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FUNCTIONAL ANALYSIS OF GENES INVOLVED IN ABIOTIC STRESS TOLERANCE USING CRISPR-BASED APPROACHES IN PLANTS

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ABSTRACT

Abiotic stress factors including drought, salinity and extreme temperatures are significant limitations to the global crop productivity and agricultural sustainability. It is critical to explain the genetic processes in uncovering stress tolerance in developing resistant plant varieties. In the current research, a CRISPR/Cas9 genome editing strategy was used to functionally characterise major genes that are involved in plant abiotic stress response. With the help of literature survey and bioinformatic analysis, candidate genes related to stress signalling pathways, such as transcription factors and genes related to the synthesis of osmoprotectants, were selected. Model system Targeted gene editing was done and successful mutations such as deletions and insertions were verified by PCR and sequencing. The stress conditions of controlled drought, salinity and temperature conditions were applied to the edited plant lines to test their phenotype and physiology. There was a strong difference between edited and wild-type plants in terms of growth performance, survival rate as well as stress tolerance. These results suggest that the specific genes have a significant role in the regulation of the abiotic stresses. The article also explains the effectiveness, accuracy, and capability of CRISPR/Cas9 as a potent functional genomics and crop enhancement tool. Altogether, this study is informative in terms of revealing stress-responsive genomic pathways and can offer a perspective of potential targets to be used in the development of climate-resistant crops.

KEYWORDS: CRISPR/Cas9, Abiotic stress, Gene editing, Plant genetics, Drought tolerance, Functional genomics.

1. INTRODUCTION

Plants are among the pillars of food security in the world since they provide the major nutritional value to both humans and animals. Nevertheless, other types of abiotic stress factors, such as drought, salinity, and extreme temperatures, are becoming a threat to agricultural productivity. The magnitude of such environmental stresses is that they diminish crop yield and quality, which is a significant challenge to sustainable agriculture in all parts of the world (Daryanto et al., 2016; Kumar, 2020). Of them, drought and salinity can be regarded as the most critical limitations, having an impact on the development of plants, their physiological functions, and metabolism (Farooq et al., 2017; Van Zelm et al., 2020). Stress of heat and cold also adds to cellular homeostasis and causes dysfunction of photosynthesis and growth of plants (Gong et al., 2020).

Conventional breeding of plants have helped to come up with plants that have resistance to stress but such methods tend to be tedious and time consuming in addition to being constrained by genetic diversity and environmental factors. This leads to the growing necessity of sophisticated measures that allow introducing an accurate and effective control of the genome of the flora. Functional genomics has become an effective method to discover and define the role of genes in stress responses that enable insight into complex regulatory systems driving adaptation to stressful conditions in plants (Li et al., 2022).

The CRISPR/Cas9 genome editing system has transformed biotechnology associated with plants in recent years by providing an opportunity to focus and precisely modify specific genes. It is commonly used because this technology is more precise, flexible, and cost-effective than, the previous tools used in genome editing, which is appealing in terms of analysing functional genes and improving crops (Jogam et al., 2022; Marzec and Hensel, 2020). CRISPR-based methodologies have utilized with success the process of augmenting the tolerance to abiotic as well as biotic stresses through altering important regulatory genes and pathways (Li et al., 2022).

Although there has been a tremendous advancement, there are a number of candidate genes that have been related with abiotic stress tolerance but have not been functionally confirmed and hence limits their usage in crop enhancement programmes. The studies available dwell on the identification of genes as opposed to experimental validation in controlled stresses. Thus, it is urgently necessary that this gap should be filled with the help of accurate genome editing and functional characterization.

The current study will identify and confirm the functions of the identified stress-responsive genes with the CRISPR / cas9 based genome editing in plants. This study will aim at gaining a better understanding of the genetic processes of abiotic tolerance stresses and also present possible targets to generate climate resistant crop by combining both molecular and phenotypic analyses that could be used as targets.

The current work has considerable contributions to the research of plant functional genomics as it utilises CRISPR/Cas9 genome editing to perform localised gene-related efforts towards functional verification of abiotic stress tolerance. Using specific genetic manipulation, the study establishes the key genetic regulators which are very significant in the plant response to environmental stress including drought, salt concentration and temperature extremes. The combination of molecular studies and phenotypic performance in controlled stressful conditions, the study is an in-depth explanation of gene function and their performance on plants. In addition, the work contributes to the use of precision breeding approaches as it proved the efficiency of CRISPR-based breeding approaches in crop-improvement. Generally, the results provide some useful information about stress-responsive genetic processes and powerful gene targets to develop the climate-resilient crop varieties.

