200 mM) treatment followed by calculating the alive plants percentage as described earlier [30].

Drought stress tolerance analysis was done by calculating the survival rate and water loss. Survival percentage was measures by subjecting 14-day old soil grown plants (WT and over-expression lines) to dehydration by withholding water supply for 20 days and then re-watered. Survival percentages were recorded 6 days after the recovery and experiment was repeated thrice [31]. Water loss analysis from detached leaves was performed as described earlier [32]. Briefly, ~0.5 g rosette leaves of three weeks old plants were weighed (FW₀) and incubated at 23˚C. The samples were then reweighed at 1, 2, 3, 4, and 5 hours (FWᵢ), whereas the dry weight (DW) was measured by drying the detached leaves at 80˚C for 5 hr. The water loss was calculated based on the initial fresh weight of plants using the formula as:

\[
\text{Water loss} = \left( \frac{\text{FW}_0 - \text{DW}}{\text{FW}_0 - \text{FW}_i} \right) \times 100
\]

For heat stress tolerance analysis, the survival rate was measured after subjecting the plants to heat stress of 45˚C for 1 hr.

Statistical analysis. Data of the physiological and biochemical parameters between the transgenic and non-transgenic lines were analyzed in triplicate through analysis of variance (ANOVA) using student t-test.

**Results**

**Identification of GRPL1 as a multi-abiotic stress-responsive gene**

Adverse environmental conditions have been a major threat to global food security. The condition got worsens with the persistent reduction in the arable land, limiting water resources and climate change trends [33]. Therefore, we aimed to utilize the publicly available transcriptome data i.e. SRA009031 [22], SRP035234 [24] and GSE72806 [23] of plants exposed to different abiotic stresses in order to identify potential stress tolerance genes. Our analyses of these public transcriptome datasets identified a total of 760 genes that were commonly up regulated in response to heat, drought, salt and the combined heat and drought stress (Fig 1A and S2 Table). The top 10 multi-stress-induced genes of that list contained some heat shock proteins along with other known abiotic stress-responsive genes (Fig 1A). However, we selected GRPL1 i.e. AT4G18280 as a candidate because it showed strong induction in response to these abiotic stresses and was not studied earlier in the context of multi abiotic stress tolerance (Fig 1A).

The expression level of GRPL1 in response to different abiotic stresses is shown in Fig 1B. Though, all tested abiotic stresses showed induction of GRPL1, the PEG-induced osmotic and salt stress showed comparably higher levels of GRPL1 mRNA than heat stress (Fig 1B). Consistent with the RNA-seq results, our qPCR analyses showed similar expression pattern for the given abiotic stresses (Fig 1C). Precisely, the mRNA levels of GRPL1 gene was up-regulated by ~5.8 folds under salt stress, ~6.1 folds in the drought stress and ~4.1 folds in heat stress (Fig 1C). To further confirm this induction of GRPL1 by abiotic stress-induced, we generated promoter GRPL1-driven GUS transgenic lines (pGRPL1::GUS). pGRPL1::GUS seedlings showed weak GUS staining under control conditions, indicating the weak promoter activity of GRPL1.
(Fig 1D), however, under abiotic stresses, we observed strong induction of pGRPL1 promoter activity represented by the strong GUS staining. Overall, these results show that transcription of GRPL1 is strongly induced under abiotic stresses, suggesting a potential role of GRPL1 in modulating plant’s response to abiotic stresses.

**Cloning and expression analysis of AT4G18280**

To validate the potential role of GRPL1 in abiotic stress response, we generated transgenic plants overexpressing HA-tagged GRPL1. First, the overexpression was analyzed at the transcript level by RT-PCR and at protein level by protein gel blot. Indeed, the three independent transgenic lines 1–4, 4–3 and 6–2 showed higher mRNA (Fig 2A) and protein accumulation (Fig 2B). Also, the transgenic plants overexpressing GRPL1 showed better growth and had significantly higher fresh weight compared to the WT plants (Fig 2C and 2D). These results confirmed the overexpression of GRPL1 at both transcript and protein levels. The confirmed overexpression lines were tested under salt, drought and heat stress conditions to confirm their tolerance level.

