



OPEN Genotype-dependent salt tolerance mechanisms in wheat–*Thinopyrum* introgression lines revealed by ion transporter gene expression and seedling phenotyping

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Soil salinity significantly impairs wheat growth and yield worldwide. Wild relatives of wheat, such as *Thinopyrum* species, harbor valuable salt tolerance traits that can improve cultivated wheat via introgression. This study investigates the salt tolerance of three wheat–*Thinopyrum* introgression lines and their wheat parents during germination under salt stress (0, 100, 200 mM NaCl). Molecular cytogenetic analyses confirmed the presence and stability of *Thinopyrum* chromatin in these lines. Morphophysiological traits, including germination rate, radicle and coleoptile length, root system architecture, and biomass were assessed. Salt stress curtailed root and shoot growth across genotypes, though *Thinopyrum* introgression lines showed varied resilience. Gene expression patterns of key ion transporters (*HKT*, *SOS*, *NHX*) involved in Na⁺ exclusion and homeostasis were also evaluated in radicle and coleoptile tissues. Results showed significant genotype-dependent differences, with the 3St(3D) substitution line exhibiting strong upregulation of *TaSOS1* and *TaNHX1* linked to improved salt tolerance mechanisms. Coordinated expression of ion transporter genes suggests enhanced sodium extrusion and sequestration pathways in tolerant genotypes. These findings highlight the potential of wheat–*Thinopyrum* introgression lines as genetic resources for breeding salt-tolerant wheat cultivars.

Keywords Salt tolerance, Wheat–*Thinopyrum* introgression lines, GISH and FISH, Gene expression

Wheat ranks among the top staple crops worldwide, largely because of its broad adaptability and reliable grain yields. Beyond caloric supply, it also contributes substantially to protein intake, which explains its central role in global food security. It is thought to have been domesticated in the Fertile Crescent about 10,000 years ago. Since then, it has spread over the world and is currently grown across a variety of geographical locations and climatic conditions¹. Although wheat is a resilient cereal, biotic and abiotic stresses both lower its yield and deteriorate the quality of grain². A substantial proportion of yield losses can be attributed to environmental stressors, including salinity³. Salt stress impacts between 20 and 50% of the world's arable land particularly in arid and semi-arid regions. Its prevalence is steadily increasing due to climate change and anthropogenic activity (salt-rich irrigation water, employing poor irrigation and inadequate drainage systems, excessive use of certain fertilizers)⁴.

Studies have demonstrated that salinity stress impacts plant growth by disrupting water intake resulting in short-term in osmotic stress. In long-term this causes ion toxicity, oxidative stress, and nutritional imbalances^{5–7}. To tolerate salinity, wheat activates multiple responses, including osmotic adjustment, antioxidant defenses, and relocalization of excess Na⁺ within plant tissues. These mechanisms act at multiple biological levels, including adjustment of the gene expression levels. Together they allow plants to sustain growth under otherwise inhibitory salt concentrations⁸. Plants generally exhibit three main mechanisms contributing to salinity tolerance: (1)

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restriction of Na^+ uptake and transport in roots and shoots; (2) Na^+ exclusion from cytosol; and (3) protection against osmotic stress through the accumulation of compatible solutes^{9–11}.

Exclusion of Na^+ is a major strategy used by many *Triticum* species, particularly durum wheat, to tolerate salinity. Although crucial, it is insufficient for bread wheat on its own; additional strategies (osmotic adjustment, compartmentalization) are required. From a breeding perspective, introgressing Na^+ exclusion genes into elite cultivars, is one of the most effective ways to improve salinity tolerance. Major genes for Na^+ exclusion in wheat include *HKT* gene family (high-Affinity K^+ transporters)¹², *SOS* (salt overly sensitive) pathway genes^{13,14} and *NHX* genes (encode antiporter proteins that exchange Na^+ or K^+ for H^+ across membranes)¹⁵. *HKT* genes in wheat primarily maintain Na^+ homeostasis during salt stress by retrieving Na^+ from the xylem. This process prevents excessive Na^+ accumulation in leaves and indirectly supports K^+ nutrition and growth¹⁶. The *SOS* pathway involves plasma membrane sodium-proton antiporter *SOS1*, protein kinase *SOS2* and calcium sensor *SOS3*. Under salt stress, *SOS3* binds to elevated calcium levels, form a complex with *SOS2*. This complex phosphorylates *SOS1* to release sodium from the cell and restore equilibrium¹⁷. *SOS1* was proved to be mainly active in root cells and also contributes to long-distance Na^+ transport; *NHX* genes encode Na^+/H^+ antiporters which are mainly responsible for moving Na^+ and H^+ ions between the cytosol and the vacuole in order to maintain ion homeostasis and pH regulation¹⁸. These antiporters are essential for salt stress tolerance by sequestering excess Na^+ in the vacuole, preventing toxic levels in the cytosol. These gene families were well described previously for hexaploid wheat (*Triticum aestivum*); however, little research was conducted on the wheat/wild relatives introgression lines that resulted from wheat crosses with related species.

Elite bread wheat cultivars typically show only moderate tolerance to salinity and often fail under highly saline soils. In contrast, wild relatives - including halophytic grasses - harbor traits that breeders can exploit to enhance wheat's stress resilience¹⁹. Of the 1554 species that²⁰ classified as halophytic, 135 were grasses, comprising 38 species from the subfamily *Pooideae* and 13 *Triticeae* species. Particularly species from *Thinopyrum* genus are highly salt tolerant or even halophytic^{19,21}. *Th. ponticum* ($2n=10x=70$, JJJJJJJ^sJ^sJ^s) is a perennial grass that can withstand salt, several accessions maintained reasonable development at an Electrical Conductivity of the Saturation Extract (ECe) of 13.9 decisiemens per meter (dS m^{-1})²² and survived exposure to 750 mM NaCl ²³. Decaploid tall wheatgrass has the ability to limit the accumulation of Na^+ and Cl^- in shoots^{24,25}. It also accumulates glycine betaine in leaf tissues²⁶, a trait associated with salt tolerance. Introgression of chromosomes 3E and 7E from diploid *Triticum elongatum* ($2n=2x=14$, EE) into bread wheat has resulted in improved salt tolerance^{27–30}. Wheat/*Thinopyrum* introgression lines carrying small chromosomal segments from wheatgrass may therefore exhibit enhanced salt tolerance without negatively affecting productivity or grain quality. Despite the availability of several wheat/*Thinopyrum* introgression lines and their prior cytogenetic identification, their functional evaluation under salt stress conditions has remained largely unexplored. Moreover, due to the cytogenetic instability of wheat–alien introgressions, functional analyses without prior cytological verification may lead to ambiguous conclusions regarding the contribution of alien chromatin. Therefore, an integrated approach combining cytogenetic validation with salt stress–related phenotyping and gene expression analysis is required to reliably link stress responses to the presence of *Thinopyrum* chromatin.

Utilizing the allelic diversity of wild relatives of wheat to develop salt-tolerant wheat varieties would be a practical and sustainable solution to cope with salinity stress. The germination phase is particularly sensitive to environmental stress, as failure at this stage directly reduces plant development and ultimately yield. Even moderate salinity or water limitation can delay or inhibit germination^{31,32}. In soils with high salt concentrations or insufficient water, seeds may be unable to absorb the moisture necessary for proper germination. Evaluating germplasm responses to salinity stress during germination enables the identification of genotypes with enhanced physiological tolerance mechanisms. The genotypes with proper responses can subsequently be exploited in wheat breeding programs.

Salt tolerance is a complex, polygenic trait controlled by multiple genes, which complicates its characterization. Consequently, the development of robust salt-tolerant wheat cultivars depends on identifying and characterizing novel genes within the wheat gene pool^{33,34}. In the present study, using cytogenetically verified wheat/*Thinopyrum* introgression lines, we aimed (1) to investigate the salt tolerance of wheat/*Thinopyrum* introgression lines and their wheat parental genotypes assessing germination performance under salinity stress; (2) to examine the expression patterns of key gene families involved in Na^+ exclusion, including *HKT*, *SOS*, and *NHX*, to elucidate the molecular mechanisms involved in salt tolerance; (3) to identify introgression lines with enhanced tolerance mechanisms that could serve as valuable genetic resources for wheat breeding programs.

