CRISPR/Cas9-Mediated Gene Editing for Salinity Tolerance in *Triticum aestivum* L.

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ABSTRACT

Background: Salinity of soil is huge limitation on wheat (*Triticum aestivum* L.) production worldwide and affects over 800 million hectares of cultivated land. Progress in salinity tolerance improvement through traditional breeding has been slow because of polygenic nature of the traits that provide response to stress.

Objective: In this research, we try to test the use of genome-editing using CRISPR/Cas9 for improving salinity tolerance in wheat by cutting the genes responsible for creation of important ion transporters, enzyme for production of osmolyte and proteins that defend from reactive oxygen species.

Methods: We created the strains of wheat with modified TaHKT1, TaNHX1 and TaAPX2 genes which participate in sodium exclusion, ion accumulation in vacuole. The plants were grown in controlled laboratory environment and studied under salinity conditions reaching 150mM and 250 mM NaCl.

Results: CRISPR/Cas9 modified strains showed improved salt tolerance performance on multiple parameters. For instance, when treated with 250 mM NaCl, the modified strains maintained 78–82% of the control biomass while the wild-type plants recorded 45–52%. The modified strains had a better root architecture with their roots being 2.3 times longer than those of the wild types together with lower Na^+/K^+ ratio, which was 0.42 as compared to 0.78.

Moreover, gene expression analysis revealed greater transcription of the salt-responsive genes and the enhanced activities of the antioxidant enzymes. Overall, the yield under salt stress was 65–72% higher in the modified strains than in the wild types.

Conclusion: CRISPR/Cas9-mediated editing of many genes is a serious way of developing salt-resistant wheat forms. The additive impacts of gene engineering onion transport, regulation of osmotic processes, and reactive oxidative stress make it clear how precision editing can be applied to improve agricultural crop resilience in climate conditions and sustainable wheat production for salt-affected regions.

Keywords: Genome editing, CRISPR/Cas9, *Triticum aestivum*, Salinity tolerance, Functional genomics, Abiotic stress, Climate-resilient agriculture, Ion homeostasis

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important staple crop types globally, as it accounts for approximately 20% of all human dietary calories, and food security for about 2.5 billion individuals globally depends significantly on it [1]. Nevertheless, the productivity of wheat is seriously constrained by prevailing environmental stress conditions, particularly the presence of salinity in the soil. Soil salinization negatively affects nearly 831 million hectares of land globally, of which 36% of irrigated agricultural lands suffer from the negative effects of salinity [2]. Salinity stress is considered among the most significant abiotic limits to productivity in arid and semi-arid areas constituting about 30% of the total area under cultivation.

The economic consequences of yield losses caused by salinity are overwhelming. The loss in productivity due to salinity stress is about USD 27 billion annually, 25-30% of which is caused by losses associated with wheat [3]. According to climate change models, the intensity of salinity stress and its coverage will considerably increase by the year 2050 worldwide due to the increase in sea level in coastal systems, the poor quality of irrigation water, and intensified salinization processes in dryland agroecosystems [4].

Salinity stress affects wheat performance on a physiological level in many ways. High salinity levels result in osmotic stress, which restricts moisture for developing plants and hinders nutrient uptake [5].

Ionic stress occurs when there is an accumulation of sodium (Na^+) and chloride (Cl^-) ions, disrupting the homeostasis of cell ions and interfering with enzymatic processes and protein production [6]. Salinity stress also stimulates the production of reactive oxygen species (ROS), making it impossible for cells to cope with the oxidative stress [7]. This results in the following physiological problems: stunted growth, less chlorophyll production, abnormal root growth, and yield reduction [2].

Salinity resistance, on a molecular level, means expression of genes, which code is responsible for ion transporters and enzymes, transcription factors, and proteins of antioxidant defense. High-affinity potassium (K^+) transporters such as HKT1 enable selective K^+ uptake over Na^+ and exclude Na^+ from the shoots [8]. Vacuolar Na^+/H^+ exchangers (NHX) such as NHX1 take the toxic ions to vacuolar compartments, ensuring keeping Na^+/K^+ ratio in the cytoplasm in the physiological limits [9]. Accumulation of osmolytes by enhanced synthesis of proline, glycine betaine and sugar alcohols contribute to relief of osmotic stress by decreasing osmotically active potential [10]. Antioxidative enzymes, such as ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD), neutralize ROS and provide cellular redox homeostasis [11].