**Function of AT4G18280 overexpression under salt, drought and heat stress**

To assess the effect of GRPL1 overexpression, the transgenic lines were tested under salt, drought and heat stress conditions. As salinity is one of the major abiotic stresses that compromises crop productivity [34], we evaluated the performance of overexpression lines under 100 mM and 200 mM salt stress. Under control conditions, all lines showed 96–100% germination
However, the WT seeds grown on MS media supplemented with 100 mM and 200 mM NaCl showed reduced germination of ~55% and ~33%, respectively. Whereas, the overexpression lines showed better germination under both concentrations of salt stress i.e. about 75–85% of seeds successfully germinated under low (100 mM) while ~50–60% seeds germinated under high concentration (200 mM NaCl) salt stress (Fig 3A).

Consistent with the previous reports [35], we observed about 65% relative root elongation in WT plants whereas the overexpression lines showed significantly higher root elongation under stress condition i.e. up to 80% relative root elongation (Fig 3B). Salt stress induces production of reactive oxygen species (ROS), which damage the cellular components [36] including chlorophyll [37]. WT plants showed over 50% reduction in the overall chlorophyll contents under salt stress (Fig 3C). However, the GRPL1 overexpressing plants showed significant tolerance towards salt stress as indicated by the reduced degradation of chlorophyll contents (~30–45%), highlighting the tolerance potential of the overexpression lines (Fig 3C). To further test the salt tolerance of GRPL1 overexpression plants, we performed proline and MDA contents quantification. Proline acts as an osmolyte plays important roles in stabilizing macromolecules and membranes in cells by a higher accumulation [38]. The WT plants under salt stress accumulated ~2-fold higher proline contents whereas the overexpression lines showed ~4-fold higher proline accumulation (Fig 3D) implying that the transgenic plants are more tolerant to salt stress. However, performance of the transgenic line 1–4 was somewhat comparable with WT plants. The Melondialdehyde (MDA) level is an indicator of the cellular damage caused by stress conditions [39]. Although the GRPL1-mediated tolerance mechanism is still elusive, there is a possibility that overexpression of GRPL1 may do so by the accumulation of proline and the efficient scavenging of ROS as represented by reduced quantities of MDA in transgenic plants (Fig 3D and 3E). Salinity is a serious threat to plants, disturbing all the physiological processes and even causes death of the plants [34]. Our results demonstrated that the overexpression of GRPL1 enhanced the survival rate of plants under higher dose of salinity (Fig 3F). All of the overexpression lines showed significantly higher survival rates.
Drought stress is also one of the biggest threats to food productivity. To examine the response of overexpression lines to drought stress, we subjected 14 days old seedlings to dehydration as no water was given to the plants for 20 days, followed by re-watering and checking the survival rates. Under water-deficient conditions for 20 days, only 22–27% of WT plants survived, whereas the overexpression lines showed significantly higher survival rates of 34–42% (Fig 4A and 4B). Furthermore, we also found the overexpression plants tend to retain more water contents compared to WT plants. After three hours of dehydration, the overexpression lines lost significantly lower amounts of water compared to WT plants and the difference between water loss got more significant with the increase in dehydration time (Fig 4C).
These results suggest that overexpression of GRPL1 results in reduced water loss under drought stress compared to WT plants and hence showed higher survival rates. Re-analysis of public transcriptome data and qPCR analysis revealed induction of GRPL1 under heat stress (Fig 1) which prompted us to check the performance of these overexpression lines under heat stress. Interestingly, the overexpression lines showed significantly higher survival rates compared to WT plants (Fig 4D and 4E) suggesting the possible role of GRPL1 in heat stress response too. However, the role of GRPL1 in heat stress response requires further study.

Tolerance to abiotic stresses in plants is often mediated by ABA [39]. In order to check if GRPL1 acts in the same pathway, we checked the expression of GRPL1 in a collection of public microarray data. Interestingly, GRPL1 showed similar expression patterns with the ABA INSENSITIVE 1 (ABI1), 2, and 5 (Fig 5A), suggesting a possibility that GRPL1 might act in the ABA pathway. To further support this hypothesis, we used public transcriptome dataset (GSE75933) of ABA-deficient (aba2-1) mutant [40]. Reanalysis of RNA-seq data revealed GRLP1 to be induced (~2.4-fold) by drought stress in WT background (Fig 5B), however, such stress treatment induction of GRLP1 was compromised in the ABA-deficient mutants (aba2-1). Taken together, these results suggest that GRPL1 may act downstream of ABA signaling.