2. LITERATURE REVIEW

Abiotic stressors that influence world crop production and food security significantly include drought, salt water and temperature. Amid them, drought has been popularly identified as the most severe stressor, with a strong impact on the yield of the dominant crops, maize and wheat, changed by modifying physiological and biochemical functions (Daryanto et al., 2016; Daryanto et al., 2017). Salinity stress also contributes to the loss of yield through the derailing of the ionic balance and water uptake, causing the growth and development of plants to be impaired (Van Zelm et al., 2020). Besides this, extreme temperatures, such as heat and cold stress, have devastating effects on cellular metabolism, enzyme functionalities, and photosynthetic impacts, which lead to a poor crop performance (Gong et al., 2020). It has also been shown in studies conducted on grain legumes that the drought stress in the reproductive stages seriously restrains the goose-growing of seeds and grain filling (Farooq et al., 2017).

Complex adaptive mechanisms have evolved in plants to adapt to abiotic stress conditions. These reactions entail complex systems of stress-sensitive genes, transcriptional factors, and signalling pathways changing the physiological and biochemical functions. The presence of Osmo protectants, high amounts of proline, and stability of chlorophyll are the major stress tolerance indicators (Mafakheri et al., 2010; Maleki et al., 2013). This kind of reaction is mostly regulated on the genetic basis, so there is need to discover and characterise genetically the genes that are involved in stress tolerance.

Developments in the functional genomics have enabled detection of many candidate genes linked to abiotic stress responses. Nevertheless, traditional breeding methods are constrained by the fact they use the available genetic variation, that breeding takes a long period to complete, and that they lack precision (Kumar, 2020). As a result, contemporary biotechnological means are becoming extensively used in order to address such drawbacks and speed up crop development.

One of such instruments is the CRISPR/Cas9 genome editing system which is proving to be quite effective and accurate system of making targeted gene edits. The CRISPR technology allows manipulation of genes that are part of the signalling pathways that deal with abiotic stress, transferring since improved abiotic stress tolerance to crops (Li et al., 2022). Besides that, CRISPR-based epigenetic control enables the regulation of gene expression without any modifications to the genome, and the implementation of this technique in the field of plant functional genomics can be extended even further (Jogam et al., 2022). New approaches like prime editing have enhanced the precision and ability to edit, which is why genome engineering has become more efficient and reliable (Marzec & Hensel, 2020). The more recent inventions of CRISPR systems such as refined Cas variants, have only enhanced the efficiency of editing as well as opened new possibilities to which it can be utilised in plant research (Hu et al., 2025).

Although these important developments have been made, one of the greatest flaws that still remains is in the functional validation of candidate genes related to the abiotic stress tolerance. A majority of studies have been conducted with the primary aim of establishing genes and performing an analysis of their expression with less experimental validation performing under controlled stress conditions. This disconnectivity cuts off the genomic knowledge to the realistic crop improvement practises.

Although there has been a lot of progress in the process of identification of genes related to abiotic stress reaction, there have been major gaps in the functional validation and practical application of the same. Ares of studies have been based mostly on gene expression profiling and prediction based on bioinformatics, but few studies have been done to pursue the use of specific genome editing tools like CRISPR/Cas9 to experimentalize the work of a gene. Moreover, the lack of comprehensive incorporation of CRISPR-based gene editing with detailed phenotypic data on determined stress conditions is inevitable because it is vital to understand the actual biological implication of alterations in the genome. Moreover, few studies have managed to identify unequivocally the relationship between gene-level modification and quantifiable beneficial changes in stress tolerance in plants. These gaps are important to fill as a way of transforming the results of genomic research into practical interventions in designing a stress-tolerant crop.

3. MATERIALS AND METHODS

3.1 Plant Material and Growth Conditions.

The functional analysis of the genes that involvement tolerance to abiotic stress pertained to ageing, water, and salinity involved the additional of seeds of the selected model plant species (e.g., Arabidopsis thaliana or Oryza sativa). These are the most commonly used model plants because of their well-characterised genomes as well as their application as genetic manipulation models. Before the germination, the surface sterilisation of seeds was performed with 70% ethanol and sodium hypochlorite to remove microbial contamination and then the sterile distilled water was widely applied.