Materials and methods

Plant material

Three wheat–Agropyron glael [*Thinopyrum intermedium* (StStJ^TJ^{VS}.J^{VS}.) × *Th. ponticum* (JJJJJJJ^sJ^sJ^s) synthetic hybrid] introgression lines developed in the Martonvásár pre-breeding program were used in this study: (1) the 4StS.1^{VS}S disomic addition line ($2n=6x+2=44$)³⁵. All 42 wheat chromosomes are present in this genotype, along with an extra Robertsonian translocation brought about by the centric fusion of the short arms of chromosomes 4St and 1^{VS}S³⁵. (2) The 6DS.6J^{VS}. Robertsonian translocation line: the 6DL wheat chromosome arm is absent from this genotype ($2n=6x=42$) whereas the 6DS chromosome arm forms a Robertsonian translocated chromosome with the 6J^{VS}. chromosome arm³⁸; (3) the 3St(3D) disomic substitution line ($2n=6x=42$), where the 3D chromosome pair is substituted by the 3St pair³⁶. The crossing strategy for developing these genetic stocks, along with their molecular cytogenetic characterisation has been described in detail^{35–38}. When developing the introgression lines, both wheat genotypes were employed: Mv9kr1 for the development of the wheat × A. glael hybrid, and Mv Karizma for the backcrosses. Therefore, both wheat varieties were used as controls in the experiments.

Molecular cytogenetic analysis

The presence of the added *Thinopyrum* chromatin was checked using multicolour genomic and fluorescence in situ hybridization (mcGISH and FISH) on sister plants of salt stressed individuals of the three wheat/*Thinopyrum* introgression lines. For molecular cytogenetic examination, germinating seedlings were grown in a hydroponic system, and root tip meristem cells were synchronized with hydroxyurea (Sigma-Aldrich, St. Louis, MO, USA) in Hoagland solution, followed by metaphase arrest using amiprophos-methyl (Wako Pure Chemicals, Osaka, Japan)³⁹. Chromosome spreads were prepared by the air-dry drop method⁴⁰. To identify *Thinopyrum* chromatin in the wheat background, mcGISH was performed using genomic DNA from *Ps. spicata* (St genome) and *Th. bessarabicum* (J genome)³⁷. Probes were labeled with digoxigenin-11-dUTP (Roche, Basel, Switzerland) or biotin using the BioPrime™ DNA Labeling System (ThermoFisher Scientific, Waltham, MA, USA). Hybridization was carried out overnight at 42 °C with wheat genomic DNA as a block. Signals were visualized using anti-digoxigenin Rhodamine Fab fragments (Roche, Basel, Switzerland) and streptavidin-Alexa Fluor 488 conjugate (Molecular Probes, Waltham, MA, USA). Fluorescence was preserved with Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Newark, CA, USA), and slides were analyzed using a Zeiss Axio Imager Z2 epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a CoolCube 4 camera and Metafer 4/ISIS software (MetaSystems, Altlußheim, Germany). After mcGISH, slides were washed and re-hybridized using FISH probes pSc119.2, Afa-family, and oligo-pTa71^{41–43}. The probes pSc119.2 and Afa-family were labeled with the BioNick™ DNA Labeling System (ThermoFisher Scientific, Waltham, MA, USA), using digoxigenin-11-dUTP (Roche, Basel, Switzerland), while pTa71 was synthesized with biotin/digoxigenin tags (Integrated DNA Technologies, Coralville, IA, USA). Hybridizations were performed at 37 °C, and signals were re-examined under the same microscope system.

Growth conditions, salt treatment and root phenotyping

Salt tolerance was assessed during seed germination and in young seedlings. For germination assays, seeds (3 × 40 per genotype and treatment) were surface-sterilized in 1% (v/v) sodium hypochlorite for 5 min, rinsed twice in distilled water, and placed on moistened filter paper containing 0, 100, or 200 mM NaCl in Petri dishes. Germination was carried out for 3 days at 24 °C in darkness. Germination percentage (%), germination rate^{44,45}, radicle and coleoptile length, and fresh and dry biomass ($n = 3 \times 5$ per genotype and treatment) were determined, and samples were collected for RNA isolation. For early seedling assays, plants with uniform root length were transferred to aerated hydroponic systems (three 96-well plates; 16 plants per genotype and treatment) containing 0, 100, or 200 mM NaCl in 2250 mL water, and grown for 3 days (Fig. 1). On the 7th day after germination, total root length, diameter, root area, and volume of the entire root system were measured using the WinRHIZO Pro image analysis system (Regent Instruments Ltd., Quebec, Canada). Root systems were scanned without additional staining. During image acquisition, roots were spread in distilled water to facilitate separation and enhance contrast. Images were acquired with an Epson P. V85 Pro scanner at 800 dpi resolution and subsequently analysed. Root detection in WinRHIZO was based on grey level differences with automatic thresholding (≈ 128), where roots were defined as darker than the background. A minimum object size of 44 pixels was used. To exclude debris, a length-to-width ratio filter was applied to remove objects with a ratio less than 4, and rough edge removal was set to medium. These parameter settings followed the manufacturer's recommendations and ensured reproducible measurements of root characteristics. Shoot length was also determined by WinRHIZO using the same settings as for root detection.

Real-time PCR

Total RNA was isolated from radicle and coleoptile tissues of wheat plants per treatment using TRIzol reagent, following the manufacturer's protocol. The concentration of RNA samples was measured with a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). Subsequently, RNA samples underwent DNase I treatment and purification using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) synthesis was carried out with M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The *TaActin* gene, shown to be stable across various

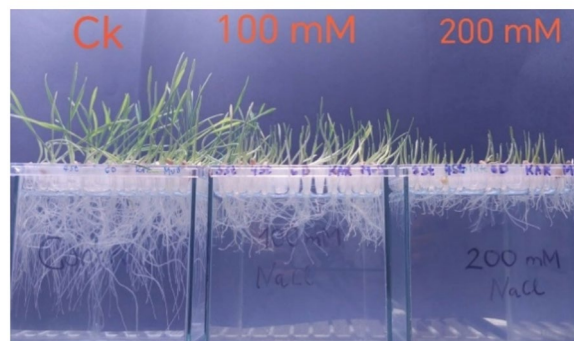


Fig. 1. Experimental design and morphological differences of 7-day-old seedlings grown in a hydroponic system using 96-well perforated plates under three different salt treatments (from left to right 0 mM, 100 mM, and 200 mM salt concentration).

conditions, served as the reference gene in RT-qPCR assays. RT-qPCR was performed using PCRBIO SyGreen Mix (PCR Biosystems, London, UK) on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each treatment included three biological replicates and three technical replicates, gene-specific and housekeeping primers (listed in Table 1) and the relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method⁴⁶.

Statistical analysis

The statistical analysis of data was performed based on the subsequent LSD's range test at a significance level of $P \leq 0.05$ with five replicates, using SAS software version 9.4. All data were performed by two-way analysis of variance (ANOVA). The effect of genotypes (G), environments (salt stress, SS, 0, 100, and 200 mM NaCl), and their combinations (G x SS) on morpho-physiological traits (radicle length, coleoptile length, fresh weight, dry weight, germination percentage, germination rate) were calculated by two-way ANOVA, using SAS software version 9.4 (SAS Institute, Cary, NC, USA). Correlation analyses and principal component analyses (PCA) were assessed to determine the relationships between the traits using OriginPro 9.1 software⁵⁰. GraphPad Prism (version 9.0.1) was employed to create visual representations of the data associated with gene expression patterns.

Results and discussions

Molecular cytogenetic confirmation of the presence of *Thinopyrum* chromosomes in the introgression lines

As introgression lines can exhibit a certain degree of genetic instability resulting in the loss of alien chromosomes, it was necessary to verify the presence of *Thinopyrum* chromosomes in the experimental plants. Multicolour genomic in situ hybridization (mcGISH) was performed on mitotic cells of the three introgression lines using genomic DNA from *Th. bessarabicum* and *Pseudoroegneria spicata* as probes. By the use these probes to the mitotic cells of the three wheat-*Thinopyrum* introgression lines, a pair of *Thinopyrum* chromosome were unambiguously detected in 4StS.1J^S disomic addition and 3St(3D) disomic substitution lines, while a wheat-*Thinopyrum* Robertsonian translocation was discriminated in the 6DS.6J^{vs}. line (Fig. 2a, c,e). The multicolour GISH performed on the experimental populations confirmed that the seeds indeed carried the respective alien chromosome (or chromosome segment) pairs. Thus, plants carrying the specific *Thinopyrum* chromosomes were used to evaluate the reported salt stress responses.