Discussion

Plants are continuously exposed to a wide range of biotic and abiotic stresses in their native environment. Extensive research in model plants and crops has aimed to understand the plant responses to a range of biotic and abiotic stresses, as these stresses reduce harvest yields [41]. In response to abiotic stresses, hundreds of plant genes are differentially regulated as
demonstrated by RNA-seq analyses. In this study, our basic understanding of the plant acclimation to abiotic stresses was extended through whole transcriptome (RNA-Seq) analysis based on the utilization of the publicly available transcriptome data sets. A total of 760 genes were commonly up regulated in response to heat, drought, salt and the combined heat and drought stress based on our analysis of the selected transcriptomic datasets. In order to identify the potentially multi stress responsive genes, we sorted the genes based on their fold-change values. The top 10 multi-stress-induced genes contained some heat shock proteins along with other known abiotic stress-responsive genes. However, we selected GRPL1 i.e. AT4G18280 as a candidate because it showed strong induction in response to these abiotic stresses and was not studied earlier in the context of multi abiotic stress tolerance. The RNA-seq predicted expression of the selected gene was confirmed by qRT-PCR, which showed that the results of both are significantly comparable to each other, and RNA-Seq results are reliable and can be used for further analysis. These results are consistent with the previous finding that the mRNA levels of glycine rich proteins (GRPs) are affected by different abiotic stresses, in a number of plant species [42].

The selected multi-stress induced gene was over-expressed in Arabidopsis thaliana, and its expression pattern was confirmed at transcript and protein level. The overexpression transgenic Arabidopsis lines with AT4G18280 functional attributes were studied to confirm their role in different stress conditions. Significant differences in the AT4G18280 overexpression lines and wild-type plants were observed under salt, drought and heat stresses. To confirm the tolerance level of over-expressed transgenic plants with AT4G18280 gene under salt stress it was tested for some of the physiological parameters. The biological role of transgenic plants over-expressing the AT4G18280 gene under salt stress was confirmed by measuring the fresh weight (mg/plant). Salt stress causes the reduction in fresh weights due to the relative increase in Na+ concentration by means of ionic stress. In our study, highest fresh weight was shown by all of the overexpression lines as compared to the wild type (Col-0) plants.

Germination is the most feasible approach used for selecting salt tolerance in plants [43]. A number of scientific studies demonstrated the negative effect of increasing dose of salinity on germination percentage of plants [44,45]. In our study, the overexpression lines evaluated for germination rate (%) under control and stress condition. The overexpression lines showed significantly high germination rate under salt stress condition than the wild type plants suggesting that the overexpression lines are able to germinate under salt stress and hence tolerant to reduce the detrimental effect on germination under increase dose of salt stress. This reduction in germination of WT seeds is consistent with the previous reports of salt stress negatively affecting germination rates, most probably by repressing biosynthesis of two phytohormones,
gibberellic acid (GA) and salicylic acid (SA) [46,47]. Overexpression of GRPL1 exaggerated germination most probably through biosynthetic regulation of these two phytohormones. Being in direct contact with the soil, root length is considered one of the most important parameters to determine the salt stress tolerance of plants. It is well known that salt stress inhibits root meristem activity, cell cycle, and elongation of root cells in Arabidopsis and other plant species [48,49], resulting in retarded primary root growth [50,51]. It has been demonstrated that high dose of salt stress may reduce the root length by decelerating the water uptake by plants [52]. The relative root lengths determined in our study revealed that under stress condition (100 mM and 200 mM NaCl), the overexpression lines showed more root growth as compared to wild type plants which implies that the roots of overexpression lines are capable to grow even in high dose of salinity and hence are more tolerant. While similar pattern of root length was observed for overexpression lines as well as wild type plants under normal growth conditions. Soil salinity is a serious threat to plants, disturbing all the physiological processes and even causes death of the plants [34]. To know if the AT4G18280 overexpression can rescue the low survival rate, survival rates were recorded for both the overexpression transgenic lines and wild-type plants after exposure to a salt stress of 200 mM NaCl. Our results showed that the overexpression lines were able to survive under higher dose of salinity as compared to wild type control plants and hence they are more tolerant to salinity.