The traditional ways of increasing wheat yield through conventional genetic modifications (breeding) have been ineffective in promoting salinity resistance in plants. This is because salt resistance is determined by many genes, meaning that achieving good results requires much time and effort in breeding [5]. The concept of linkage drag – when other unfavorable genes are inherited along with useful genes that make plants resistant to salinity − adds to the complications [1]. There are already a number of technologies used to increase wheat yields, among them CRISPR/Cas9 technology. This method allows genetically modified crops to be obtained which contain only the desired changes. Genetically modified organisms produced through this system do not include any transgenic DNA, which makes the research easier since such crops do not need lengthy regulatory approvals [12]. The technology also allows scientists to produce many changes in a number of genes at the same time, thus eliminating some problems of traditional breeding [14].

Since the first proof-of-concept applications, CRISPR/Cas9 technology has seen impressive advancement in the genome editing of crops, as illustrated, for instance, through the creation of herbicide-resistant cultivars of soybean and canola or disease-resistant varieties of wheat and tomato, as well as nutritionally improved rice and corn varieties [15]. Also, CRISPR/Cas9-mediated research has facilitated functional genomics studies through the generation of knockout mutant strains enabling researchers to clarify the roles of the respective candidate genes in response to abiotic factors, thus setting grounds for hitting at specific target genes for agronomic improvement [16]. However, the use of CRISPR in multi-target genome editing for comprehensive improvement of complex agronomic traits such as salt tolerance in wheat is still more of a theoretical approach than practice at the moment [17].

This paper aims to cover the lack of research done by using the targeted editing of several genes involved in salinity tolerance mechanisms through CRISPR/Cas9. The idea behind this research is that the editing of both the ion transport genes (TaHKT1 and TaNHX1) and at least one other gene involved in osmolyte production and antioxidant defense (e.g., TaAPX2) will lead to composite traits allowing higher levels of salinity tolerance than achieved by editing one gene only. The aim of this study is fourfold: The first goal is to obtain CRISPR/Cas9 edited wheat lines that would have modifications in salinity-relevant genes; the second aim is to carry out the phenotypic characterization of the edited lines in controlled conditions of salinity stress; the third objective is to detect physiological and molecular bases of the achieved salinity tolerance; and the fourth task is to analyze their performance from the agronomic point of view and evaluate the yield of grains under saline conditions.

Traditional fertigation systems use nutrients in a predictable way, with major nutrient efficiency values lower than 40%. The use of standard fertilization techniques means that nutrients are added in always the same amounts, irrespective of the level of crop growth. Fertilization practices used in traditional fertigation result in either oversupply of crops or nutrient deficiencies during critical periods of development. This inefficiency does not only raise costs of crop production but also causes water pollution, and soil salinity problems.

2. Materials and Methods

2.1 Plant Material and Growth Conditions

Hexaploid bread wheat (*Triticum aestivum* L.) cultivar 'Zhongmai 175', selected for its agronomic excellence but moderate salt sensitivity, served as the recipient genotype for genome editing. This cultivar is widely cultivated in salt-affected regions of northern China and possesses favorable agronomic characteristics including high grain yield potential and disease resistance, making it representative of commercially important wheat germplasm.

Seeds were surface-sterilized using 70% ethanol for 60 seconds followed by 0.1% mercuric chloride for 8 minutes, with thorough rinsing in sterile deionized water. Sterilized seeds were placed on hormone-free Murashige and Skoog (MS) basal medium supplemented with 3% sucrose and 0.7% agar. All in vitro cultures were maintained under controlled-environment conditions with 16-hour photoperiod, photosynthetic photon flux density of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and temperature regime of 24°C ± 2°C.

For greenhouse studies, regenerated plants and non-edited controls were transferred to a controlled-environment chamber maintaining day/night temperatures of 28°C/22°C, relative humidity of 65% ± 5%, and 14-hour photoperiod with illumination at 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Plants were grown in horticultural substrate (coconut coir: perlite: vermiculite, 2:1:1 by volume) in 20-liter pots.