After GISH analysis confirmed the presence of individual *Thinopyrum* chromosomes in the three different introgression lines, the GISH fluorescent signals were detected under the microscope, subsequently removed, and the same slides were subjected to FISH using three repetitive DNA probes (Afa-family, pSc119.2, and oligo-pTa71). The simultaneous application of these three probes generated distinct and characteristic hybridization patterns, enabling the unambiguous identification of wheat chromosomes. The analysis confirmed the presence of all 42 wheat chromosomes in the 4StS.1J^S disomic addition line, with no visible wheat-wheat rearrangements (Fig. 2b). In the 6DS.6J^{vs}. Robertsonian translocation line, the presence of the short arm of chromosome 6D was verified (Fig. 2d). In the 3St(3D) disomic substitution line, the absence of chromosome 3D was detected, again without evidence of intra-wheat rearrangements (Fig. 2f). The names of the wheat/*Thinopyrum* introgression lines, their introgression types, and chromosomal constitutions are summarized in Fig. 2g.

Through wheat-alien introgression, hexaploid wheat's agronomic traits and resistance to biotic and abiotic stressors can be significantly enhanced or deteriorated⁵¹. Thus, the genetic stability of introgression lines is crucial, particularly when testing for salt tolerance or other agronomic traits. Alien chromosome elimination is a common phenomenon in wheat × alien species hybrids (e.g. *Thinopyrum*, *Aegilops*, etc.), which can be a consequence of several processes, e.g. improper chromosome pairing, asymmetric segregation^{52,53}, centromere and kinetochore-related issues⁵⁴, absence of homologous recombination or faulty recombination⁵⁵, epigenetic mechanism⁵⁶, the presence of gametocidal genes⁵⁷, and environmental (e.g. heat) stress⁵⁸. In practice, monosomic addition, and substitution lines are particularly prone to instability^{59,60}, which is why GISH/FISH analysis is routinely used to confirm the stable retention of alien chromosomes⁶⁰.

The FISH patterns (Afa-family, pSc119.2, and pTa71) provide a reliable reference for assessing the structural integrity of wheat chromosomes. Comparison with the standard wheat FISH pattern^{61,62} revealed no detectable wheat-wheat translocations within the resolution limits of the technique, and the introgression did not induce any random genomic rearrangements in this generation. The absence of chromosome 3D in the 3St(3D) disomic substitution line was anticipated, confirming that the genetic background of the experimental material is well defined.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	References
<i>TaActin</i>	GTGTACCCTCAGAGGAATAAGG	GTACCACACAATGTCGCTTAGG	⁴⁷
<i>TaNHX1</i>	CAGTATGTTGGTATGTTTCATGGTC	GATAGAAGCAACAAGAGCAG	⁴⁸
<i>TaNHX2</i>	AATAAGCTGGAGGCAGCAA	GTGCTAAACAGAACGACAGT	⁴⁹
<i>TaSOS1</i>	CATGCTGGGAGAGTCCACT	ACACGCGGCTCTGCTCT	⁴⁹
<i>TaSOS2</i>	GAAAACCTGCTTCTTGATTACG	GCTGCAGATCCATCATAGCC	⁴⁹
<i>TaSOS3</i>	GTTCGACCTCTTCGATCTCAAG	GAACGTCGTCGTAATGTCCTG	⁴⁸
<i>TaHKT9-7D</i>	GCTGGTGATATGGGCACTGA	AGCACAAAGAGTGTGATGGCA	¹²

Table 1. Genes and primers used for RT-qPCR analyses in the present study.

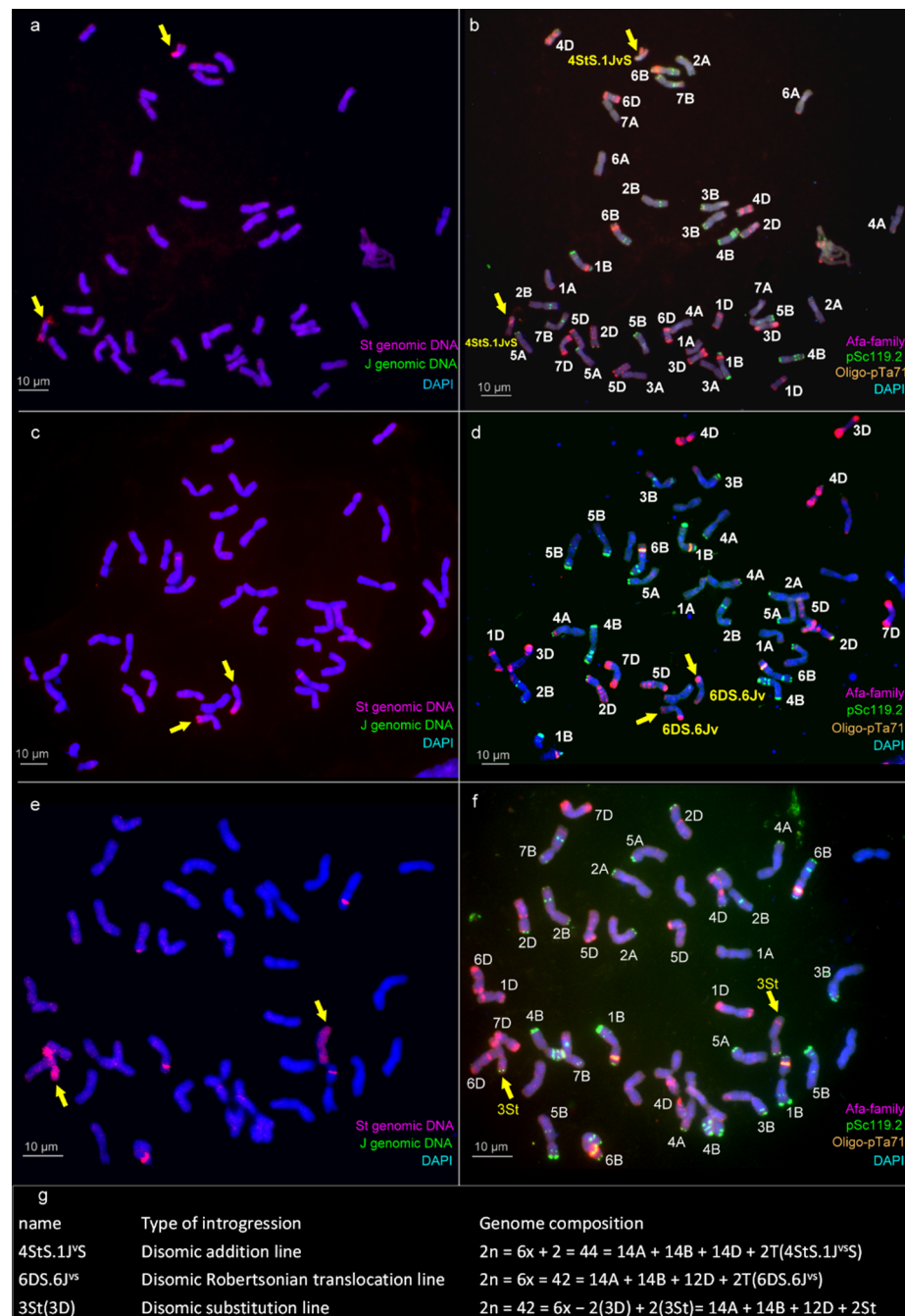


Fig. 2. Genomic in situ hybridization (GISH, **a,c,e**) and fluorescence in situ hybridization (FISH, **b,d,f**) patterns of metaphase cells from three wheat/*Thinopyrum* introgression lines. (**a,b**) 4StS.1J^vS disomic addition line: *Thinopyrum* chromosomes are indicated by yellow arrows. Repetitive DNA FISH with Afa-family, pSc119.2, and oligo-pTa71 probes enables the identification of all 42 wheat chromosomes and a pair of *Thinopyrum* chromosomes carrying Afa-family signals on the long arm. (**c,d**) 6DS.6J^vS. Robertsonian translocation line: GISH reveals a *Thinopyrum* segment fused to the short arm of chromosome 6D, corroborated by the corresponding FISH signal distribution. (**e,f**) 3St(3D) disomic substitution line: GISH confirms the presence of a *Thinopyrum* chromosome pair and the absence of a wheat chromosome pair. FISH patterns indicate the loss of wheat 3D chromosome pair. Scale bar = 10 μ m. (**g**) Names of the three wheat/*Thinopyrum* introgression lines, their introgression types, and corresponding chromosomal constitutions shown in the panel.