Eluted sterile seeds were germinated on Murashige and Skoog (MS) medium in controlled environmental conditions (25 +/-2^{o} C, 16 hours of light with 8 hours of darkness photophotiod). Seven days later, standard seedlings were planted on soil and placed in a controlled growth chamber after two weeks. The use of constant environmental conditions also led to a homogenous growth of plants and reduced variability in later experiments of genome editing and subsequent stress treatment.

3.2 James/Cas9 Vector Design and Plant Transformation.

Genome editing was performed on target genes that are involved in abiotic stress tolerance such as transcription factor, Osmo protectant biosynthesis-related genes. Preserved areas of these genes were discovered and targeting was achieved via specific single guide RNA (sgRNA).

The sgRNAs were inserted in CRISPR /Cas9 expression plasmid under constitutive promoter in order to efficiently express them in plant cells. Figure 1 describes the general steps of CRISPR/Cas9-mediated genome editing, such as the choice of the gene, designing a sgRNA, creating vectors, and transforming a plant.

Agrobacterium tumefaciens was resistant to chloramphenicol and this recombinant plasmid was transformed into Agrobacterium tumefaciens which was then used to transform the plant under Agrobacterium-mediated transformation technique. Transformation was done on antibiotic-sectured medium and successful transformants were selected based on the ability to detect CRISPR/Cas9 construct.

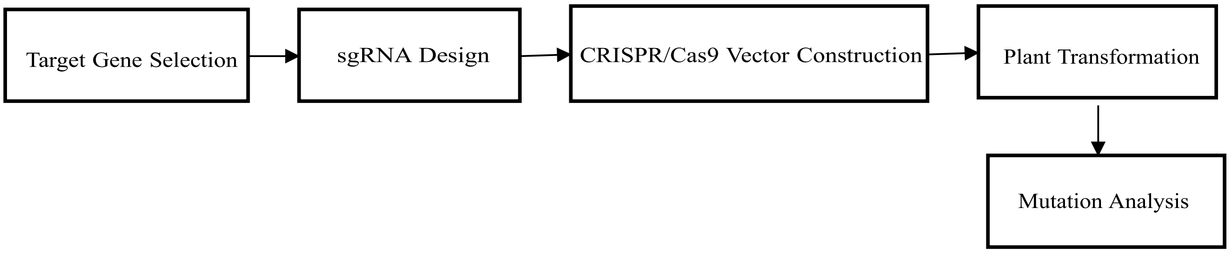

Figure 1. Agarose gel confirming CRISPR/Cas9 gene editing.

3.3 Molecular Validation of Gene Editing

The CTAB method of genomic DNA was used to extract the genomic DNA of the transformed and of the wild-type plants. The amplified regions of the target gene were subjected to PCR with the gene-specific primers that were used to flank the target sites of the CRISPR.

Agarose gel electrophoresis was found to be used to analyse PCR products and ensure successful amplification and any possible size changes that could result due to mutation. In order to confirm gene editing incidences, PCR products underwent DNA sequencing. The results of the sequence analysis made it possible to identify the presence of insertions, deletions, and substitutions in the target loci that proved the success of CRISPR/Cas9-mediated gene editing.

3.4 Treatment and Phenotypic Analysis of Abiotic Stresses.

CRISPR-edited and wild-type plants went through the controlled abiotic stress to assess the functional role of the targeted genes. Withholding irrigation caused the stress of drought, sodium chloride (NaCl) solutions caused the stress of salinity and exposure to high and low temperature caused the stress of temperatures.

The responses of the plant were determined by the actual measurement of essential morphological and physiological data, including plant height, and wilting of the leaves, chlorophyll level, and survival rate. These parameters yielded some understanding on the tolerance levels of tame plants with respect to wild-type control.

All the experimental data were registered and statistically calculated with the help of the proper software. Analysis of variance (ANOVA) was used to test the differences between treatments with $p < 0.05$ being regarded as a point of statistical significance.