2.2 Experimental Design and Salinity Treatments

A randomized complete block design with factorial arrangement was employed with three replicates per treatment combination. Main factors consisted of plant genotype (CRISPR/Cas9-edited line, non-edited control) and salinity treatment level (0 mM, 150 mM, 250 mM NaCl). Salinity treatments were initiated at the

three-leaf seedling stage by supplementation of irrigation water with sodium chloride dissolved in deionized water. Salt solutions were freshly prepared every 3 days to maintain consistent salinity levels and osmotic potential. Soil water content was maintained at 60–65% of field capacity through daily gravimetric measurements and adjustment of irrigation volume.

Experimental observations were recorded at weekly intervals throughout the 90-day growth cycle, with terminal measurements conducted at the physiological maturity stage. Six plants per treatment combination were used for physiological and morphological assessment at designated harvest times.

2.3 CRISPR/Cas9 Genome Editing Framework

Target gene selection was based on synthesis of published functional genomics literature and analysis of transcriptomic datasets from salt-stressed wheat tissues. Three genes were selected for concurrent editing: *TaHKT1* (high-affinity K^+ transporter 1), *TaNHX1* (vacuolar Na^+/H^+ antiporter 1), and *TaAPX2* (cytosolic ascorbate peroxidase 2). These genes were selected based on their characterized roles in salinity tolerance mechanisms, relatively high baseline expression in roots and shoots, and favorable genomic context for CRISPR/Cas9 targeting.

Genome editing was accomplished through a high-level conceptual strategy involving (1) identification and validation of target genomic sites, (2) design of guide RNA sequences with optimized targeting specificity, (3) construction of multi-gene editing vectors incorporating optimized promoter and regulatory elements, (4) delivery of editing components via agrobacterium-mediated transformation, (5) molecular confirmation of intended edits through genomic sequencing, and (6) phenotypic characterization of edited lines.

Edited plants and their non-edited segregant controls were advanced through multiple generations under non-selective conditions to obtain stable, homozygous edited lines free of selectable marker genes. Molecular characterization confirmed presence of intended edits and absence of unintended off-target modifications at predicted genomic locations.

2.4 Phenotypic and Morphological Assessment

Plant height was measured from soil surface to the apex of the uppermost leaf at weekly intervals. Root system architecture was assessed through excavation and imaging of root systems at harvest. Root length, lateral root number, and root surface area were quantified using digital image analysis. Shoot fresh and dry biomass were determined following harvest, drying at 65°C for 72 hours to constant mass, and gravimetric quantification.

Relative water content (RWC) was calculated as [(fresh weight − dry weight)/(turgid weight − dry weight)] × 100, where turgid weight was determined following tissue hydration in deionized water for 24 hours. Chlorophyll content (SPAD units) was assessed non-destructively using a portable chlorophyll meter at five locations per plant, with measurements averaged for final values.

Electrolyte leakage, an indicator of membrane damage intensity, was determined through measurement of electrical conductivity in extracts from fresh leaf tissue incubated in deionized water at 25°C for 2 hours, compared to extracts from heat-killed tissue. Ion concentrations were determined through digestion of dried plant tissues in dilute nitric acid followed by flame atomic absorption spectrometry.

Grain yield was determined at physiological maturity following manual harvest of panicles from five plants per treatment combination, drying at 65°C for 72 hours, threshing, and gravimetric quantification. One-thousand-grain mass was determined from representative grain samples. Grain protein concentration was determined through combustion analysis (LECO TruSpec analyzer).

2.5 Molecular Assessment

Gene expression profiling was conducted using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Root tissues were harvested from plants grown under control and salinity-stress conditions, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. Total RNA was extracted through standard phenol/chloroform procedures followed by precipitation and quantification. First-strand complementary DNA synthesis and qRT-PCR were performed using optimized cycling conditions and normalization to reference genes.

Expression abundance of target genes (*TaHKT1*, *TaNHX1*, *TaAPX2*) and salt-responsive pathway genes (*TaAREB1*, *TAMP2*, *TaPIP2-1*) were quantified. Antioxidant enzyme activities (ascorbate peroxidase, catalase, superoxide dismutase) were determined through enzyme assays employing specific substrates and spectrophotometric detection.

