Supporting Information

Yang et al. 10.1073/pnas.1412839111

SI Materials and Methods

Plant Material. A synthetic allohexaploid wheat genotype "Allo-960" [produced by crossing a *Triticum turgidum*, accession Blackbird (genome BBAA) with a *Aegilops tauschii*, accession 30A (genome DD) (1)], its two parental genotypes, and a natural hexaploid bread wheat (*Triticum aestivum*, cv. Chinese Spring) were used in the present study. The initial seeds of Allo-960 were procured from George Fedak (Agriculture and Agri-Food Canada), and the plants were then self-pollinated for seven generations in our hands. In this study, Allo-960 was labeled as neo-6*x*, the natural hexaploid wheat as nat-6*x*, the tetraploid wheat (*T. turgidum*) as 4*x*, and the diploid goat-grass (*Ae. tauschii*) as 2*x*. We defined two types of salt-stress treatments, type I and type II, to the four genotypes listed above. Type I salt stress was planned to determine germination and survival rates of the four genotypes, whereas type II salt stress was planned to assess the physiological bases underlying salt tolerance by measuring morphophysiological differences between 4*x* and 6*x*. In type I treatment, we subjected plants of each genotype to a constant salt stress, from seed germination to seedling growth for a defined duration, which mimics conditions in the natural saline habitats. In type II treatment, salt was applied to the 30-d-old seedlings (starting from the day of sprouting) grown under normal conditions.

Determination of Germination Rate. Three biological replicates with 50 seeds each were germinated under normal and salt-stressed conditions. For this purpose, the seeds were sown in 8.5-cm tight-fitting Petri dishes and submerged in 10 mL of nutrient solution supplemented with 100–500 mM of NaCl. The dishes were placed in a vitreous greenhouse maintained at 25 °C day and 18 °C night temperatures under 16 h light at 300 μmol $m^{-2}{\cdot}s^{-1}$. Germination was recorded daily for 8 d, and the germination rate was calculated following Brenchley and Probert (2). The nutrient solution in the Petri dishes was replaced daily during the course of study.

Survivorship Under Type I Salt Stress. Six biological replicates with 20 seeds each were used per treatment. Seeds of each of the four lines were sown in plastic pots containing thoroughly washed sand. The pots were watered with a half-strength Hoagland nutrient solution with or without 150 mM of NaCl daily. Plants were continuously subjected to salt stress from seed germination to the 30 d of growth, a treatment defined as type I salt stress. The experiment was performed from mid-April to early June, under a temperature regime of 19–25 °C/14–17 °C. All pots were placed outdoors with protection from rain.

Plant Growth Under Type II Salt Stress. Seeds of the four genotypes were sown in plastic pots each containing five seedlings, which served as biological replicates. The seedlings were supplied daily with a half-strength Hoagland nutrient solution to saturation for 30 d, after which (at about the four-leaf stage), salt stress was applied for 32 or 36 d by supplementing the nutrient solution with 150 mM of NaCl, a treatment defined as type II salt stress. All pots were placed outdoors with protection from rain. The experiment was performed from the mid-April to early July, under a temperature range of 19–26 °C/14–19 °C.

Measurement of Photosynthesis-Related Parameters and Assessment of Stomatal Anatomy. After 32 d of salt stress (type II), anatomical analysis of stomata and measurement of photosynthetic parameters were performed on 15–25 seedlings of each genotype. Net photosynthetic rate (P_N), internal CO_2 concentration (C_i), and stomatal conductance (g_s) of leaves were determined during 8:30–10:30 AM on fully expanded leaf blades, using a portable open flow gas exchange system LI-6400 (LI-COR). The photosynthetic active radiation (PAR) was 1,200 μmol $m^{-2}{\cdot}s^{-1}$ (i.e., at saturation). Stomatal length and number were measured using the nail polish impress method (3). Eight to 15 stomata for each individual were recorded. The measurements and counts were made using a light microscope (BX61; Olympus). Starch and chlorophyll amounts in the leaves were determined according to the method reported in ref. 4.

