Functional & Integrative Genomics (2020) 20:261–275
https://doi.org/10.1007/s10142-019-00707-x

ORIGINAL ARTICLE

Characterization of natural genetic variation identifies multiple genes involved in salt tolerance in maize

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Received: 2 July 2019 / Revised: 2 August 2019 / Accepted: 8 August 2019 / Published online: 14 September 2019

Abstract

Progressive decline in irrigation water is forcing farmers to use brackish water which increases soil salinity and exposes the crop plants to salinity. Maize, one of the most important crops, is sensitive to salinity. Salt tolerance is a complex trait controlled by a number of physiological and biochemical processes. Scant information is available on the genetic architecture of salt tolerance in maize. We evaluated 399 inbred lines for six early vigor shoot and root traits upon exposure of 18-day seedlings to salinity (EC_{iw} = 16 dS m^{-1}) stress. Contrasting response of shoot and root growth to salinity indicated a meticulous reprogramming of resource partitioning by the plants to cope with the stress. The genomic analysis identified 57 single nucleotide polymorphisms (SNP) associated with early vigor traits. Candidate genes systematically associated with each SNP include both previously known and novel genes. Important candidates include a late embryogenesis protein, a divalent ion symporter, a proton extrusion protein, an RNA-binding protein, a casein kinase 1, and an AP2/EREBP transcription factor. The late embryogenesis protein is associated with both shoot and root length, indicating a coordinated change in resource allocation upon salt stress. Identification of a casein kinase 1 indicates an important role for Ser/Thr kinases in salt tolerance. Validation of eight candidates based on expression in a salt-tolerant and a salt-sensitive inbred line supported their role in salt tolerance. The candidate genes identified in this investigation provide a foundation for dissecting genetic and molecular regulation of salt tolerance in maize and related grasses.

Keywords Salinity · *Zea mays* · Maize · Salt tolerance · Gene expression · GWA

Introduction

Maize (*Zea mays*), the third major cereal crop worldwide after wheat and rice, is a staple food for humans and a primary source of nutrients for animal feed. Maize is also used to produce various industrial products including vegetable oil, industrial and beverage alcohol, condiments, and biofuels. The worldwide maize production in 2017 was 1134 million metric tons with 371 million metric tons produced in the USA followed by 259 million metric tons in China (FAOSTATS; http://www.fao.org/faostat/en/#data/QC). Maize growth, development, and production potential are severely affected by abiotic stress factors including drought (Nepolean et al. 2018) and salinity (Farooq et al. 2015; Sun et al. 2018). Meeting the food and energy needs for an increasing global population will force cultivation of maize on marginally productive lands with progressively higher exposure to drought and salinity. The predicted increase in global surface temperature and irregular weather patterns are expected to further

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10142-019-00707-x) contains supplementary material, which is available to authorized users.

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exacerbate the losses in maize productivity due to these abiotic stresses (Ummenhofer et al. 2015).

High salinity is detrimental to plant growth and development, resulting in severe yield losses. Salt interferes with germination, vegetative and reproductive growth, and nutrient balance (Sandhu and Kaundal 2018; Shrivastava and Kumar 2015). Twenty percent of the global agricultural land is estimated to be highly saline, and this number is expected to increase due to the scarcity of irrigation water, agricultural practices, and increasing instability of weather patterns (Flowers 2004; Jamil et al. 2011). Limited availability of water due to sub-optimal rainfall, scarcity of good quality irrigation reservoirs, excessive evapotranspiration, and poor water and soil management practices leads to increased residual salts in the soil. Reduced availability of freshwater forces farmers to use low-quality recycled or degraded water that is often high in salt content, and such practice further increases the salinity experienced by crop plants. If no major correction steps were taken, about half of the arable land is expected to be affected with salinity by 2050 (Wang et al. 2003).

Salinity is a genetically complex abiotic stress affected by several physiological and biochemical processes. Several physiological mechanisms that improve salt tolerance include ion exclusion from roots, ion compartmentalizing into vacuoles, regulation of ion transport from root to shoot, accumulation of organic compatible solutes in tissues, and increased tissue tolerance to toxic ions (Munns and Tester 2008; Sandhu and Kaundal 2018). Therefore, it is important to identify various component traits contributing to the aforementioned salt tolerance mechanism, investigate the relative importance of these traits in various crops and production systems, and understand the genetic architecture of these component traits. Furthermore, the determination of the component traits should also consider the growth stage that is most affected by salt stress.

Maize is moderately sensitive to salinity with a soil electrical conductivity (EC_e) threshold of 1.7 dS m^{-1}, and every 1 dS m^{-1} increase in EC_e results in 12% loss in grain yield (Maas and Hoffman 1977). Therefore, soils or irrigation water with high salt (NaCl) concentrations pose a serious threat to global maize production, particularly in arid and semi-arid regions. Sodium in salt interferes with potassium uptake, resulting in misregulation of stomatal opening, excessive evapotranspiration, and, ultimately, necrosis of the leaves (Fortmeier and Schubert 1995). In addition, higher concentrations of Na^+ and Cl^- interfere with the uptake of other ions affecting the function of other transport proteins such as potassium, zinc, and electron transporters (Sandhu and Kaundal 2018). Salt stress also leads to excessive production of reactive oxygen species and causes oxidative damage in maize (de Azevedo Neto et al. 2006).

Progress in characterization of the genetic architecture of salt tolerance and identification of underlying genes and genetic elements has been slow in maize and other crops. While some single genes have been identified for some of the component traits, most display characteristics of a multigenic inheritance (Campbell et al. 2017; Farooq et al. 2015; Morton et al. 2019; Sandhu et al. 2017, 2018). Several quantitative trait loci (QTL) underlying the component traits have been identified (Luo et al. 2017, 2019). However, isolation of genes underlying quantitative traits has been challenging due to high linkage disequilibrium and the resulting lack of resolution in the biparental populations. Genome-wide association (GWA) studies, which take advantage of historical recombination in a large number of diverse genotypes, provide enhanced resolution resulting in the identification of single nucleotide polymorphisms (SNP) and the associated causal genes. GWA studies have been successfully used in maize to identify single nucleotide polymorphisms associated with a number of loci regulating quantitative traits (Buckler et al. 2009; Mazaheri et al. 2019; McMullen et al. 2009; Sekhon et al. 2019; Xiao et al. 2017). In a recent study, 49 candidate genes associated with survival rate under salinity stress in maize seedlings were identified (Luo et al. 2019).

