RAPID BREEDING, GENOME-WIDE CHARACTERIZATION AND MORPHO-PHYSIOLOGICAL EVALUATION OF A SALT TOLERANT RICE

Rana Md Masud

Doctoral Program in Life and Food Sciences
Graduate School of Science and Technology
Niigata University

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Rana Md Masud

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Academic Advisor of Dissertation

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Toshiaki Mitsui

Professor of Department of Life and Food Sciences

Co-advisors

Kimiko Itoh

Professor of Department of Life and Food Sciences

Kuni Sueyoshi

Professor of Department of Life and Food Sciences

Dissertation Committee

Kimiko Itoh, Professor of Department of Life and Food Sciences

Kuni Sueyoshi, Professor of Department of Life and Food Sciences

Naoki Harada, Professor of Department of Life and Food Sciences

Kentaro Kaneko, Assistant Professor of Department of Life and Food Sciences

CONTENTS

ABBREVIATIONS

cDNA	complementary DNA
ANOVA	analysis of variance
CIA	chloroform/isoamyl alcohol (24:1)
CO_2	carbon dioxide
CTAB	hexadecyltrimethylammonium bromide
DAG	days after germination
EC_e	electrical conductivity of saturated extract
EMS	ethyl methanesulfonate
GWAS	gene-based genome-wide association study
hst1	*hitomebore salt tolerant 1*
InDel	small insertion/deletion
K^+	Potassium ion
MAS	marker-assisted selection
mRNA	messenger RNA
Na^+	sodium ion
NaCl	sodium chloride
OCT	optimal cutting temperature
PCI	phenol/chloroform/isoamyl alcohol (25:24:1)
PCR	polymerase chain reaction
QC	quality control
qPCR	quantitative real-time PCR
QTL	quantitative trait loci
ROS	reactive oxygen species
RWC	relative water content
SD	standard deviation
SDS	sodium dodecyl sulfate
SNP	single-nucleotide polymorphism
SSR	simple sequence repeats
t ha^{-1}	tonne per hectare
WGS	whole-genome sequencing
WT	wild type

1. INTRODUCTION

Projected climate change will aggravate a variety of abiotic stresses of rice plants, including salinity, heat, drought, and submergence, thus reducing world rice production (Wassmann *et al*., 2008; Welch *et al*., 2010; Sreenivasulu *et al*., 2015; Calanca, 2017). At the same time, we must increase global rice production by at least 70% to feed the anticipated 9.6×10^9 people by 2050 (Godfray *et al*., 2010; United Nations, 2013). Under these conditions, the improvement of the salinity tolerance of locally grown high-yielding rice cultivars is one of the most promising breeding objectives by which to meet global food demand.

Rice is considered the most salt-sensitive cereal crop (Munns and Tester, 2008), with a threshold EC_e (electrical conductivity of saturated extract) of 3 dSm^{-1}, above which yield starts to decline (Rao *et al*., 2008; Negrão *et al*., 2011; Marcos *et al*., 2018). Salinity imposes osmotic effects, ion toxicity, and nutritional imbalance and substantially affects almost all phases of growth (Munns and Tester 2008; Todaka *et al*., 2012). Possible salt tolerance mechanisms in rice involve ion homeostasis and compartmentalization, ion transport and uptake, biosynthesis and accumulation of osmoprotectants, osmolytes, and compatible solutes, activation of antioxidant enzymes for ROS detoxification, and hormone modulation (Horie *et al*., 2012; Roy *et al*., 2014; Deinlein *et al*., 2014; Hanin *et al*., 2016; Reddy *et al*., 2017; Chen *et al.*, 2017). The *Saltol* (Bonilla *et al*., 2002; Thomson *et al*., 2010) and *SHOOT* K^+ *CONCENTRATION 1* (Lin *et al*., 2004; Ren *et al*., 2005) genes have been identified from major quantitative trait loci (QTLs) of salt-tolerant landraces Pakkali and Nona Bokra, respectively. These QTLs have been introgressed into some widely grown, high-yielding rice cultivars to improve salt tolerance (Vu *et al*., 2012; Linh *et al*., 2012; Gregorio *et al*., 2013; Babu *et al*., 2017; Quan *et al*., 2018), but the rate of improvement is slow.

Rice biotechnology has made advances in identifying single nucleotide polymorphisms (SNPs) controlling salinity tolerance (Negrão *et al*., 2013; Jain *et al*., 2014; Rahman *et al*., 2016; Tiwari *et al*., 2016; Mishra *et al*., 2016). Mutant lines of 'Hitomebore' were generated by treatment with ethyl methanesulfonate (EMS), an

inducer of nucleotide substitutions, and isolated a salt-tolerant line carrying the *hitomebore salt tolerant 1* (*hst1*) gene. The causative SNP conferring the high salinity tolerance of the *hst1* mutant line corresponded to the third exon of the *Os06g0183100* gene, which is predicted to encode a B-type response regulator designated *OsRR22*. We backcrossed the *hst1* line with 'Hitomebore' to breed the salt-tolerant cultivar 'Kaijin', with a yield ability of 5.88 t ha^{-1} (Takagi *et al*., 2015; Abe *et al.*, 2016). 'Yukinko-mai' is an early-maturing standard cultivar derived from a cross between 'Yukino-sei' and 'Domannaka' at the Niigata Agricultural Research Institute's Crop Research Center (Ishizaki *et al*., 2008); it has a high yield potential of 6.84 t ha^{-1} (Ishizaki *et al*., 2008) and is tolerant to high temperatures during grain filling (Shiraya *et al.*, 2015).

To combat earthquake- and tsunami-induced soil salinity in Japan, it is crucial to improve the salt resistance of locally grown popular rice cultivars, most of which are salt sensitive (Lee *et al*., 2003; Roy *et al*., 2014, Takagi *et al*., 2015; Kurotani *et al*., 2015; Rahman *et al.*, 2016). In addition, developing Japanese cultivars for international appeal and fine-tuning their yield performance under various ecosystems around the world are time-demanding tasks. To generate new rice cultivars quickly in response to evolving consumer preferences and crises, the salt-tolerant 'Kaijin' was crossed with 'Yukinko-mai' to develop a salt-tolerant line with elite agronomic traits through the use of marker-assisted selection (MAS). MAS is the most advanced tool yet developed for the precise introgression of genes of interest into elite rice cultivars (Collard and Mackill, 2008; Miah *et al*., 2013), and allows breeders to recover most of the recurrent parent genome in only two or three generations (Miah *et al*., 2015). Salt, heat, and drought stress-responsive genes or QTLs revealed by recent advances in genomics and biotechnology are being used for MAS of rice all over the world (Fukuoka *et al*., 2010; Luo *et al*., 2016; Das *et al*., 2017).

In recent years, rapid generation-advance technology called 'speed-breeding' has been used to shorten the generation cycle, accelerating the progress of genomics and breeding studies in multiple crops (Ohnishi *et al*., 2011; Tanaka *et al*., 2016; Collard *et al*., 2017; Watson *et al.*, 2018). This technique has been used for the genetical improvement of rice, such as recombinant inbred lines, backcrossed inbred lines, and isogenic cultivars (Tanaka *et al*., 2016; Collard *et al*., 2017). The speed breeding method

has been reported for six major crops such as spring wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*), chickpea (*Cicer arietinum*), pea (*Pisum sativum*), and canola (*Brassica napus*), that uses a prolonged photoperiod to reduce the generation time (Watson *et al.*, 2018). Nagatoshi and Fujita (2019) developed a breeding technique for short-day soybean plant applying supplemental CO_2 in combination with long-day and appropriate temperature cycles. Using speed-breeding, which combines temperature, light duration, and humidity control, tiller removal, and embryo rescue, breeders can obtain four to five advanced generations in a year (Ohnishi *et al*., 2011; Tanaka *et al*., 2016).

In this study, a new rice germplasm with salt tolerance was developed, obtained by precise transferring the known salt tolerance gene *hst1* (*OsRR22*) from a cultivar 'Kaijin' (tolerant) into high-yielding cultivar 'Yukinko-mai' (susceptible). The SNP marker-assisted selection coupled with speed-breeding was used to transfer the target gene and to shorten the generation cycle. After three backcrosses to the latter, progeny homozygous for the target gene was selected, fully sequenced and SNP genotyped to examine the genomic resemblance to the recurrent parent. Salinity tolerance of the newly developed material was evaluated under controlled conditions at seedling and heading stages by assessing a series of morpho-physiological parameters which all validated the salt tolerance expressed by the new germplasm.

2. MATERIALS AND METHODS

2.1 Planting materials

Seeds of 'Yukinko-mai' (elite cultivar) and 'Kaijin' (salt tolerant) were obtained from the Niigata Agricultural Research Institute's Crop Research Center (Nagaoka city, Niigata, Japan) and the Iwate Biotechnology Research Center (Kitakami city, Iwate, Japan), respectively.

2.2 Speed-breeding – modified controlled-biotron breeding conditions

The advanced generations were developed using the protocol described by Ohnishi e*t al.* (2011) with some modifications. Plants were grown in a growth chamber (CFH-415; Tomy Seiko, Tokyo, Japan) equipped with temperature, light, and humidity controls. Seeds were sterilized in 2.5% sodium hypochlorite and incubated at 30 °C in the dark for 2 days. They were then placed on seedling nursery trays and cultured. Ten-day-old seedlings were transplanted (1 per pot) into 230-mL plastic pots filled (4/5) with granulated rice nursery culture soil. Plants were grown under a long daylength (14/10 h light/dark) for 30 days to accelerate vegetative growth and then under a short daylength (10/14 h light/dark) to accelerate reproductive development. The temperature was maintained at 30/25 °C light/dark. Relative humidity was set to 70% and light intensity was set to 350 μmol m^{-2} s^{-1} (**Figure 1**). Each plant was restricted to the main culm by removing tillers. The flowers of the female parent were emasculated and pollinated according to Ohnishi *et al.* (2011). At 10 days after pollination, embryos were rescued from developing seeds and cultured them for 10 days according to the same protocol. Healthy rice seedlings were then transplanted and raised to the next breeding step.

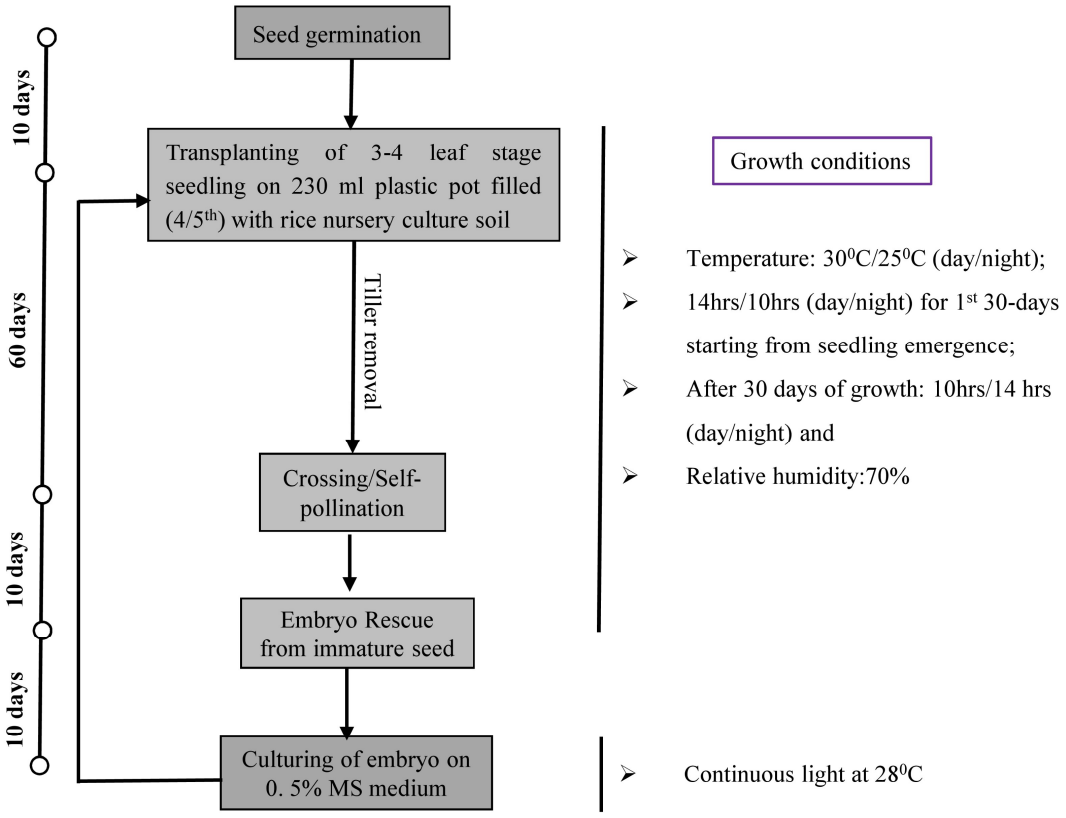

Figure 1| Schematic representation of speed-breeding technique based on a biotron that controls temperature, daylength, and humidity, tiller removal, and embryo rescue.

2.3 Developing salt-tolerant line by backcrossing 'Kaijin' to 'Yukinko-mai'

The backcrossing was performed to develop an advanced line for salinity tolerance due to the *hst1* gene derived from 'Kaijin' using the recurrent parent 'Yukinko-mai'. F_1 plants were confirmed as heterozygous at the *hst1* (*OsRR22*) locus by Sanger sequencing, and were backcrossed to 'Yukinko-mai' to produce BC_1F_1 plants. The same strategy of selecting plants heterozygous at *hst1* and backcrossing was followed to develop BC_2F_1 and BC_3F_1 generations. Selected BC_3F_1 heterozygous plants were self-pollinated to generate BC_3F_2 lines with the donor allele in the homozygous state. The genome of BC_3F_2 line #31-2-4 was sequenced to compare with the genomes of the parents. Self-pollinated seeds of line #31-2-4 were named 'YNU31-2-4' (BC_3F_3 generation) and used for phenotypic evaluation.

2.4 Confirmation of genotypes by Sanger sequencing

A PCR primer set was used to amplify a 545-bp region around the selected SNP (nucleotide 1975 of the *OsRR22* locus) (Takagi *et al.*, 2015) from genomic DNA extracted from young leaves of 20-day-old plants using the CTAB method (Doyle and Doyle, 1987). Well defined PCR product was gel-purified with a High Pure PCR Product Purification Kit (Roche Applied Science, Japan). Sanger sequencing was performed using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a Prism 3130 Genetic Analyzer (Applied Biosystems). Sequence chromatogram data were visualized in FinchTV software (Geospiza, Inc., Seattle, Washington, USA) to determine the genotype at the SNP position.

2.5 DNA extraction for whole-genome sequencing

The nuclear pellet method (Walbot and Warren,1988) was optimized through the integration of simple purification steps to extract total genomic DNA from leaves of 'Yukinko-mai' and BC_3F_2 line #31-2-4. The optimized nuclear pellet method is described as follows:

2.5.1 Reagents

- Solution I: 15 % Sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0) and 500 mM NaCl
- Resuspension buffer: 20 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)
- 20 % Sodium dodecyl sulfate (SDS)
- 7.5 M Ammonium acetate
- 5 M NaCl
- Isopropanol
- TE buffer: 10 mM Tris-HCl (pH 7.6), 0.1 mM EDTA
- RNase A, a working solution of 1 µg/µL (final concentration ≈ 10 ng/µL)
- Proteinase K, a working solution of 2 µg/µL (final concentration ≈ 80 ng/µL)
- PCI: Phenol/Chloroform/Isoamyl alcohol (25:24:1)
- CIA: Chloroform/Isoamyl alcohol (24:1)
- 100 % Ethanol (v/v)
- 70 % Ethanol (v/v)
- Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA)

2.5.2 Equipment

- Mortar and pestle
- Dry block heater
- Table-top high-speed micro-centrifuge
- Freezer (-20 °C)
- Gel electrophoresis system
- Eppendorf BioSpectrometer® fluorescence (Eppendorf AG, Hamburg, Germany)

Note: Prepare all reagents using Milli Q water; autoclave glassware before use.

2.5.3 Protocol

The protocol for gDNA extraction using the optimized NP method is as follows and is shown schematically in **Figure 2**:

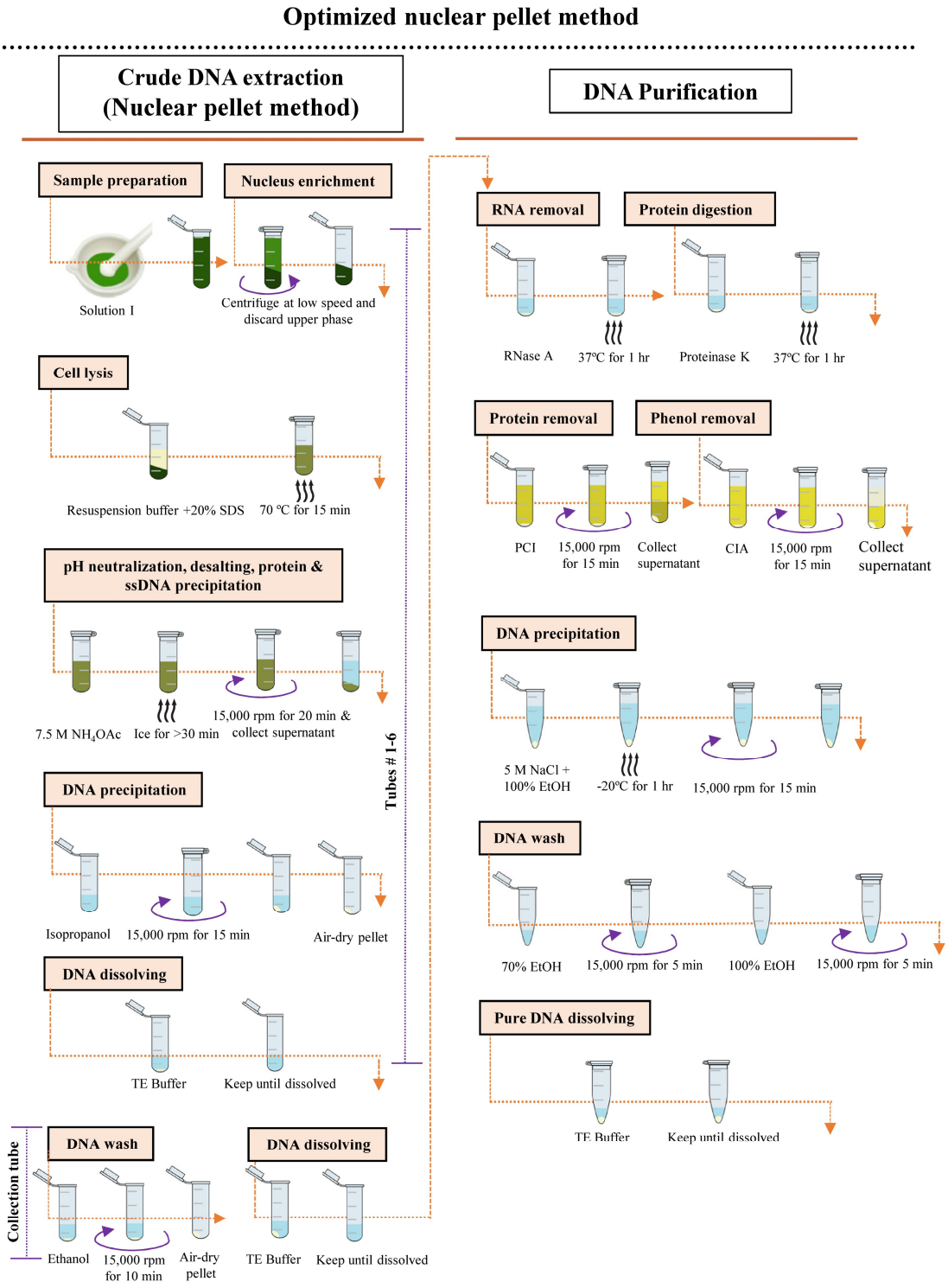

Figure 2| Schematic illustrating the procedures of genomic DNA extraction using the optimized nuclear pellet method.