Biochemical Measurements. After 32 d of type II salt stress, plant materials were harvested from each genotype under normal and salt-stressed conditions for biomass determination and biochemical analysis. Root, leaf, culm, and spike of all genotypes were collected and assayed. Spikes of *Ae. tauschii* (2*x*) were not included in these analyses for reasons explained in the main text. For each treatment, four biological replicates were included in the analyses. To minimize plant-to-plant variations, five seedlings were pooled and triturated for biochemical measurements in each replicate. The dried leaves were hydrolyzed with HCl, and subsequently amino acid content was measured using an automated amino acid analyzer (Sykam; S-433D). Free amino acids were separated and also measured by an automated amino acid analyzer (5). Dry samples of the plant material were suspended in 10 mL of deionized water maintained at 100 °C, and the extracts were used to determine the contents of free inorganic anions. The contents of NO_3^-, Cl^-, $H_2PO_4^-$, and SO_4^{2-} were determined by ion chromatography with the DX-300 ion chromatographic system (Thermal Scientific) using an AS4A-SC ion-exchange column and a CD M-II electrical conductivity detector (Dionex). The mobile phase consisted of $Na_2CO_3/NaHCO_3$ = 1.7/1.8 mM. Proline and soluble sugars were measured using ninhydrin and anthrone, respectively (6). Dry samples were steeped for 48 h in 65% HNO_3, then digested three times using 65% HNO_3 at 100 °C, and the extracts were used to determine the content of mineral elements. A flame photometer was used to determine K^+ and Na^+ contents, and an inductively coupled plasma emission spectrometer was used to measure the contents of Ca, Mg, Fe, Mn, Cu, Zn, B, Mo, and P. Membrane permeability, represented by electrolyte leakage rate (ELR), was determined by an ameliorated method (7).

Fitness Measurements. The spikelet development of 20 individuals of each genotype was assessed. For this purpose, after 36 d of type II salt stress, the spikes were photographed, and spikelet development was examined. As *Ae. tauschii* (2*x*) requires vernalization for flowering, it was excluded from the analyses.

Coupled TaqMan SNP Genotyping and Quantitative RT-PCR Assays. After 10 d of type II salt stress, the leaves and roots harvested from the control and salt-stressed plants were collected to extract total RNA from five plants per genotype grown under salt-stress or normal conditions using TRIzol reagent (Invitrogen). The RNA was treated with DNaseI (Invitrogen), reverse transcribed using SuperScriptTM RNase H-Reverse Transcriptase (Invitrogen), and then subjected to TaqMan SNP Genotyping Assay and quantitative RT-PCR (qRT-PCR) analysis using gene-specific primers (Dataset S5). The wheat *Actin*, *β-tubulin*, and *RLI* genes were used as internal controls in the assay. The qRT-PCR amplification conditions were 95 °C for 5 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative expression levels of

the targeted genes were calculated using the ΔΔCt method (8). Subsequently, a TaqMan SNP Genotyping Assay (9, 10) was performed to assess the relative expression levels of the *HKT1;5* homeologs, *HKT1;5-D* and *HKT1;5-B* in neo-6*x* and nat-6*x*. For this purpose, the gene-specific primers were designed from the regions conserved between *HKT1;5-D* and *HKT1;5-B*, and TaqMAN-MGB probes were designed to target homeolog-specific SNPs. In neo-6*x* and nat-6*x*, homeolog-specific gene expression was calculated from the ratio of homeolog-specific transcripts to the total transcripts, and dividing this value by the total gene expression level determined by qRT-PCR. The TaqMan SNP Genotyping Assay and qRT-PCR was performed on the ABI StepOnePlus PCR apparatus (Applied Biosystems). The reaction mixture contains the master mixture supplied with the Taqman Universal Master Mix kit (ABI), 200 nM of each primer, 200 nM of *HKT1;5-B*–specific TaqMan probe labeled with FAM dye, and 200 nM of *HKT1;5-D*–specific probe labeled with VIC dye. The conditions of PCR amplification were 50 °C for 30 s and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for a 1-min extension. At the end of PCR amplification, terminal fluorescence signal was detected to determine the respective expression levels of *HKT1;5-D* and *HKT1;5-B*. Notably, in parental genotypes (2*x* and 4*x*), we observed a weak amplification of the homeologous gene. For instance, in case of 2*x* we observed a weak amplification of *HKT1;5-B*, and in 4*x* we observed a weak amplification of *HKT1;5-D*. However, these weak nonspecific amplifications were unlikely to affect the results because they only constitute small proportions of the total amplified product and therefore have negligible effects on the relative expression ratio of *HKT1;5-D* vs. *HKT1;5-B*. The amplification plots are shown in Dataset S4.

Statistical Analysis. Statistical analysis was performed using the statistical program SPSS 13.0. All data were from 3–25 biological replicates. Statistical significance was determined by *t* test or least significant difference (LSD).