Free proline content can increase upon exposure of plants to drought, salinity, cold, heavy metals or certain pathogens. Determination of free proline levels is a useful assay to monitor physiological status and to assess stress tolerance of higher plants [53]. Our results demonstrated the increase in proline content by all of the overexpression lines as compared to the wild type plants suggesting that they are more tolerant to salt stress. A large number of scientific studies have been conducted in Arabidopsis thaliana by overexpressing the native or antonym genes that resulted in higher proline content accumulation [54]. Different plants produce higher free proline levels in response to salinity and many scientists have cited the potential roles of proline such as stabilizing proteins, osmolyte, scavenging of hydroxyl radicals and regulating cytosolic pH [55–57]. It is known that salt stress causes membrane lipid peroxidation which is used as an indicator for salt stress induced oxidative membranes damage [58]. It is therefore used as an important criterion for plant stress tolerance evaluation under stress condition. In our study the melondialdehyde (MDA) content was determined both in control and salt stress conditions. Under salt stress condition all of the overexpression lines showed significantly lower MDA content as compared to the wild type plants. This imply that the transgenic plants overexpressing the AT4G18280 gene confers tolerance to salt stress as they provide better protection against the oxidative damage. Plant tolerant to salt stress would accumulate less MDA contents compared to the susceptible plants [59]. Our results are consistent with the previous reports of reduced MDA accumulation in tolerant transgenic plants under salt and osmotic stresses [59]. Salt stress has been demonstrated to damage the chlorophyll contents of plants and hence reduces the photosynthesis of plants in different plant species [60,61]. In this study the overexpression lines and control plants were evaluated for chlorophyll content (mg/g FW) under control and salt stress condition. The overexpression lines maintain the chlorophyll content at high dose (200 mM NaCl) of salinity which means that they can withstand in saline environment by maintaining its chlorophyll content and hence more tolerant. Prolonged drought stress causes dehydration and a result death of the plants. To know the role of AT4G18280 overexpression under drought stress, the overexpression lines with AT4G18280 gene were tested for the survival rate. Significantly higher survival rate was recorded for all of the overexpression lines as compared to wild type control plants under drought stress. These results imply that the overexpression of Arabidopsis with AT4G18280 gene results in greater tolerance to drought stress than the wild-type (Col-0) plants. Further, to support that AT4G18280 overexpression is indeed involved
in providing drought tolerance to transgenic plants, they were tested for water loss at different intervals of dehydration. Our results showed that all of the overexpression lines were able to maintain its water content over increasing hours of dehydration. Significantly less water loss was recorded for the overexpression lines as compared to wild-type control plants; suggests that they are more tolerant to drought conditions.

After knowing the potential role of the over-expressed transgenic Arabidopsis plants with AT4G18280 gene in salt and drought stress, it was evaluated to confirm its role in heat stress as well. As the heat stress is lethal to plants, causing a number of changes in plant’s metabolism and even causes the mortality in plants. For this, the survival rate of the overexpression lines and wild type plants were recorded after subjected to heat stress of 45˚C for 1hr. The survival rate recorded for all the overexpression lines was significantly higher than the wild type plants which means that they are able to withstand heat stress and hence more tolerant.

The expression of GRPL1 was checked in a collection of public microarray data and interestingly, GRPL1 showed similar expression pattern with the ABA INSENSITIVE 1 (ABI1), 2, and 5, suggesting a possibility that GRPL1 might act in the ABA pathway. This hypothesis was further supported by analyzing public transcriptome dataset (GSE75933) of ABA-deficient (aba2-1) mutant [40]. GRPL1 was highly induced under drought stress in WT background, however, such stress treatment induction of GRPL1 was compromised in the ABA-deficient mutants (aba2-1). ABA2 gene is an alcohol dehydrogenase and mutation in this gene results in blockage of xanthoxin to ABA aldehyde, a key step in ABA-biosynthesis resulting in low ABA production. ABA positively regulates the induction of drought-inducible genes. The signaling mechanism is well-known [62]. Briefly, ABA inhibits the PP2C-dependent negative regulation of the SnRK2 protein kinase [63], as a result the SnRK2 can phosphorylate its downstream target transcription factors (for instance ABFs) which in turn induces the target genes. The fact that the ABA-deficient mutant is unable to induce the expression of GRPL1 in response to drought stress suggests that ABA is required for the induction of GRPL1 and that it acts downstream of ABA. Taken together; these results suggest that GRPL1 may act downstream of ABA signaling, however, further experiments are required to validate this hypothesis.

Conclusions

In conclusion, using a number of publicly available transcriptomic datasets and transgenic approaches, we identified and characterized a previously unknown GRPL1 as an abiotic stress-responsive gene. Constitutive expression of GRPL1 in Arabidopsis increased plant’s tolerance to salt, drought and heat stresses. Further understanding of the GRPL1 function could be helpful in the devising breeding strategies for abiotic stress tolerance and can contribute to the global food security.