Goals of this study were (1) to characterize the natural genetic variation for early vigor traits in response to salt stress and (2) to identify and validate genes and genetic elements governing the effect of salinity on early vigor of maize. A high-resolution view of the genetic architecture underlying salt tolerance would provide a better understanding of biological underpinnings of salt tolerance and facilitate the development of salt-tolerant genotypes in maize and related grasses.

Materials and methods

Plant materials and growth conditions

A subset of the maize diversity panel (Mazaheri et al. 2019) consisting of 420 inbred lines (Table S1) was evaluated in the greenhouse lysimeters at the US Salinity Laboratory, Riverside, CA (33.973265 latitude, − 117.321158 W longitude). The dimensions of the lysimeter sand tanks were 120 cm (L) × 60 cm (W) × 50 cm (D). Thirty maize inbred lines including two reference lines, B73 and Mo17, were planted in every tank (Figure S1). The reference lines were used for adjustment of the data for microenvironment differences experienced by inbred lines in individual tanks. The experiment was replicated twice. Six seeds of each line were germinated and thinned to three plants once the plants had one fully extended leaf. Seeds were allowed to germinate and grow with irrigation water containing basic macronutrients (control, Table 1) and micronutrients in the following composition: Fe-DTPA (Sprint 330®), $ZnSO_4.7H_2O$ 1.2 μmol L^{-1}, $CuSO_4.5H_2O$ 0.3 μmol L^{-1}, $(NH_4)_6Mo7O_{24}.4H_2O$ 0.1 μmol L^{-1}, H_3BO_3 23 μmol L^{-1}, and $MnSO_4$ 15 μmol L^{-1}. Constant

Table 1 Composition of irrigation water

Treatment	EC_{iw} (dS m^{-1})	Ion concentration ($mmol_c$ L^{-1})							
		Cl^-	SO_4^{2-}	NO_3^-	PO_4^{3-}	Na^+	K^+	Ca^{2+}	Mg^{2+}
Control	1.46	1.41	1.44	5.39	1.5	1.88	6.59	3.35	2.1
Saline	13.65	128.35	27.32	5.39	1.5	106.88	6.59	29.6	23

nutrients were maintained in irrigation water stored in 890-L reservoirs. Water was pumped to sand tanks through PVC pipes twice a day, and the excess water was drained back into the reservoir. Of the 420 inbred lines originally planted, 399 lines with optimal germination and stand count were used for further evaluation.

Plants were allowed to grow for 18 days before exposure to salinity. To avoid any osmotic shock to the plants, the target salinity of irrigation water (EC_{iw} = 16 dS m^{-1}) was achieved over a 4-day period by slowly increasing salt in steps. To represent the natural composition of irrigation water, mixed cation composition was used and the ratio between cations (Ca = 1.25 Mg = 0.25 Na) was maintained. Cl^- was kept as the predominant anion. Nutrient compositions were retained at the same level in control and salinity treatments.

Collection and processing of phenotypic data

Plants were harvested 2 weeks after initiation of salt treatment. Root length (RL) was measured from the scutellar node to the tip of the primary root and shoot length (SL) was measured from the scutellar node to the tip of the topmost non-elongated leaf in the whorl. The length was measured in centimeters (cm) and weight was measured in grams (g). These two organs were separated by cutting at the scutellar node, and the resulting samples obtained from three plants of each inbred line were dried separately at 70 °C for 96 h to determine the shoot weight (SW) and root weight (RW).

The raw phenotypic data for the four traits of interest (SL, SW, RL, and RW) for each inbred line were adjusted for environmental differences induced by the microenvironment of each of the tank (including vicinity to cooling pads, sunlight, and location of fans). The adjustments were based on the augmented or incomplete block design concepts (Federer and Raghavarao 1975). Mo17 and B73 served as the check inbreds to estimate the random tank effects. The significant random tank effects were added to the raw phenotypic data to produce the adjusted values of each trait for each inbred line by calculating the best linear unbiased predictions (BLUP) for each line. The statistical calculations were performed using the mixed linear model platform of JMP Pro v13.2.0 (SAS Institute Inc., Cary, NC, USA). The BLUP values were used for all the subsequent analyses and data presented in the study.

Salt tolerance index (ST), an index indicative of the tolerance of a plant to salt stress (Munns et al. 2002; Sandhu et al. 2017), was calculated for each trait by dividing the adjusted phenotypic value of an inbred line in salt-treated tanks by the adjusted phenotypic value of the inbred line in the control tanks. ST for the ratio of root length and shoot length and weight was calculated by dividing RL(ST) by SL(ST), and ST for the ratio of root weight and shoot weight was obtained by dividing RW(ST) by SW(ST).

Genome-wide association analysis

Genome-wide association (GWA) analysis was performed on ST for each trait using mixed linear model (MLM) (Yu et al. 2006) implemented in Genome Association and Prediction Integrated Tool (GAPIT) (Lipka et al. 2012). Kinship was calculated using EMMA, the number of principle components was set at three, and minor allele frequency cutoff of 0.01 was used. The SNP markers used in this study were originally derived from RNA sequencing (RNA-seq) of a panel of 942 diverse inbred lines that produced 899,784 SNPs (Mazaheri et al. 2019). From the larger set, we extracted a subset of 587,982 polymorphic SNPs for the 399 inbred lines evaluated in the current study and used this smaller set for the GWA analysis. Since many SNPs in this dense set are expected to be in linkage disequilibrium, to reduce multiple testing for calculation of p value threshold, we calculated the effective number of markers for significance testing by Genetic type I Error Calculator (Li et al. 2012). This analysis resulted in 220,047 independent markers, a suggestive threshold of 4.50E-6, a significant ($\alpha \leq 0.05$) threshold of 2.25E-7 and a highly significant ($\alpha \leq 0.01$) threshold of 4.50E-9. The suggestive p value, which controls the expected false-positive rate to one per genome scan (Lander and Kruglyak 1995), was used to allow discovery of maximum possible SNPs that can be subjected to functional validation, thus increasing chances of identifying causal genes.