1. Zhao N, et al. (2011) Extensive and heritable epigenetic remodeling and genetic stability accompany allohexaploidization of wheat. *Genetics* 188(3):499–510.
2. Brenchley JL, Probert RJ (1998) Seed germination responses to some environmental factors in the seagrass *Zostera capricorni* from eastern Australia. *Aquat Bot* 62(3): 177–188.
3. Maherali H, Walden AE, Husband BC (2009) Genome duplication and the evolution of physiological responses to water stress. *New Phytol* 184(3):721–731.
4. Ni Z, et al. (2009) Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* 457(7227):327–331.
5. Dluzniewska P, et al. (2007) Nitrogen uptake and metabolism in Populus x canescens as affected by salinity. *New Phytol* 173(2):279–293.
6. Guo J (2006) *Laboratory Manual of Plant Physiology* (Higher Education Press, Beijing).
7. Lutts S, Kinet J, Bouharmont J (1996) NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Ann Bot (Lond)* 78(3):389–398.
8. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402–408.
9. Tomás G, et al. (2012) Development and validation of a TaqMan-MGB real-time RT-PCR assay for simultaneous detection and characterization of infectious bursal disease virus. *J Virol Methods* 185(1):101–107.
10. Alonso R, Mateo E, Cisterna R (2007) Detection of *parC* mutations in *Streptococcus pneumoniae* by real-time PCR and Taqman-MGB probes. *J Microbiol Methods* 69(1): 214–217.

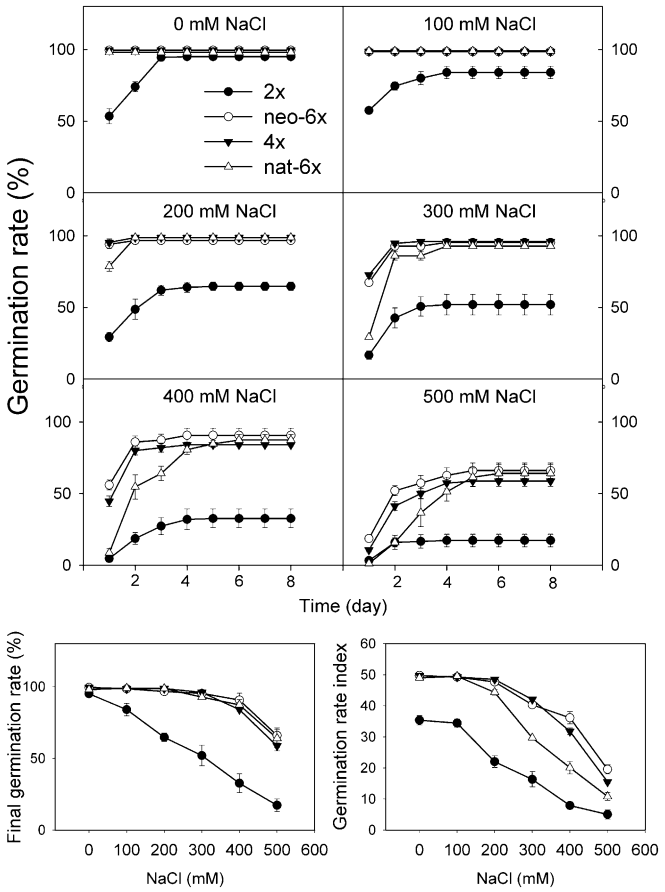

Fig. S1. Effects of salt stress (type I salt stress) on germination in the synthetic allohexaploid wheat (neo-6*x*), its diploid (2*x*) and tetraploid (4*x*) parents, and natural hexaploid wheat (nat-6*x*). The values are means (±SE) of three biological replicates.

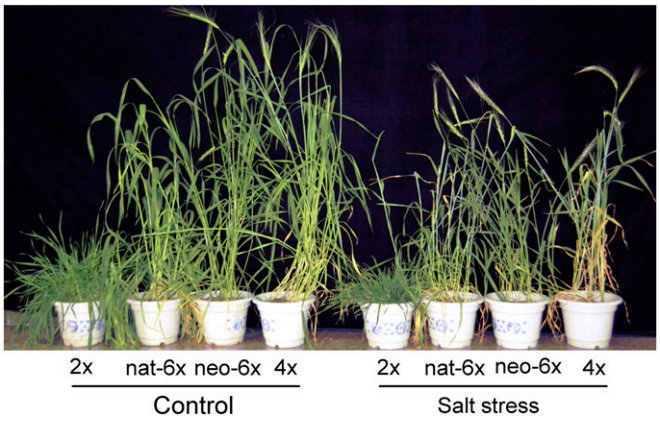

Fig. S2. An example of plants subjected to type II salt stress for 32 d (150 mM NaCl treatment to 30-d-old seedlings) to demonstrate the effects of salt stress on plant growth of neo-6*x*, its diploid (2*x*) and tetraploid (4*x*) parents, and nat-6*x*.