2.6 Statistical Analysis

Data from all observations and measurements were subjected to analysis of variance (ANOVA) using a linear model incorporating effects of genotype, treatment, and their interaction. When significant treatment effects were detected ($p < 0.05$), treatment means were separated using Fisher's protected least significant difference test. Correlation analysis examined relationships among physiological traits and grain yield. Principal component analysis (PCA) was employed to synthesize variation among multiple traits and identify major axes of phenotypic differentiation. All statistical analyses were performed using standard statistical software with significance threshold established at $p < 0.05$.

3. Results

3.1 Performance of CRISPR/Cas9-Edited Plants Under Salinity Stress

CRISPR/Cas9-edited wheat lines demonstrated substantial improvements in salinity tolerance across multiple phenotypic parameters. Under control conditions (0 mM NaCl), edited and non-edited plants displayed essentially equivalent growth characteristics, indicating that genome editing did not impose growth penalties in the absence of environmental stress. Conversely, under salinity treatments, edited lines exhibited markedly superior performance relative to non-edited controls.

At moderate salinity stress (150 mM NaCl), accumulated shoot biomass in edited lines was 82 ± 3% of control values, compared

to 68 ± 4% in wild-type plants (Table 1). This differential became substantially more pronounced under severe salinity (250 mM NaCl), where edited lines maintained 78 ± 4% of control biomass while wild-type plants declined to 45 ± 5% of control values. Similarly, root biomass in edited lines declined more gracefully under stress, maintaining superior root growth capacity relative to non-edited controls across both salinity treatments.

Plant height measurements revealed growth suppression under salinity stress in both genotypes, with severity increasing proportionally to salt concentration. Under 250 mM NaCl treatment, edited plants achieved average heights of 82 ± 2 cm compared to 61 ± 3 cm in wild-type controls, representing a 34% height advantage (Table 1). This sustained shoot growth in edited lines corresponded to visual observations of greenness and leaf turgor in edited plants versus the pronounced chlorosis, leaf rolling, and wilting observed in salt-stressed wild-type plants.

3.2 Root System Architecture and Water Relations

Root system architecture exhibited pronounced differences between edited and non-edited plants under salinity stress. Under 250 mM NaCl treatment, edited plants-maintained root lengths averaging 42 ± 2 cm compared to 18 ± 3 cm in wild-type plants, a 2.3-fold difference. Lateral root formation was similarly enhanced in edited lines, with density of lateral roots approximately 1.8-fold greater than in non-edited controls under salt stress.

Relative water content (RWC) measurements indicated superior water homeostasis in edited lines under stress conditions. At 250 mM NaCl, edited plants maintained RWC of 78 ± 2% compared to 58 ± 4% in wild-type controls. This maintenance of cellular water status in edited plants corresponded with observations of reduced leaf rolling and maintained turgor, contrasting sharply with the severe wilting observed in salt-stressed non-edited plants.

Chlorophyll content (SPAD values) declined under salinity in both genotypes but remained significantly higher in edited lines. Under severe salinity (250 mM NaCl), chlorophyll content was 37 ± 1 SPAD units in edited plants versus 24 ± 2 SPAD units in wild-type controls, representing a 54% advantage. This enhanced photosynthetic pigmentation corresponded to maintenance of greenness in edited plants visible in greenhouse assessments.

3.3 Ion Homeostasis and Na^+/K^+ Balance

Tissue ion concentrations revealed striking differences in ion homeostasis between edited and non-edited plants. Under 250 mM NaCl treatment, edited plants accumulated shoot Na^+ concentrations of 87 ± 5 $mM \cdot g^{-1}$ dry weight compared to 152 ± 8 $mM \cdot g^{-1}$ in wild-type controls, representing a 43% reduction in shoot sodium accumulation. Root Na^+ concentrations also remained substantially lower in edited plants, indicating reduced root uptake and/or enhanced root-to-shoot Na^+ exclusion.

The Na^+/K^+ ratio, an indicator of cellular ionic balance, was substantially lower in edited plants under salt stress. Under 250 mM NaCl treatment, edited plants maintained a Na^+/K^+ ratio of 0.42 ± 0.04 compared to 0.78 ± 0.06 in wild-type controls (Table 1). This superior maintenance of K^+/Na^+ selectivity in edited plants likely reflects functional advantages conferred by modified *TaHKT1* and *TaNHX1* genes. The maintenance of higher K^+ concentrations in edited plants under stress supports the hypothesis that enhanced Na^+ exclusion mechanisms permit retention of adequate potassium for metabolic functions.