Changes sources	DF	Mean Squares					
		Radicle length	Coleoptile length	Fresh weight	Dry weight	Germination percentage	Germination rate
Block	2	0.086 ^{ns}	0.006 ^{ns}	0.0001 ^{ns}	0.00001 ^{ns}	16.25 ^{ns}	1.75 ^{ns}
Salt Stress (SS)	2	29.36**	2.83**	0.001**	0.00002**	482.91**	2091.02**
Genotypes (G)	4	0.620**	0.97**	0.0002**	0.00003**	199.44**	56.43**
G × SS	8	0.344**	0.217**	0.0001**	0.00001**	57.56**	18.11**
Error	28	-	-	-	-	-	-

Table 2. Analysis of variance of the effects of salt stress (0, 100 and 200 mM NaCl) on morpho-physiological traits of wheat genotypes (Mv Karizma wheat cultivar and Mv9kr1 winter wheat line) and wheat/*Thinopyrum* introgression lines (4St.1J^{vs} disomic addition line, 6DS.6J^{vs} Robertsonian translocation line and 3St(3D) disomic substitution line).

Cultivars	Treatments	Radicle length (cm)	Coleoptile length (cm)	Fresh weight (g)	Dry weight (g)	Germination percentage	Germination rate
Mv Karizma	Control	6.2 ^a	2.28 ^c	0.05 ^b	0.0033 ^{de}	95.83 ^{ab}	39.65 ^c
	100 mM NaCl	4.2 ^d	2.3 ^c	0.037 ^f	0.0036 ^{de}	90 ^{dc}	22.53 ^f
	200 mM NaCl	2.5 ^{gh}	1.4 ^e	0.024 ^g	0.0030 ^e	90 ^{cd}	18.49 ^{gh}
Mv9kr1	Control	5.6 ^b	2.27 ^c	0.06 ^a	0.0043 ^{bc}	100 ^a	44.33 ^b
	100 mM NaCl	4.3 ^{dc}	1.9 ^d	0.04 ^c	0.0033 ^{de}	95.83 ^{ab}	29.77 ^d
	200 mM NaCl	2.3 ^h	1.09 ^f	0.024 ^g	0.0021 ^f	86.66 ^d	20.21 ^{gf}
4St.1J ^{vs}	Control	5.5 ^b	2 ^d	0.04 ^c	0.0023 ^f	98.33 ^{ab}	39.44 ^c
	100 mM NaCl	4.1 ^{de}	2.36 ^c	0.045 ^{de}	0.0036 ^{de}	95.83 ^{ab}	29.94 ^d
	200 mM NaCl	2.96 ^{fg}	1.2 ^{ef}	0.024 ^g	0.0036 ^{de}	90 ^{cd}	17.66 ^h
6DS.6J ^{vs}	Control	5.5 ^b	2.27 ^c	0.048 ^{cd}	0.0036 ^{de}	95.83 ^{ab}	49.27 ^a
	100 mM NaCl	4.3 ^{cd}	2.8 ^a	0.051 ^{bc}	0.0046 ^b	85 ^d	30.77 ^d
	200 mM NaCl	3.11 ^f	1.81 ^d	0.033 ^f	0.0031 ^e	70.83 ^e	20.60 ^{fg}
3St(3D)	Control	5.8 ^{ab}	2.48 ^{bc}	0.054 ^b	0.004 ^{bc}	97.500 ^{ab}	42.60 ^b
	100 mM NaCl	4.8 ^c	2.7 ^{ab}	0.052 ^{bc}	0.0055 ^a	95 ^{bc}	26.51 ^e
	200 mM NaCl	3.5 ^e	2.46 ^{bc}	0.048 ^{cd}	0.0045 ^b	93.33 ^{bc}	22.01 ^f

Table 3. The effect of salt stress (0, 100 and 200 mM NaCl) on morpho-physiological traits of wheat genotypes (Mv Karizma wheat cultivar and Mv9kr1 winter wheat line) and wheat/*Thinopyrum* introgression lines (4St.1J^{vs} disomic addition line, 6DS.6J^{vs} Robertsonian translocation line, and 3St(3D) disomic substitution line). In each column, values followed by the same letter(s) do not have a significant difference at $\alpha = 0.05$.

In this study, the GISH/FISH analyses demonstrated that none of the three wheat–*Thinopyrum* introgression lines showed detectable unexpected deletions, wheat–wheat translocations, or other chromosomal rearrangements that could interfere with the interpretation of results in subsequent studies. The stability of the three wheat /*Thinopyrum* introgression lines has been clearly confirmed, and the differences in salt tolerance were not influenced by cytogenetic instability.

Germination assay

Five genotypes were tested for germination and early seedling growth in both control and two NaCl stress levels (100 and 200 mM). NaCl treatments decreased radicle and coleoptile length, fresh and dry weight, and germination percentage and rate across all genotypes, demonstrating the usual inhibitory effects of salinity. However, the ANOVA results indicated that salt stress (SS) and genotypes (G), and their interactions significantly influence of morpho-physiological traits in wheat genotypes (Mv Karizma wheat cultivar and Mv9kr1 winter wheat line) and wheat/*Thinopyrum* introgression lines (4St.1J^{vs} disomic addition line, 6DS.6J^{vs} Robertsonian translocation line and 3St(3D) disomic substitution line) at germination stage (Table 2). Salt Stress (SS), has a highly significant effect on all traits (radicle length, coleoptile length, fresh weight, dry weight, germination percentage, and germination rate). Salt stress clearly affects the plants' growth and germination. Genotypes (G), also have a highly significant effect on all traits, meaning the different wheat genotypes respond differently in these morpho-physiological traits under the experimental conditions. The interaction between genotype and salt stress is significant for all traits. This means the effect of salt stress depends on the genotype; different genotypes react differently to salt stress (Table 3).

With significant decreases in radicle length (from 6.2 cm in control to 2.5 cm at 200 mM NaCl) and germination rate (from 39.65 to 18.49), Mv Karizma demonstrated a high susceptibility to salt stress. Under moderate stress, Mv9kr1 maintained comparatively high germination percentages (95.8% at 100 mM); nevertheless, radicle and coleoptile development significantly declined at 200 mM NaCl, indicating intermediate tolerance. The 4St.1J^{vs} disomic addition line demonstrated considerable tolerance, especially at 100 mM NaCl, where it maintained

radicle length and germination above 4 cm and 95%, respectively, whereas reductions were more pronounced at higher salinities. The 6DS.6J^{vs} Robertsonian translocation line demonstrated the best coleoptile elongation (2.8 cm) and germination rate at 100 mM NaCl, but at 200 mM NaCl, its characteristics significantly declined. However, even at 200 mM NaCl, 3St(3D) disomic substitution line showed the most consistent performance under salt stress, with only slight decreases in radicle and coleoptile length, fresh and dry weight, and germination characteristics. In terms of salt tolerance at germination stage, the genotypes were ranked as follows overall: 3St(3D) > 4St.1J^{vs} ≈ 6DS.6J^{vs} > Mv9kr1 > Mv Karizma.

Root and shoot phenotyping under increasing salinity at seedling stage

Using the WinRHIZO Pro image analysis system, the total root length, diameter, area, and volume of the complete root system were measured on the seventh day following germination. The increase in salt concentration significantly reduced root length in all genotypes, but the reduction was more drastic between the control and 100 mM treatment (53.8% compared to the control) than between the 100 mM and 200 mM salt concentrations (73.34% compared to the control). Under control conditions, we observed significant differences between genotypes in the root length of seedlings grown in Hoagland nutrient solution.