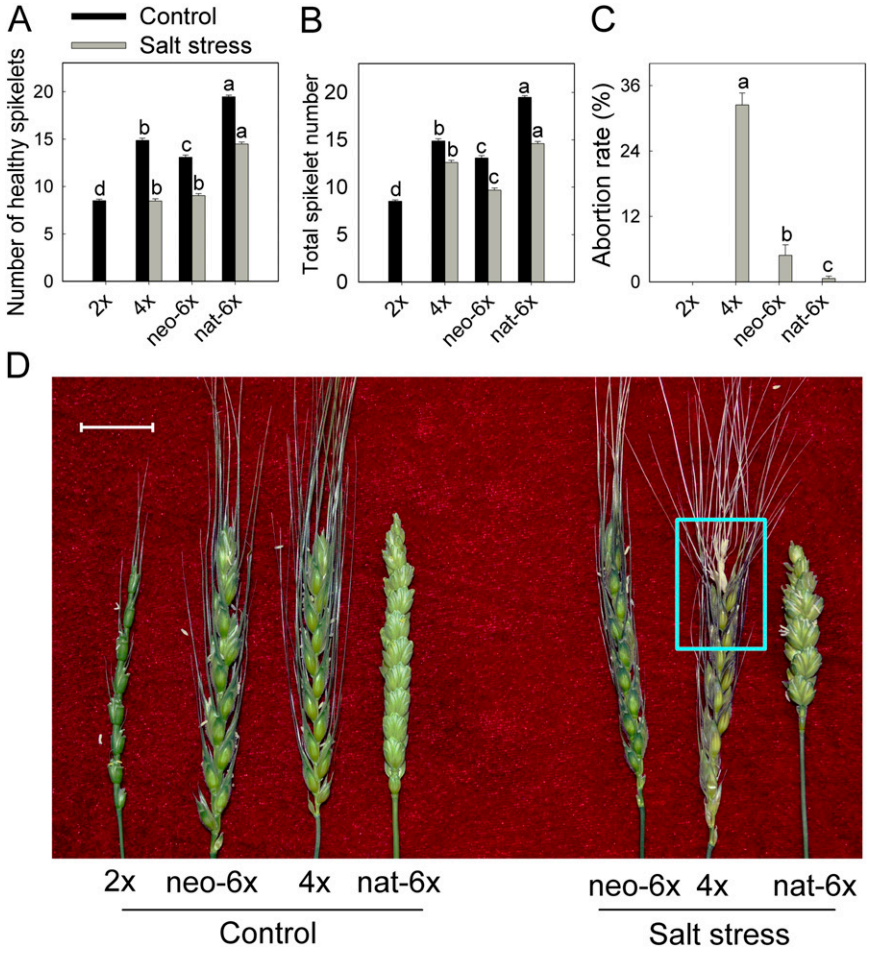

Fig. S3. Effects of salt stress on spikelet development in neo-6*x*, its diploid (2*x*) and tetraploid (4*x*) parents, and nat-6*x*. Means labeled by different letters among the genotypes are significantly different according to LSD ($P < 0.05$) (*A*–*C*). The 30-d-old seedlings grown under normal conditions were subjected to salt stress (150 mM NaCl) for 36 d (type II salt stress), and 20 individuals of each genotype were measured. Rectangle in *D* shows typical phenotype of salt-injured spikelet. (Scale bar, 2 cm.) Abortion rate = number of salt-injured spikelet/total number of spikelet × 100%.