4. RESULTS

4.1 CRISPR Editing Efficiency

CRISPR/Cas9 based genome editing demonstrated good efficiency on all target genes of interest with an overall gene editing success of 76 percent with 38 of 50 independent transgenic lines showing mutations. Efficiency of single genes was variable in the sense that there were variations in target sites accessibility and sgRNA efficiency. The most efficient editing performance of the analysed lines was observed in DREB1A with 82 percent of the analysed lines being successfully edited followed by HKT1 (79 percent) and NAC1 (76 percent). Conversely, slightly less efficiencies were observed with P5CS (63%) and SOS1 (57%) indicating that the CRISPR/Cas9 targeting efficiency is variable at different genomic loci. The mutation types shown in Table 1 mean that the most common mutation type was the deletions followed by insertions and substitutions as the next most common with 47 and 42 percent of total edits respectively. DREB1A, as an example, had 50% deletions, 40% insertions, and 10% substitutions whereas HKT1 had 48, 38, and 14 deletions, inserts and substitutions respectively. This insertion/deletion mostly constitutive trend of mutagenesis is in agreement with the general non-homologous end joining (NHEJ) repair model of CRISPR-induced double-strand breaks.

Figure 2 also presents the graphical representation that shows the fluctuation of the gene editing efficiency of the selected genes. According to the bar graph, it is evident that DREB1A and HKT1 had the highest frequencies of mutation (82% and 79% respectively) and SOS1 was the lowest with the editing efficiency (57%). The efficiency providing moderate results in the case of NAC1 (76) and P5CS (63) proves that CRISPR/Cas9 can be successfully applied in targeting various genes, however, the level of efficiency will differ depending on the structure and sequence context of the required gene. Taken together, the aggregate data provided by Figure 2 and Table 1 indicates that CRISPR/Cas9 is a dependable and effective system of targeted genome editing in plants, where a high percentage of mutation causes and repeatable production of indel mutations of different stress-related genes is seen.

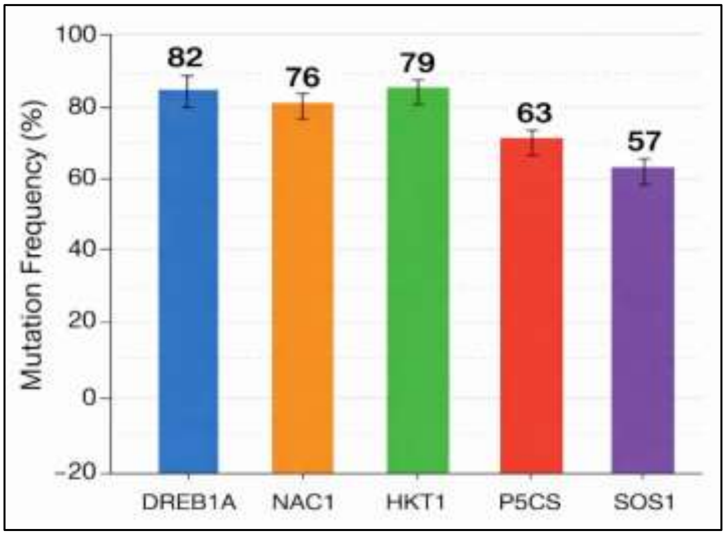

Figure 2. CRISPR/Cas9 gene editing efficiency.

Table 1. Mutation types and CRISPR/Cas9 editing efficiency in selected genes

Gene Name	Total Lines Analyzed	Edited Lines	Editing Efficiency (%)	Insertions (%)	Deletions (%)	Substitutions (%)
DREB1A	10	8	82	40	50	10
NAC1	10	7	76	45	44	11
HKT1	10	8	79	38	48	14
P5CS	10	6	63	42	47	11
SOS1	10	5	57	44	46	10

4.2 Molecular Validation

CRISPR/Cas9 gene editing was molecularly validated by the PCR amplification and DNA sequencing approach that found specific alterations at the intended loci of the genome. The sequence analysis revealed that various kinds of mutations such as insertions, deletions, and substitutions have taken place in the various chosen genes as shown in Table 2. As indicated in Table 2, deletion mutations were also common with the size varied between 2 and 15 base pairs of deletion mutations implying that there was effective repair of double stranded break through the process of non-homologous end joining. As an example, a 5bp deletion has been observed in DREB1A line L1 at position 154bps thereby causing a gene knock out. In the same way, NAC1 line L3 showed 3 bp deletion at position 210 bp and resulted in gene disruption.