1. Weigh 1 g of young fresh leaf tissue and cut into small pieces using clean and sharp scissors. Place the cut tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Add 12 ml Solution I to the fine tissue powder and suspend well. Transfer the suspended tissue (2 ml in each) into six individual 2 ml micro-centrifuge tubes.
2. Centrifuge the tubes containing the suspended tissue at 500 rpm for 3 min at 4 °C. Discard the upper phase carefully. Centrifuge at 1,000 rpm for 1 min at 4 °C and discard the upper phase.
3. Add 450 µl of resuspension buffer and 30 µl of 20 % SDS into each tube containing tissue, shake briefly and incubate at 70 °C for 15 min.
4. Add 230 µl of 7.5 M NH_4OAc into each tube, shake vigorously and incubate the reaction mixture on ice for at least 30 min.
5. Centrifuge the reaction mixture at 15,000 rpm for 20 min at 4 °C and divide equal amounts of the cleared supernatant into six individual micro-centrifuge tubes, labeled # 1–6. Repeat this step (once).
6. Add 1 volume of isopropanol to the supernatant of each tube, gently mix by inverting and centrifuge at 15,000 rpm for 15 min at 25 °C. Discard the supernatant and air-dry pellet for 5–10 min (do not excess dry).
7. Add 50 µl of TE buffer individually to tubes # 1–5 and wait until the *pellet has dissolved*. Transfer the dissolved DNA from tubes # 1–5 into tube # 6. Add 50 µl TE buffer to the original tube # 1, allow to dissolve then transfer it to tube # 2. Repeat this transfer and dissolving process until tube # 5, then transfer the DNA to tube # 6. The final volume of the DNA sample in tube # 6 will be 300 µl.
8. Add 600 µl of 100 % ethanol to the sample in tube # 6, centrifuge at 15,000 rpm for 10 min at 25 °C and decant the supernatant. Again, add 600 µl of 100 % ethanol to the DNA pellet in tube # 6, centrifuge at 15,000 rpm for 1 min and decant the supernatant.
9. Air-dry the pellet for 5–10 min. Resuspend the *pellet* in 50 µl of TE buffer (do not vortex).
10. Add 0.5 µl of RNase A to the solution and incubate at 37 °C for 1 h.
11. Add 2.2 µl of Proteinase K to the solution and incubate at 37 °C for 1 h.

12. Check gDNA quality by electrophoresis using a 0.7 % agarose gel (optional).
13. Add 400 µl of TE buffer to the digested sample containing the DNA and mix gently by pipetting.
14. Add 450 µl of PCI to the DNA solution, gently mix and centrifuge at 15,000 rpm for 15 min at 4 °C. Transfer the cleared supernatant to a new 1.5 ml collection tube. Repeat this step (once).
15. Add 450 µl of CIA to the DNA solution, mix gently and centrifuge at 15,000 rpm for 15 minutes at 4 °C. Transfer the cleared supernatant to a new 1.5 ml collection tube. Repeat this step (once).
16. Add 27 µl of 5 M NaCl and 1 ml of 100 % ethanol to the solution, gently mix and incubate at -20 °C for 1 hr.
17. Centrifuge at 15,000 rpm for 15 min at 4 °C and discard the supernatant.
18. Wash the pellet with 100 µl of 70 % ethanol, centrifuge at 15,000 rpm for 5 min and discard the ethanol.
19. Wash the pellet with 100 µl of 100 % ethanol, centrifuge at 15,000 rpm for 5 min and discard the supernatant. Again, add 100 µl of 100 % ethanol to the pellet, wash and discard the ethanol.
20. Air-dry the pellet for 5–10 min. Resuspend the *pellet* in 11 µl of TE buffer.
21. Dilute the DNA with TE buffer (as required) for downstream analysis.

2.6 DNA library construction and whole-genome sequencing

The quantity of genomic DNA was tested with a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and the quality was tested by 0.8% agarose gel electrophoresis. The DNA was sent to Macrogen Japan Corp. (Sakyo-Ku, Kyoto, Japan) for Illumina HiSeq X Ten sequencing with NGS libraries prepared by the TruSeq DNA PCR-Free Library Prep Kit (Illumina, Inc., San Diego, CA, USA). Finally, 183 119 379 paired-end 150-bp reads of 'Yukinko-mai' and 211 937 727 of BC_3F_2 #31-2-4 were obtained (**Figure 3**).

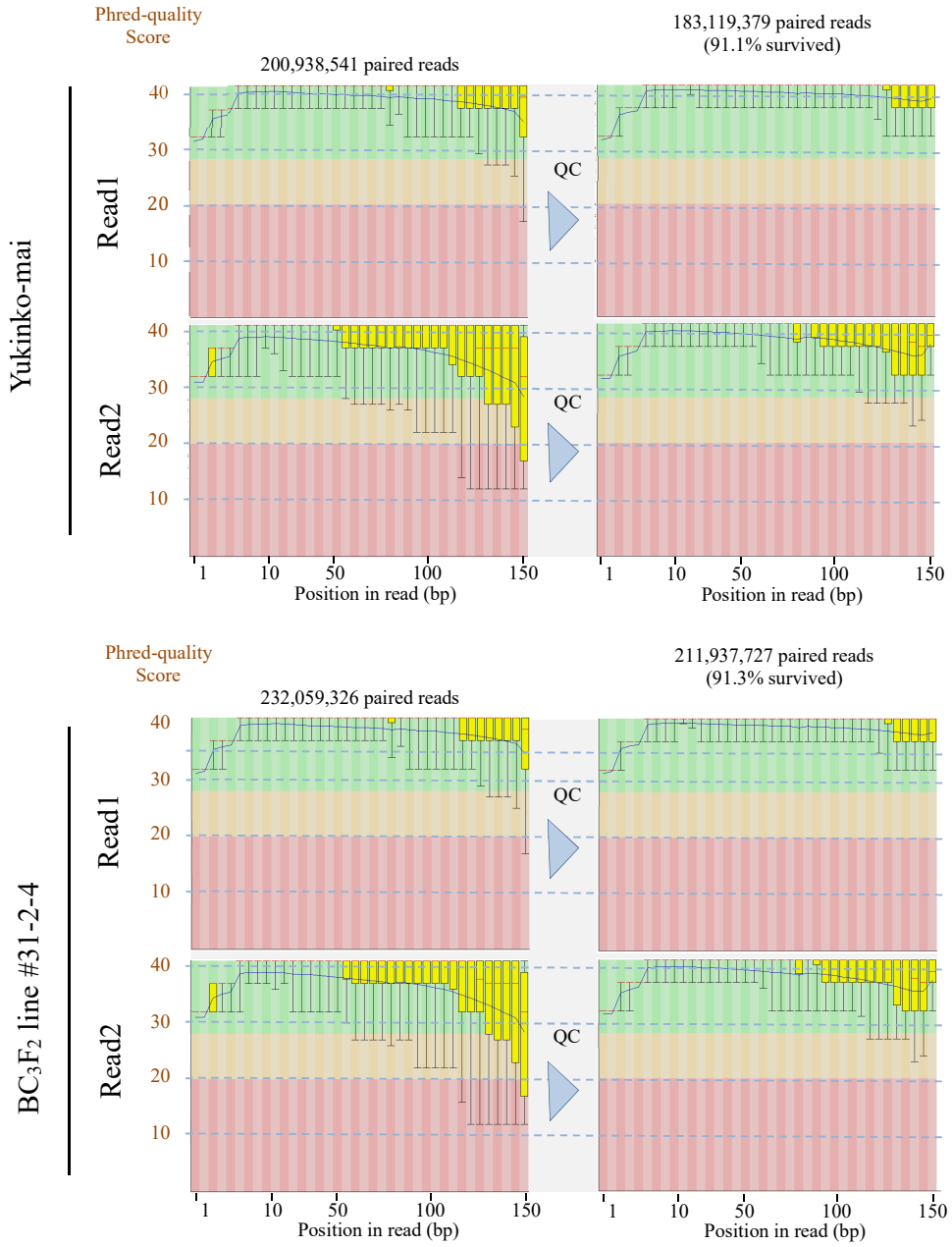

Figure 3| Per base sequence quality of Illumina sequencing reads

Phred quality scores across paired-end reads before (left columns) and after (right columns) quality control (QC) are shown in Box plots. The blue and red lines represent the mean quality and median value, respectively. The yellow box represents the first quartile and third quartile. The whiskers extend from the ends of the box to the top 10 % value and 90 % value. The background of the graph in the y-axis shows sequence quality. Green, very good quality calls; orange, reasonable quality; red, poor quality. Quality control was performed using Trimmomatic v. 0.33 software (Bolger *et al.*, 2014) with the following parameters: SLIDINGWINDOW: 8:20; TRAILING: 30; MINLEN: 70.

2.7 Read mapping, variant calling, and variant annotation

'Kaijin' whole-genome sequencing reads (DRR021949, DRR021950, DRR021951, DRR021952) were downloaded from public databases. The raw paired-end reads from 'Yukinko-mai', BC_3F_2 #31-2-4, and 'Kaijin' sequences were trimmed in Trimmomatic v. 0.33 software (Bolger *et al.*, 2014) with the following parameters: SLIDINGWINDOW, 8:20; TRAILING, 30; MINLEN, 70. The processed reads were mapped to the Nipponbare reference genome (IRGSP-1.0) by using the BWA-MEM v. 0.7.15 algorithm (Li and Durbin, 2009). PCR duplicates in the binary alignment map (BAM) file of 'Kaijin' were marked in Picard Tools v. 1.68 software (http://broadinstitute.github.io/picard/). Then indel realignment and base recalculation were done in Genome Analysis Toolkit (GATK) v. 3.6 software (DePristo *et al.*, 2011). For multi-sample variant calling, GATK HaplotypeCaller in gVCF mode followed by GATK GenotypeGVCFs was used. The variants with missing data, multi-allelic sites, heterozygous sites in 'Kaijin' and 'Yukinko-mai', low coverage depth (DP < 6), and low quality (QUAL < 20) were filtered out. Further heterozygous variants in BC_3F_2 #31-2-4 outside the range of 40%–60% allele frequency were filtered out by a custom script, and then visualized the genotype map of BC_3F_2 #31-2-4 in the gtrellis package of R software (Gu *et al.*, 2016). The variants were annotated in SnpEff v. 4.0e software (Cingolani *et al.*, 2012) and summarized the results in the Python programming language. Sequentially, I extracted 'HIGH'- and 'MODERATE'-impact variants flagged by SnpEff and performed functional annotation analysis based on two agronomic data sets: data set 1, the Overview of Functionally Characterized Genes in Rice Online (OGRO) database (Yamamoto *et al.*, 2012); and data set 2, the potential agronomic functional gene set selected by gene-based GWAS of the Japanese rice population (Yano *et al.*, 2016). One of non-synonymous variant in *Os09g0356200* was sequenced by Sanger sequencing using forward primer: 5'-cactggaggtcgaaactgct-3' and reverse primer: 5'-tccggtcccagaaatgaagc-3'. The analyses were all based on gene annotation information and genome sequences from the Rice Annotation Project Database (RAP-DB: http://rapdb.dna.affrc.go.jp).

2.8 Estimation of genome recovery rate

The genome recovery rate of BC_3F_2 #31-2-4 was estimated by calculating the 'Yukinko-mai'-type allele frequency out of total variants as:

$$\text{Genome recovery rate} = \frac{YY + YK/2}{YY+YK+KK}$$

where YY = number of 'Yukinko-mai' homozygous variants, YK = number of heterozygous variants, and KK = number of 'Kaijin' homozygous variants.

2.9 Phenotypic evaluation under field condition

'YNU31-2-4' plants were grown in paddy fields of the Crop Research Center, Niigata University, Japan (37°51′20.75″N 138°57′37.9″E), during May-September in 2018, to evaluate the major agro-morphological traits. The experiment was laid out in a randomized complete block design with three replications. 'Kaijin' and 'Yukinko-mai' were used as salt tolerant and high yielding check cultivars, respectively. Thirty-day-old seedlings were transplanted at a spacing of 20 cm × 15 cm. All agronomic practices were performed uniformly for all the genotypes following the local cultural practices. Four uniform looking plants of each genotype from the central row of each replication were selected to determine the phenotype. The major agronomic traits such as (1) flag leaf color, (2) flag leaf length (3) flag leaf width, (4) heading date, (5) plant height, (6) tiller number, (7) panicle number, (8) panicle length, (9) spikelet number, (10) grain number, (11) 1000-grain weight, (12) seed setting rate, (13) grain yield, and (14) above ground biomass were determined. Grain length, width, and thickness were determined with a rice grain grader (RGQI20A; Satake, Hiroshima, Japan).

2.10 Growth conditions and evaluation of salinity tolerance at seedling stage

'YNU31-2-4' and the parents were evaluated for seedling-stage salt tolerance in the growth chamber at 26/23 °C (12/12 h) and a relative humidity of 70%. Pre-germinated seeds were placed in 230-mL plastic pots filled with rice nursery culture soil containing 0.5 g N, 0.9 g P, and 0.5 g K/kg. The experiment consisted of four treatments: 0 (control), 50, 75, and 125 mM NaCl (pH 5). The salt stress was imposed ten days after germination. The experiment used four biological replicates, each with 10 seedlings. Phenotype was evaluated 2 weeks after salt was imposed.

2.11 Evaluation of salt-stress tolerance at reproductive stage

'YNU31-2-4', salt-tolerant 'Kaijin', and susceptible 'Yukinko-mai' plants were evaluated for salt-stress tolerance at the reproductive stage in a semi-controlled greenhouse. Thirty-day-old seedlings were transplanted (1 per bucket) into 2.5-L plastic bucket and each treatment had six replicates. Plants were subjected to 0 or 50 mM NaCl in irrigation water (pH 5) at 60 days after germination (DAG). After 2 weeks, the salt concentration was increased to 75 mM until booting stage (95 DAG), and then plants were recovered by irrigating with fresh water. The net assimilation rate of penultimate leaves was measured with an LI-6400 gas exchange system (LI-COR Inc., USA) 4 weeks after salt was imposed. Gas exchange was determined at 25 °C at a photosynthetic photon flux density of 350 μmol m^{-2} s^{-1}. Yield and its attributes, particularly panicle number, spikelet number, and 1000-spikelet weight were determined at harvest.

2.12 Determination of Leaf Relative Water Content, Chlorophyll, and proline

A separate experiment was conducted to measure biochemical and physiological traits related to salinity tolerance. 'YNU31-2-4', 'Kaijin', and 'Yukinko-mai' seedlings were cultured in nutrient solution (Yoshida *et al*., 1976). Ten-day-old rice seedlings were subjected to 0 or 125 mM NaCl (pH 5.0). Samples were collected 10 days after salt was imposed.

The relative water content (WC%) of control and salt-treated leaves was determined according to Sade *et al*. (2009) as:

$$\%WC = (\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight}) \times 100$$

Total chlorophyll content was determined according to Lichtenthaler (1987). Fresh leaf samples (0.1g) were frozen in liquid nitrogen and ground to a fine powder using mortar and pestle. After extraction with 2.0 ml of 80% (v/v) acetone, plant debris were removed by centrifugation at 18,800g for 1 minute. The absorbance of the supernatant was read at 663.2 and 646.8 nm (UH5300 spectrophotometer, Hitachi, Tokyo, Japan), using 80% (v/v) acetone as blank. The total chlorophyll content was calculated and expressed as mg g^{-1} FW.

Free proline content was measured by a colorimetric assay as described by Bates *et al*. (1973). Frozen leaf tissue (0.1g) was homogenized in 5 ml of 3% sulphosalicylic acid

and the plant residues were removed by centrifugation at 12000g for 10 minutes. The reaction mixture, supernatant, glacial acetic acid, acid-ninhydrin (1:1:1) was incubated at 100°C for 1 hour and the reaction was terminated in an ice bath. The chromophore was extracted using 2 ml of toluene and its absorbance was measured at 520 nm (UH5300 spectrophotometer, Hitachi, Tokyo, Japan) using toluene as blank.

2.13 Measurement of Na^+ and K^+ concentrations

Sodium and potassium ions in shoots and roots were quantified by a wet digestion method (Pequerul *et al*., 1993). Dried, finely powered plant samples (50 mg) were digested in HNO_3/H_2O_2 solution (2:1) in a microwave oven for 4–5 min until the solution became clear. The digested solution was shaken gently and filtered through 0.2-µm filters (Whatman, Maidstone, England), and the solid fraction was discarded. The contents of Na^+ and K^+ in the extract were quantified by atomic absorption spectrophotometry (Z-6100, Hitachi, Tokyo, Japan).

For localization of sodium and potassium ions, samples were prepared according to the protocol of Mitsui *et al.* (1999). Harvested basal portions of shoots were immediately frozen and embedded in OCT compound medium (Sakura Finetek USA, Inc., Torrance, CA, USA), which contained 10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, and 85.50% w/w of a nonreactive ingredient. Then 5-µm sections were scanned with an electron probe microanalyzer (EPMA-1605; Shimadzu, Kyoto, Japan).

2.14 mRNA analysis

Total RNA from rice shoot tissue (100 mg) was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), and the cDNA was synthesized from 1 µg of the total RNA with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan) according to the manufacturer's instructions. The qPCR analysis was performed using SsoFast Eva Green Supermix (Bio-Rad) and CFX96 real time PCR system/C1000TM Thermal Cycler (Bio-Rad). Gene-specific primer pairs for qPCR were as follows: 5'-TGGCATGCATAACAGAAAGGA -3' and 5'-AGTGGCATCAGGCTTCAACA-3' for *Os04t0607500-01,* and 5'-ACAAGGACCAGAGCGTGCAA-3' and 5'-ATCGTGATTCGGGGTCAATC-3' for *Os04t0607500-02*. The expression level of the

18S rRNA gene (Accession no. AK059783) was used as an internal control (reference gene). Relative expression of the target genes was calculated using the comparative Ct method.