Annotation of candidate genes

Protein sequences of candidate genes were used as a query in Ensembl Plants (http://plants.ensembl.org/index.html) (Kersey et al. 2017), MaizeGDB (https://www.maizegdb.org/) (Andorf et al. 2016), NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), and NCBI Conserved Domain Database (Marchler-Bauer and Bryant 2004) to identify key domains and assign annotations to candidate genes. The putative annotations of candidate genes were used as query to search in the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) for prior published work on respective genes.

Quantitative reverse transcription-PCR analyses

One salt-tolerant (B84) and one salt-sensitive (NC326) inbred line were selected based on ST for four traits (SL, RL, SW, and RW). The plants were allowed to grow for 18 days before the salinity treatment (EC_{iw} = 16 dS m^{-1}) was initiated. The experiment was conducted in three replications and, from each replication, root and leaf samples were pooled from two plants. Following the same approach used for phenotyping and explained above, the samples were harvested at 0 h, 24 h, 48 h, and 72 h after initiation of the salinity treatment. Samples were frozen instantly in liquid nitrogen and RNA isolation was carried out using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were treated with DNase I to remove any DNA contamination (Thermo Scientific, Waltham, MA, USA).

Expression of candidate genes was examined using qRT-PCR (Pfaffl 2004). Genes for qRT-PCR analysis were selected based on the GWA analysis. Primers were designed based on the maize reference genome (B73_RefGen_v4) retrieved from https://plants.ensembl.org/Zea_mays/Info/Index. To avoid amplification from residual DNA in RNA samples, at least one primer out of each pair was designed from two exons flanking an intron. The qRT-PCR analysis was performed using iTaq™ Universal SYBR® Green One-Step Kit in a BioRad CFX96 machine (Bio-Rad Laboratories, Hercules, CA, USA). Each PCR was conducted in 10-μl volume consisting of 1 ng total RNA, 0.75 μM of each of the primers, 5 μl of 2× one-step SYBR® Green Reaction mix, and 0.125-μl iScript™ Reverse Transcriptase. The thermocycler program was as follows: 50 °C for 10 min, 95 °C for 1 min, then 40 cycles of 95 °C denaturation for 10 s, 57 °C annealing for 30 s, and 68 °C extension for 30 s. Quantification cycle (Cq) values were calculated by subtracting the baseline from the well data (Table S2). Gene expression was normalized using maize *leunig*, *ubiquitin carrier protein*, and *cyclophilin* reference genes. The relative expressions were determined using the following formula:

$$\text{Relative expression}_{\text{sample(GOI)}} = \left\{\text{RQ}_{\text{sample (GOI)}}\right\} / \left\{\text{RQ}_{\text{sample (ref 1)}} \times \text{RQ}_{\text{sample (ref 2)}} \times \ldots \text{RQ}_{\text{sample (ref } n\text{)}}\right\}^{1/n}$$

where RQ is the relative quantity of a sample, ref is the reference target in a run that includes one or more reference targets in each sample, and GOI is the gene of interest. The melt curve analysis was used to test the amplification specificity by ramping the temperature to 95 °C for 10 s and back to 65 °C for 5 s followed by increments of 0.5 °C/cycle up to 95 °C. For each gene, PCR was repeated twice resulting in two technical replicates.

A series of students *t* tests were used to compare the significance of difference based on $p \leq 0.05$, among control and salt treatments or between two genotypes at different stages. The statistical calculations were performed using JMP (Statistical Discovery from SAS, Cary, NC, USA) and the online version of GraphPad Prism (https://www.graphpad.com/quickcalcs/ttest1/).

Results

Natural variation for salt tolerance in maize

We evaluated natural variation for salt tolerance in a set of 399 diverse dent maize inbred lines that represent the three heterotic groups, Iowa Stiff Stalk Synthetic (SS), Non-Stiff Stalk (NSS), and Iodent (IDT) maintained and bred in isolation from one another (Mikel and Dudley 2006). Since plant growth is an important indicator of plant response to salt stress (Negrão et al. 2017), we recorded the effect of salt on SL, SW, RL, and RW and the root/shoot ratios (RW/SW and RL/SL). Salt treatment resulted in a significant decrease in RL (10%), SL (29%), and SW (16%) while RW registered a relatively small increase (10%) (Fig. 1a). To understand the change in intrinsic relationship among these traits upon the salt treatment, we examined Pearson's correlation among various trait combinations. SL and SW were significantly and highly correlated in control plants and this correlation was even stronger in plants grown under salinity (Fig. 1b). Interestingly, RL and RW showed a relatively low albeit significant correlation in control plants and this relationship was stronger in plants under salinity. Since ST is a reliable parameter to quantify the effect of salt treatment on a trait, we found substantial variation for ST of the six traits (Fig. 1c). Based on ST for all four traits, NC290A, N545, NC326, NC362, and NC368 were most salinity-tolerant inbred lines while W59E, B84, YE_4, W552, C68, and B90 were most salinity-susceptible inbred lines (Table S1).

Identification of loci associated with salt tolerance

To identify the genes underlying salt tolerance, we tested the association of 587,982 RNA-seq SNP markers of the maize diversity panel (Mazaheri et al. 2019) with ST of six early response traits of 22 days old seedlings. GWA analysis identified 57 unique SNPs on all 10 maize chromosomes that were associated with one or more of the traits (Fig. 2, Figure S2, Table 2). Of all the SNP markers, one was highly significant, 13 were significant, and the remaining 43 were suggestive SNP markers (Lander and Kruglyak 1995; Li et al. 2012). Only SNP with the lowest *p* value within a 200-kilobase pairs (kb) window (100 kb ± SNP position) was considered. A total of 1, 7, 11, 13, and 27 SNPs were associated with RL/SL, RL, RW/SW, SW, and SL, respectively (Table 2). No significant association of any SNP was found with RW (not shown). Two SNPs, rs3_974864 and rs4_207876796, were associated with