Supporting information

S1 Fig. (TIFF)

S2 Fig. (TIFF)

S1 Table. Primers used for RT-PCR. (DOCX)

S2 Table. List of commonly up-regulated genes in the selected datasets. (DOCX)
Acknowledgments

Authors are thankful to the contributions of each member of the Biochemical and Analytical Division in experiments, data analysis and manuscript preparation.

Author Contributions

Conceptualization: Faiza Tawab, Iqbal Munir.
Data curation: Faiza Tawab.
Formal analysis: Zeeshan Nasim, Aqib Iqbal.
Funding acquisition: Iqbal Munir.
Methodology: Zeeshan Nasim.
Project administration: Iqbal Munir.
Supervision: Iqbal Munir, Mohammad Sayyar Khan.
Visualization: Adnan Nasim.
Writing – original draft: Faiza Tawab, Adnan Nasim, Mian Afaq Ahmad, Waqar Ali.
Writing – review & editing: Mohammad Sayyar Khan, Saleha Tawab, Raheel Munir, Maria Munir, Noreen Asim.

References

1. De Leonardis AM, Marone D, Mazzucotelli E, Neffar F, Rizza F, Di Fonzo N, et al. Durum wheat genes up-regulated in the early phases of cold stress are modulated by drought in a developmental and genotype dependent manner. Plant Sci. 2007; 172: 1005–1016. https://doi.org/10.1016/j.plantsci.2007.02.002.

2. Zhu Jian-Kang. 2016. Abiotic stress signaling and responses in plants. Cell 167, 313–324. https://doi.org/10.1016/j.physbeh.2017.03.040 PMID: 27716505

3. Lobell DB, Schlenker WS, Costa-Roberts J. Climate trends and global crop production since 1980. Sci.2011; 333: 616–620. https://doi.org/10.1126/science.1204531 PMID: 21551030

4. Fedoroff N V., Battisti D.S., Beachy R.N., Cooper P.J.M., Fischhoff D.A., Hodges C.N., et al, 2010. Radically rethinking agriculture for the 21st century. Science (80-.). 327, 833–834. https://doi.org/10.1126/science.1186834 PMID: 2150494

5. Hirayama T, Shinozaki K. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Plant Sci. 2007; 12:343–351. https://doi.org/10.1016/j.tplants.2007.06.013 PMID: 17629540

6. Takeda S, Matsuoka M. Genetic approaches to crop improvement: responding to environmental and population changes. Nat. Rev. Genetics. 2008; 9: 444–457. https://doi.org/10.1038/nrg2342 PMID: 18475268

7. Newton AC, Johnson SN, Gregory PJ. Implications of climate change for diseases, crop yields and food security. Euphytica. 2011; 179: 3–18.

8. Mahajan S, Tuteja N. Cold, salinity and drought stresses: An overview. Arch BiochemBiophys. 2005; 444: 139–58. https://doi.org/10.1016/j.abb.2005.06.018 PMID: 16309626

9. Zeller G, Henz SR, Widmer CK. Stress-induced changes in the Arabidopsis thaliana transcriptome analyzed using whole-genome tiling arrays. Plant. J. 2009; 58: 1086–1082. https://doi.org/10.1111/j.1365-313X.2009.03835.x PMID: 19222804

10. Yu S, Zhang F, Yu Y, Zhang D, Zhao X. Transcriptome profiling of dehydration stress in the Chinese cabbage (Brassica rapa L. ssp. pekinensis) by tag sequencing. Plant Mol. Biol. Rep. 2012; 30: 17–28.