2.15 Statistical analysis

Values are presented as mean ± standard deviation (SD). Means were tested by analysis of variance (ANOVA) followed by Tukey's or Duncan's multiple range test at $P < 0.05$ in SPSS software (SPSS Inc., Chicago, IL, USA).

2.16 Accession codes

The sequence data have been deposited in the DDBJ Sequence Read Archive: DRR151851 (BC_3F_2) and DRR151852 ('Yukinko-mai').

3. RESULTS

3.1 Biotron speed-breeding system accelerated the breeding cycle

The salt-tolerance *hst1* gene from 'Kaijin' was introgressed into the genetic background of the high-yielding 'Yukinko-mai' by three backcrosses followed by two rounds of self-fertilization (**Figure 4A**). To accelerate the breeding cycle, a biotron speed-breeding system was used, with controlled temperature and daylength, restriction of tillers, and embryo rescue (**Figure 1**). At each cross, the plants produced a good quantity of fertilized seed; the cross success rate ranged between 54% and 69% (**Table 1**), and seeds from 3 or 4 plants were sufficient to develop new progeny. Each advanced generation took approximately 70 days from germination to flowering and 10 days from pollination to embryo rescue (**Figure 1**). The embryo rescue was used at 10 d after pollination to raise new plants; the total duration of each generation varied according to days to flowering. Using this speed-breeding technique, the BC_3F_3 population, carrying our desired allele in the homozygous state was developed, in 6 generations and 17 months.

3.2 Introgression of *hst1* into 'Yukinko-mai' (WT) and genotyping of advanced progeny using SNP marker

SNP-based genotyping by Sanger sequencing was used to identify plants harboring the donor allele in each breeding round. A SNP in the *OsRR22* (*hst1*) gene confers the salinity tolerance of the donor parent 'Kaijin'. The breeding lines were selected on the basis of target peaks of G/A heterozygosity (nucleotide 1975 of the *OsRR22* locus) in the F_1, BC_1F_1, BC_2F_1, and BC_3F_1 generations and of A/A homozygosity at the same locus in the BC_3F_2 generation (**Figure 4B**). In the F_1 to BC_3F_1 generations two genotypes were obtained: either homozygous, lacking the donor allele (G/G), or heterozygous (G/A) (**Figure 4A**). The heterozygous BC_3F_1 population was self-pollinated to develop BC_3F_2 lines that carried the donor allele in the homozygous state (A/A). BC_3F_2 plants (A/A) morphologically similar to the recurrent parent were self-pollinated to develop the BC_3F_3 generation. The whole genome of BC_3F_2 line #31-2-4 was sequenced to compare it with the parental genome and characterize it.

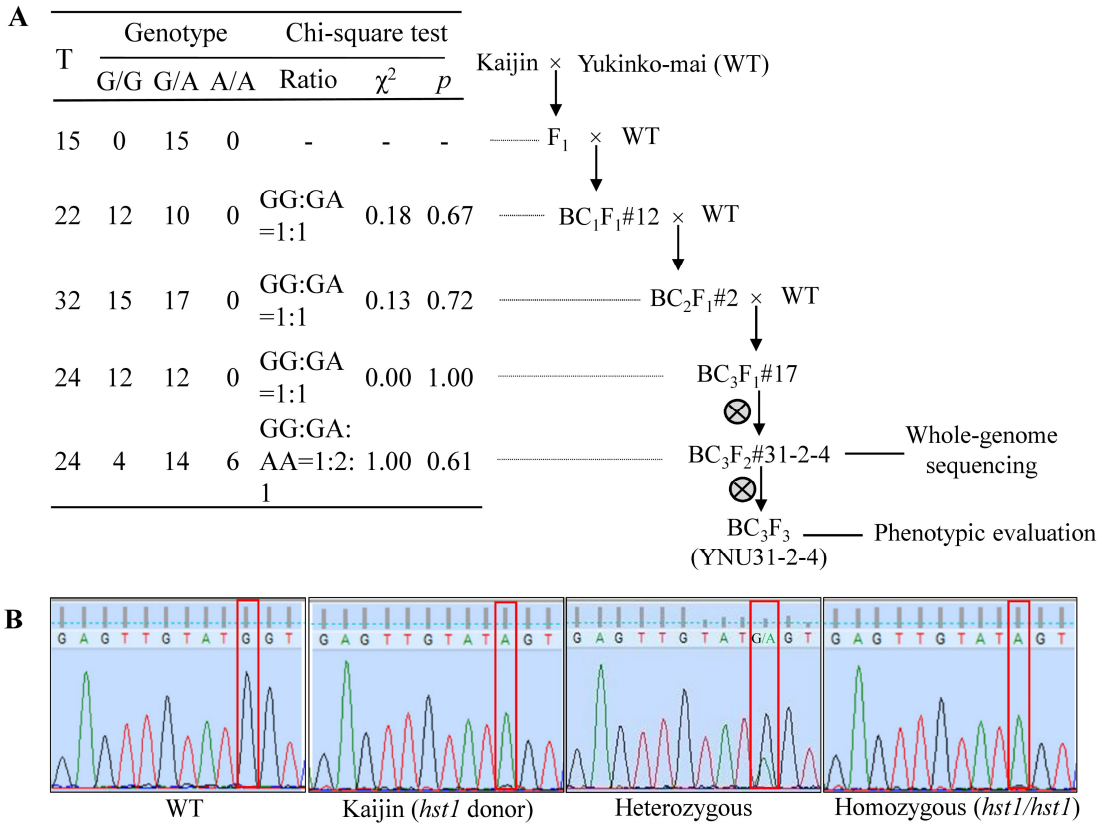

Figure 4| SNP marker-aided introgression of *hst1* from 'Kaijin' into 'Yukinko-mai'. (**A**) The *hst1* gene was transferred from highly salt-tolerant 'Kaijin' into 'Yukinko-mai'. 'Kaijin' was backcrossed to 'Yukinko-mai' (WT) 3 times followed by 2 rounds of self-pollination to breed the advanced homozygous (*hst1*) BC_3F_3 generation. The table shows the selection results at each generation: T, total number of tested plants; G/G, number of plants not carrying donor allele; G/A, number of plants carrying donor allele in heterozygous state; A/A, plants carrying donor allele in homozygous state. The number after "#" is the individual plant number used in backcrossing or self-pollination. (**B**) Advanced breeding individuals were genotyped by direct sequencing. The red box represents nucleotide 1975 of the *OsRR22* locus, which is responsible for salt tolerance. F_1, BC_1F_1, BC_2F_1, and BC_3F_1 plants showing peaks of G/A heterozygosity and a BC_3F_2 plant showing a peak of A/A homozygosity were selected.

Table 1 | Cross-efficiency rate and advanced generation duration in biotron breeding system.

Breeding event	No. of spikelets crossed	No. of seed developed	Success rate (%)	Days to flowering	Total duration (days)
'Kaijin' × 'Yukinko-mai'	9.00 ± 3.24 (81)	4.66 ± 2.44 (42)	53.84 ± 25.83	67.0 ± 1.58	77.4 ± 1.14
F_1 × 'Yukinko-mai'	8.38 ± 2.33 (67)	5.00 ± 2.45 (40)	57.82 ± 13.37	69.4 ± 1.14	79.4 ± 1.14
BC_1F_1 × 'Yukinko-mai'	9.15 ± 3.02 (119)	6.15 ± 2.12 (80)	68.85 ± 16.22	70.4 ± 1.67	80.6 ± 1.51
BC_2F_1 × 'Yukinko-mai'	9.41 ± 2.31 (113)	6.25 ± 2.70 (75)	64.95 ± 14.37	70.0 ± 1.41	80.0 ± 1.41
BC_3F_1-selfing	–	–	–	71.2 ± 1.30	81.2 ± 1.30
BC_3F_2-selfing	–	–	–	70.6 ± 1.67	107.8 ± 4.15†

Data are mean ± SD (n = 5-13). Days to flowering refers to the flowering of 'Yukinko-mai' (1st breeding event), F_1, BC_1F_1, BC_2F_1, BC_3F_1, and BC_3F_2 individuals. Data in parentheses in columns 2 and 3 represent the total numbers. †Embryo rescue and tiller removal were not performed so as to obtain the maximum number of true seeds.

3.3 Recovery rate and characterization of BC_3F_2 #31-2-4 genome

To investigate the genetic similarities between the advanced line and the parents, BC_3F_2 #31-2-4 was analyzed using whole-genome sequencing. After filtered out low-reliability SNPs/indels, #31-2-4 showed 118 454 SNPs/indels, comprising 106 288 'Yukinko-mai'-type homozygous alleles, 3157 'Kaijin'-type homozygous alleles, and 9009 heterozygous alleles (**Table 2**). These SNPs/indels lie across the genome with deep coverage (**Figure 5**, dots), indicating high resolution and successful genome-wide genotyping. Allele types formed dense blocks on chromosomes (**Figure 5**, vertical bars), clearly showing recovered regions ('Yukinko-mai' homozygous blocks), 'Kaijin' genome segments ('Kaijin' homozygous blocks), and unfixed segments (heterozygous blocks) (**Figure 5**, horizontal bar). 'Yukinko-mai' chromosomes (Chrs.) 5, 11, and 12 were recovered almost completely. There were small 'Kaijin' segments in Chrs. 1, 4, 7, 9, and 10, large 'Kaijin' segments in Chrs. 2, 3, and 6, small heterozygous segments in Chr. 4, and large heterozygous segments in Chrs. 3, 6, 8, and 9. Interestingly, some genotype blocks overlapping other genotype blocks (**Figure 5**, chr08, 5–10 Mb; chr09, 10–12 Mb) were identified, resulting from continuous recombination events in these extremely short regions (de Haas *et al.*, 2017; Si *et al.*, 2015). The high-resolution analysis with massive numbers of SNP/indel markers not only enabled accurate genome-wide genotyping, but also highlighted potential recombination hotspots. The genome recovery rate was calculated from the number of 'Yukinko-mai' alleles out of the total number; the BC_3F_2 genome recovered 93.5% of the 'Yukinko-mai' genome, from 89.7% homozygous alleles and 7.6% heterozygous alleles (**Table 2;** section 2.7). This score is close to the theoretical value of 93.7% following three backcrosses and one self-fertilization. In addition, 2.7% of the BC_3F_2 genome was 'Kaijin' homozygous and 7.6% remained unfixed as heterozygous (**Table 2**).

Table 2 | SNP/indel detection in BC_3F_2 #31-2-4.

Genotype	Number of variants			Genome %
	SNPs	Indels	Total	
'Yukinko-mai' (homo)	84 927	21 361	106 288	89.7
'Kaijin' (homo)	2329	828	3157	2.7
Hetero	7556	1453	9009	7.6
Total	94 812	23 642	118 454	100.0

SNPs/indels in BC_3F_2 #31-2-4 are classified into 'Yukinko-mai'-type (homo[zygous]), 'Kaijin'-type (homo[zygous]), and Hetero(zygous) alleles.

To estimate the effects of variants on phenotypes, variants causing protein sequence alterations (e.g. frameshift and in-frame indels, non-synonymous SNPs, SNPs/indels at splice donor/acceptor sites etc.) in SnpEff software for Sequence Ontology and effect prediction were listed (**Figure 6**). Functional annotations of the genes harboring significant SNPs were retrieved from variant annotations in two agronomic-trait-related gene datasets (Yamamoto *et al.*, 2012; Yano *et al.*, 2016) (**Table 3**). In BC_3F_2, 207 'Kaijin' homozygous and 536 heterozygous variants were found in exon and splice sites. Only 71 and 230, respectively, of those caused protein sequence alterations (**Figure 6; Table 4**). These non-synonymous changes occurred in only four genes for agronomic traits (**Table 3**). This result suggests that BC_3F_2 has similar agronomic phenotypes to the recurrent parent, 'Yukinko-mai'. However, one of two non-synonymous changes in *Os09g0356200*, a putative heading-date-associated gene identified in a gene-based genome-wide association study (GWAS) (Yano *et al.*, 2016), caused a frameshift deletion probably causing loss-of-function. BC_3F_3 progeny of BC_3F_2 plants harboring the *Os09g0356200* gene in the heterozygous state likely shows a wide range of heading date.

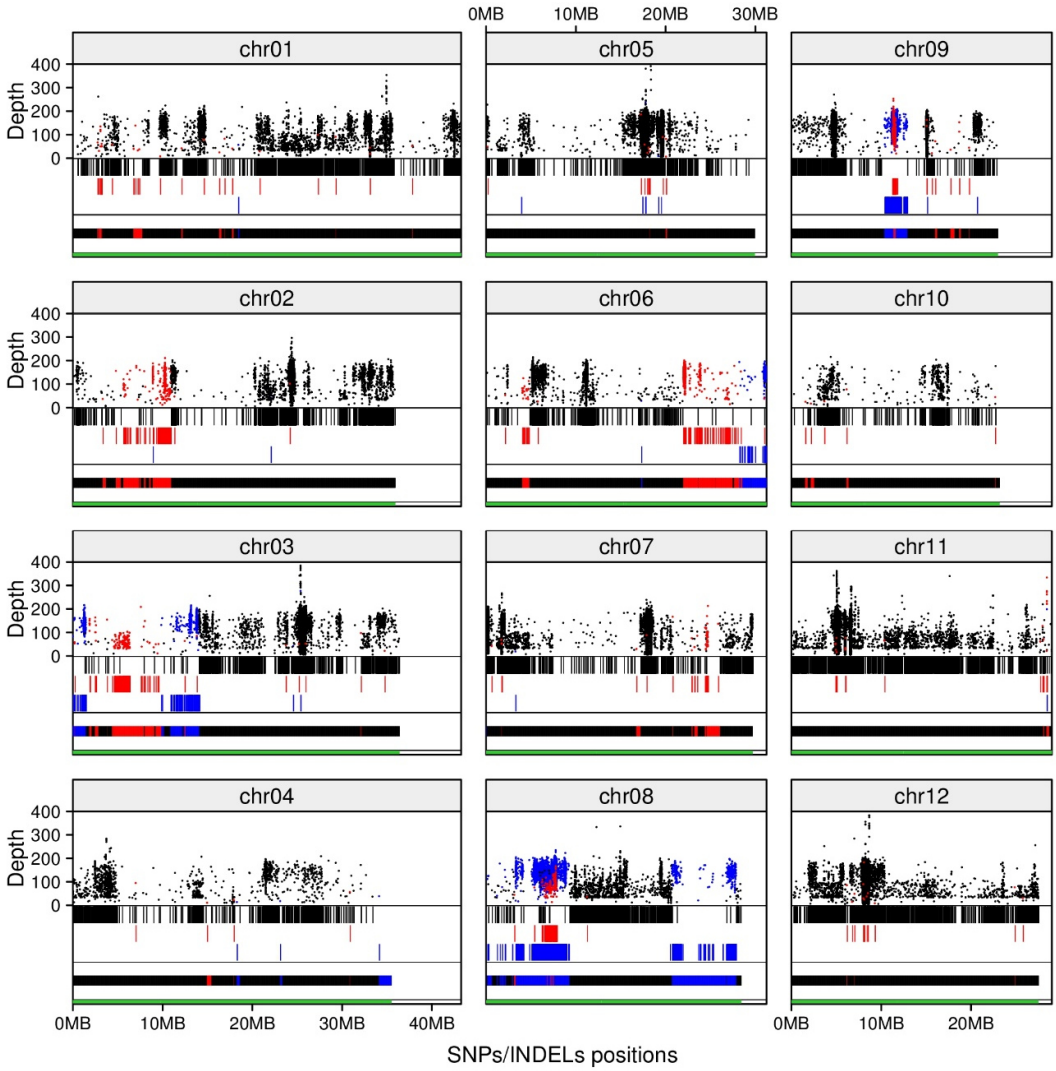

Figure 5| Positions and depths of SNPs/indels and genotype blocks on BC_3F_2 #31-2-4 genome. Dots show coverage depth of SNPs/indels. Vertical bars show their positions by genotype (color). Horizontal bar shows densities of SNPs/indels densities in 10 000-nt sliding window by genotype (colour). Black, 'Yukinko-mai'-type homozygous; red, 'Kaijin'-type homozygous; blue, heterozygous. Green bars represent chromosome length.

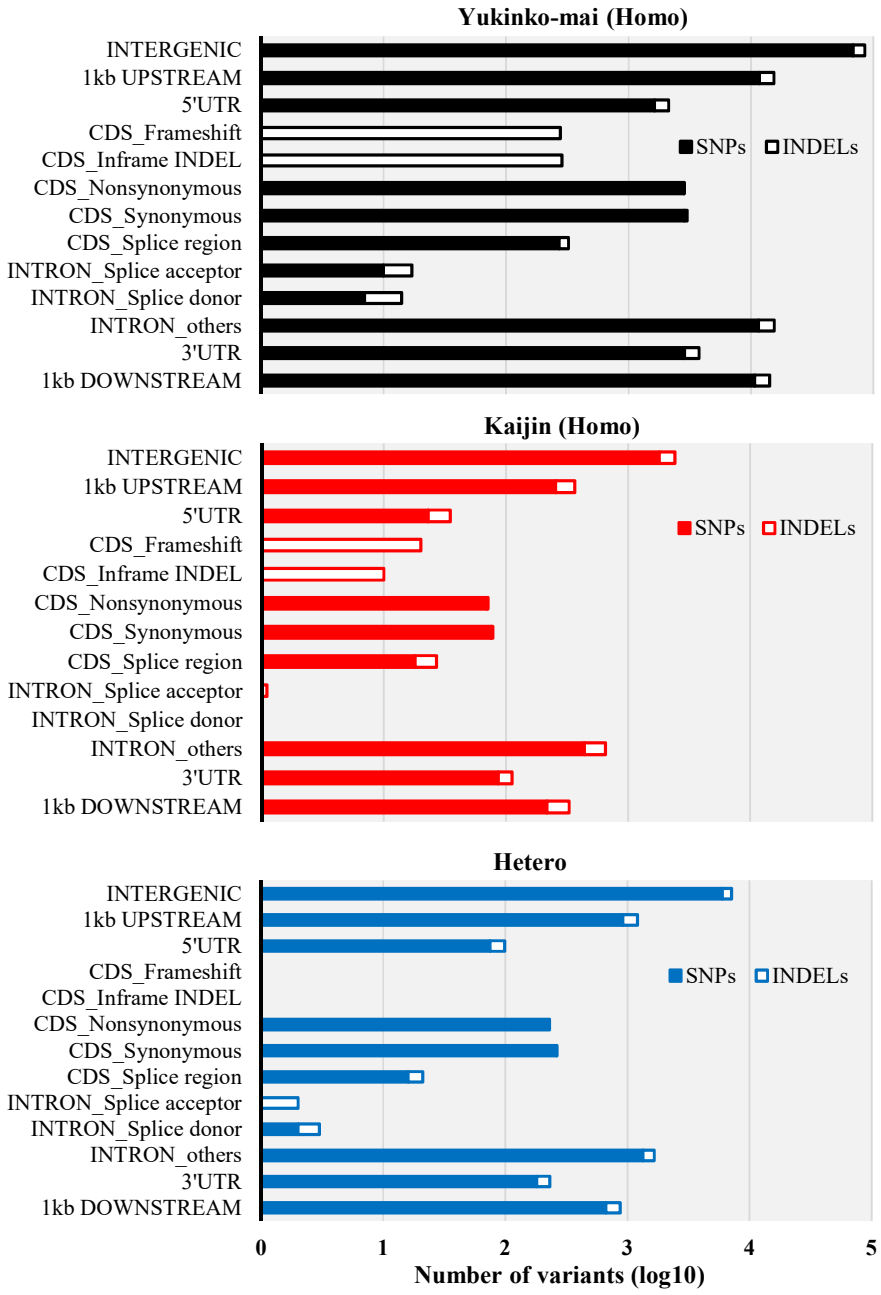

Figure 6| Sequence Ontology analysis of BC_3F_2 SNPs/indels. Sequence Ontology is based on SnpEff terms with minor modifications. Closed bars, SNPs; open bars, indels. Black, 'Yukinko-mai'-type homozygous, red, 'Kaijin'-type homozygous; blue, heterozygous.