The 4St.1J^{vs} substitution line and Mv Karizma had the longest root systems, while the root length of the Mv9kr1 parent line was significantly shorter than that of the other genotypes. In the 100 mM stress treatment, the longest root length was measured in the 6DS.6J^{vs} translocation line, but there was no significant difference between the 6DS.6J^{vs}, 4St.1J^{vs}, and Mv9kr1 genotypes. Even at a salt concentration of 200 mM, the 6DS.6J^{vs} translocation outperformed the other genotypes, but its root length did not differ significantly from the Mv Karizma parent. The root system of the Mv9kr1 line and the 4St.1J^{vs} substitution line lagged significantly behind the other genotypes. The decrease in root length at a salt concentration of 200 mM was smallest in the 6DS.6J^{vs} translocation line (69.0%), while the greatest decrease in root length was observed in the 4St.1J^{vs} substitution line (79.42%) (Fig. 3).

When examining the shoot length of seedlings, we determined that shoot length decreased significantly in parallel with increasing salt concentration. Shoot length decreased by 38.4% at a salt concentration of 100 mM and by 67% at a concentration of 200 mM, so it was concluded that the effect of increased salt concentration was significantly greater on root length and root surface area than on shoot length. In the control treatment, the longest shoot length was measured in the Mv Karizma variety, but from the introgression lines only the 4St.1J^{vs} genotype differed significantly from this parent. The drive length of the 6DS.6J^{vs} Robertsonian translocation and 3St(3D) disomic substitution lines was significantly greater than that of the Mv9kr1. There was no significant

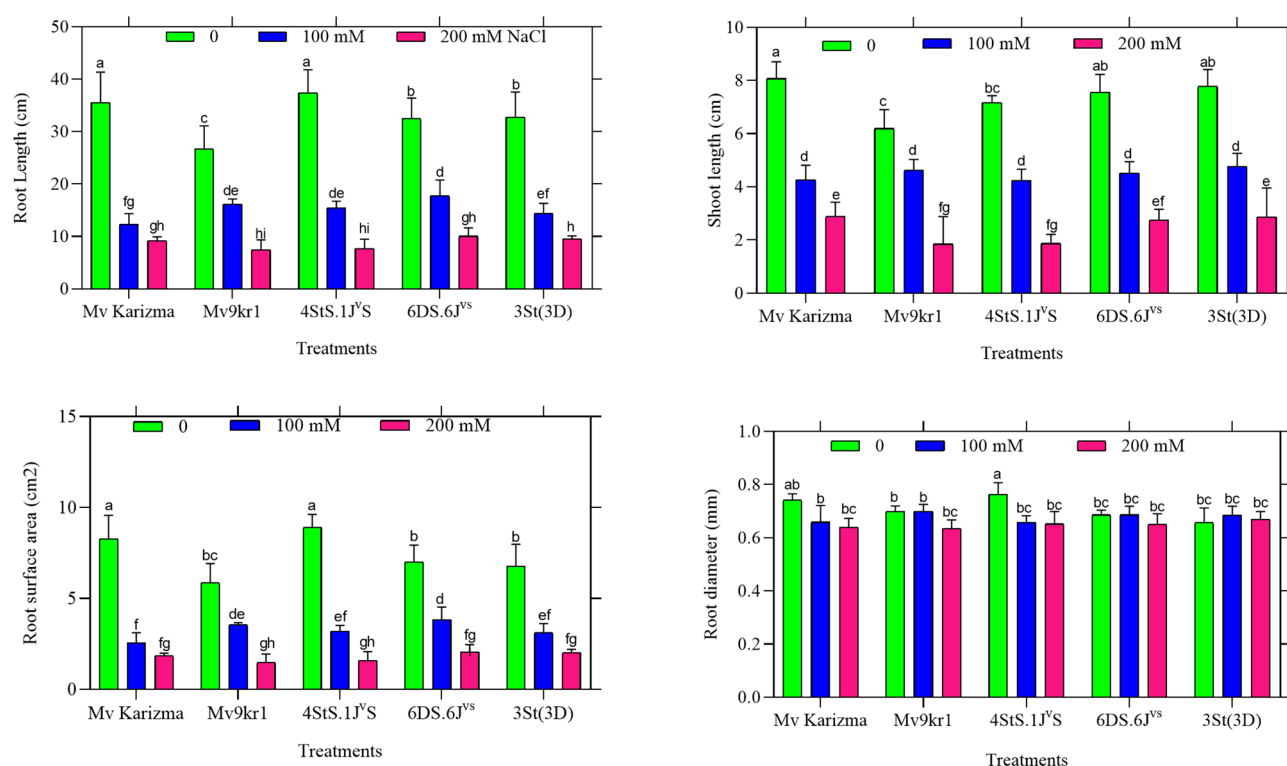


Fig. 3. The effect of salt stress (0, 100 and 200 mM NaCl) on total root length, shoot length, root surface, and root diameter, and of wheat genotypes (Mv Karizma wheat cultivar and Mv9kr1 winter wheat line) and wheat/*Thinopyrum* introgression lines (4St.1J^{vs} disomic addition line, 6DS.6J^{vs} Robertsonian translocation line, and 3St(3D) disomic substitution line). Each column represents the mean of five technical and biological replicates, and error bars indicate standard deviations. Lower-case letters above the bars denote significant differences between genotypes within each salt treatment (LSD test at $p < 0.05$).

difference between genotypes at a salt concentration of 100 mM, but at 200 mM, the shoot length of the 6DS.6J^{vs}. and 3St(3D) lines were statistically identical to that of Mv Karizma, while the shoot length of 4StS.1J^{vs} and Mv9kr1 was significantly shorter. Osmotic stress at an intensity of 200 mM reduced shoot length in the 6DS.6J^{vs}. and 3St(3D) lines to the smallest extent compared to the control (63.6% and 60%, respectively), while the largest reduction in shoot length was observed for 4StS.1J^{vs} line (73.9%).

Significant genotypic differences were observed in root surface area at each salt concentration, while generally, the salt stress significantly reduced this parameter for each genotype. On average of the genotypes, the 100 mM salt stress reduced the total root surface area by 66%, while in the 200 mM treatment this value was 75.4% compared to the control treatment. In the control treatment (0 mM), the largest root surface area was observed in the 4StS.1J^{vs} disomic addition line, which did not differ significantly from the Mv Karizma variety. In the case of the 6DS.6J^{vs}. and 3St(3D) genotypes, we did not detect any significant differences compared to either parent (Mv Karizma and Mv9kr1). At a salt concentration of 100 mM, the largest root surface area was measured in the 6DS.6J^{vs}. genotype, but this line did not differ significantly from the Mv9kr1 genotypes wheat parent. Among the lines, 100 mM stress treatment caused the smallest decrease in root surface area in the case of 6DS.6J^{vs}. (45.5%), while among the parent lines, we observed a 39.4% decrease in the case of Mv9kr1 and a 69.1% decrease in the case of Mv Karizma. At a salt concentration of 200 mM, the largest root surface area was also measured in the 6DS.6J^{vs}. and 3St(3D) lines, but no significant differences were detected between the investigated lines.

ANOVA confirmed that the salt stress had a significant effect on root diameter. Based on the evaluation of the results, we found that, on average, salt stress reduced root diameter by 4.46% and 9.5% at concentrations of 100 mM and 200 mM, respectively, compared to the control. Significant differences between the genotypes can be determined only under control conditions (0 mM NaCl). The 4StS.1J^{vs} disomic addition line has the thickest roots, which were paired with the average root diameter of Mv Karizma. The smallest root diameter was observed for the 3St(3D) disomic substitution line. The greatest decrease in root diameter induced by salinity was observed in Mv Karizma and 4StS.1J^{vs} line, while no changes in root diameter were detected in 3St(3D) line.

These results are in accordance with earlier findings in wheat germination stages where correlation analysis found that all growth traits (radicle length, coleoptile length, radicle weight, coleoptile weight, germination percentage, and germination rate) were positively and significantly correlated with each other under drought stress. Increased germination was significantly associated with longer and heavier radicles and coleoptiles⁴⁵.

Salinity significantly reduced root and shoot growth parameters in all investigated genotypes. Increasing NaCl concentration led to a progressive reduction of root length, root surface area and shoot length, while root diameter remained largely unchanged in different salt treatments. To investigate the connections between germination characteristics and early seedling growth metrics under different salt concentrations, Pearson correlation and principal component analysis (PCA) for every genotype were conducted.