11. Wang XS, Liu Y, Jia YY, Gu HY, Ma HY. Transcriptional responses to drought stress in root and leaf of chickpea seedling. Mol. Biol. Rep.2012; 39: 8147–8158. https://doi.org/10.1007/s11033-012-1662-4 PMID: 22562393
12. Shen Y, Zhang Y, Chen J, Lin H, Zhao M. Genome expression profile analysis reveals important transcripts in maize roots responding to the stress of heavy metal Pb. Physiol. Plant. 2013; 147: 270–282. https://doi.org/10.1111/j.1399-3054.2012.01670.x PMID: 22747913

13. Nachappa P, Levy J, Tamborindeguy C. Transcriptome analyses of Bactericera cockerellii adults in response to “Candidatus Liberibacter solanacearum” infection. Mol. Genet. Genomics. 2012; 287: 803–817. https://doi.org/10.1007/s00438-012-0713-9 PMID: 22945464

14. Zhou Y, Gao F, Liu R, Feng J, Li H. De novo sequencing and analysis of root transcriptome using 454 pyro-sequencing to discover putative genes associated with drought tolerance in Ammopiptanthus mongolicus. BMC.Genomics. 2012; 13: 266. https://doi.org/10.1186/1471-2164-13-266 PMID: 22721448

15. Fan XD, Wang JQ, Yang N, Dong YY, Liu L. Gene expression profiling of soybean leaves and roots under salt, saline-alkali and drought stress by high-throughput Illumina sequencing. Gene. 2012; 512: 392–402. https://doi.org/10.1016/jgene.2012.09.100 PMID: 23063936

16. Park H.J., Kim W.Y., Yun D.J., 2016. A new insight of salt stress signaling in plant. Mol. Cells 39, 447–459. https://doi.org/10.14348/molcells.2016.0083 PMID: 27339814

17. Qin F., Shinozaki K., Yamauchi-Shinozaki K., 2011. Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant Cell Physiol. 52, 1569–1582. https://doi.org/10.1093/pjpc/prr074 PMID: 21828105

18. Kim Joo Yeol, Kim W.Y., Kwak K.J., Oh S.H., Han Y.S., Kang H., 2010. Glycine-rich RNA-binding proteins are functionally conserved in arabidopsis thaliana and oryza sativa during cold adaptation process. J. Exp. Bot. 61, 2317–2325. https://doi.org/10.1093/jxb/erq058 PMID: 20231330

19. Sachetto-Martins G., Franco L.O., De Oliveira D.E., 2000. Plant glycine-rich proteins: A family or just proteins with a common motif? Biochim. Biophys. Acta—Gene Struct. Expr. 1492, 1–14. https://doi.org/10.1016/S0167-4781(00)00064-6 PMID: 10858526

20. Yang D.H., Kwak K.J., Kim M.K., Park S.J., Yang K.Y., Kang H., 2014. Expression of Arabidopsis glycine-rich RNA-binding protein AtGRP2 or AtGRP7 improves grain yield of oryza sativa under drought stress conditions. Plant Sci. 214, 106–112. https://doi.org/10.1016/j.plantsci.2013.10.006 PMID: 24268168

21. Adams E., Diaz C., Matsu M., Shin R. 2014. Overexpression of a Novel Component Induces HAK5 and Enhances Growth in Arabidopsis. Hindawi Publishing Corporation ISRN Botany.https://doi.org/10.1155/2014/490252.

22. Filichkin SA, Henry D, Scott AG, Rongkun S, Douglas WB, Samuel EF, et al. Alternative splicing in Arabidopsis thaliana. Genome Res. 2010; 20: 45–58. https://doi.org/10.1101/gr.093302.109 PMID: 19853648

23. Suzuki N, Bassil E, Hamilton JS, Inupakutika MA, Zandalinas SI, Tripathy D, et al. ABA is required for plant acclimation to a combination of salt and heat stress. PLoS One. 2016; 11: 0147625. https://doi.org/10.3738/journal.pone.0147625 PMID: 26824246

24. Ding F, Cui P, Wang Z, Zhang S, Ali S, Xiong L. Genome-wide analysis of alternative splicing of pre-mRNA under salt stress in Arabidopsis.BMC Genomics. 2014; 15: 431. https://doi.org/10.1186/1471-2164-15-431 PMID: 24897929

25. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 2012; 7: 562–78. https://doi.org/10.1038/nprot.2012.016 PMID: 22383036

26. Li X. Histostaining for Tissue Expression Pattern of Promoter-driven GUS Activity in Arabidopsis. BIO-PROTOCOL 1. 2011. https://doi.org/10.21769/bioprotoc.93.

27. Aron DI. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol. 1949; 24: 1–15. https://doi.org/10.1104/pp.24.1.1 PMID: 16654194

28. Bates LS, Waldren RP, Teare ID. Rapid determination of free proline for water stress studies. Plant Soil. 1973; 39, 205.