Table 3 | List of agronomic-trait-related genes harboring alternative protein sequences in BC_3F_2 #31-2-4.

Chr./ position	Ref.	Genotype			Alteration type	RAP ID	Protein encoded	Dataset 1	Dataset 2
		BC_3F_2	'Kaijin'	'Yukinko-mai'					
Chr06/ 26277010	G	A	A	G	missense SNP	Os06g0644200	Vacuolar HD-translocating inorganic pyrophosphatase 1	Cold tolerance	–
Chr08/ 6268486	GCACGG-CCACGGC	Hetero	GCACGG-CCACGGC	G	in-frame deletion	Os08g0207500	Zn-regulated transporter, iron (Fe)-regulated transporter–like protein 4	Other soil stress tolerance	–
Chr08/ 26913261	G	Hetero	G	A	missense SNP	Os08g0538300	Chitin elicitor receptor kinase 1	Blast resistance	–
Chr09/ 11449688	G	Hetero	G	A	missense SNP	Os09g0356200	Malectin-like carbohydrate-binding domain–containing protein	–	Days to heading
Chr09/ 11449800	AGG	Hetero	AGG	AC	frameshift deletion			–	

Chr., chromosome; Ref., Nipponbare reference allele; Hetero, heterozygous. Dataset 1, Overview of Functionally Characterized Genes in Rice Online database (OGRO) (Yamamoto *et al.*, 2012); Dataset 2, potential agronomic functional gene set by gene-based GWAS of Japanese rice population (Yano *et al.*, 2016).

Table 4 | Annotation of SNPs and indels in BC_3F_2 #31-2-4.

Location/Variant type	Yukinko-mai (Homo)		Kaijin (Homo)		Heterozygous	
	SNPs	Indels	SNPs	Indels	SNPs	Indels
1kb DOWNSTREAM	10,715	3,547	216	113	666	204
3'UTR	2,877	882	86	26	180	51
INTRON_others	11,570	3,902	438	215	1335	325
INTRON_Splice donor	7	7	-	-	2	1
INTRON_Splice acceptor	10	7	-	1	1	1
CDS_Splice region	272	51	18	9	16	5
CDS_Synonymous	2,856	141	75	3	262	2
CDS_Nonsynonymous	2,839	16	70	1	230	-
CDS_Inframe INDEL	-	285	-	10	-	7
CDS_Frameshift	-	277	-	20	-	9
5'UTR	1,629	493	23	12	75	24
1kb UPSTREAM	11,735	3,640	255	111	914	289
INTERGENIC	68,341	16,751	1,807	611	5,984	1,143

3.4 'YNU31-2-4' shares most agronomic traits with the recurrent parent but has better yield than the donor parent in the field

'YNU31-2-4' was evaluated in the field to compare its major agronomic traits with those of the parents. A significant difference was observed in days-to-heading between 'YNU31-2-4' and WT: some plants headed significantly earlier than WT (**Table 5; Figure 7B**). Heading date was associated negatively with the *Os09g0356200* genotype and positively with the *hst1* genotype. These results support the accuracy of gene-based GWAS (Yano *et al.*, 2016), and also suggest the potential early flowering function of *hst1* or the effect of unknown flowering-related genes. Grain width, grain thickness, and 1000-grain weight were significantly higher in 'YNU31-2-4' plants than WT (**Table 5; Figure 8E-F**). The *hst1* mutant also had a wider grain than WT (Takagi *et al.*, 2015). Together these results, at least partly, suggest that *hst1* might be involved in the increase of grain width. In contrast, other important morphological

traits of ‘YNU31-2-4’ were highly similar to those of WT, particularly flag leaf color, length, and width, plant height, tiller number per plant, panicle number per plant, panicle length, spikelet number per panicle, grain yield per plant, aboveground biomass per plant, and grain length (**Table 5; Figure 8A–D**). Interestingly, ‘YNU31-2-4’ had significantly higher yield potential than the donor parent ‘Kaijin’ owing to the higher number of panicles per plant and 1000-grain weight.

3.5 ‘YNU31-2-4’ showed increased tolerance to salt stress at the seedling stage

The salinity tolerance of ‘Kaijin’, ‘Yukinko-mai’ (WT), and ‘YNU31-2-4’ seedlings was evaluated during 3 weeks at 0, 75, and 125 mM NaCl. At 0 mM NaCl, there was no phenotypic difference between ‘YNU31-2-4’ and WT (**Figure 9A**). Under salt stress, however, the WT leaves were rolled, whereas those of ‘Kaijin’ and ‘YNU31-2-4’ remained flat and stayed green even at 125 mM NaCl. Further, 125 mM NaCl reduced the survival of WT seedlings to 52.5%, whereas all seedlings of ‘YNU31-2-4’ survived (**Figure 9B**). Moreover, ‘Kaijin’ and ‘YNU31-2-4’ had significantly better shoot and root growth under salinity vs. WT (**Figure 9C–F**); at 125 mM NaCl, ‘YNU31-2-4’ had 30% and 38% better shoot and root dry weight, respectively than WT. Notably, ‘Kaijin’ and ‘YNU31-2-4’ had a larger root system than WT at 0 mM NaCl, suggesting a function of *hst1* in root elongation and branching (**Figure 10A–C**). These results clearly indicate that ‘YNU31-2-4’ has stronger salt tolerance than ‘Yukinko-mai’ (WT) at the seedling stage.

Table 5| Growth and yield performance of 'Kaijin', WT, and 'YNU31-2-4' genotypes under normal field conditions.

Agronomic traits	Genotype		
	'Kaijin'	WT	'YNU31-2-4'
Flag leaf greenness (SPAD value)	42.93 ± 2.63 a	43.49 ± 1.82 a	43.24 ± 2.42 a
Flag leaf length (cm)	27.21 ± 2.53 b	29.85 ± 2.13 a	29.12 ± 1.85 a
Flag leaf width (cm)	1.11 ± 0.06 b	1.23 ± 0.10 a	1.21 ± 0.04 a
Days-to-heading (day)	105.17 ± 1.40 a	105.08 ± 1.38 a	103.75 ± 1.76 b
Plant height (cm)	98.42 ± 3.56 a	94.75 ± 3.49 b	95.83 ± 2.41 ab
Tiller number per plant	20.40 ± 3.31 b	23.80 ± 3.22 a	24.40 ± 3.57 a
Panicle number per plant	20.40 ± 3.31 b	23.80 ± 3.22 a	24.20 ± 3.12 a
Panicle length (cm)	18.71 ± 0.69 b	19.79 ± 0.52 a	19.11 ± 0.97 ab
Spikelet number per panicle	71.99 ± 8.91 a	73.55 ± 6.27 a	72.66 ± 5.80 a
Grain number per panicle	63.61 ± 8.20 a	67.36 ± 5.81 a	60.59 ± 7.29 a
1000-grain weight (g)	22.42 ± 0.14 b	21.56 ± 0.22 c	23.29 ± 0.43 a
Seed setting rate (%)	88.34 ± 3.71 a	91.61 ± 2.52 a	83.43 ± 7.58 b
Grain yield (g per plant)	33.77 ± 4.24 b	40.07 ± 4.59 a	37.84 ± 4.46 a
Aboveground biomass (g per plant)	61.80 ± 8.50 b	72.30 ± 7.63 a	67.13 ± 9.14 ab

Grain yield is weight of filled spikelets at 14% moisture content. Data are mean ± SD (n =10-12). Values with the same letter within a row are not statistically different (Duncan's multiple range *test*, $P < 0.05$).

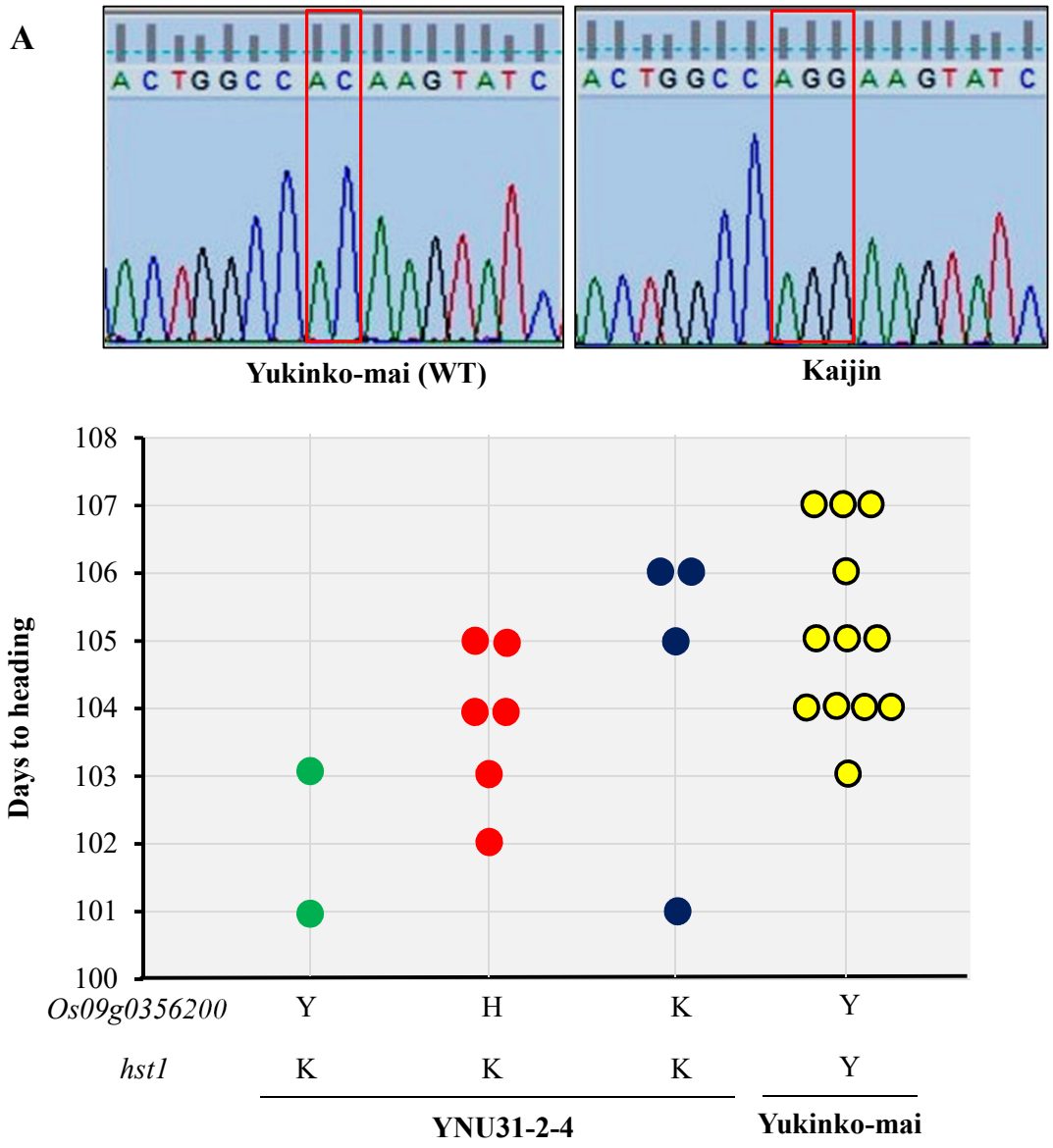

Figure 7| Frameshift deletion in *Os09g0356200* affects days-to-heading of 'YNU31-2-4' plants. (**A**) Frameshift deletion in *Os09g0356200*; red box indicates indel position (Chr. 9:1144980). (**B**) Distribution of days-to-heading. Genotypes: Y, 'Yukinko-mai' homozygous; H, heterozygous; K, 'Kaijin' homozygous. 'YNU31-2-4' plants were grown under normal field conditions to assess differences in heading within the population.

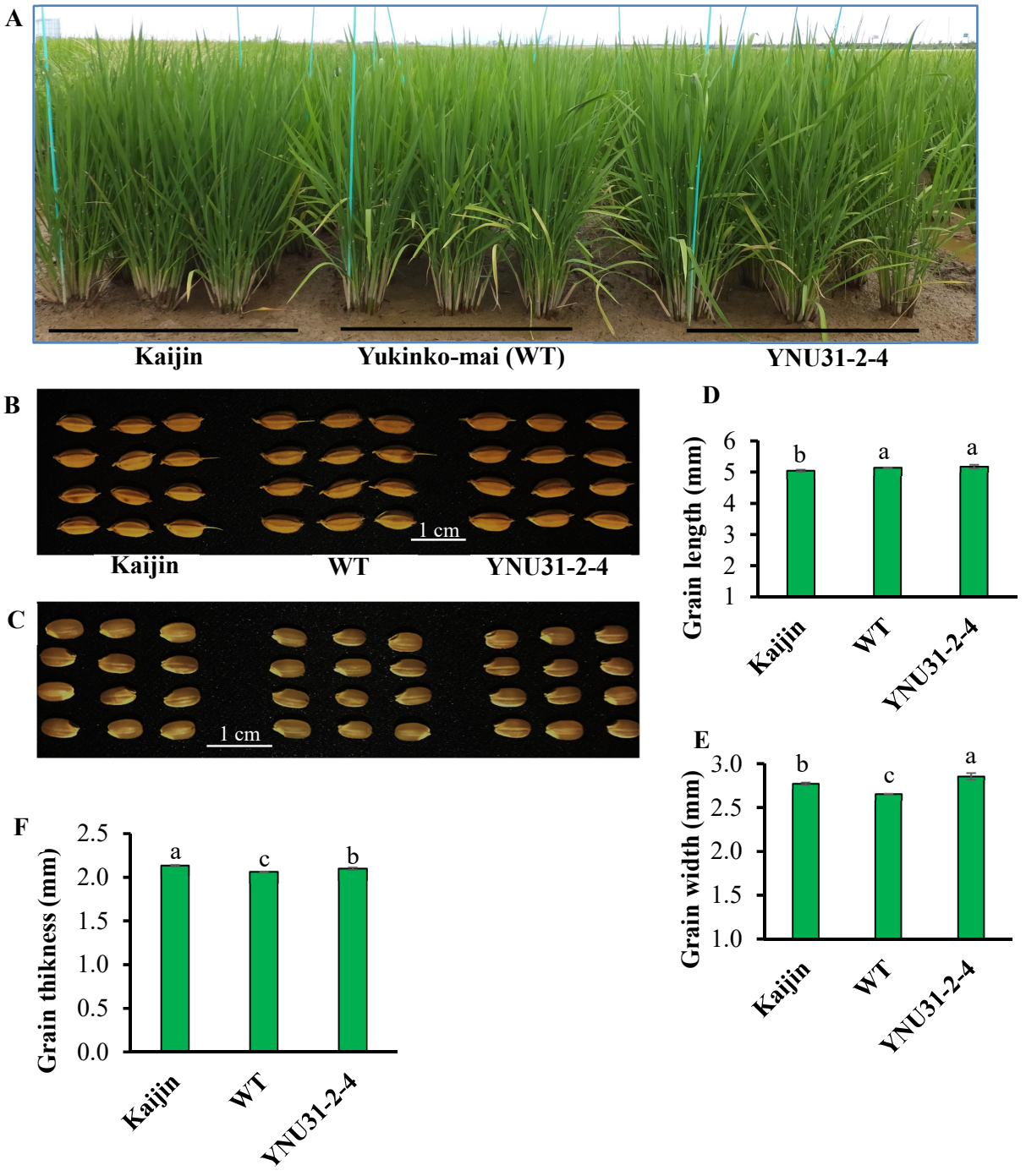

Figure 8| Agronomic trait assessment of 'Kaijin', WT, and 'YNU31-2-4' plants under control field condition. (**A**) Phenotypes of plants after flag leaf emergence (95 days after germination); (**B**) mature rice seeds and (**C**) brown rice grains. (**D–F**) Comparison of (**D**) grain length, (**E**) width, and (**F**) thickness. Data are mean ± SD of four biological replicates, each being a pool of 400 grains. Bars labeled with different letters are statistically different (Duncan's multiple range test, $P < 0.05$).