The correlation pattern (Fig. 4) in the introgression lines and parents indicates close linkage or coordinated regulation of shoot and root growth parameters during germination and early seedling development. However, higher germination success (GP) might trade-off with the extent of root system growth at this stage. The data

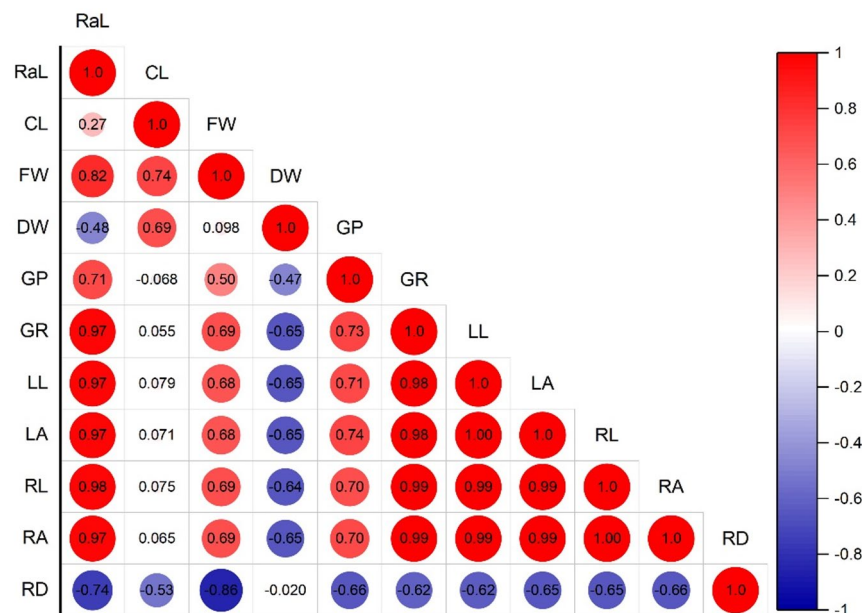


Fig. 4. Pearson correlation between germination characteristics and effect of salt stress (0, 100 and 200 mM NaCl) of wheat genotypes (Mv Karizma wheat cultivar and Mv9kr1 winter wheat line) and wheat/*Thinopyrum* introgression lines (4St.1J^{vs} disomic addition line, 6DS.6J^{vs}. Robertsonian translocation line and 3St(3D) disomic substitution line). Radicle Length (RaL), coleoptile length (CL), fresh weight (FW), dry weight (DW), germination percentage (GP), germination Rate (GR), leaf length (LL), leaf surfarea (LA), root length (RL), root surface area (RA), root diameter (RD).

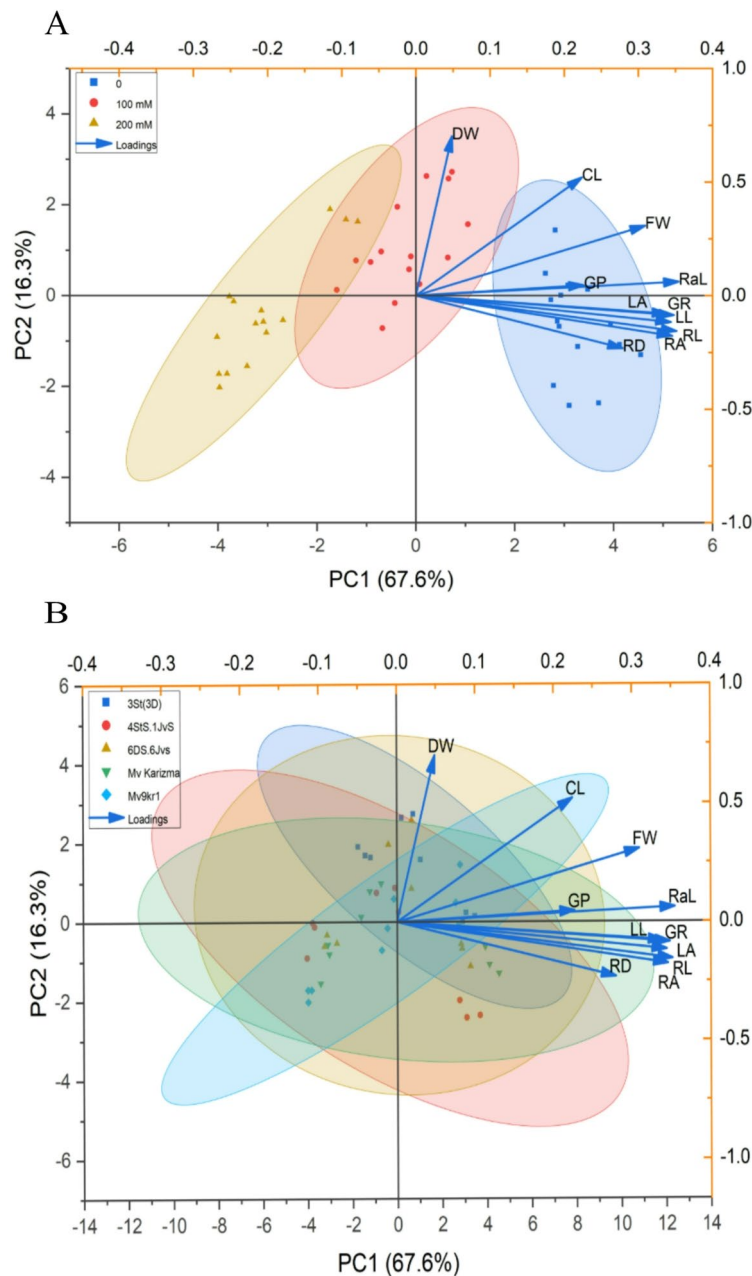


Fig. 5. Principal component analysis. (A) In different introgression lines and their parent (Mv Karizma, Mv9kr1). (B) Effect of salt stress (0, 100 and 200 mM NaCl). Radicle Length (RaL), coleoptile length (CL), fresh weight (FW), dry weight (DW), germination percentage (GP), germination Rate (GR), leaf length (LL), leaf surface area (LA), root length (RL), root surface area (RA), root diameter (RD).

can help identify which traits can be potentially selected in breeding lines to improve seedling vigour and establishment. The correlation results showed, strong positive correlations exist among several growth and size traits such as fresh weight (FW), coleoptile length (CL), radicle length (RaL), leaf length (LL), leaf surface area (LA), root length (RL), root surface area (RA), and root diameter (RD) (Fig. 4). This suggests these traits tend to increase together. Germination percentage (GP) and germination rate (GR) have moderate to strong positive correlations with each other (0.78) but negative correlations with many root-related traits such as radicle length, root length, and root diameter (Fig. 4). This may indicate germination success is not directly coupled with extensive root growth at the early stage. Dry weight (DW) showed a weak or no correlation with fresh weight (FW) and other traits, possibly reflecting variability in water content or seedling moisture status across lines. There are strong positive correlations (> 0.97) between radicle length (RaL) and various shoot-related traits such as coleoptile length (CL), leaf length (LL), and leaf surface area (LA), indicating root elongation is linked with better shoot development. Root Diameter (RD) show very strong positive correlation (0.99), as expected since thicker roots generally contribute to greater volume (Fig. 4).