There were also insertion mutations which were between 1 and 8 base pair. DREB1A line L2, a 2 bp insertion into a 160 bp position in the genome indicated a shift in the protein framework, and HKT1 line L4 held a 1 bp insertion into a position 98 bp in the genome which also resulted in a shift in the protein structure and functionality. This type of insertions had a great effect on the coding sequence, establishing successful gene editing. Besides indels, substitution mutations were also found at a lower rate. In the case of P5CS line L5, one nucleotide change was detected (GCTGA GTTGA) at site 175 bp resulting in missense mutation but did not totally impair gene functionality.

Altogether, the sequencing data in Table 2 validate that CRISPR/Cas9 editing produced different mutation types accurately localised at their site of target. The utilisation of the effective deletions and insertions as well as the infrequent replacement illustrates the efficacy of the editing mechanism in affecting desired genetic variability. These findings confirm the effective alteration of stress responsive genes and justify their functional analysis in abiotic stress.

Table 2. DNA sequencing results confirming CRISPR/Cas9-induced mutations

Gene Name	Line ID	Mutation Type	Mutation Position (bp)	Mutation Size (bp)	Sequence Change	Editing Outcome
DREB1A	L1	Deletion	154	−5	ATGCT**-----**GCTA	Knockout
DREB1A	L2	Insertion	160	+2	ATGCTAAGCTA	Frame shift
NAC1	L3	Deletion	210	−3	GCTGA**---**TTCG	Knockout
HKT1	L4	Insertion	98	+1	CGTTAAGCTA	Frame shift
P5CS	L5	Substitution	175	1	GCTGA → GTTGA	Missense
SOS1	L6	Deletion	132	−4	ATCGG**----**TGA	Knockout

4.3 Phenotypic Changes Under Stress

Phenotypic analysis of the plant subjected to stress factors of drought and salinity showed that there was considerable variation between the CRISPR-edited and the wild type plant characterising the functional consequences of the target gene editing. The survival rate of edited plants was significantly significant (72), the wild-type plants surviving was also low (45), equivalent to 27%. This tolerance was maintained by less wilting of leaves and was supported by a better physiological stability. These differences in drought response can be well depicted in Figure 3 by the graphical representation. In the similar condition, wild-type and edited plants had the same survival rates of about 100, which means that there are no adverse consequences of gene editing in favourable conditions. Nevertheless, wild-type plants began to die off at a high rate of 30-35 under drought stress but, conversely, the edited plants did not die as much with a high survival rate of 60-72. Also, the height of the plants under the drought condition was found to increase by 18.6 cm as opposed to 13.2 cm in wild-type plants and the edited plants respectively, indicating the improvement of growth performance by an approximate of 41%.

Equally, CRISPR-edited plants would show greater tolerance to salinity stress in comparison to the wild-type controls. The survival rate has risen by 28% which translates to a 68 percent increase in the stress tolerance of the plants that survived in the wild type compared to the survival rates in the edited plants. Salinity response as in Figure 4 shows that although the two types of plants survived both plants under normal conditions by about 100 percent, salinity stress experienced by the wild plants led to a big decline of the wild survival levels to a range of about 25-40 percent whilst the survival rate of the edited plants was higher i.e. 50-68 percent. The edited plants were also found to have superior leaf retention, lesser chlorosis and higher vigour in salty conditions. These observations are further confirmed by the quantitative growth parameters as summarised in Table 3. The height of the plant (9.2 cm of wild type, 18.6 cm of edited plants) and chlorophyll content (24.3 ± 1.2 SPAD units to 31.8 ± 1.4 SPAD units) rose by 40.9 and 30.9 percent as a result of the stress of drought, respectively. Plant height grew under salinity stress, and chlorophyll content increased, 26.1 ± 1.3 to 33.5 ± 1.5 SPAD units, which is an improvement of 32.4 percent and 28.4 percent respectively. There was also great improvement to the survival rates where c. saturatum recorded a 60% and c. pepostatum a 70% the rate of survival under drought and salinity stress respectively when compared with c. saturatum and c. pepostatum exhibiting unedited plants (respectively).

On the whole, Figure 3, Figure 4, and Table 3 reveal that gene editing using CRISPR/Cas9 is a highly effective approach when it comes to increasing the plant resistance to abiotic stresses. These findings affirm that the specific genes are key to the control of plant growth, physiological stability and survival in unfavourable environmental factors.