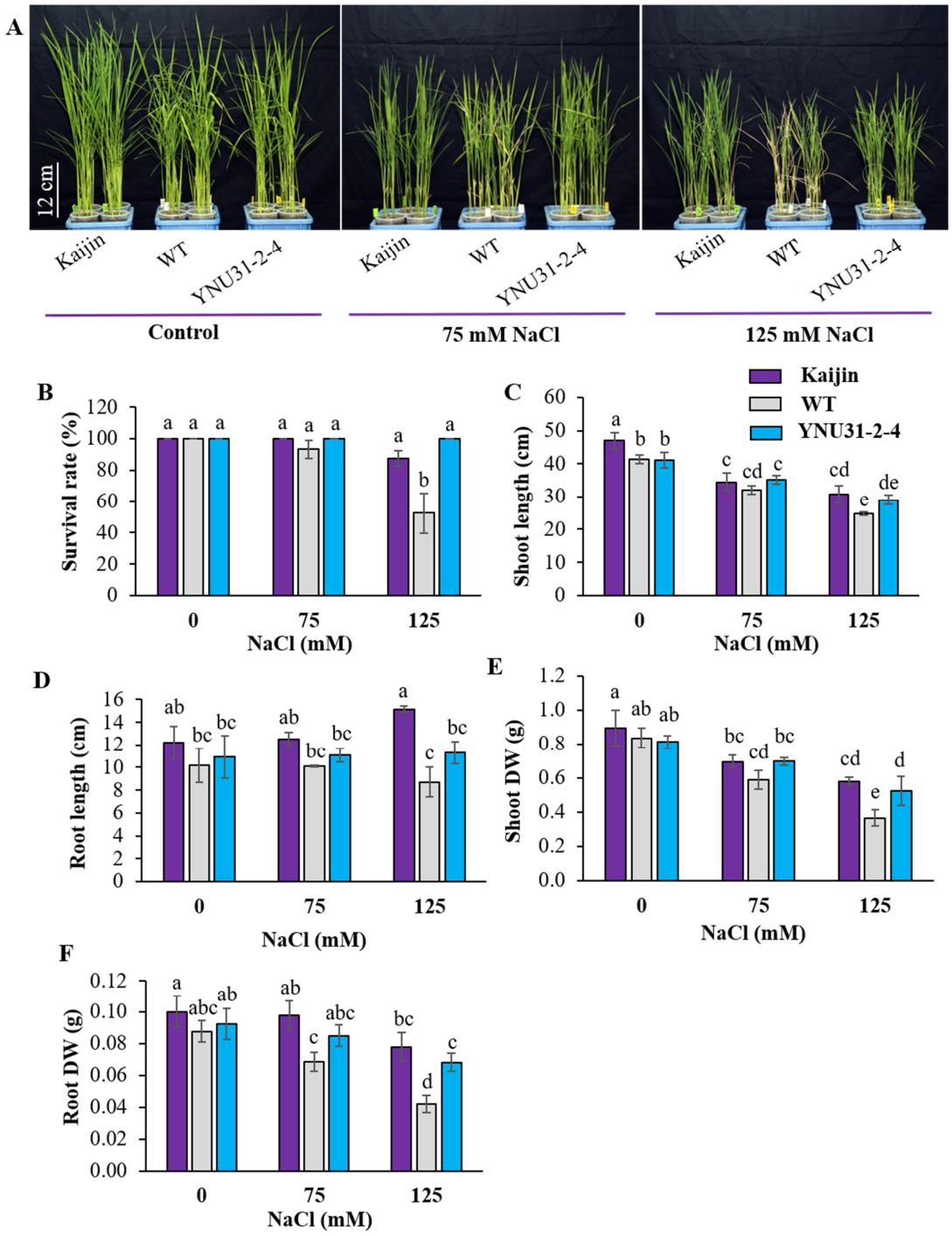

Figure 9| Salinity tolerance of BC_3F_3 line 'YNU31-2-4' at seedling stage. (A) Phenotypic comparison of 'Kaijin', WT, and 'YNU31-2-4' seedlings grown in 0, 75, or 125 mM NaCl for 2 weeks. (**B**) Survival rates, (**C**) shoot length, (**D**) root length, (**E**) shoot dry weight, and (**F**) root dry weight of seedlings shown in **A**. Data in **B–F** are mean ± SD of four independent biological replicates; data in **E** and **F** are dry weight (DW) of 10 plants in each treatment. Bars labeled with the same letter are not statistically different (Tukey's test, $P < 0.05$).

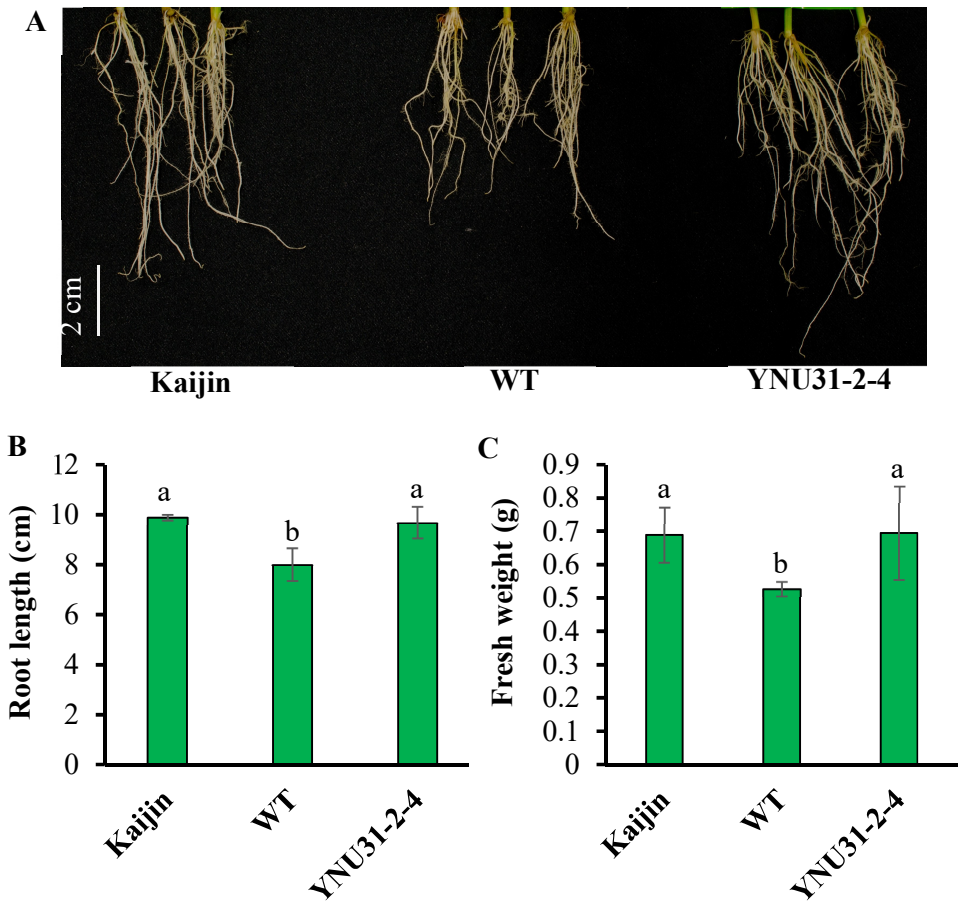

Figure 10| 'YNU31-2-4' plants have an enlarged root system. (**A**) Root phenotype comparison of 'Kaijin', WT, and 'YNU31-2-4'. Seedlings were cultured in Yoshida's nutrient solution and photographed at 2 weeks. (**B**) Root length and (**C**) root fresh weight of plants grown under stress-free condition for 2 weeks. Data are mean ± SD of four independent biological replicates, each being a pool of 5 individuals. Bars labeled with the same letter are not statistically different (Duncan's multiple range test, $P < 0.05$).

3.6 YNU31-2-4 has better plant growth and less yield loss under salt stress at the reproductive stage

Under control condition at the reproductive stage, there was no obvious phenotypic difference between WT and 'YNU31-2-4' plants (**Figure 11A**). Salt stress for 5 weeks

caused severe burning and wilting symptoms in WT, but 'YNU31-2-4' and 'Kaijin' plants maintained green leaves. Under control condition, the penultimate leaves of 'YNU31-2-4' plants maintained a slightly higher net CO_2 assimilation rate than the parents (**Figure 11B**). Salt stress for 4 weeks significantly reduced the net CO_2 assimilation rate of WT plants relative to 'YNU31-2-4' and 'Kaijin'. Under control condition, WT and 'YNU31-2-4' plants had similar phenotypic characters and yield attributes except for a higher 1000-spikelet weight than the parents (**Figure 11C–G**). Under salt stress, in contrast, 'YNU31-2-4' had higher plant height, yield characters, and aboveground biomass than WT (**Figure 11C–G, I**). Relative to control condition, salt stress reduced grain yield by 68% in WT but by 38% in 'YNU31-2-4' (**Figure 11H**). As a result, the grain yield of 'YNU31-2-4' was 10% higher than that of the donor parent 'Kaijin' under control condition and 45% higher than that of WT under saline condition. Thus, the yield improvement was due to the significantly higher number of panicles per plant and 1000-spikelet weight, comparable to the field evaluation results (**Figure 11D, G**).

3.7 'YNU31-2-4' maintains higher relative water, chlorophyll, proline content at the seedling stage under salt stress

In a separate experiment (**Figure 12**), at 0 mM NaCl, there were no significant differences in leaf relative water content or chlorophyll content between WT and 'YNU31-2-4' plants. Under salt stress, the 'YNU31-2-4' plants were able to maintain significantly higher relative water content and chlorophyll levels than WT (**Figure 12A, B**). Assay of soluble proline levels is a useful way to monitor physiological status and to assess stress tolerance, since plants under salt stress accumulate this osmoprotectant against ion-dependent protein degradation (Hayat *et al.,* 2012). Under control condition, 'YNU31-2-4' had significantly higher proline content than the parents. Exposure to salinity led to a considerable increase in proline levels in all genotypes, and to 1.6× the WT level in 'YNU31-2-4' (**Figure 12C**).

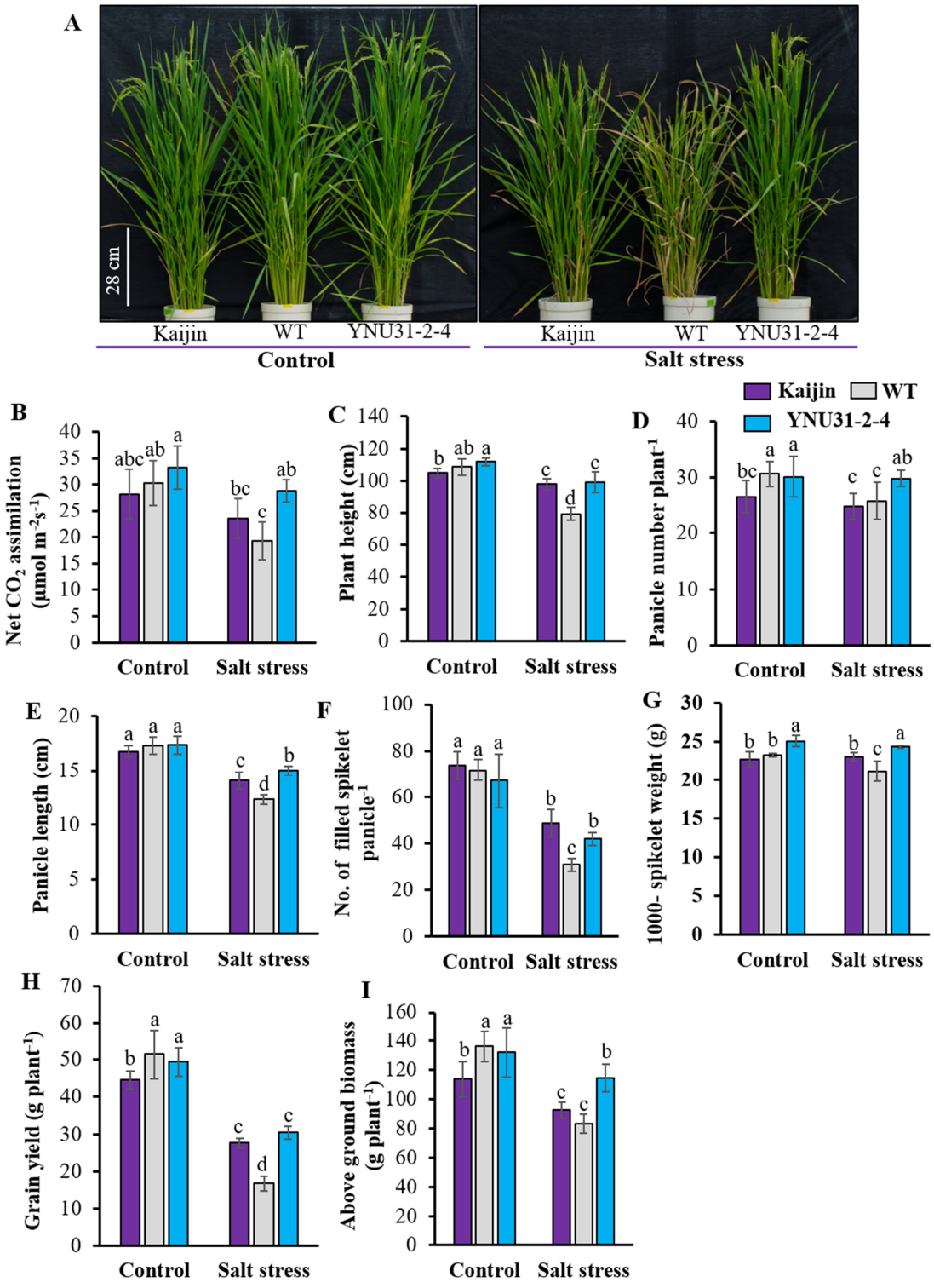

Figure 11| Salinity tolerance of BC_3F_3 line 'YNU31-2-4' at heading. (A) Phenotypic comparison of 'Kaijin', WT, and 'YNU31-2-4' plants grown with or without salt stress. Salt-stressed plants were grown in 50 mM NaCl from 60 days after germination (DAG) and then in 75 mM from 74 DAG until 95 DAG (booting stage), and then in fresh water until 110 DAG

(heading). **(B)** Net CO_2 assimilation rate of the penultimate leaf 4 weeks after imposition of salt treatment. **(C)** Comparison of plant height at 110 DAG. (**D–I**) Comparisons of **(D)** number of panicles/plant, **(E)** panicle length, **(F)** number of filled spikelets/panicle, **(G)** weight of 1000 filled spikelets, **(H)** grain yield/plant, and **(I)** dry weight of aboveground biomass at harvest. Grain yield is weight of filled spikelets at 14% moisture content. Data are mean ± SD of 6 individuals. Bars with the same letter are not statistically different (Duncan's multiple range test, $P < 0.05$).

3.8 YNU31-2-4 avoided Na^+ accumulation under salinity stress

The K^+ and Na^+ contents in shoots and roots of seedlings were assayed under salinity, since the degree of stress depends on their uptake and translocation. Under control condition, shoot K^+ was significantly lower in 'YNU31-2-4' than in its parent (**Figure 13A**), whereas under salt stress, it was 1.4× the WT level in shoot and 2.6× in root (**Figure 13A, D**). Under control condition, Na^+ levels in leaves (**Figure 13B**) and roots (**Figure 13E**) of all genotypes remained similarly low. Salinity stress increased Na^+ concentration in WT shoots relative to the other two genotypes, reaching 6.5× that in 'YNU31-2-4' plants (**Figure 13B**). Under control conditions, the Na^+/K^+ ratio did not differ significantly among the tested genotypes in shoots (**Figure 13C**) and roots (**Figure 13F**). Under salinity, it was 9.2× the 'YNU31-2-4' level in WT shoots and 2.9× in WT roots (**Figure 13C, F**).

Under control condition, electron probe micro-analysis revealed a dense distribution of K^+ in the basal portion of the shoot of all genotypes, but only a very sparse distribution of Na^+ (**Figure 14**). Under salt stress, the Na^+ distribution in cells was increased in all genotypes, and the salt-sensitive WT accumulated significantly more Na^+ than the other two genotypes (**Figure 14**).

Hence, the milder stress symptoms and salinity tolerance in 'YNU31-2-4' leaves, similar to the *hst1* donor parent 'Kaijin', correlate with higher K^+ and lower Na^+ in the tissue.

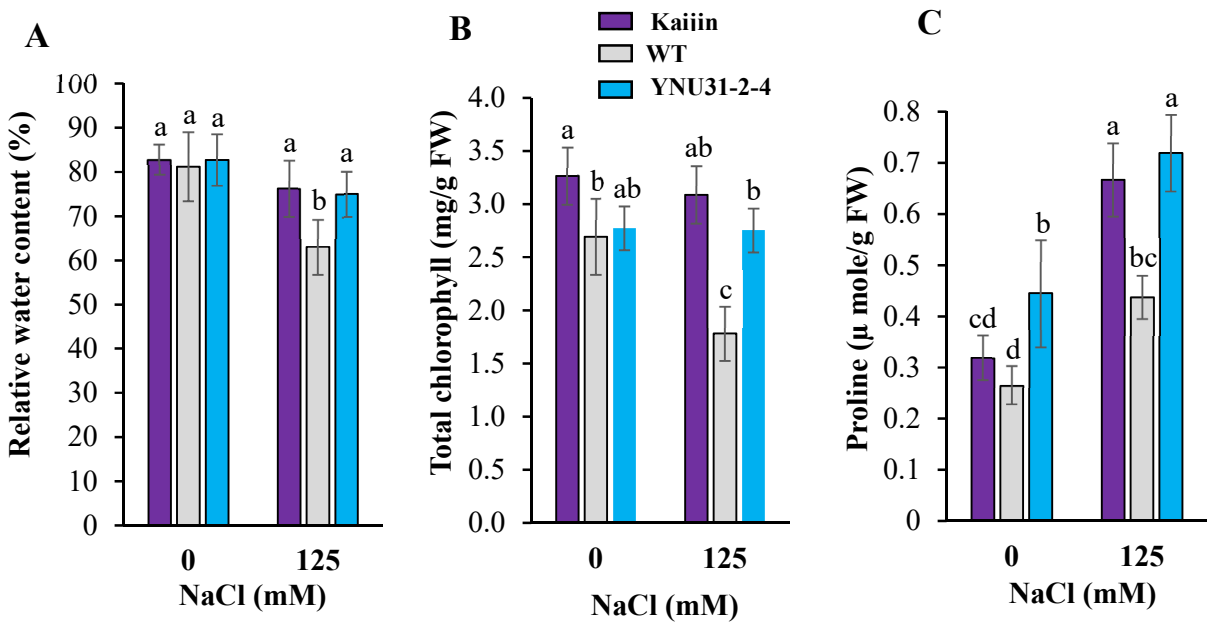

Figure 12| 'YNU31-2-4' maintains higher relative water, chlorophyll, and proline contents at the seedling stage under salt stress. (**A**) Relative water content, (**B**) total chlorophyll content, and (**C**) proline content in shoots of 20-day-old seedlings. Data are mean ± SD of three independent biological replicates. Bars labeled with the same letter are not statistically different (Duncan's multiple range test, $P < 0.05$).