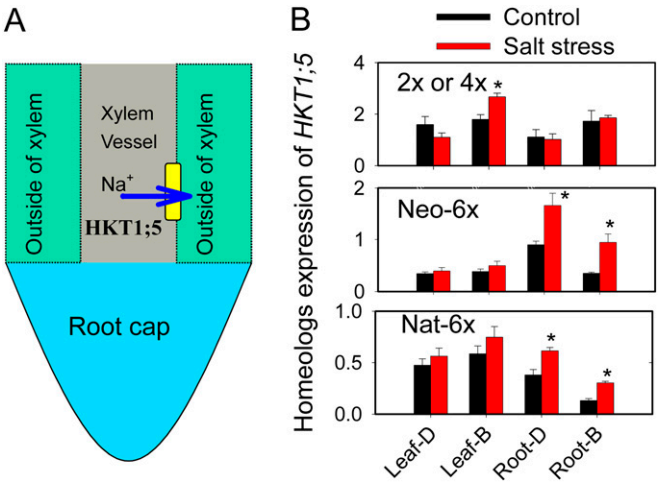

Fig. S4. Diagrammatic illustration of the in planta function of the HKT1;5 gene in retaining Na^+ within wheat roots (*A*), and steady-state transcript levels of the two homeologs (*HKT1;5-B* and *HKT1;5-D*) of the HKT1;5 gene, in the four genotypes, neo-6*x*, 2*x*, 4*x*, and nat-6*x*, under control and salt-stressed conditions (*B*). Coupled qRT-PCR and TaqMan SNP Genotyping Assay was used to quantify the homeolog-specific transcripts. The values are means of three to five biological replicates. RNAs were isolated from seedlings of mock control or plants subjected to type II salt stress for 10 d. Significant difference (*t* test, $P < 0.05$) in the quantity of homeolog-specific transcripts between control and salt-stressed plants for a given genotype is indicated by asterisks.

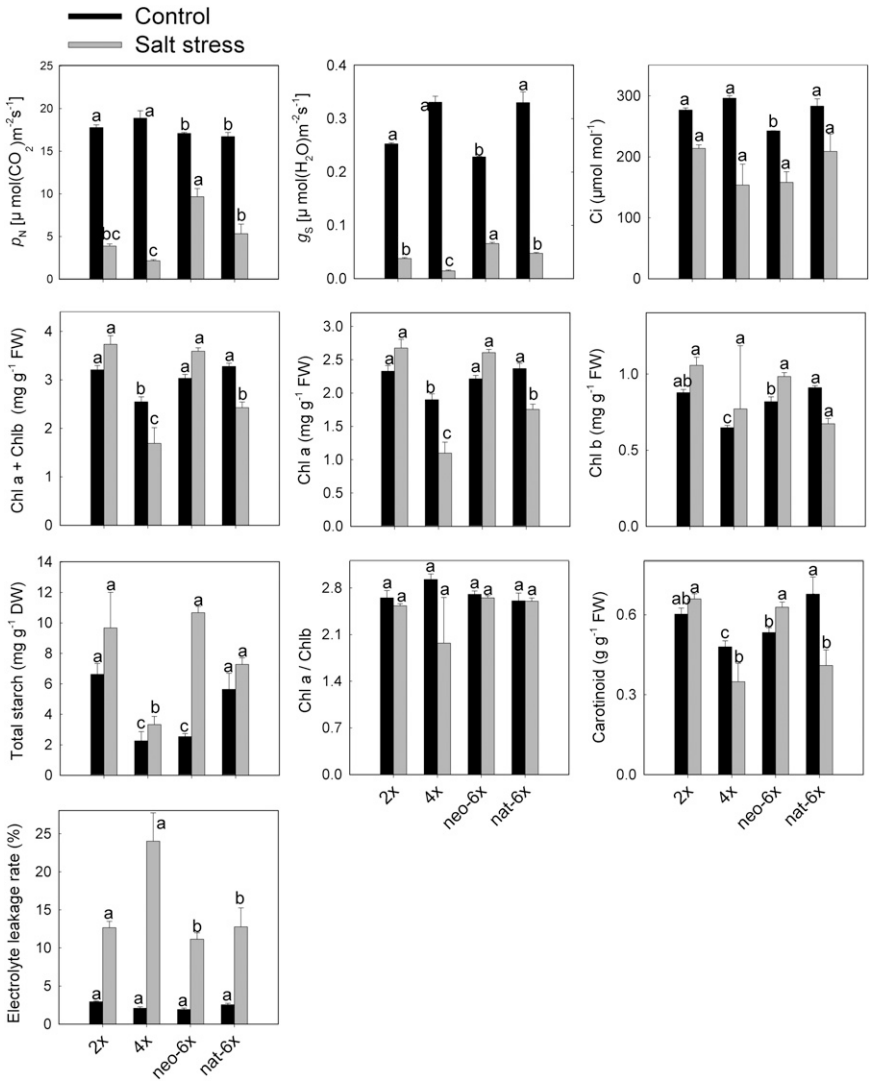

Fig. S5. Effects of salt stress on photosynthesis in neo-6*x*, its diploid (2*x*) and tetraploid (4*x*) parents, and natural allohexaploid (nat-6*x*). The values are means of four biological replicates. g_s, stomatal conductance; P_N, net photosynthetic rate; C_i, internal CO_2 concentration; and Chl, chlorophyll. Means followed by different letters are significantly different according to LSD ($P < 0.05$). The 30-d-old seedlings grown under normal conditions were subjected to type II salt stress for 32 d.