29. Zhang S, Gan Y, Xu B. Application of plant-growth-promoting fungi trichodermalongibachium i enhances tolerance of wheat to salt stress through improvement of antioxidative defense system and gene expression. Front. Plant Sci. 2016; 7: 1405.

30. Zhao W.T., Feng S.J., Li H., Faust F., Kleine T., Li L.N., et al. 2017. Salt stress-induced FERROCHELASE 1 improves resistance to salt stress by limiting sodium accumulation in Arabidopsis thaliana. Sci. Rep. 7, 1–16. https://doi.org/10.1038/s41598-017-13593-9 PMID: 28127051

31. Ziyuan L, Chunfei W, Jianjun Y, Xian L, Liangjun L, Libao C, et al. Molecular cloning and functional analysis of lotus salt-induced NnDREB2C, NnPIP1-2 and NnPIP2-1 in Arabidopsis thaliana. Mol Biol Rep. 2020; 47: 497–506. https://doi.org/10.1007/s11033-019-05156-0 PMID: 31654214

32. Niu CF, Wei W, Zhou QY et al. Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. Plant, Cell Environ. 2012; 35: 1156–1170. https://doi.org/10.1111/j.1365-3040.2012.02480.x PMID: 22220579
33. Lobell DB, Schlenker WS, Costa-Roberts J. Climate trends and global crop production since 1980. Sci. 2011; 333: 616–620. https://doi.org/10.1126/science.1204531 PMID: 21551030

34. Munns R, Tester M. Mechanisms of salinity tolerance. Annu. Rev. Plant Biol. 2008; 59: 651–81. https://doi.org/10.1146/annurev.arplant.59.032607.092911 PMID: 18444910

35. Neumann PM. The role of cell wall adjustment in plant resistance to water deficits (Review and Interpretation). Crop Sci. 1995; 35: 1258–1266.

36. Van Breusegem F., Dat J.F., 2006. Reactive oxygen species in plant cell death. Plant Physiol. https://doi.org/10.1104/pp.106.078295 PMID: 16760492

37. Smirnoff N. The Function and Metabolism of Ascorbic Acid in Plants. Ann. Bot. 1996: 78: 661–669.

38. Wang W, Vinocur B, Altman A. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 2003; 218: 1–14. https://doi.org/10.1007/s00425-003-1105-5 PMID: 14513379

39. Sharma P, Jha AB, Dubey RS, Pessarakli M, Alsar T, Razak S, et al. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanisms in Plants under Stressful Conditions [WWW Document]. BMC Complement. Altern. Med. 2016; https://doi.org/10.1186/s12906-016-1240-8.

40. Shinde S, Villamarom JG, Lin W, Sharma S, Verslues PE. Proline coordination with fatty acid synthesis and redox metabolism of chloroplast and mitochondria. Plant Physiol. 2016; 172: 1074–1088. https://doi.org/10.1104/pp.16.01097 PMID: 27512016

41. Hirayama T, Shinozaki K. Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J. 2010; 61: 1041–52. https://doi.org/10.1111/j.1365-313X.2010.04124.x PMID: 20409277

42. Czolpinska M, Rurek M. Plant glycine-rich proteins in stress response: An emerging, still prospective story. Front. Plant Sci. 2018; 9. https://doi.org/10.3389/fpls.2018.00302 PMID: 29568308

43. Huang J, Redmann RE. Salt tolerance of Hordeum and Brassica species during germination and early seedling growth. Can. J. Plant Sci. 1995; 75: 815–819.

44. Gulzar S, Khan MA, Ungar IA. Effect of salinity and temperature on the germination of Urochondras etulosa (Trin.) C. E. Hubbard. Seed Sci. Technol. 2001; 29: 21–29.

45. Jeannette S, Craig R, Lynch JP. Salinity tolerance of Phaseolus species during germination and early seedling growth. Crop Sci. 2002; 42: 2019–2029.