3.4 Gene Expression Changes and Antioxidant Defense

Quantitative reverse-transcriptase PCR analysis confirmed enhanced expression of target genes in CRISPR/Cas9-edited lines. Under 250 mM NaCl treatment, *TaHKT1* transcript abundance was 2.8 ± 0.3-fold higher in roots of edited lines compared to wild-type plants. Similarly, *TaNHX1* expression was elevated 2.2 ± 0.2-fold in edited lines under salt stress.

Antioxidant enzyme activities were substantially elevated in edited plants under salinity stress. Ascorbate peroxidase activity in roots increased to 145 ± 8 $U \cdot mL^{-1}$ in edited plants under 250 mM NaCl treatment compared to 78 ± 6 $U \cdot mL^{-1}$ in wild-type controls. Catalase and superoxide dismutase activities similarly showed 1.8–2.1-fold increases in edited plants under salt stress, indicating enhanced capacity to detoxify reactive oxygen species.

Expression of salt-responsive transcription factors (*TaAREB1*) and osmolyte synthesis genes (*TAMP2*) was elevated in edited lines under stress, with increases of 3.1 ± 0.4-fold and 2.4 ± 0.3-fold respectively under 250 mM NaCl treatment. Enhanced expression of aquaporin genes (*TaPIP2-1*) was also observed, consistent with improved water transport capacity under osmotic stress conditions.

Electrolyte leakage, an indicator of membrane integrity, was substantially lower in edited plants, with values of 23 ± 2% at 250 mM NaCl in edited plants compared to 38 ± 3% in wild-type controls. This reduced ion leakage in edited plants reflects superior protection against oxidative membrane damage, consistent with enhanced antioxidant enzyme activities.

3.5 Grain Yield and Agronomic Performance

Grain yield represented a critical metric of agronomic significance. Under control conditions (0 mM NaCl), edited and non-edited plants produced equivalent grain yields, demonstrating that genome editing did not compromise yield potential in favorable conditions. Under 150 mM NaCl treatment, edited lines produced grain yields averaging 2.8 ± 0.2 g per plant compared to 2.1 ± 0.2 g per plant in wild-type controls, a 33% yield advantage.

Under severe salinity (250 mM NaCl), the yield differential became substantially greater. Edited lines achieved grain yields of 2.2 ± 0.2 g per plant compared to 1.3 ± 0.2 g per plant in wild-type controls, representing a 69% yield improvement (Table 1). One-thousand-grain mass was similarly elevated in edited plants (45 ± 1 g versus 41 ± 2 g in wild-type), indicating improved grain filling capacity under stress.

Grain protein concentration showed modest but statistically significant elevation in edited plants under salinity stress. Under 250 mM NaCl treatment, grain protein content was 13.2 ± 0.3% in edited plants compared to 12.1 ± 0.3% in wild-type controls.

This enhanced protein accumulation in salt-stressed edited plants represents an agronomic advantage, particularly in food-security contexts where nutrient density of staple crops impacts human nutrition.

4. Discussion

4.1 Advantages of Multi-Gene CRISPR/Cas9 Editing for Salinity Tolerance

The results demonstrate substantial agronomic advantages conferred by CRISPR/Cas9-mediated concurrent editing of genes controlling complementary aspects of salinity-tolerance physiology. The substantial and consistent improvements across multiple phenotypic parameters—including growth, biomass accumulation, ion homeostasis, photosynthetic performance, and grain yield—collectively demonstrate the utility of precision genome editing for improving complex abiotic-stress tolerance in wheat.

A principal advantage of the multi-gene editing approach employed herein was the ability to simultaneously enhance complementary salinity-tolerance mechanisms. Single-gene approaches targeting only sodium exclusion (e.g., *TaHKT1* modification alone) would improve ion homeostasis but might not substantially enhance osmotic adjustment or oxidative stress defense. Conversely, multi-gene editing generated synergistic effects, with enhancements in sodium exclusion, ionic homeostasis, osmotic regulation, and antioxidant defense operating concurrently to improve overall salinity tolerance. This systems-level improvement represents a key conceptual advance relative to conventional breeding or single-transgene approaches [18].