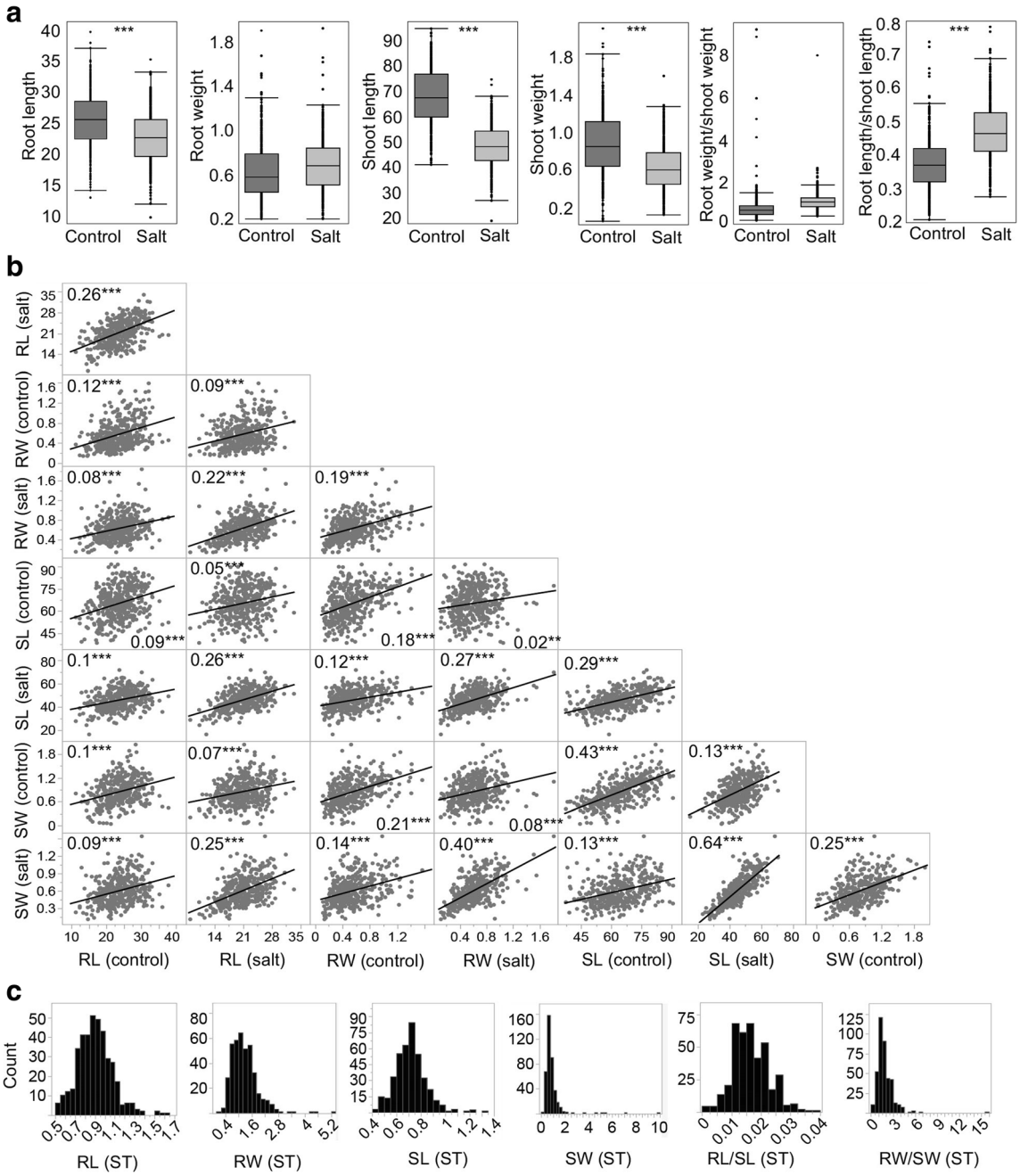

Fig. 1 Natural variation effect of salt on the early vigor in maize. **a** Variation in six traits in normal and saline plants. Best linear unbiased predictions (BLUP) of each trait are presented. **b** Scatter plots showing the association between the phenotypic traits. Number in each scatter plot represents Pearson's correlation (R^2); two and three asterisks indicate p value ≤ 0.01 and 0.001, respectively. **c** Frequency distribution of salt tolerance index (ST) of the six traits. Bins of trait values are shown on x-axis and counts of inbred lines with the phenotypic values for these bins are shown on y-axis. RW, root weight; RL, root length; SW, shoot weight; SL, shoot length

two traits each. SNP rs3_974864 was associated with SL and RL and rs4_207876796 was associated with SL and SW (Table 2). Total phenotypic variance (R^2) explained by the identified SNPs ranged between 5.5 and 12.4% with a median value of 6.4%.

To understand the role of these SNPs in salt tolerance, we identified genes within a 200-kb region (100 kb ± SNP position), which is a reasonable window size based on linkage disequilibrium used in similar diversity panels of maize (Diepenbrock et al. 2017; Li et al. 2012, 2016). This approach resulted in the identification of 404 annotated genes within the 200-kb windows surrounding the significant SNPs with a range of 1 to 18 and a median of 6 genes per window (Table S3). To identify the most likely candidate gene within