46. Kim SG, Park CM. Gibberelic acid-mediated salt signaling in seed germination. Plant Signal. Behav. 2008; 3: 877–879. https://doi.org/10.4161/psb.3.10.6247 PMID: 19704528

47. Lee S, Kim SG, Park CM. Salicylic acid promotes seed germination under high salinity by modulating antioxidant activity in Arabidopsis. New Phytol. 2010; 188: 626–637. https://doi.org/10.1111/j.1469-8137.2010.03378.x PMID: 20663063

48. Burssens S, Himanen K, Van De Cotte B, Beeckman T, Van Montagu M, Inze D, et al. Expression of cell cycle regulatory genes and morphological alterations in response to salt stress in Arabidopsis thaliana. Planta. 2000; 211: 632–640. https://doi.org/10.1007/s004250000334 PMID: 11089675

49. Zhu JK. Abiotic stress signaling and responses in plants. Cell. 2016; 167: 313–324. https://doi.org/10.1016/j.physbeh.2017.03.040 PMID: 27716505

50. Bostos D, Lascano R, Villasuso AL, Machado E, Senn ME, Córdoba A, et al. Reductions in maize root-tip elongation by salt and osmotic stress do not correlate with apoplastic O2•- levels [WWW Document]. Ann. Bot. 2008; https://doi.org/10.1093/aob/mcn141.

51. Jiang K, Moe-Lange J, Hennet L, Feldman LJ. Salt stress affects the redox status of Arabidopsis root meristems. Front. Plant Sci. 2016; 7: 81. https://doi.org/10.3389/fpls.2016.00081 PMID: 26904053

52. Werner JE, Finkelstein RR. Arabidopsis mutants with reduced response to NaCl and osmotic stress. Physiol. Plant. 1995; 93: 659–666.

53. Ábrahám E, Hourton-Cabassa C, Erdei L, Szabados L. Methods for Determination of Proline in Plants. In: Sunkar R, editor. Plant Stress Tolerance. Methods in Molecular Biology (Methods and Protocols), Humana Press; 2010. https://doi.org/10.1007/978-1-60761-702-0_20.

54. Liu W, Tai H, Li S, Gao W, Zhao M, Xie C, et al. bHLH122 is important for drought and osmotic stress resistance in Arabidopsis and in the repression of ABA catabolism. New Phytol. 2014; 201: 1192–1204. https://doi.org/10.1111/nph.12607 PMID: 24261563

55. Koca H, Bor M, Özdemir F, Türkân I. The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. Environ. Exp. Bot. 2007; 60: 344–351.

56. Hazman M, Hause B, Eiche E, Nick P, Riemann M. Increased tolerance to salt stress in OPDA-deficient Rice Allene Oxide Cyclase mutants is linked to an increased ROS-scavenging activity. J. Exp. Bot. 2015; 66: 3339–3352. https://doi.org/10.1093/jxb/erv142 PMID: 25873666
57. Dar MI, Naikoo MI, Rehman F, Naushin F, Khan FA. ‘Proline accumulation in plants: roles in stress tolerance and plant development’. In Iqbal N, Nazar R, Khan NA, Editors. Osmolytes and Plants Acclimation to Changing Environment: Emerging Omics Technologies, Springer, New Delhi. 2016; pp. 155–166.

58. Hernández JA, Almansa MS. Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves. Physiol. Plant. 2002; 115: 251–257. https://doi.org/10.1034/j.1399-3054.2002.1150211.x PMID: 12060243

59. Ullah A, Sun H, Hakim, Yang X, Zhang X. A novel cotton WRKY gene, GhWRKY6-like, improves salt tolerance by activating the ABA signaling pathway and scavenging of reactive oxygen species. Physiol. Plant. 2018; 162: 439–454. https://doi.org/10.1111/ppl.12651 PMID: 29027659

60. Taffouo VD, Wamba OF, Youmbi E, Nono GV, Akoa A. Growth, yield, water status and ionic distribution response of three bambara groundnut (vigna subterranea (L.) verdc.) landraces grown under saline conditions. Int. J. Bot. 2010; 6: 53–58. https://doi.org/10.3923/ijb.2010.53.58.

61. Turan MA, Turkmen N, Taban N. Effect of NaCl on stomatal resistance and proline, chlorophyll, Na, Cl and K concentrations of lentil plants. J. Agron. 2007; 6: 378–381. https://doi.org/10.3923/ja.2007.378.381.

62. Vishwakarma K., Upadhyay N., Kumar N., Yadav G., Singh J., Mishra R.K., et al. (2017) Abscisic acid signaling and abiotic stress tolerance in plants: a review on current knowledge and future prospects. Front. Plant Sci. 8: 161. https://doi.org/10.3389/fpls.2017.00161 PMID: 28265276

63. Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, et al. Molecular basis of the core regulatory network in abscisic acid responses: sensing, signaling, and transport. Plant Cell Physiol. 2010; 51:1821–39. https://doi.org/10.1093/pcp/pcq156 PMID: 20980270