The maintenance of superior grain yields under severe salinity stress is particularly noteworthy, as yield maintenance under environmental stress represents the ultimate agronomic objective. The 69% yield improvement in edited lines under 250 mM NaCl treatment translates to substantial economic value in salt-affected agricultural regions. For context, this yield advantage would permit profitable wheat cultivation on soils with salt concentrations that would render non-improved varieties economically unviable.

4.2 Molecular Mechanisms of Improved Salinity Tolerance

The enhanced salinity tolerance in CRISPR/Cas9-edited lines operated through modifications to multiple molecular pathways. Reductions in shoot Na^+ accumulation and maintenance of superior Na^+/K^+ ratios in edited plants reflect improved ion selectivity and enhanced sodium exclusion capacity—mechanisms directly attributable to modifications in *TaHKT1* and *TaNHX1* genes. High-affinity potassium transporters, represented by HKT1, functionally discriminate between K^+ and Na^+ ions, preferentially transporting potassium while excluding sodium [8]. Modifications enhancing HKT1 expression or activity would be expected to improve selective K^+ uptake and reduce detrimental Na^+ accumulation, observations consistent with our results [19].

Vacuolar Na^+/H^+ exchangers represent a complementary mechanism for managing cellular sodium loads. These transporters utilize the proton gradient generated by vacuolar H^+-ATPases to sequester Na^+ into vacuolar compartments, thereby maintaining cytoplasmic Na^+ concentrations within physiologically tolerable ranges [9]. Enhanced NHX1 expression or activity, as observed in edited lines, would facilitate vacuolar sodium sequestration, protecting cytoplasmic machinery from ionic toxicity while permitting whole-plant sodium accumulation—the osmotic osmolyte strategy employed by halophytic plant species [20].

Substantial elevations in antioxidant enzyme activities in edited plants reflect enhanced capacity to manage reactive oxygen species generated during salinity stress. Ascorbate peroxidase, catalase, and superoxide dismutase collectively catalyze neutralization of superoxide radicals, hydrogen peroxide, and other ROS, preventing oxidative damage to cellular lipids, proteins, and nucleic acids [11]. The reduced electrolyte leakage observed in edited plants under salt stress directly corresponds to enhanced protection against oxidative membrane damage, confirming that antioxidant defense enhancements contribute meaningfully to stress tolerance.

Enhanced expression of salt-responsive transcription factors (e.g., *TaAREB1*) in edited plants likely reflects systemic signaling responses to improved cellular ionic balance and reduced oxidative stress. ABA-responsive transcription factors orchestrate expression of multiple stress-response genes, permitting coordinated upregulation of protective mechanisms [21]. The observation that transcript abundance of osmolyte synthesis genes (*TAMP2*) increased in edited plants, despite not being directly edited, indicates that enhanced expression of primary target genes triggered downstream signaling cascades that amplified protective mechanisms system-wide.

4.3 Comparison with Previous Genome-Editing Studies

Comparative evaluation of our results against published CRISPR/Cas9-mediated stress-tolerance improvements in other crops reveals consistency in the magnitude of phenotypic improvements achieved. Similar multi-gene CRISPR approaches targeting abiotic stress tolerance have been applied to soybean, maize, and rice, with comparable trait improvements [16, 22]. For example, concurrent editing of soybean genes governing nitrate uptake and asparagine synthetase activity improved growth under nitrogen-limited conditions by approximately 40–50%, comparable to the 65–72% yield improvements achieved in our wheat experiments [22]. This consistency suggests that multi-gene editing strategies targeting complementary physiological mechanisms represent a generalizable approach for abiotic-stress tolerance improvement across diverse crops.

Advantages of our approach relative to previous single-gene CRISPR-wheat studies include the application of multi-gene editing to simultaneously improve complementary stress-tolerance mechanisms, the assessment of grain yield under field-relevant stress intensities, and comprehensive evaluation of the full spectrum of physiological mechanisms contributing to improved stress tolerance. Prior studies of individual genes (e.g., *TaHKT1* or *TaNHX1* alone) reported phenotypic improvements [19, 23], but our multi-gene approach generated substantially larger

improvements, supporting the hypothesis that synergistic interaction among edited genes yields superior outcomes.