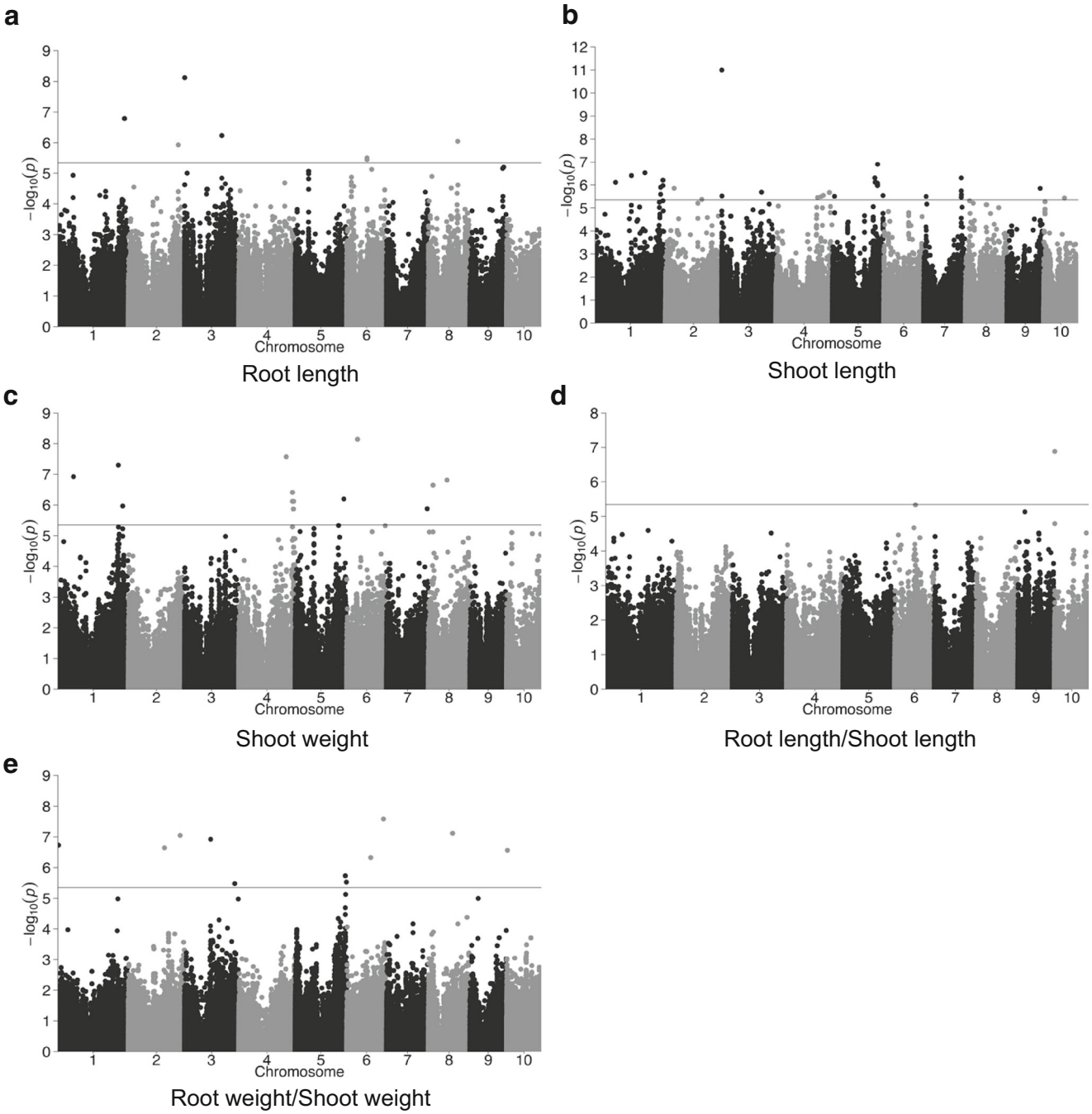

Fig. 2 Manhattan plots for genome-wide association (GWA) analysis of three phenotypic traits. Chromosomes are shown on *x*-axis and *p* values are shown on *y*-axis. The solid horizontal line represents suggestive *p* value threshold

each SNP window, annotation of the genes in the 200-kb window (100 kb ± SNP), if available, was used to examine the role of each gene or the gene family in literature. In the absence of available annotations or published evidence of involvement in salinity stress of the 200-kb window genes, the gene closest to the significant GWA SNP was reported. Using the above strategy, each of the 57 unique GWA SNPs identified in the study was linked to a likely candidate gene (Table 2, Table S3).

Many of the candidate genes putatively encode for proteins either known or expected to be involved in salt tolerance including a sodium/hydrogen exchanger, a CemA-like proton extrusion protein, divalent symporter, zinc transporter, S-type anion channel, ion transporters, and ethylene response factors. Importantly, there were several proteins with no obvious direct association with salt tolerance, including a zinc transporter, a DnaJ protein, phospholipid-transporting ATPase, a MYB DNA-binding domain protein, and an