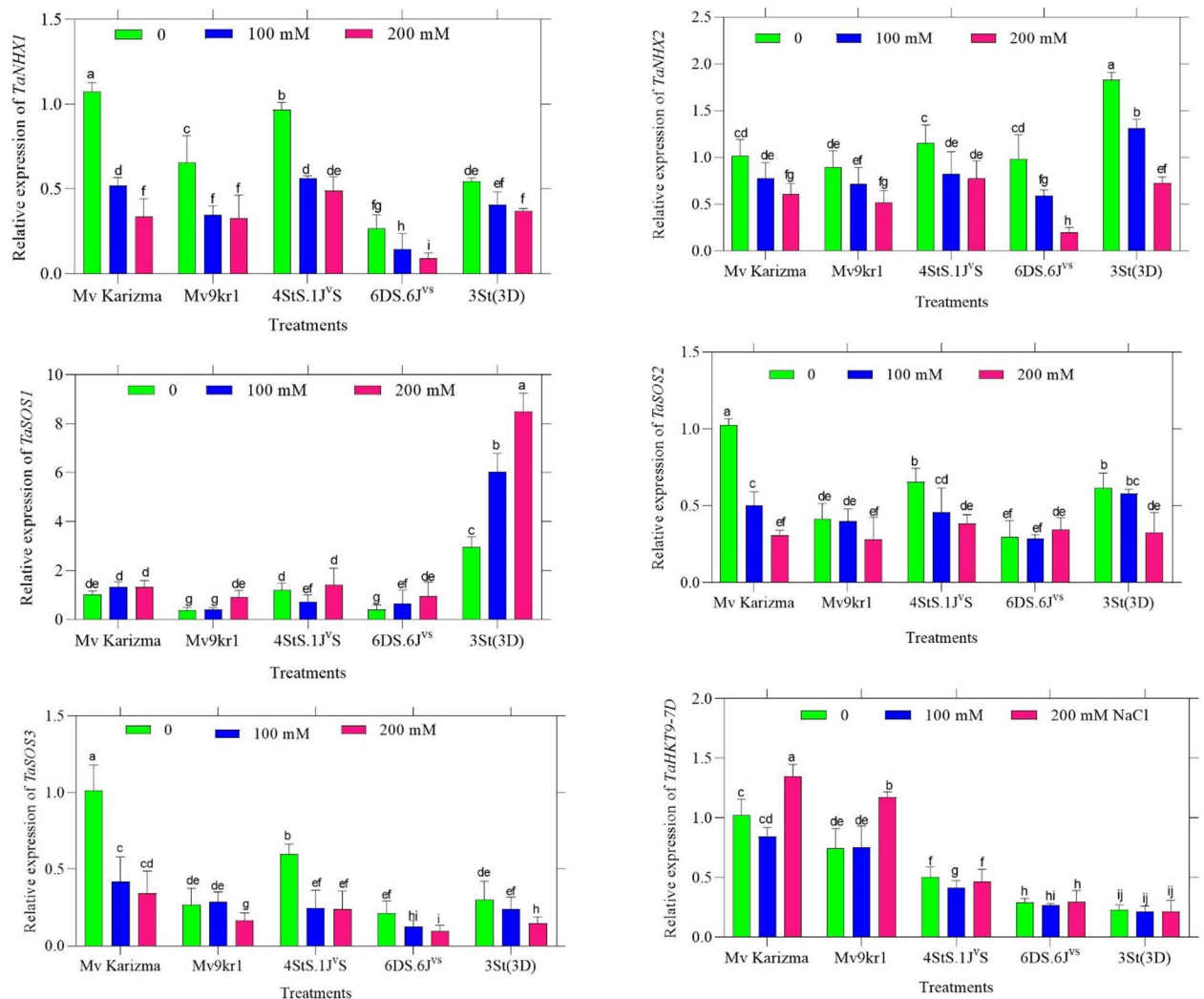


Fig. 6. The effect of salt stress (0, 100 and 200 mM NaCl) on the expression patterns of *TaNHX1*, *TaNHX2*, *TaSOS1*, *TaSOS2*, *TaSOS3*, and *TaHKT9-7D* genes in coleoptile tissue in different wheat/*Thinopyrum* introgression lines (4StS.1J^{VS} disomic addition, 6DS.6J^{VS} Robertsonian translocation, and 3St(3D) disomic substitution) and their parent (Mv Karizma and Mv9kr1). Each column is the mean expression of three technical and biological replicates. Error bars are standard deviations of biological replicates. Lower-case letters above bars indicate mean comparisons from LSD test at $p < 0.05$.

The PCA revealed that radicle length, coleoptile length, fresh weight, and germination traits largely differentiate genotypes along PC1. Dry weight contributes more distinctly to PC2. The introgression lines showed varied trait performance separating them well from each other and their parents in seedling growth and morphology traits based on this multivariate analysis (Fig. 5).

Gene expression

The expression levels of *TaNHX1*, *TaNHX2*, *TaSOS1*, *TaSOS2*, *TaSOS3*, and *TaHKT9-7D* genes in coleoptile tissue under varying NaCl salinity levels (0, 100, 200 mM) for the three introgression lines (4StS.1J^{VS} disomic addition, 6DS.6J^{VS} Robertsonian translocation, and 3St(3D) disomic substitution), as well as their wheat parents (Mv Karizma, Mv9kr1), reflect coordinated molecular mechanisms mediating salt stress response and tolerance. Collectively, these genes are known to participate in ion transport, compartmentalization, and signalling processes that contribute to salinity tolerance; however, it should be emphasized that transcript abundance alone does not provide direct evidence of ion fluxes or Na⁺/K⁺ homeostasis. In coleoptile tissues, *TaNHX1* and *TaNHX2*, which encode Na⁺/H⁺ antiporters involved in intracellular ion compartmentalization, generally downregulated by increasing salinity in most wheat varieties, except some showing moderate expression at 100 mM (Fig. 6). This pattern is consistent with previous reports indicating tissue- and genotype-specific regulation of *NHX* genes during salt stress^{15,63}. *TaSOS1*, *TaSOS2*, and *TaSOS3* are components of the salt overly sensitive (SOS) signalling pathway that regulates Na⁺ efflux and intracellular ion balance. The *TaSOS1* gene encodes a plasma membrane Na⁺/H⁺ antiporter extruding Na⁺ from cells, typically downregulated by increasing salinity in