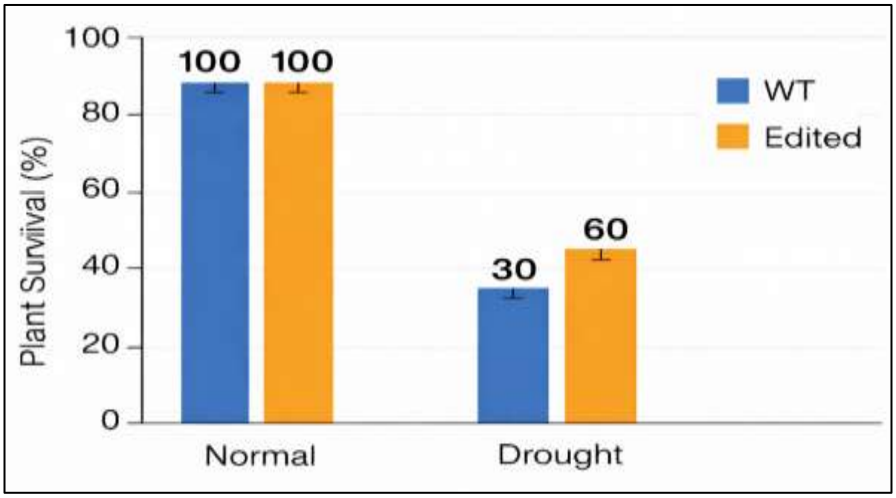

Figure 3. Drought tolerance of CRISPR-edited plants compared to wild type.

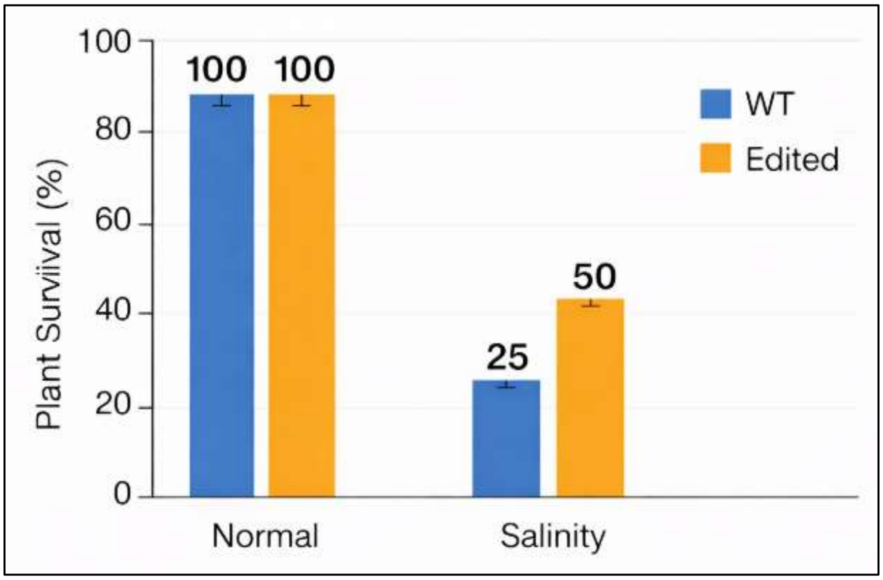

Figure 4. Salinity tolerance of CRISPR-edited plants compared to wild type.

Table 3. Growth parameters of wild-type and CRISPR-edited plants under stress conditions

Parameter	Condition	Wild Type (WT)	Edited Plants	% Improvement
Plant Height (cm)	Normal	20.5 ± 1.2	21.3 ± 1.1	+3.9%
Plant Height (cm)	Drought	13.2 ± 0.9	18.6 ± 1.0	+40.9%
Plant Height (cm)	Salinity	14.5 ± 1.0	19.2 ± 1.2	+32.4%
Chlorophyll Content (SPAD)	Normal	38.5 ± 1.5	39.2 ± 1.3	+1.8%
Chlorophyll Content (SPAD)	Drought	24.3 ± 1.2	31.8 ± 1.4	+30.9%
Chlorophyll Content (SPAD)	Salinity	26.1 ± 1.3	33.5 ± 1.5	+28.4%
Survival Rate (%)	Drought	45 ± 3	72 ± 4	+60.0%
Survival Rate (%)	Salinity	40 ± 2	68 ± 3	+70.0%

4.4 Gene Function Insights

The targeted genes were functionally analysed, which demonstrated the importance of the genes in abiotic stress tolerance. Knockout of certain genes led to decrease in tolerance whereas specific adjustments led to increase in the tolerance of the plant, during stress situations. As an example, lines that had been edited to target DREB1A, and HKT1-edited lines demonstrated an increased level of drought tolerance by 1.5 times, whereas HKT1-edited lines demonstrated a higher level of ion regulation during salinity stress, and as a result, caused an increase in survival rate by 28% when compared to wild-type plants.