Table 2 SNPs and candidate genes associated with salt tolerance in maize

#	Trait	SNP	Chr	Position	*p* value	LOS	MAF	R^2 (%)	Candidate gene	Annotation
1	RW/SW	rs1_2885189	1	2885189	1.86E-07	SIG	0.01	7.3	Zm00001d027320	CemA-like proton extrusion protein-related
2	SW	rs1_67928754	1	67928754	1.20E-07	SIG	0.01	7.6	Zm00001d029372	Serine carboxypeptidase-like 4
3	SL	rs1_89617280	1	89617280	7.79E-07	S	0.01	6.3	Zm00001d029842	Divalent ion symporter
4	SL	rs1_159018481	1	159018481	3.93E-07	S	0.01	6.7	Zm00001d030774	RNA-binding (RRM/RBD/RNP motifs) protein
5	SL	rs1_217341999	1	217341999	2.96E-07	S	0.02	6.8	Zm00001d032212	GDT1-like protein 4
6	SW	rs1_263016045	1	263016045	5.10E-08	SIG	0.04	8.1	Zm00001d033446	Zinc transporter 7
7	SW	rs1_281970910	1	281970910	1.08E-06	S	0.03	6.4	Zm00001d034039	Two-pore potassium channel 1
8	SL	rs1_286080132	1	286080132	1.27E-06	S	0.04	6.1	Zm00001d034191	Electron transporter
9	RL	rs1_290274747	1	290274747	1.62E-07	SIG	0.01	7.2	Zm00001d034350	IAP-like protein 1
10	SL	rs1_295602061	1	295602061	1.07E-06	S	0.01	6.2	Zm00001d034517	Serine/threonine-protein phosphatase
11	SL	rs1_295877489	1	295877489	6.24E-07	S	0.01	6.5	Zm00001d034538	Rubredoxin-like protein
12	SL	rs2_37436632	2	37436632	1.39E-06	S	0.01	6.0	Zm00001d003239	IRX15-LIKE
13	RW/SW	rs2_156819053	2	156819053	2.29E-07	S	0.03	7.2	Zm00001d005062	Vacuolar-sorting receptor 1
14	SL	rs2_158414576	2	158414576	4.26E-06	S	0.01	5.5	Zm00001d005105	Protein kinase superfamily protein
15	RL	rs2_217599385	2	217599385	1.17E-06	S	0.01	6.2	Zm00001d006836	Alpha/beta-hydrolase
16	RW/SW	rs2_225584357	2	225584357	9.03E-08	SIG	0.01	7.7	Zm00001d007242	Thioredoxin superfamily protein
17	SL, RL	rs3_974864	3	974864	1.00E-11	HSIG	0.01	12.4	Zm00001d039279	Harpin-induced protein
18	SL	rs3_1396745	3	1396745	3.04E-06	S	0.02	5.6	Zm00001d039314	S-type anion channel SLAH3
19	RW/SW	rs3_114162779	3	114162779	1.20E-07	SIG	0.01	7.5	Zm00001d041356	Alpha-(14)-fucosyltransferase
20	RL	rs3_162248006	3	162248006	5.84E-07	S	0.02	6.5	Zm00001d042342	Serine/arginine-rich splicing factor SR45a
21	SL	rs3_174100728	3	174100728	2.06E-06	S	0.01	5.8	Zm00001d042621	Magnesium transporter NIPA4
22	RW/SW	rs3_218788183	3	218788183	3.34E-06	S	0.43	5.8	Zm00001d044090	HD domain containing protein
23	SL	rs4_182563452	4	182563452	3.54E-06	S	0.01	5.6	Zm00001d052186	Pre-mRNA-splicing factor prp45
24	SL	rs4_196533703	4	196533703	3.26E-06	S	0.01	5.6	Zm00001d052666	Retinoblastoma-related protein 2
25	SW, SL	rs4_207876796	4	207876796	2.72E-08	SIG	0.02	8.4	Zm00001d052990	Cysteine-rich receptor-like protein kinase 6
26	SL	rs4_233640028	4	233640028	2.11E-06	S	0.01	5.8	Zm00001d053544	U-box domain-containing protein 17
27	SL	rs4_233795850	4	233795850	2.11E-06	S	0.01	5.8	Zm00001d053554	Peroxidase 52
28	SW	rs4_234741978	4	234741978	3.91E-07	S	0.02	7.0	Zm00001d053568	Unknown
29	SW	rs4_234904952	4	234904952	7.67E-07	S	0.03	6.6	Zm00001d053569	Cytochrome P450 711A1
30	SW	rs4_238808254	4	238808254	1.36E-06	S	0.03	6.3	Zm00001d053675	Lipoxygenase1
31	SW	rs4_238978350	4	238978350	7.68E-07	S	0.04	6.6	Zm00001d053687	MYB-related-transcription factor 88
32	SL	rs5_9183779	5	9183779	3.10E-06	S	0.02	5.6	Zm00001d013348	Thioredoxin superfamily protein
33	SL	rs5_185086111	5	185086111	4.94E-07	S	0.01	6.6	Zm00001d017091	Isocitrate dehydrogenase (NAD) regulat. sub. 3
34	SL	rs5_186108001	5	186108001	7.79E-07	S	0.01	6.3	Zm00001d017121	Glyceraldehyde-3-phosphate dehydrogenase4
35	SL	rs5_186850708	5	186850708	7.41E-07	S	0.18	6.4	Zm00001d017144	CMP-sialic acid transporter 3
36	SL	rs5_196842776	5	196842776	1.26E-07	SIG	0.04	7.3	Zm00001d017466	Ethylene-responsive transcription factor ERF15
37	SW	rs5_211185397	5	211185397	6.37E-07	S	0.02	6.7	Zm00001d017977	Histidine kinase1
38	RW/SW	rs5_218097150	5	218097150	1.85E-06	S	0.08	6.1	Zm00001d018288	Ethylene-responsive transcription factor ERF18
39	SL	rs5_220891489	5	220891489	2.90E-06	S	0.05	5.7	Zm00001d018440	Casein kinase 1
40	RW/SW	rs5_222670061	5	222670061	2.97E-06	S	0.04	5.8	Zm00001d018537	Pentatricopeptide repeat-containing protein
41	SW	rs6_47636979	6	47636979	7.31E-09	SIG	0.01	9.2	Zm00001d035775	Phospholipid-transporting ATPase 9
42	RL	rs6_88912441	6	88912441	3.10E-06	S	0.02	5.7	Zm00001d036449	MATE efflux family protein
43	RL	rs6_89092235	6	89092235	3.54E-06	S	0.04	5.6	Zm00001d036455	Leucine-rich repeat (LRR) protein
44	RW/SW	rs6_105215129	6	105215129	4.73E-07	S	0.01	6.8	Zm00001d036871	4S ribosomal protein S21
45	RW/SW	rs6_160510264	6	160510264	2.63E-08	SIG	0.01	8.3	Zm00001d038584	AP2/EREBP transcription factor
46	SL	rs7_11060930	7	11060930	3.18E-06	S	0.01	5.6	Zm00001d018967	Transcription factor bHLH7
47	SL	rs7_164028184	7	164028184	1.83E-06	S	0.01	5.9	Zm00001d021831	Zinc finger CCCH domain protein 56
48	SL	rs7_164314716	7	164314716	4.94E-07	S	0.01	6.6	Zm00001d021844	Sodium/hydrogen exchanger 2
49	SW	rs7_177216931	7	177216931	1.32E-06	S	0.01	6.3	Zm00001d022416	Early response to dehydration 15-like
50	SW	rs8_20247967	8	20247967	2.29E-07	S	0.03	7.3	Zm00001d008788	Eukaryotic translation initiation factor 3 sub. a
51	SW	rs8_80846046	8	80846046	1.56E-07	SIG	0.01	7.5	Zm00001d009783	DnaJ protein ERDJ3B
52	RW/SW	rs8_105842645	8	105842645	7.56E-08	SIG	0.02	7.8	Zm00001d010248	Electron transporter
53	RL	rs8_128256373	8	128256373	8.97E-07	S	0.04	6.3	Zm00001d010798	Oxidative stress 3
54	SL	rs9_144523482	9	144523482	1.41E-06	S	0.01	6.0	Zm00001d047893	Phosphoenolpyruvate carboxykinase homolog2
55	RL/SL	rs10_2016026	10	2016026	1.32E-07	SIG	0.12	7.3	Zm00001d023282	MYB DNA-binding domain protein
56	RW/SW	rs10_3595739	10	3595739	2.74E-07	S	0.02	7.1	Zm00001d023331	Pentatricopeptide repeat-containing protein
57	SL	rs10_90682553	10	90682553	3.74E-06	S	0.02	5.5	Zm00001d024853	1-aminocyclopropane-1-carboxylate oxidase15

Chr, chromosome; *MAF*, minor allele frequency; *LOS*, level of significance indicated as HSIG, highly significant; *SIG*, significant; *S*, suggestive (see text)

RNA-binding protein. These novel genes are valuable targets for enhancing our understanding of salinity response in plants.