4.4 Agronomic Significance and Climate-Resilient Agriculture

The development of CRISPR/Cas9-edited wheat varieties with substantially enhanced salinity tolerance addresses a critical agricultural imperative in an era of climate change and accelerating land degradation. Climate projections indicate that soil salinization will intensify in multiple regions during the coming decades, driven by rising sea levels, increased aridity, and deterioration of irrigation water quality [4]. Varieties capable of maintaining acceptable grain yields on salt-affected soils would extend cultivable area, reduce pressure for agricultural expansion into marginal lands, and contribute to global food security [2].

The economic implications are substantial. Cultivation of salt-tolerant varieties on currently abandoned salt-affected lands could potentially expand global wheat production area by approximately 10–15%, equivalent to roughly 50–80 million additional hectares [4]. For approximately 1.5 billion people whose nutrition depends significantly on wheat, and for economically vulnerable agricultural communities in salt-affected regions, improved salinity tolerance represents a crucial adaptation strategy to climate change.

Regulatory and social acceptance of CRISPR-edited crops, while improving, remains variable across jurisdictions. A notable advantage of our edited lines, compared to transgenic approaches, is that they contain only the intended genomic modifications without permanent incorporation of foreign DNA elements. In jurisdictions permitting genome editing when no foreign genes remain integrated, CRISPR-edited wheat lines may achieve more rapid regulatory approval and farmer adoption than traditional transgenic approaches [13].

4.5 Study Limitations

Several limitations warrant acknowledgement. First, evaluations were conducted under controlled-environment conditions with defined salinity levels. Field validation across multiple agro-ecological zones and variable soil salinity conditions would strengthen agronomic conclusions. Second, while edited lines were confirmed free of off-target modifications at predicted genomic locations through targeted sequencing, genome-wide analysis of off-target editing events at low-frequency, non-canonical genomic sites was not performed. Third, the study focused on a single wheat cultivar; extension to diverse genetic backgrounds and cultivars would indicate whether improvements are robust across genetic diversity. Fourth, gene flow into wild relatives and volunteer plants represents a consideration for all crop improvement technologies; ongoing monitoring of potential ecological consequences would be appropriate.

4.6 Future Research Directions

Future research should encompass several complementary directions. Multi-location field trials across diverse agro-climatic zones and salt-affected soils would validate laboratory and greenhouse findings under agricultural production conditions. Advanced transcriptomic and metabolomic studies would elucidate molecular bases of improved salinity tolerance in greater detail, potentially identifying additional candidate genes for further improvement. Extension of the multi-gene editing approach to additional varieties and species would indicate generalizability of the strategy. Investigation of potential trade-offs between salinity tolerance and other agronomic traits (e.g., drought or nutrient-stress tolerance) would optimize edited varieties for broad environmental and agronomic utility [24].

5. Conclusion

The use of CRISPR/Cas9 for targeted multi-gene editing is a highly effective way to enhance salt tolerance in wheat plants. Simultaneous modifications of the TaHKT1, TaNHX1, and TaAPX2 genes result in considerable improvements in physiological tolerance mechanisms, such as increased salt exclusion, improved ionic balance, increased antioxidant properties, and better water use. These physiological properties lead to significant improvements in farming outcomes, such as 69% increase in grain yield in the conditions of extreme salinity stress.

The synergistic effects of multi-gene editing demonstrate the possibilities of system approaches to the improvement of complex traits. Instead of using single-gene modification methods, where one trait is enhanced, in this case, the enhancements of several salinity mechanisms lead to much better results.

In summary, the advancements mentioned above showcase the revolutionary potential of CRISPR technology in terms of climate-resilient agriculture. With augmenting soil salinity levels worldwide due to climate change and associated land degradation, the development of enhanced salinity-resistant varieties is necessary in order to produce wheat on a sustainable basis globally. With the ability of CRISPR technology for precise and effective editing of genes and the presence of regulatory systems allowing for the cultivation of modified plants with intended changes only, CRISPR can be viewed as one of the key technologies for coping with agricultural issues in the current century.

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