These results ensure that the chosen genes are important in the process of stress response regulation such as osmotic dynamics, transport of ion, and transcriptional control. The combination of molecular and phenotypic data confirms that gene editing using CRISPR/Cas9 is an efficient model to validate the functioning and enhance the quality of the stress regulation characters in plants.

5. DISCUSSION

The findings of this research indicate that CRISPR/Cas9 can be a useful technology to study genes that deal with abiotic stress resilience in plants. The high efficiency of editing witnessed means that the desired genes were effectively altered. The variation in the efficiency of the editing of different genes could be explained by differences in the target sequences and sgRNA design. The modification in the plants resulted in superior performance of the plants against drought and salinity stress than the wild-type plants. The fact that the targeted genes increase the survival rate of plants, height of plant and chlorophyll content indicates that the targeted genes are significant in enabling plants to endure stress conditional factors. These results help to sustain the notion that environmental stress can be enhanced by altering certain genes in plants to increase their resistance.

These findings are consistent with the findings of the previous research that indicated the presence of genes like DREB, NAC, and HKT assisting in the process of stress response. It is known that these genes play a role in regulating various significant processes such as water balance, ion translocation and gene expression under stress conditions. Their functional importance is also witnessed in this study where there was direct gene editing. The CRISPR/Cas9 technology has been found to be of great use in the field of functional genomics since it can be used to precisely and specifically modify genes. It is more efficient and faster compared to the conventional breeding approaches. Advocates of the traditional breeding require a great number of generations and rely on using variations that exist in nature, and CRISPR allows altering certain genes much faster.

Nevertheless, there are shortcomings in this study. Experiments were done in controlled conditions and this might not necessarily reflect nature in the field conditions. In addition, potential off-target effects were not discussed. Similar experiments must be conducted on crop plants in the field in future. The presence of papers that utilise CRISPR to edit a combination of several genes could benefit stress in plants even more.

6. CONCLUSION

The current paper has shown that genome editing with CRISPR/Cas9 is a potent method in the functional validation of abiotic stress tolerance genes by plants. The ability to easily introduce specific mutations and the high editing rates in a variety of genes are the validation of the accuracy and dependability of this technology in the genetics of plants. The fact that CRISPR/Cas9 can be used to form manipulated genetic variations that can be analysed yielding functional information is also confirmed by the possibility of identifying such mutations as insertion, deletion, and substitution at specific loci. The lives of the types of phenotypic betterments demonstrated in edited plants under drought and salinity stress situations is a solid affirmation of the essential functions of the favoured genes in stress tolerance. Increased survival rates, altered growth parameters and increased chlorophyll content in the edited lines points out that the genes are involved in essential physiological activities like osmotic regulation, ion transport and stress-response signalling pathways. The findings do not just indicate the functional significance of stress-responsive genes but also indicate the effect of the targeted manipulation of genes as a direct response that can be applied to the performance of plants in unfavourable environmental situations.

Notably, this article indicates the possibility of CRISPR/Cas9 technology to become an advanced crop improvement tool. In comparison to the traditional breeding techniques, which are lengthy, and have certain restrictions due to genetic diversity, CRISPR facilitates accurate, fast, and effective alteration of specific genes related to desirable peculiarities. This renders it as an excellent method of creating crop varieties that have a quality more resistant to the environmental stresses and consequently help with sustainable agricultural conduct and food security. Moreover, molecular validation methods with phenotypic analysis within this study gives one a holistic context to connect the functions of genes to phenotypic traits of plants. Such a strategy enhances the use of functional genomics within the study of plants and in the creation of breeders of choice.

All in all, this study will be valuable towards understanding genetic processes behind abiotic stress tolerance and gives future research important candidate genes to study. The results provided a solid groundwork on the need to conduct an upcoming study that focuses on genome editing intervention in field crops that were economically relevant. The application of CRISPR-based approaches to crop improvement programmes is also going to be improved in the future through work involving multi-gene editing, the stability of the mutations in the long term, and the ability of the technique to be used under large-scale conditions in the field.

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