Validation of candidate genes

Of the 399 maize lines evaluated for their biomass traits, two inbred lines, B84 and NC326, showed significant differences for response to salt stress (Fig. 3). B84, a sensitive line, had a significant (over 50%) reduction in SW, RW, and SL while NC326 did not show a significant reduction for any of the traits recorded (Fig. 3a–D). Furthermore, NC326 had substantially higher ST for all four biomass traits (SW, SL, RW, and RL) compared with B84 (Fig. 3e). Therefore, these two inbred lines were designated as salt-sensitive (B84) and salt-tolerant (NC326) and selected for the validation of candidate genes discovered in the GWA experiment. The expression of seven candidate genes was examined in B84 and NC326 grown under salinity and control conditions. One candidate gene, *Zm00001d039279* (a putative hairpin-induced protein), identified by a SNP associated with both SL and RL, was upregulated in both root and leaf tissues of control and salt-treated NC326 compared with that of B84 (Fig. 4a, B). The change in *Zm00001d039279* expression in salt with respect to the control was significantly higher for NC326 roots at 24 h after treatment, whereas such upregulation was delayed and appeared at 48 h in B84 (Fig. 4c). In leaf, *Zm00001d039279* relative expression was higher in NC326 at 0 h, 24 h, and 48 h although the expression did not increase significantly upon salt treatment (Fig. 4d).

Four genes associated with SL or SW including *Zm00001d052990*, *Zm00001d018440*, *Zm00001d029842*, and *Zm00001d032212* had higher expression in leaves of control and salt-treated NC326 plants compared with those of B84 (Fig. 5a–d). *Zm00001d052990* (putative cysteine-rich receptor-like kinase), a candidate gene associated with SL and SW, was upregulated at 24 h upon salt treatment in NC326 while downregulated in B84 (Fig. 5F). *Zm00001d018440* (putative casein kinase 1), a candidate gene associated with SL, was induced at 24 h and 48 h after salt treatment in NC326, whereas it was downregulated in B84, with a significant reduction at 48 h (Fig. 5g). *Zm00001d029842* (putative divalent ion symporter), another candidate gene associated with SL, displayed substantially increased relative expression at 72 h after salt treatment, although the level of upregulation was not statistically different from that observed in B84 (Fig. 5h). Likewise, *Zm00001d032212* (putative GDT1-like protein), a candidate gene associated with SL, was downregulated in B84 and upregulated in NC326 at 24 h and 48 h after salt treatment (Fig. 5i). *Zm00001d029372* (putative serine carboxypeptidase), a candidate gene associated with SW, had substantially lower expression in control and salt-treated plants of NC326 as compared with those of B84 through all four stages (Fig. 5e). This gene showed significant downregulation in B84 at 48 h upon salt treatment (Fig. 5j). Interestingly, all five candidate genes associated with SL and/or SW exhibited similar relative expression between NC326 and B84 at 72 h after salt treatment (Fig. 5f–j).

Zm00001d010798 (putative ortholog of *OXIDATIVE STRESS 3*), a candidate gene associated with root length, showed at least twofold upregulation in the roots of control and salt-treated NC326 plants compared with those of B84 (Fig. 6a). This gene was downregulated at 24 h and 48 h after salt treatment in both inbred lines, albeit the level of downregulation was less in NC326 (Fig. 6b). Interestingly, this gene was significantly upregulated in B84 at 72 h after salt treatment.

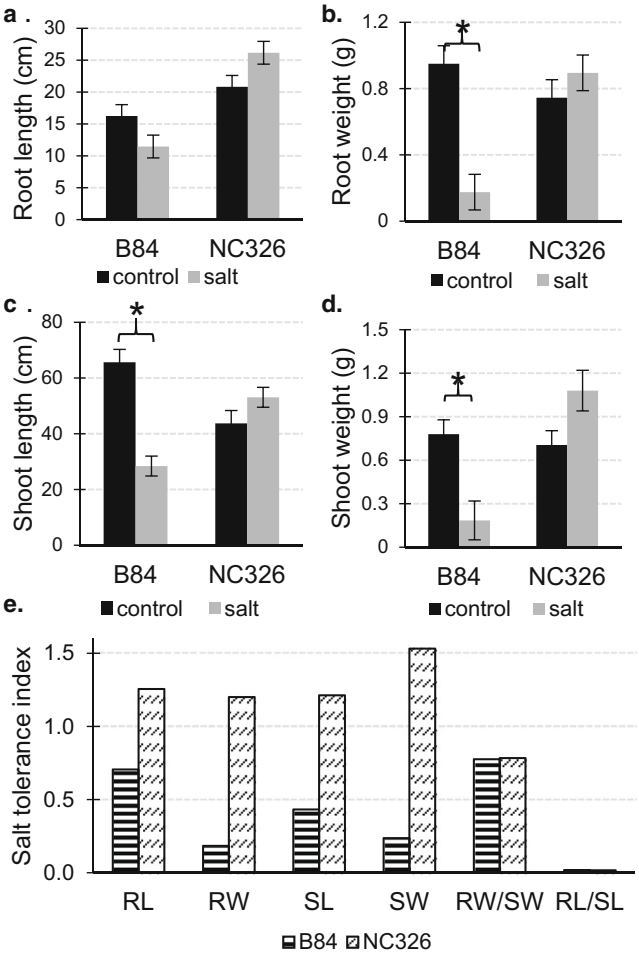

Fig. 3 Phenotypic differences between a salt sensitive (B84) and tolerant (NC326) maize inbred upon salt treatment. **a** Root length of B84 and NC326 under control and salinity. **b** Root weight of B84 and NC326 under control and salinity. **c** Shoot length of B84 and NC326 under control and salinity. **d** Shoot weight of B84 and NC326 under control and salinity. **e** Salt tolerance index of B84 and NC326 for different traits. The asterisks indicate significant ($p \leq 0.05$) difference. Error bars represent standard error. RW, root weight; RL, root length; SW, shoot weight; SL, shoot length; RW/SW, root weight/shoot weight; RL/SL, root length/shoot length