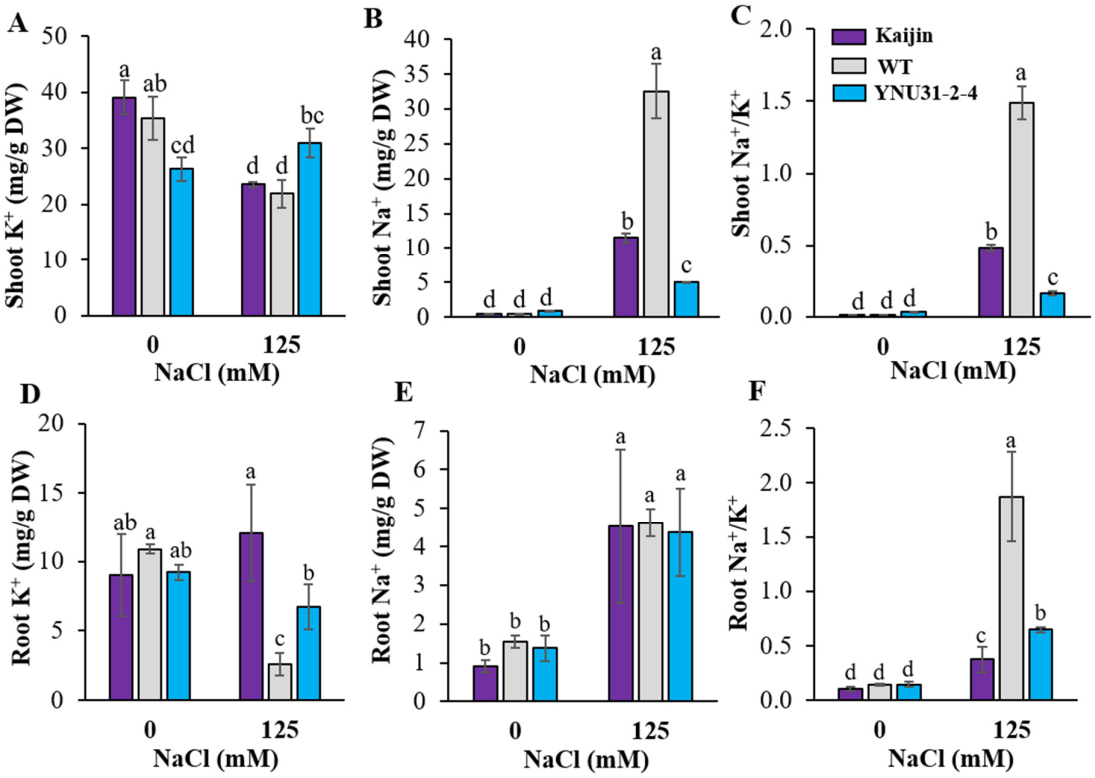

Figure 13| 'YNU31-2-4' maintains lower Na^+/K^+ ratio in both shoot and root under salt stress at seedling stage. (**A**) Shoot K^+, (**B**) shoot Na^+, (**C**) shoot Na^+/K^+, (**D**) root K^+, (**E**) root Na^+, and (**F**) root Na^+/K^+ of 20-day-old seedlings. Data are mean ± SD of three independent biological replicates. Bars with the same letter are not statistically different (Duncan's multiple range test, $P < 0.05$).

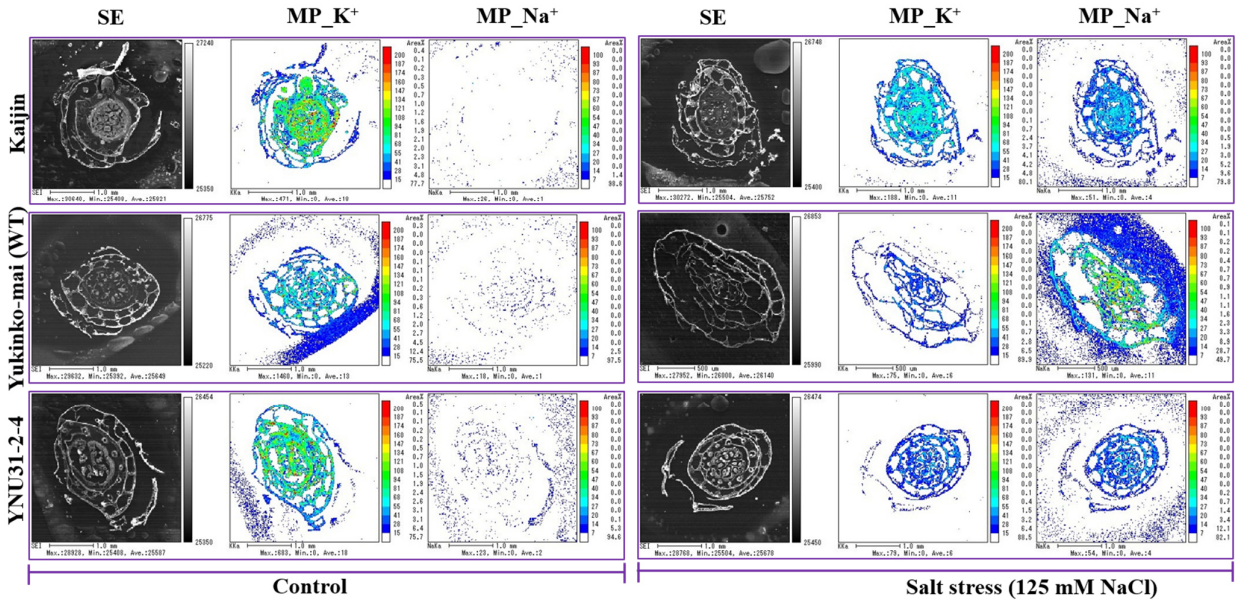

Figure 14| Accumulation and distribution of K^+ and Na^+ in cell clusters of 20-day-old seedlings. Harvested basal portions of shoots were immediately frozen and embedded in OCT compound; 5-µm sections were scanned with an electron probe microanalyzer. Relative amounts of K^+ and Na^+ are indicated by color coding. SE, secondary electron image; MP_K^+, mapping pattern of K^+; MP_Na^+, mapping pattern of Na^+.

3.9 *OsHKT1;1* up-regulated in the shoot tissue of 'YNU31-2-4' under salinity stress

The K^+/Na^+ homeostasis is an important salt tolerant mechanism in plant. The gene *OsHKT1;1*/ *Os04g0607500*, coding for a member of the high-affinity K^+ transporter family is known to function as a Na^+ transporter in rice (Garciadeblás *et al.,* 2003; Takagi *et al.,* 2015). The relative expression levels of high-affinity K+ transporter 4 (*OsHKT1;1*) transcripts was analyzed in rice shoot grown with or without salt stress. In WT, the relative expression levels of *Os04t0607500-01* and *Os04t0607500-02* were not increased under salt stress, relative to the control condition (**Figure 15A, B, D, E**).

Under salt stress condition, ‘YNU31-2-4’ had significantly higher relative expression levels of *Os04t0607500-01* than the control condition and reached 4.9× and 1.9 × the control level after 24 h and 48 h salt imposed, respectively (**Figure 15A, B**). Similarly, under salt stress condition, ‘YNU31-2-4’ had significantly higher relative expression levels of *Os04t0607500-02* than the non-stress condition, and reached 4.5× and 1.6 × the control level after 24 h and 48 h salt imposed, respectively (**Figure 15D, E**). A similar up-regulation pattern of *OsHKT1;1* was observed in donor parent ‘Kaijin’ under salt stress condition in shoot tissue (**Figure 15A, B, D, E**). The relative expression levels of *Os04t0607500-01*and *Os04t0607500-02*, 7-day after salt treatment were very low or could not be detected in all the tested genotypes (**Figure 15C, F**), indicates early salt response of *OsHKT1;1* gene.

Therefore, salinity tolerance in ‘YNU31-2-4’, similar to the *hst1* donor parent ‘Kaijin’, might associate with the function of *OsHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue.

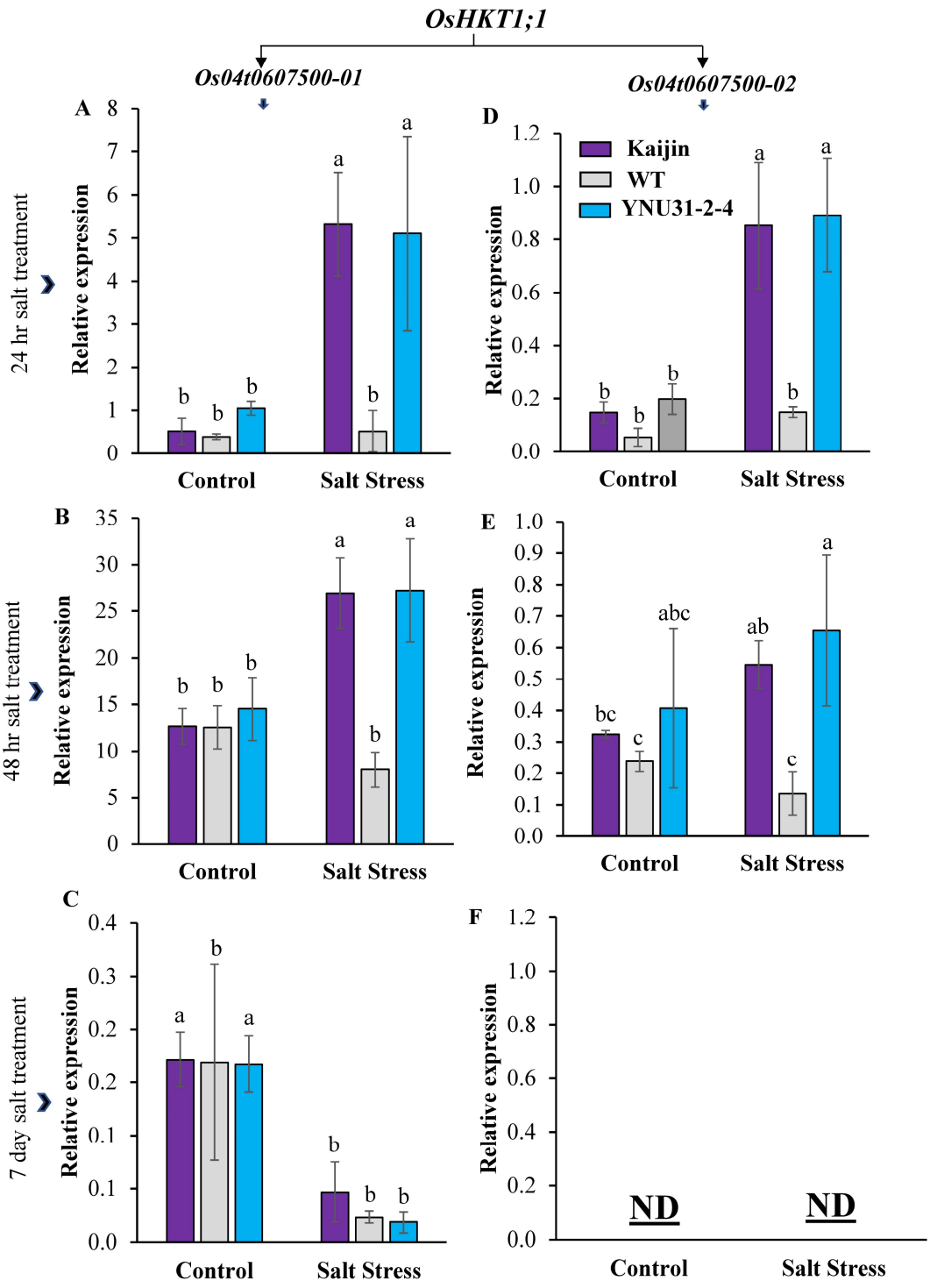

Figure 15| Relative expression levels of high-affinity K+ transporter 4 (*OsHKT1;1*) transcripts in rice shoot grown with or without salt stress. Relative expression level of *Os04t0607500-01* is shown in panel **A-C** and, *Os04t0607500-02* in panel **D-F** at 24h, 48h and 7-day salt treatment, respectively. The salt stress (125mM NaCl) was imposed ten days after germination. Shoot samples were collected 24h, 48h and 7-day after salt was imposed. Data are mean ± SD of three independent biological replicates. Bars with the same letter are not statistically different Tukey's test, $P < 0.05$). ND; not detected.

4. DISCUSSION

Soil salinity is a major threat to future food production, affecting more than 6% of the total land area (Munns, 2005). The rapid global warming and sea level rise pose threats to rice yield and quality in South Asian rice-growing countries. In addition, a tsunami contaminated paddy fields in Miyagi prefecture, Japan, with salt in 2011 (Inui *et al*., 2012, Roy *et al*., 2014, Takagi *et al*., 2015). Therefore, it is important to introgress genes/QTLs/SNPs conferring salt tolerance in locally grown popular rice cultivars, focusing on higher grain yield, to ensure food security under changing climatic conditions. Cultivar improvement through conventional breeding is feasible, but it takes a long time to minimize linkage drag through phenotypic screening (Iftekharuddaula *et al.*, 2012; Hasan *et al*., 2015). For these reasons and to achieve breeding goals, the *hst1* gene from 'Kaijin' was introgressed into 'Yukinko-mai', which has excellent yield stability. The BC_3F_3 generation, named 'YNU31-2-4' was developed, through SNP marker-aided selection (**Figure 4A**).

To accelerate the breeding cycle, a biotron speed-breeding system (**Figure 1**) without a CO_2 supply was used, since the application of 475 ppm CO_2 in growth chambers did not greatly change the breeding cycle (Ohnishi *et al*., 2011), and many rice breeders do not have CO_2 regulation facilities owing to the high cost. By using a longer daylength (14/10 h light/dark) for first 30 days to accelerate the vegetative growth followed by a shorter daylength (10/14 h light/dark) to induce reproduction, tiller removal, and embryo rescue to decrease the period before seed maturity (**Figure 1**), four breeding generations were achieved within 11 months (**Table 1**). Developing four to five generations a year is the ultimate objective (Tanaka *et al*., 2016; Collard *et al*., 2017; Watson *et al*., 2018). This simplified, faster, efficient method for reducing the duration and number of breeding cycles will contribute significantly to genomic studies and the deployment of superior rice.

The whole-genome sequencing (WGS) was used to characterize the advanced breeding lines and genome recovery rate, genotype blocks, and putative phenotypes were revealed (**Figures 5, 6; Tables 2, 3**). WGS identified 118 454 SNPs/indels as markers. As WGS provides higher resolution of genome blocks than conventional SSR marker methods (Tanweer *et al.*, 2015), it can thus be used for advancing generations

from parents with low genetic variation, as here. Furthermore, in subsequent selection, knowledge of these genotype blocks helped us to rapidly fix heterozygous regions into the recipient allele through the MABC selection method (**Figure 7A)**.

Functional annotation was able to predict five SNP/indels (**Table 3**) that may affect agronomic traits and thus phenotype in our advanced line 'YNU31-2-4'. Indeed, the field assessment results demonstrate the difference in the distribution of heading dates between 'YNU31-2-4' and WT ('Yukinko-mai') (**Table 5; Figure 7B**). Additionally, the heading date of the BC_3F_3 population was associated with the genotype of the putative heading-date-associated gene *Os09g0356200* (**Table 3; Figure 7B**). This finding indicates the potential value and reliability of gene-based GWAS among the Japanese rice population data set (Yano *et al.*, 2016) in breeding by incorporating the genetic variations into those cultivars, and its practical value in predicting genes governing complex traits of agronomic phenotypes. The results of whole-genome sequencing have demonstrated the success in developing improved cultivars using our rapid breeding system. They also reveal undesirable genome regions and genes from the donor parent 'Kaijin', valuable information for estimating agronomic properties without further phenotyping study. Identifying heterozygous genes can provide mechanistic insights toward homogenizing phenotypes for ongoing breeding. It is important to note that the heterozygote disadvantage has been overcome by taking advantage of the SNP-based selection of the homozygous WT allele from the YNU31-2-4 population (**Figure 7**). Furthermore, other morphological traits of 'YNU31-2-4' were similar to those of WT (**Table 5; Figure 8A–D**). The field assessment results along with the high-resolution genotyping data indicate no apparent grain yield reduction in 'YNU31-2-4' in relation to the presence of the target *hst1* gene. In fact, 'YNU31-2-4' increased ca. 11% yield than the donor parent 'Kaijin' owing to the higher number of panicles per plant and 1000-grain weight.

Rice is very sensitive to salt stress at the seedling stage (Munns and Tester, 2008), and its sensitivity varies with the developmental stage (Jagadish *et al*., 2012). To assess the practical utility of *hst1* in the introgression line, seedlings were exposed to 75 and 125 mM salt stresses. Salt tolerance at this stage is of importance in saline environments, as crop establishment is fundamentally determined during the earliest stages of development. The findings revealed that under high salt stress, the 'YNU31-

2-4' plants had significantly higher survival rate, shoot and root biomass than WT (**Figure 9A–F**), which suggest strong tolerance similar to that of the donor parent. The 'YNU31-2-4' plants maintained significantly higher plant growth, proline content, and plant water status under salinity, which could indicate a physiological and biochemical tolerance mechanism (Munns, 2005; Ma *et al*., 2017). In fact, previous studies showed that salinity might reduce the fertility of the spike and the translocation of assimilates to the grain in bread wheat and rice. Physiologically, the 'YNU31-2-4' maintain its fully hydrated state under saline condition, which could at least partially have rapid and large effects on cell expansion, cell division, stomatal opening, maintain normal rates of transpiration, abscisic acid (ABA) accumulation, etc. Proline is accumulated in taxonomically diverse sets of plants (Saxena *et al*. 2013), providing stress tolerance by protecting the cell membrane and maintaining osmotic balance within the cell, and also serves as an organic nitrogen reserve during stress recovery (Szabados and Savouré, 2010; Hayat *et al.*, 2012; Gupta and Huang, 2014).

The yield traits of 'YNU31-2-4' was also assessed under salt stress at the early reproductive to booting stages, when salt stress reduces panicle and spikelet numbers per plant, leading to significant yield losses (Zeng and Shannon, 2000; Walia *et al*., 2007). The improvement of rice grain yield under salt stress is the focus of breeding (Zhou *et al*., 2018). Owing to the significant increases in panicle number per plant, spikelet number per panicle, and 1000-spikelet weight, the final grain yield of 'YNU31-2-4' plants was 45% higher than WT under salt stress at the reproductive stage (**Figure 11D, F–H**). Under control condition, there was no yield difference between 'YNU31-2-4' and WT (**Figure 11H**). Interestingly, 'YNU31-2-4' has higher yield potential than the donor parent under control condition owing to the higher number of panicles per plant and 1000-spikelet weight, comparable to the field evaluation results (**Figure 11D, G**). The higher number of panicles can be attributable to the WT background. The higher seed weight of 'YNU31-2-4' could be due, at least partly, to the improved photosynthetic efficiency (**Figure 11B**) due to coordination of leaf morphological and physiological traits, which has great potential for use in breeding for higher yield. Accordingly, 'YNU31-2-4' showed larger flag leaf than the donor 'Kaijin', which could play an important role to grain filling and hence determining yield potential. The superior tiller growth with higher

leaf size rendered the source, sink, and flow stronger and more harmonized and consequently increased the cereal yield (Murchie *et al.*, 2002; Zhai *et al.*, 2002; Kasai *et al.*, 2008; Sanchez-Bragado *et al.*, 2014; Zhou *et al.*, 2017). Thus, this study clearly shows that the introgression of *hst1* to the WT significantly increased salt resistant without any reduction in grain yield. Thus, 'YNU31-2-4' has significant breeding value without a noticeable yield penalty under normal and salt stress conditions.