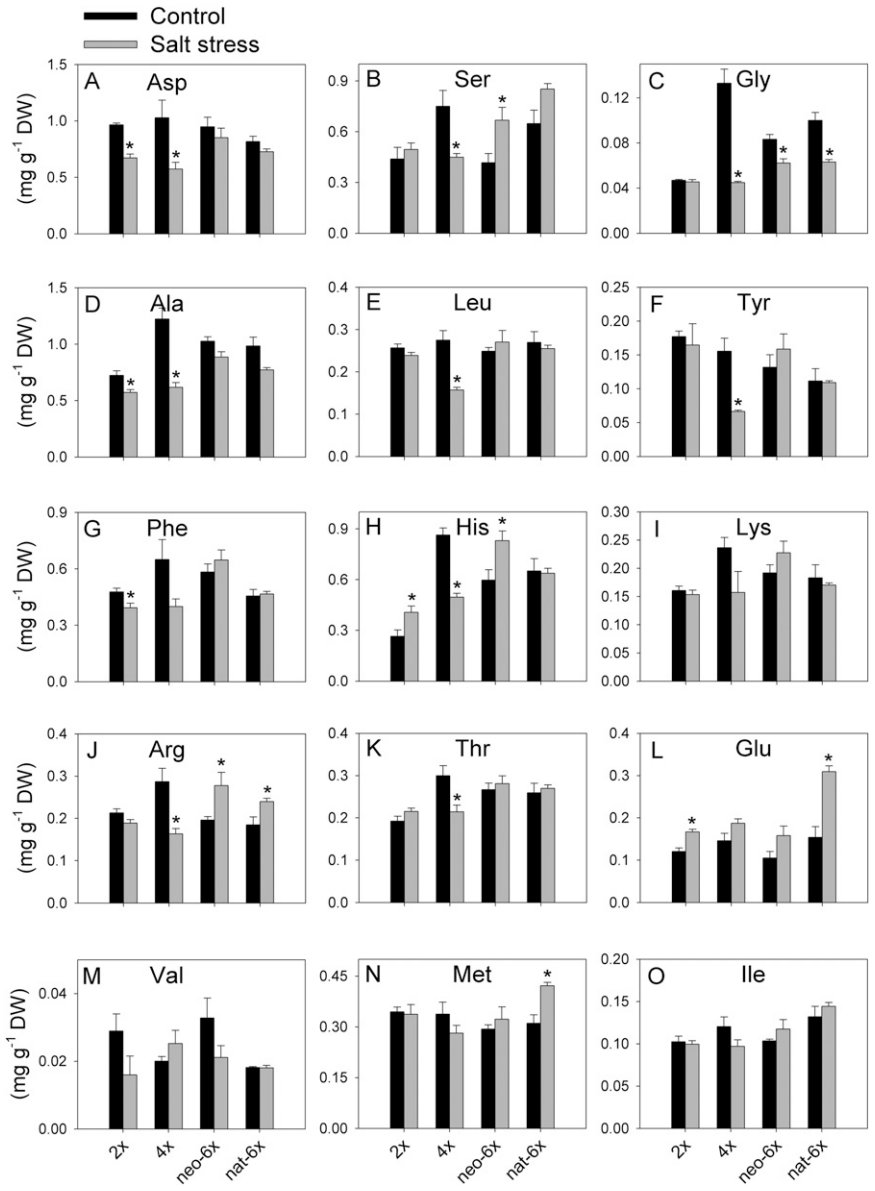

Fig. S6. Effects of salt stress on free amino acid contents in leaves of neo-6*x*, its diploid (2*x*) and tetraploid (4*x*) parents, and natural allohexaploid (nat-6*x*). The values are means of four biological replicates, and each replicate consisted of a pool of five plants. The 30-d-old seedlings were subjected to type II salt stress for 32 d.

Other Supporting Information Files

Dataset S1 (PDF)
Dataset S2 (XLS)
Dataset S3 (PDF)
Dataset S4 (PDF)
Dataset S5 (XLS)