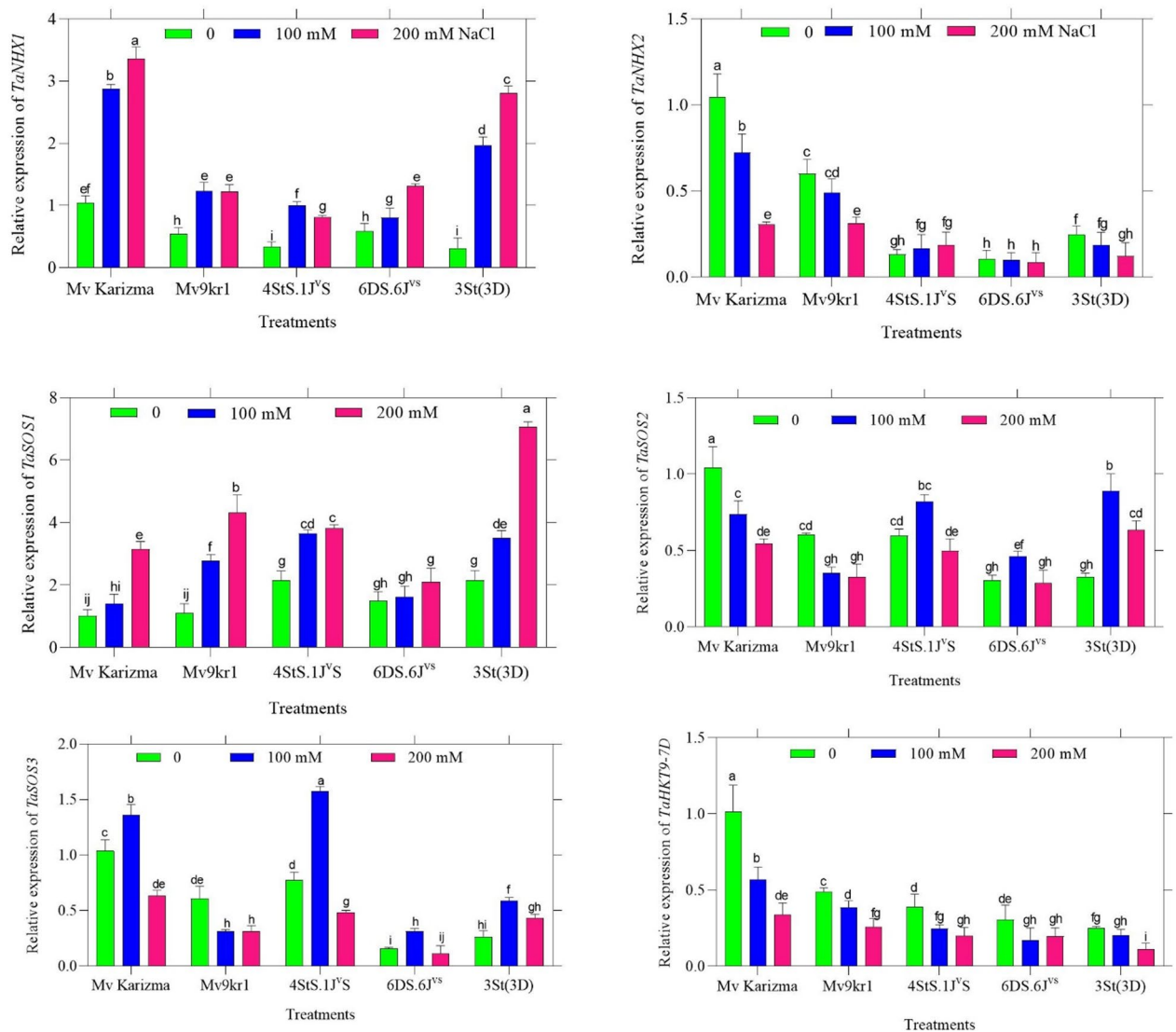


Fig. 7. The effect of salt stress (0, 100, and 200 mM NaCl) on the expression patterns of *TaNHX1*, *TaNHX2*, *TaSOS1*, *TaSOS2*, *TaSOS3*, and *TaHKT9-7D* genes in radicle tissue in different wheat/*Thinopyrum* introgression lines (4StS.1JVS disomic addition, 6DS.6JVS Robertsonian translocation, 3St(3D) disomic substitution) and their parent (Mv Karizma and Mv9kr1). Each column is the mean expression of three technical and biological replicates. Error bars are standard deviations of biological replicates. Lower-case letters above bars indicate mean comparisons from LSD test at $p < 0.05$.

most varieties except 3St(3D), which strongly upregulates *TaSOS1* in response to salinity stress (Fig. 6). *TaSOS2* and *TaSOS3* act as regulatory kinases sensing Na^+ and activating *TaSOS1* to maintain Na^+ homeostasis. The up- or downregulation of SOS genes in response to salinity varies by genotype but consistently coordinate exclusion of Na^+ from the cytosol^{64,65}. *TaHKT9-7D*, a member of the *HKT* transporter family, shows upregulation at higher salinity primarily in Mv Karizma and Mv9kr1 varieties. *HKT* transporters control Na^+ uptake and distribution between root and shoot tissues, thereby modulating shoot Na^+ accumulation and salt tolerance (Fig. 6). The upregulated expression of *HKT* genes has been widely associated with salt stress tolerance in cereals, although functional outcomes depend on tissue specificity and ion dynamics^{12,63}. Overall, 3St(3D) showed strong *TaSOS1* induction and heightened SOS pathway activity under salinity, suggesting the activation of molecular pathways associated with Na^+ extrusion; Mv Karizma and Mv9kr1 exhibit higher *TaHKT9-7D* expression affording better Na^+ homeostasis; and *TaNHX1/2* genes exhibit conserved expression patterns linked to vacuolar Na^+ sequestration (Fig. 6). This integrated gene expression network supports salt tolerance via combined cellular compartmentalization, exclusion, and signalling mechanisms, consistent with findings in wheat and other crops^{12,15,63,65}. The expression levels of *TaNHX1*, *TaNHX2*, *TaSOS1*, *TaSOS2*, *TaSOS3*, and *TaHKT9-7D* genes in radicle tissue under varying NaCl salinity levels (0, 100, 200 mM) across different introgression lines (4StS.1JVS disomic addition, 6DS.6JVS Robertsonian translocation, and 3St(3D) disomic substitution) and their parental genotypes (Mv Karizma and Mv9kr1), are involved mainly in ion transport and homeostasis for salt stress

tolerance in wheat. Under 200 mM, *TaNHX1* typically exhibited elevated expression, particularly in Mv Karizma and 3St(3D) genotypes, signalling its active role in salt tolerance through compartmentalization (Fig. 7).

TaNHX2 showed reduced expression under salinity, highest at 0 mM in Mv Karizma and Mv9kr1, suggesting its role is reduced under salt stress, consistent with distinct regulatory mechanisms or tissue-specific roles. The proteins encoded by *NHX1* and *NHX2* remain crucial for maintaining ionic balance and cell turgor under stress^{66,67}. *TaSOS1* showed higher expression at 200 mM NaCl in certain varieties (3St(3D) disomic substitution and Mv9kr1 wheat line) aligns with its role in active Na⁺ efflux⁶⁸. *TaSOS2*, and *TaSOS3* showed reduced expression under salinity. *TaSOS2* decreases in Mv Karizma but rises in 3St(3D) disomic substitution line and 4StS.1J¹S disomic addition line under salt, reflecting varied activation in salt signalling while, *TaSOS3* patterns vary, with high expression at 100 mM in 4StS.1J¹S; it is part of the Ca²⁺ sensing salt stress response (Fig. 7).

SOS2 encodes a protein kinase that activates *SOS1* by phosphorylation, with varied expression indicating different regulatory dynamics among varieties. *SOS3* is a calcium sensor that senses salt-induced Ca²⁺ signals and interacts with *SOS2* to initiate the pathway, with expression patterns suggesting modulated signalling activity in response to stress. Together, these genes tightly regulate Na⁺ homeostasis and stress responses⁶⁹. *TaHKT9-7D* encodes a transporter involved in K⁺ uptake or exclusion, aiding ionic balance maintenance. Under salinity, *TaHKT9-7D* declines across all varieties, peaking highest at 0 mM in Mv Karizma, tied to K⁺ transport modulation (Fig. 7).

Under salt stress, the 3St(3D) disomic substitution line shows strong induction of the *TaSOS1* gene, which encodes a plasma membrane Na⁺/H⁺ antiporter that extrudes sodium ions from cells to sustain ion balance and avoid toxicity. *TaNHX1* expression also rises, encoding a vacuolar Na⁺/H⁺ antiporter that sequesters excess cytosolic Na⁺ into vacuoles, thus safeguarding the cytosol and preserving osmotic balance. The *Thinopyrum* chromatin in 3St(3D) probably harbors alleles or regulatory elements that boost expression of these genes or adjust the salt-responsive signalling pathway they engage. Therefore, the enhanced expression of *TaSOS1* and *TaNHX1* in the 3St(3D) genotype indicates an increased transcriptional engagement of pathways associated with sodium ion extrusion and vacuolar sequestration, driven by the introgressed *Thinopyrum* genetic material, which contributes to better salt stress tolerance in this line compared to wheat parents and other introgression lines. In summary, the substitution of chromosome 3D with 3St chromosomes introduces salt tolerance-associated genes or regulatory networks that upregulate *TaSOS1* and *TaNHX1* expression under salt stress, providing improved ionic regulation at the transcriptional level in the 3St(3D) disomic substitution line.

Its downregulation with increased salinity in all varieties indicates a role in restricting Na⁺ entry or modulating K⁺/Na⁺ transport under salt stress, preventing ion toxicity⁷⁰. Under increasing salt stress, plants activate specific genes to manage Na⁺ ions to maintain cellular homeostasis. *TaNHX1* and *TaSOS1* are upregulated to compartmentalize and extrude sodium, respectively, and these play major roles in tolerant varieties such as Mv Karizma and 3St(3D) disomic substitution line. *TaSOS2*, and *TaSOS3* modulate the signalling cascade for *TaSOS1* activation, showing variety-dependent responses. *TaNHX2* and *TaHKT9-7D* are downregulated or differently regulated, reflecting their distinct or complementary functions in ion transport balance (Fig. 7). Differential expression patterns across wheat varieties indicate varied capacities and strategies for salt tolerance at the radicle tissue level.

Conclusion

This study demonstrated that wheat carrying different *Thinopyrum* chromosome segments exhibit differential salt responses during germination and early seedling growth, mediated by distinct morpho-physiological traits and gene expression profiles. Molecular cytogenetic analyses confirmed the genomic stability of introgression lines, ensuring reliable phenotypic evaluation. Salt stress significantly reduced growth parameters, yet introgression lines displayed variable tolerance levels, underscoring the value of wild relative alleles. The 3St(3D) substitution line, in particular, showed enhanced expression of *TaSOS1* and *TaNHX1*, facilitating effective sodium extrusion and vacuolar sequestration, key mechanisms conferring salt tolerance. These results provide molecular and physiological insights supporting the use of *Thinopyrum* species in breeding programs aimed at improving wheat resilience to salinity stress, a critical challenge for global food security in the face of increasing soil salinization.

Data availability

All the data in the present study are included in this manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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