Roots absorb minerals and water from the soil and play a key role in transporting them to leaves. In the context of salt tolerance, roots are sensitive to NaCl and are the first site of defense, directly limiting or excluding sodium uptake (Munns, 2005; Liu *et al*., 2015). Roots are often used as a biomarker of salt stress. Root architecture differed between WT and 'YNU31-2-4' plants after 2 weeks of normal hydroponic culture (**Figure 10**). Roots of 'YNU31-2-4' and 'Kaijin' exposed to high salt stress elongated more than WT roots (**Figure 9D**). Thus, this behavior as a novel effect of the *hst1* gene in response to salt stress is proposed. However, more validation trials are needed to confirm this hypothesis. The better morphophysiological and biochemical characters of 'YNU31-2-4' under salt stress demonstrate the success of introgression of *hst1* into 'Yukinko-mai'.

The genes involved in conferring salt tolerance, which is likely a complex trait controlled by a combination of multiple genes, are yet to be elucidated. Recent research advances have identified major genes conferring salinity tolerance in rice, including *OsHKT1;1*, *OsHKT2;1*, *OsSOS1*, *OsNHX1*, *OsCAX1*, *OsAKT1*, *OsKCO1*, *OsNRT1;2*, *OsCLC1*, *OsADS31* and *OsTPC1*; however, their functional pathways during salt stress are not coordinately linked for explaining the very complex phenomenon of salt tolerance (Reddy *et al*., 2017; Yu *et al.*, 2018; Ali *et al.*, 2019). The *hst1* (loss-of-function in *OsRR22*) gene primarily led to the upregulation of *OsHKT1;1* (encoding a high-affinity K^+ transporter) that functions as a Na^+ transporter contributing salt resistance of the *hst1* mutant and 'Kaijin' (Takagi *et al*., 2015). The Na^+/K^+ ratio in shoot and root was divergent between WT and 'YNU31-2-4'. The quantification and localization results demonstrate that like 'Kaijin', 'YNU31-2-4' plants maintained a very low Na^+/K^+ ratio in both shoot and root under salt stress (**Figure 13C, F**), which is one of the most important mechanisms used by plants to withstand salt stress (Okada *et al*., 2008; Hauser and Horie, 2010). Under salt stress,

the susceptible WT plants had more Na^+ densely localized in shoot tissue (**Figure 14**). An overload of Na^+ can dramatically depolarize the plasma membrane, leading to K^+ efflux via depolarization-activated outward-rectifying K^+ channels (Chen *et al*., 2007). It is notable that *hst1*-regulated salt stress resistance involved K^+ homeostasis. These results suggest that the accumulation of more K^+ with less Na^+ in 'YNU31-2-4' plants would be mediated by a mechanism of K^+ influx and Na^+ efflux.

The gene *OsHKT1;1*/ *Os04g0607500*, coding for a member of the high-affinity K^+ transporter family is known to function as a Na^+ transporter in rice (Garciadeblás *et al.,* 2003; Takagi *et al.,* 2015). The relative expression levels of high-affinity K+ transporter 4 (*OsHKT1;1*) transcripts in rice shoot grown with or without salt stress was analyzed. The *OsHKT1;1* significantly up-regulated in shoot tissue of 'Kaijin' and 'YNU31-2-4' (**Figure 15A, B, D, E**). Therefore, salinity tolerance in 'YNU31-2-4', associate with the function of *OsHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue. Further investigation will be needed to elucidate the molecular mechanisms mediating K^+ and Na^+ homeostasis in 'YNU31-2-4'.

5. CONCLUSIONS

In conclusion, the results of this study demonstrate that the modified biotron breeding system coupled with SNP MAS offers a rapid and effective way to improve single traits in rice. The precise introgression of *hst1*, combined with suitable genetic resources and phenotyping results, resulted in the selection of a line, 'YNU31-2-4', adapted to salt stress at the vegetative and reproductive stages with improved yield. Salinity tolerance in 'YNU31-2-4', similar to the *hst1* donor parent 'Kaijin', correlate with higher leaf water relations, chlorophyll, proline, photosynthesis and, function of *OsHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue. The improved salt tolerant line, 'YNU31-2-4' has a practical breeding value and could be utilized as an important source of genetical material. 'YNU31-2-4' is a potential candidate for new rice cultivar with markedly improved salinity tolerance, which might sustain grain yield and food security in a changing climate.

6. FUTURE PERSPECTIVE

Rice (*Oryza sativa* L.) is an important staple crop feeds more than half of the world's population. In this study, success was achieved in the breeding of a promising rice line ('YNU31-2-4'), carrying the *hst1* gene, highly tolerant to salt stress at vegetative and reproductive stages using SNP marker-assisted selection coupled with speed-breeding. This study demonstrated a more rapid and reliable means of implementing breeding programs for abiotic stress tolerance in rice. The newly developed rice line will be contributed significantly in achieving food security, by increasing the yield stability in the near future against a background of changing global climate and shifting arable land ranges. On the genomic perspective, the genome-wide characterization data generated here might be helpful for further development of new rice variety and genomic studies of rice. Yield variability is driven primarily by variability in the natural environment; therefore, it is necessary to evaluate the phenotype of the promising line under large-scale field trials. The root system characteristics are highly relevant for salt tolerance and correlated with agronomic performance of the plant. Further in-depth study of the function of *hst1* gene in root growth could revealed valuable information. This study explored that salinity tolerance in 'YNU31-2-4', associate with the function of *OsHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue. However, it is still unclear which pathways lead to salinity tolerance of the promising rice line. To fully understand the role of *hst1* gene in salinity tolerance, comprehensive "omics" analysis of the newly developed rice at metabolomic, transcriptomic and proteomic levels, should be major focus of further research.

7. SUMMARY

Soil salinity is a major threat to future food production, affecting more than 6% of the total land area. Salinity critically limits rice metabolism, growth, and productivity worldwide. The rapid global warming and sea level rise pose threats to rice yield and quality in South Asian rice-growing countries. In addition, a tsunami contaminated paddy fields in Miyagi prefecture, Japan, with salt in 2011. To combat earthquake- and tsunami-induced soil salinity in Japan, it is crucial to improve the salt resistance of locally grown popular rice cultivars, most of which are salt sensitive. In addition, developing Japanese cultivars for international appeal and fine-tuning their yield performance under various ecosystems around the world are time-demanding tasks.

In this study, a new rice germplasm with salt tolerance was developed, obtained by precise transferring the known salt tolerance gene *hst1* (*OsRR22*) from a cultivar 'Kaijin' (tolerant) into high-yielding cultivar 'Yukinko-mai' (susceptible). SNP marker-assisted selection coupled with speed-breeding were used to transfer the target gene and to shorten the generation cycle. After three backcrosses to the latter, progeny homozygous for the target gene was selected, fully sequenced and SNP genotyped to examine the genomic resemblance to the recurrent parent. Salinity tolerance of the newly developed material was evaluated under controlled conditions at seedling and heading stages by assessing a series of morpho-physiological parameters (**Figure 16**).

To accelerate the breeding cycle, a biotron speed-breeding system was used. By using a longer daylength (14/10 h light/dark) for first 30 days to accelerate the vegetative growth followed by a shorter daylength (10/14 h light/dark) to induce reproduction, tiller removal, and embryo rescue to decrease the period before seed maturity, the BC_3F_3 population, named 'YNU31-2-4', carrying our desired allele in the homozygous state was developed, in 6 generations and 17 months. This simplified, faster, efficient method for reducing the duration and number of breeding cycles will contribute significantly to genomic studies and the deployment of superior rice.

Whole-genome sequencing (WGS) was used to characterize the advanced breeding lines and genome recovery rate, genotype blocks, and putative phenotypes were revealed. High-resolution genotyping by WGS revealed that the BC_3F_2 genome had 93.5% similarity to the WT and fixed only 2.7% of donor parent alleles. WGS

identified 118 454 SNPs/indels as markers. To estimate the effects of variants on phenotypes, variants causing protein sequence alterations for Sequence Ontology and effect prediction were listed.

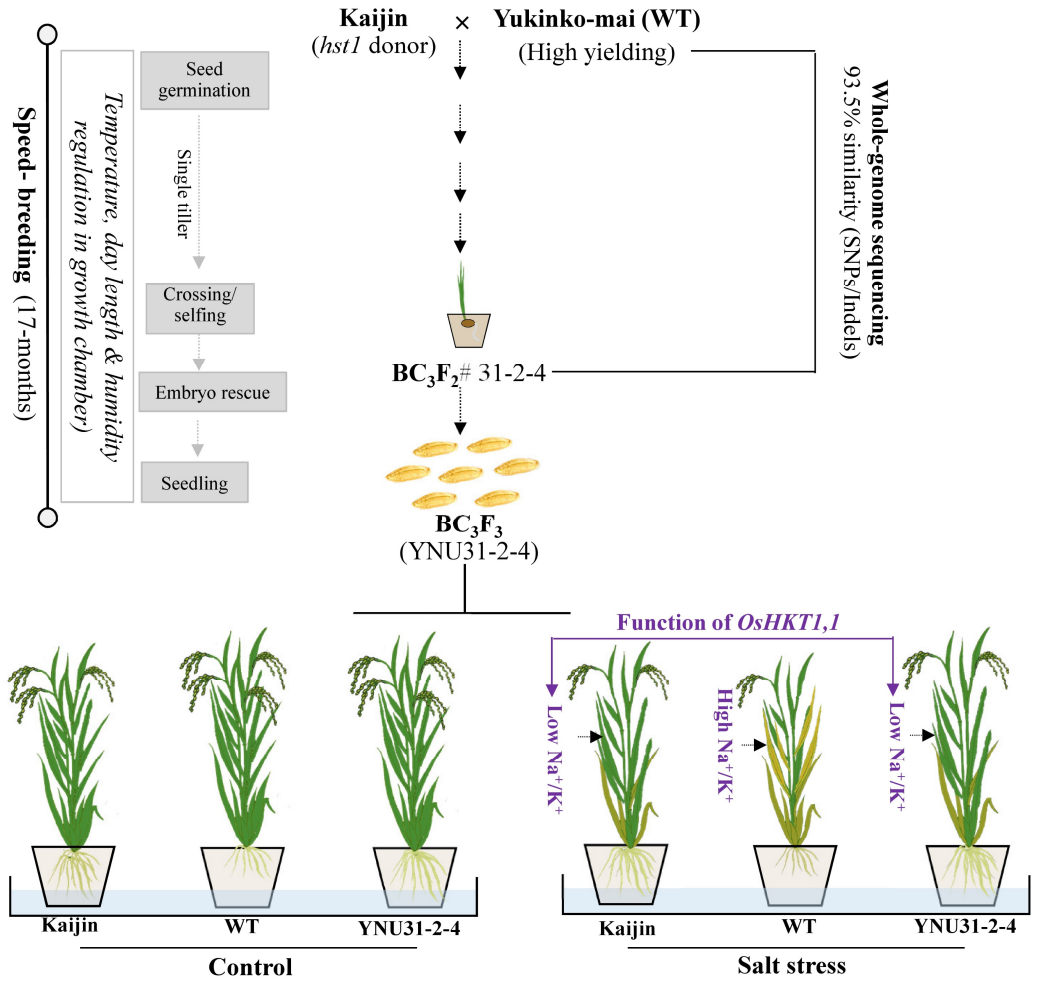

Figure 16| Schematic illustrating the rapid breeding and evaluation of a salt tolerant rice. Speed-breeding coupled with SNP marker-aided backcrossing accelerated introgression of *hst1* gene from 'Kaijin' into genetic background of 'Yukinko-mai' rice. An introgressed line significantly avoided Na^+ accumulation in shoot due to the function of *osHKT1,1*, showing strong salinity tolerance at vegetative and reproductive stages with improved yield.

In BC_3F_2, 207 ‘Kaijin’ homozygous and 536 heterozygous variants were found in exon and splice sites. Only 71 and 230, respectively, of those caused protein sequence alterations. These non-synonymous changes occurred in only four genes for agronomic traits. However, one of two non-synonymous changes in *Os09g0356200*, a putative heading-date-associated gene identified in a gene-based genome-wide association study (GWAS), caused a frameshift deletion probably causing loss-of-function.

The field assessment results under normal condition demonstrate the difference in the distribution of heading dates between ‘YNU31-2-4’ and WT (‘Yukinko-mai’). Other morphological traits of ‘YNU31-2-4’ were similar to those of WT. Additionally, the heading date of the BC_3F_3 population was associated with the genotype of the putative heading-date-associated gene *Os09g0356200*. This finding indicates the potential value and reliability of gene-based GWAS among the Japanese rice population data set in breeding by incorporating the genetic variations into those cultivars, and its practical value in predicting genes governing complex traits of agronomic phenotypes. The field assessment results along with the high-resolution genotyping data indicate no apparent grain yield reduction in ‘YNU31-2-4’ in relation to the presence of the target *hst1* gene. In fact, ‘YNU31-2-4’ increased ca. 11% yield than the donor parent ‘Kaijin’ owing to the higher number of panicles per plant and 1000-grain weight.

To assess the practical utility of *hst1* in the introgression line, seedlings were exposed to 75 and 125 mM salt stresses. The findings revealed that under high salt stress, ‘YNU31-2-4’ plants had significantly higher survival rate, shoot and root biomass than WT, which suggest strong tolerance similar to that of the donor parent. Root architecture differed between WT and ‘YNU31-2-4’ plants after 2 weeks of normal hydroponic culture. Roots of ‘YNU31-2-4’ and ‘Kaijin’ exposed to high salt stress elongated more than WT roots. Thus, this behavior as a novel effect of the *hst1* gene in response to salt stress is proposed.

The yield traits of ‘YNU31-2-4’ were assessed under salt stress at the early reproductive to booting stages, when salt stress reduces panicle and spikelet numbers per plant, leading to significant yield losses. Owing to the significant increases in panicle number per plant, spikelet number per panicle, and 1000-spikelet weight, the final grain yield of ‘YNU31-2-4’ plants was 45% higher than WT under salt stress at

the reproductive stage. Under control condition, there was no yield difference between 'YNU31-2-4' and WT. Interestingly, 'YNU31-2-4' has higher yield potential than the donor parent under control condition owing to the higher number of panicles per plant and 1000-spikelet weight. The higher seed weight of 'YNU31-2-4' could be due, at least partly, to the improved photosynthetic efficiency due to coordination of leaf morphological and physiological traits. Thus, this study clearly shows that the introgression of *hst1* to the WT significantly increased salt resistant without any reduction in grain yield.

The 'YNU31-2-4' plants maintained significantly higher plant growth, proline content, and plant water status under salinity, which could indicate a physiological and biochemical tolerance mechanism.

The K^+ and Na^+ contents in shoots and roots of seedlings were assayed under salinity, since the degree of stress depends on their uptake and translocation. The Na^+/K^+ ratio in shoot and root were divergent between WT and 'YNU31-2-4'. The quantification and localization results demonstrate that like 'Kaijin', 'YNU31-2-4' plants maintained a very low Na^+/K^+ ratio in both shoot and root under salt stress. The milder stress symptoms and salinity tolerance in 'YNU31-2-4' shoot, similar to the *hst1* donor parent 'Kaijin', correlate with higher K^+ and lower Na^+ in the tissue.

The gene *OsHKT1;1*/ *Os04g0607500*, coding for a member of the high-affinity K^+ transporter family is known to function as a Na^+ transporter in rice. The relative expression levels of high-affinity K+ transporter 4 (*OsHKT1;1*) transcripts in rice shoot grown with or without salt stress was analyzed. The *OsHKT1;1* significantly up-regulated in shoot tissue of 'Kaijin' and 'YNU31-2-4'. Therefore, salinity tolerance in 'YNU31-2-4', associate with the function of *OsHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue.

In conclusion, the results of this study demonstrate that the modified biotron breeding system coupled with SNP marker-assisted selection offers a rapid and effective way to improve single traits in rice. The precise introgression of *hst1*, combined with suitable genetic resources and phenotyping results, resulted in the selection of a line, 'YNU31-2-4', adapted to salt stress at the vegetative and reproductive stages with improved yield. Salinity tolerance in 'YNU31-2-4', similar to the *hst1* donor parent 'Kaijin', correlate with higher leaf water relations,

chlorophyll, proline, photosynthesis and, function of *osHKT1;1* in maintenance of K^+/Na^+ homeostasis in the tissue. The improved salt tolerant line, 'YNU31-2-4' has a practical breeding value and could be utilized as an important source of genetical material. 'YNU31-2-4' is a potential candidate for new rice cultivar with markedly improved salinity tolerance, which might sustain grain yield and food security in a changing climate.

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REFERENCES

Abe, A., Takagi, H., Nakajo, S., Terauchi, R., and Kuroda, E. (2016) Yielding ability and physicochemical characteristics related to eating quality in salt-tolerant rice variety “Kaijin” under normal salt-free cultivated condition. *Tohoku J Crop Sci*. 59: 19–20.

Ali, A., Maggio, A., Bressan, R., Yun, D.-J., Ali, A., Maggio, A., et al. (2019) Role and Functional Differences of HKT1-Type Transporters in Plants under Salt Stress. *Int J Mol Sci*. 20: 1059.

Babu, N.N., Krishnan, S.G., Vinod, K.K., Krishnamurthy, S.L., Singh, V.K., Singh, M.P., et al. (2017) Marker Aided Incorporation of Saltol, a Major QTL Associated with Seedling Stage Salt Tolerance, into Oryza sativa ‘Pusa Basmati 1121’. *Front Plant Sci*. 8: 1–14.

Bates, L.S., Waldren, R.P., and Teare, I.D. (1973) Rapid determination of free proline for water-stress studies. *Plant Soil*. 39: 205–207.

Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 30: 2114–2120.

Bonilla, D., Mackill, D., Deal, K., and Gregorio, G. (2002) RFLP and SSLP mapping of salinity tolerance genes in chromosome 1 of rice (Oryza sativa L.) using recombinant inbred lines. *J Agric Food Chem*. 85: 68–76.

Calanca, P.P. (2017) Effects of Abiotic Stress in Crop Production. In *Quantification of Climate Variability, Adaptation and Mitigation for Agricultural Sustainability*. pp. 165–180 Springer International Publishing, Cham.

Chen, Z., Pottosin, I.I., Cuin, T.A., Fuglsang, A.T., Tester, M., Jha, D., et al. (2007) Root plasma membrane transporters controlling K+/Na+ homeostasis in salt-stressed barley. *Plant Physiol*. 145: 1714–25.

Chen, Z.C., Yamaji, N., Horie, T., Che, J., Li, J., An, G., et al. (2017) A Magnesium Transporter OsMGT1 Plays a Critical Role in Salt Tolerance in Rice. *Plant Physiol*. 174: 1837–1849.

Cingolani, P., Platts, A., Wang, L.L., Coon, M., Nguyen, T., Wang, L., et al. (2012) A program for annotating and predicting the effects of single nucleotide

polymorphisms, SnpEff. *Fly (Austin)*. 6: 80–92.

Collard, B.C.Y., Beredo, J.C., Lenaerts, B., Mendoza, R., Santelices, R., Lopena, V., et al. (2017) Revisiting rice breeding methods – evaluating the use of rapid generation advance (RGA) for routine rice breeding. *Plant Prod Sci*. 20: 337–352.

Collard, B.C.Y., and Mackill, D.J. (2008) Marker-assisted selection: An approach for precision plant breeding in the twenty-first century. *Philos Trans R Soc B Biol Sci*. 363: 557–572.

Das, G., Patra, J.K., and Baek, K.-H. (2017) Insight into MAS: A Molecular Tool for Development of Stress Resistant and Quality of Rice through Gene Stacking. *Front Plant Sci*. 8: 985.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G., and Schroeder, J.I. (2014) . *Trends Plant Sci.*19: 371-378.

DePristo, M.A., Banks, E., Poplin, R., Garimella, K. V, Maguire, J.R., Hartl, C., et al. (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet*. 43: 491–498.

Doyle, J.J., and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull*. 19: 11–15.

Fukuoka, S., Ebana, K., Yamamoto, T., and Yano, M. (2010) Integration of Genomics into Rice Breeding. *Rice*. 3: 131–137.

Garciadeblás, B., Senn, M.E., Bañuelos, M.A., and Rodríguez-Navarro, A. (2003) Sodium transport and HKT transporters: the rice model. *Plant J*. 34: 788–801.

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Muir, J.F., et al. (2010) Food security: the challenge of feeding 9 billion people. *Science*. 327: 812–818.

Gregorio, G.B., Islam, M.R., Vergara, G. V., and Thirumeni, S. (2013) Recent advances in rice science to design salinity and other abiotic stress tolerant rice varieties. *Sabrao J Breed Genet*. 45: 31–41.

Gu, Z., Eils, R., and Schlesner, M. (2016) gtrellis: an R/Bioconductor package for making genome-level Trellis graphics. *BMC Bioinformatics*. 17: 169.

Gupta, B., and Huang, B. (2014) Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. *Int J Genomics*. 2014: 701596.

de Haas, L.S., Koopmans, R., Lelivelt, C.L.C., Ursem, R., Dirks, R., and Velikkakam James, G. (2017) Low-coverage resequencing detects meiotic recombination pattern and features in tomato RILs. *DNA Res*. 24: 549–558.

Hanin, M., Ebel, C., Ngom, M., Laplaze, L., and Masmoudi, K. (2016) New Insights on Plant Salt Tolerance Mechanisms and Their Potential Use for Breeding. *Front Plant Sci*. 7: 1–17.

Hasan, M.M., Rafii, M.Y., Ismail, M.R., Mahmood, M., Rahim, H.A., Alam, M.A., et al. (2015) Marker-assisted backcrossing: A useful method for rice improvement. *Biotechnol Biotechnol Equip*. 29: 237–254.

Hauser, F., and Horie, T. (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K +/Na + ratio in leaves during salinity stress. *Plant Cell Environ*. 33: 552–565.

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., and Ahmad, A. (2012) Role of proline under changing environments: a review. *Plant Signal Behav*. 7: 1456–66.

Horie, T., Karahara, I., and Katsuhara, M. (2012) Salinity tolerance mechanisms in glycophytes: An overview with the central focus on rice plants. *Rice*. 5: 1–18.

Iftekharuddaula, K.M., Salam, M.A., Newaz, M.A., Ahmed, H.U., Collard, B.C.Y., Septiningsih, E.M., et al. (2012) Comparison of phenotypic versus marker-assisted background selection for the SUB1 QTL during backcrossing in rice. *Breed Sci*. 62: 216–222.

Inui, T., Yasutaka, T., Endo, K., and Katsumi, T. (2012) Geo-environmental issues induced by the 2011 off the Pacific Coast of Tohoku Earthquake and tsunami. *Soils Found*. 52: 856–871.

Ishizaki, K., Matsui, T., Kaneda, S., Kobayashi, K., Kasaneyama, H, Abe, S. et al., (2008) A New Rice Cultivar 'Yukinko-mai'. *J Niigata Agric Res Inst*. 89–98.

Jagadish, S.V.K., Septiningsih, E.M., Kohli, A., Thomson, M.J., Ye, C., Redoña, E., et al. (2012) Genetic Advances in Adapting Rice to a Rapidly Changing Climate. *J Agron Crop Sci*. 198: 360–373.

Jain, M., Moharana, K.C., Shankar, R., Kumari, R., and Garg, R. (2014) Genomewide discovery of DNA polymorphisms in rice cultivars with contrasting drought and

salinity stress response and their functional relevance. *Plant Biotechnol J*. 12: 253–264.

Kasai, M. (2008) Regulation of leaf photosynthetic rate correlating with leaf carbohydrate status and activation state of Rubisco under a variety of photosynthetic source/sink balances. *Physiol Plant*. 134: 216–226.

Kurotani, K., Yamanaka, K., Toda, Y., Ogawa, D., Tanaka, M., Kozawa, H., et al. (2015) Stress Tolerance Profiling of a Collection of Extant Salt-Tolerant Rice Varieties and Transgenic Plants Overexpressing Abiotic Stress Tolerance Genes. *Plant Cell Physiol*. 56: 1867–1876.

Lee, K.-S., Choi, W.-Y., Ko, J.-C., Kim, T.-S., and Gregorio, G.B. (2003) Salinity tolerance of japonica and indica rice (Oryza sativa L.) at the seedling stage. *Planta*. 216: 1043–1046.

Li, H., and Durbin, R. (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 25: 1754–1760.

Lichtenthaler, H.K. (1987) Chlorophylls and Carotenoids: Pigments of Photosynthetic Biomembranes. *Methods Enzymol*. 148: 350–382.

Lin, H.X., Zhu, M.Z., Yano, M., Gao, J.P., Liang, Z.W., Su, W.A., et al. (2004) QTLs for Na+ and K+ uptake of the shoots and roots controlling rice salt tolerance. *Theor Appl Genet*. 108: 253–260.

Linh, L.H., Linh, T.H., Xuan, T.D., Ham, L.H., Ismail, A.M., and Khanh, T.D. (2012) Molecular Breeding to Improve Salt Tolerance of Rice (Oryza sativa L.) in the Red River Delta of Vietnam. *Int J Plant Genomics*. 2012: 1–9.

Liu, W., Li, R.-J., Han, T.-T., Cai, W., Fu, Z.-W., and Lu, Y.-T. (2015) Salt stress reduces root meristem size by nitric oxide-mediated modulation of auxin accumulation and signaling in Arabidopsis. *Plant Physiol*. 168: 343–56.

Luo, Y., Ma, T., Zhang, A., Ong, K.H., Li, Z., Yang, J., et al. (2016) Marker-assisted breeding of the rice restorer line Wanhui 6725 for disease resistance, submergence tolerance and aromatic fragrance. *Rice*. 9: 66.

Ma, Y.-C., Augé, R.M., Dong, C., and Cheng, Z.-M.M. (2017) Increased salt tolerance with overexpression of cation/proton antiporter 1 genes: a meta-analysis. *Plant Biotechnol J*. 15: 162–173.

Marcos, M., Sharifi, H., Grattan, S.R., and Linquist, B.A. (2018) Spatio-temporal

salinity dynamics and yield response of rice in water-seeded rice fields. *Agric Water Manag*. 195: 37–46.

Miah, G., Rafii, M.Y., Ismail, M.R., Puteh, A.B., Rahim, H.A., Asfaliza, R., et al. (2013) Blast resistance in rice: A review of conventional breeding to molecular approaches. *Mol Biol Rep*. 40: 2369–2388.

Miah, G., Rafii, M.Y., Ismail, M.R., Puteh, A.B., Rahim, H.A., and Latif, M.A. (2015) Recurrent parent genome recovery analysis in a marker-assisted backcrossing program of rice (Oryza sativa L.). *C R Biol*. 338: 83–94.

Mishra, S., Singh, B., Panda, K., Singh, B.P., Singh, N., Misra, P., et al. (2016) Association of SNP Haplotypes of HKT Family Genes with Salt Tolerance in Indian Wild Rice Germplasm. *Rice*. 9: 1–13.

Mitsui, T., Loboda, T., Itoh, A., and Ikarashi, T. (1999) Sugar-Controlled Ca2+ Uptake and α-Amylase Secretion in Cultured Cells of Rice (Oryza sativa L.). *Plant Cell Physiol*. 40: 884–893.

Munns, R. (2005) Genes and Salt Tolerance. *New Phytol*. 167: 645–663.

Munns, R., and Tester, M. (2008) Mechanisms of Salinity Tolerance. *Annu Rev Plant Biol*. 59: 651–681.

Murchie, E.H., Yang, J., Hubbart, S., Horton, P., and Peng, S. (2002) Are there associations between grain-filling rate and photosynthesis in the flag leaves of field-grown rice? *J Exp Bot*. 53: 2217–24.

Nagatoshi, Y., and Fujita, Y. (2019) Accelerating Soybean Breeding in a CO 2 - Supplemented Growth Chamber. *Plant Cell Physiol*. 60: 77–84.

Negrão, S., Cecília Almadanim, M., Pires, I.S., Abreu, I.A., Maroco, J., Courtois, B., et al. (2013) New allelic variants found in key rice salt-tolerance genes: an association study. *Plant Biotechnol J*. 11: 87–100.

Negrão, S., Courtois, B., Ahmadi, N., Abreu, I., Saibo, N., and Oliveira, M.M. (2011) Recent updates on salinity stress in rice: From physiological to molecular responses. *CRC Crit Rev Plant Sci*. 30: 329–377.

Ohnishi, T., Yoshino, M., Yamakawa, H., and Kinoshita, T. (2011) The biotron breeding system: A rapid and reliable procedure for genetic studies and breeding in rice. *Plant Cell Physiol*. 52: 1249–1257.

Okada, T., Nakayama, H., Shinmyo, A., and Yoshida, K. (2008) Expression of

OsHAK genes encoding potassium ion transporters in rice. *Plant Biotechnol*. 25: 241–245.

Pequerul, A., Pérez, C., Madero, P., Val, J., and Monge, E. (1993) A rapid wet digestion method for plant analysis. In *Optimization of Plant Nutrition*. pp. 3–6 Springer Netherlands, Dordrecht.

Quan, R., Wang, J., Hui, J., Bai, H., Lyu, X., Zhu, Y., et al. (2018) Improvement of Salt Tolerance Using Wild Rice Genes. *Front Plant Sci*. 8: 1–11.

Rahman, M.A., Thomson, M.J., Shah-E-Alam, M., De Ocampo, M., Egdane, J., and Ismail, A.M. (2016) Exploring novel genetic sources of salinity tolerance in rice through molecular and physiological characterization. *Ann Bot*. 117: 1083–1097.

Reddy, I.N.B.L., Kim, B.-K., Yoon, I.-S., Kim, K.-H., and Kwon, T.-R. (2017) Salt Tolerance in Rice: Focus on Mechanisms and Approaches. *Rice Sci*. 24: 123–144.

Ren, Z.-H., Gao, J.-P., Li, L.-G., Cai, X.-L., Huang, W., Chao, D.-Y., et al. (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nat Genet*. 37: 1141–1146.

Roy, K., Sasada, K., and Kohno, E. (2014) Salinity status of the 2011 Tohoku-oki tsunami affected agricultural lands in northeast Japan. *Int Soil Water Conserv Res*. 2: 40–50.

Roy, S.J., Negrão, S., and Tester, M. (2014) Salt resistant crop plants. *Curr Opin Biotechnol*. 26: 115–124.

Sade, N., Vinocur, B.J., Diber, A., Shatil, A., Ronen, G., Nissan, H., et al. (2009) Improving plant stress tolerance and yield production: is the tonoplast aquaporin SlTIP2;2 a key to isohydric to anisohydric conversion? *New Phytol*. 181: 651–661.

Sanchez-Bragado, R., Elazab, A., Zhou, B., Serret, M.D., Bort, J., Nieto-Taladriz, M.T., et al. (2014) Contribution of the ear and the flag leaf to grain filling in durum wheat inferred from the carbon isotope signature: Genotypic and growing conditions effects. *J Integr Plant Biol*. 56: 444–454.

Saxena, S.C., Kaur, H., Verma, P., Petla, B.P., Andugula, V.R., and Majee, M. (2013) Osmoprotectants: Potential for Crop Improvement Under Adverse Conditions. In *Plant Acclimation to Environmental Stress*. pp. 197–232 Springer New York, New York, NY.

Shiraya, T., Mori, T., Maruyama, T., Sasaki, M., Takamatsu, T., Oikawa, K., et al. (2015) Golgi/plastid-type manganese superoxide dismutase involved in heat-stress tolerance during grain filling of rice. *Plant Biotechnol J*. 13: 1251–1263.

Si, W., Yuan, Y., Huang, J., Zhang, X., Zhang, Y., Zhang, Y., et al. (2015) Widely distributed hot and cold spots in meiotic recombination as shown by the sequencing of rice F $_2$ plants. *New Phytol*. 206: 1491–1502.

Sreenivasulu, N., Butardo, V.M., Misra, G., Cuevas, R.P., Anacleto, R., and Kavi Kishor, P.B. (2015) *Designing climate-resilient rice with ideal grain quality suited for high-temperature stress*. *J. Exp. Bot.* 66: 1737–1748.

Szabados, L., and Savouré, A. (2010) Proline: a multifunctional amino acid. *Trends Plant Sci*. 15: 89–97.

Takagi, H., Tamiru, M., Abe, A., Yoshida, K., Uemura, A., Yaegashi, H., et al. (2015) MutMap accelerates breeding of a salt-tolerant rice cultivar. *Nat Biotechnol*. 33: 445–449.

Tanaka, J., Hayashi, T., and Iwata, H. (2016) A practical, rapid generation-advancement system for rice breeding using simplified biotron breeding system. *Breed Sci*. 66: 542–551.

Tanweer, F.A., Rafii, M.Y., Sijam, K., Rahim, H.A., Ahmed, F., Ashkani, S., et al. (2015) Introgression of Blast Resistance Genes (Putative Pi-b and Pi-kh) into Elite Rice Cultivar MR219 through Marker-Assisted Selection. *Front Plant Sci*. 6: 1002.

Thomson, M.J., de Ocampo, M., Egdane, J., Rahman, M.A., Sajise, A.G., Adorada, D.L., et al. (2010) Characterizing the Saltol quantitative trait locus for salinity tolerance in rice. *Rice*. 3: 148–160.

Tiwari, S., Sl, K., Kumar, V., Singh, B., Rao, A.R., Sv, A.M., et al. (2016) Mapping QTLs for Salt Tolerance in Rice (Oryza sativa L.) by Bulked Segregant Analysis of Recombinant Inbred Lines Using 50K SNP Chip. *PLoS One*. 11: 1–19.

Todaka, D., Nakashima, K., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2012) Toward understanding transcriptional regulatory networks in abiotic stress responses and tolerance in rice. *Rice*. 5: 1–9.

United Nations (2013) World Population Prospects: The 2012 Revision. Highlights and Advance Tables. *Popul Dev Rev*. 36: 775–801.

Vu, H.T.T., Le, D.D., Ismail, A.M., and Le, H.H. (2012) Marker-assisted backcrossing (MABC) for improved salinity tolerance in rice (Oryza sativa L.) to cope with climate change in Vietnam. *Aust J Crop Sci*. 6: 1649–1654.

Walbot, V., and Warren, C. (1988) Regulation of Mu element copy number in maize lines with an active or inactive Mutator transposable element system. *Mol Gen Genet*. 211: 27–34.

Walia, H., Wilson, C., Zeng, L., Ismail, A.M., Condamine, P., and Close, T.J. (2007) Genome-wide transcriptional analysis of salinity stressed japonica and indica rice genotypes during panicle initiation stage. *Plant Mol Biol*. 63: 609–623.

Wassmann, R., Jagadish, S.V.K., Heuer, S., Ismail, A., Redona, E., Serraj, R., et al. (2009) . Adv. Agron.

Watson, A., Ghosh, S., Williams, M.J., Cuddy, W.S., Simmonds, J., Rey, M.D., et al. (2018) Speed breeding is a powerful tool to accelerate crop research and breeding. *Nat Plants*. 4: 23–29.

Welch, J.R., Vincent, J.R., Auffhammer, M., Moya, P.F., Dobermann, A., and Dawe, D. (2010) *Rice yields in tropical/subtropical Asia exhibit large but opposing sensitivities to minimum and maximum temperatures. Proc. Natl. Acad. Sci. U. S. A.* 107: 14562–14567.

Yamamoto, E., Yonemaru, J.-I., Yamamoto, T., and Yano, M. (2012) OGRO: The Overview of functionally characterized Genes in Rice online database. *Rice*. 5: 26.

Yano, K., Yamamoto, E., Aya, K., Takeuchi, H., Lo, P.C., Hu, L., et al. (2016) Genome-wide association study using whole-genome sequencing rapidly identifies new genes influencing agronomic traits in rice. *Nat Genet*. 48: 927–934.

Yoshida, S., Forno, D.A., Cock, J.H., and Gomez, K.A. (1976) *Laboratory Manual for Physiological Studies of Rice*, 3rd ed.; International Rice Research Institute: Manila, Philippines, pp. 61-66.

Yu, J., Zhao, W., Tong, W., He, Q., Yoon, M.-Y., Li, F.-P., et al. (2018) A Genome-Wide Association Study Reveals Candidate Genes Related to Salt Tolerance in Rice (Oryza sativa) at the Germination Stage. *Int J Mol Sci*. 19: 3145.

Zeng, L., and Shannon, M.C. (2000) Salinity Effects on Seedling Growth and Yield Components of Rice. *Crop Sci*. 40: 996.

Zhai, H., Cao, S., Wan, J., Zhang, R., Lu, W., Li, L., et al. (2002) Relationship between leaf photosynthetic function at grain filling stage and yield in super high-yielding hybrid rice (Oryza sativa. L). *Sci China Ser C Life Sci*. 45: 637–646.

Zhou, Y.-B., Liu, C., Tang, D.-Y., Yan, L., Wang, D., Yang, Y.-Z., et al. (2018) The Receptor-Like Cytoplasmic Kinase STRK1 Phosphorylates and Activates CatC, Thereby Regulating H_2O_2 Homeostasis and Improving Salt Tolerance in Rice. *Plant Cell*. 30: 1100–1118.

Zhou, W., Lv, T., Yang, Z., Wang, T., Fu, Y., Chen, Y., et al. (2017) Morphophysiological mechanism of rice yield increase in response to optimized nitrogen management. *Sci Rep*. 7: 17226.