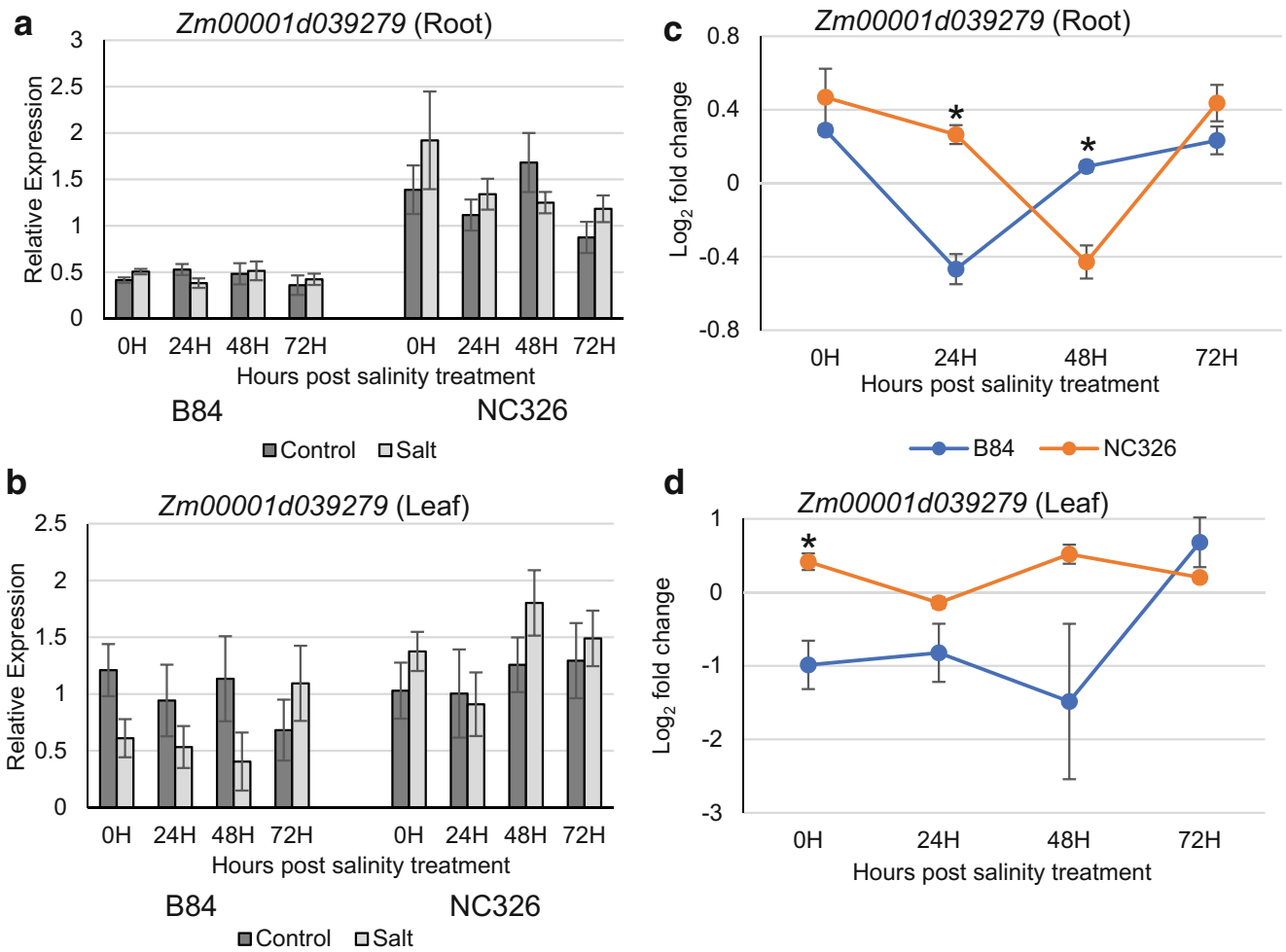

Fig. 4 Expression of a candidate gene associated with both shoot length and root length. Shown here are the expression levels of *Zm00001d039279* in the root (**a**) and leaf (**b**) tissues of a salt-sensitive (B84) and salt-tolerant (NC326) inbred line at four time points. The $\log_2$ fold expression changes in the salinity treatment relative to the control are shown in root (**c**) and leaf (**d**). Error bars represent standard error of the mean. The asterisk indicates a significant difference ($p \leq 0.05$)

Discussion

Salinity is a major abiotic stress that significantly impacts sustainable crop production and hence food security (Flowers 2004; Roy et al. 2014). Salinity decreases the production of major crops by slowing growth rates, decreasing tillering, lowering harvest index, and affecting the reproductive development and flowering (Roy et al., Saade et al. 2016). Maize is moderately sensitive to salinity (Farooq et al. 2015; Sun et al. 2018). Due to the complex nature of the salt-tolerance trait, the most efficient approach in improving salt tolerance in modern cultivars is through the exploitation of the available naturally diverse germplasm (Morton et al. 2019). Such efforts, however, have been hindered by poor understanding of the allelic diversity for salinity-related genes. While several QTLs have been mapped in relation to salt tolerance in maize (Cui et al. 2015; Luo et al. 2017; Wang et al. 2019), a low resolution of such biparental mapping limits the use of such QTLs in breeding programs. High-resolution analysis of the genetic architecture of salinity tolerance and identification of underlying genes/alleles are needed to improve salinity tolerance.

Here, we have used GWA analysis on 399 maize inbred lines evaluated using a lysimeter system that maintains constant root-zone salinity. Growth of maize seedlings is particularly hampered by salinity (Farooq et al. 2015, Sun et al. 2018). Early vigor, marked by the ability of seedling to start making assimilates independent of seed reserves, would be an important determinant of salt tolerance. Therefore, we studied the effects of salt stress at the seeding stage on six important traits (RL, RW, SL, SW, RW/SW, and RL/SL) associated with early vigor. We observed a wide range of phenotypic variation among maize genotypes under salinity treatment for the traits examined, which indicated the presence of substantial genetic variation for salt tolerance in maize. On average, maize lines displayed reduced performance under salinity for SL, SW, and RL, whereas, remarkably, RW showed an increase (Fig. 1).

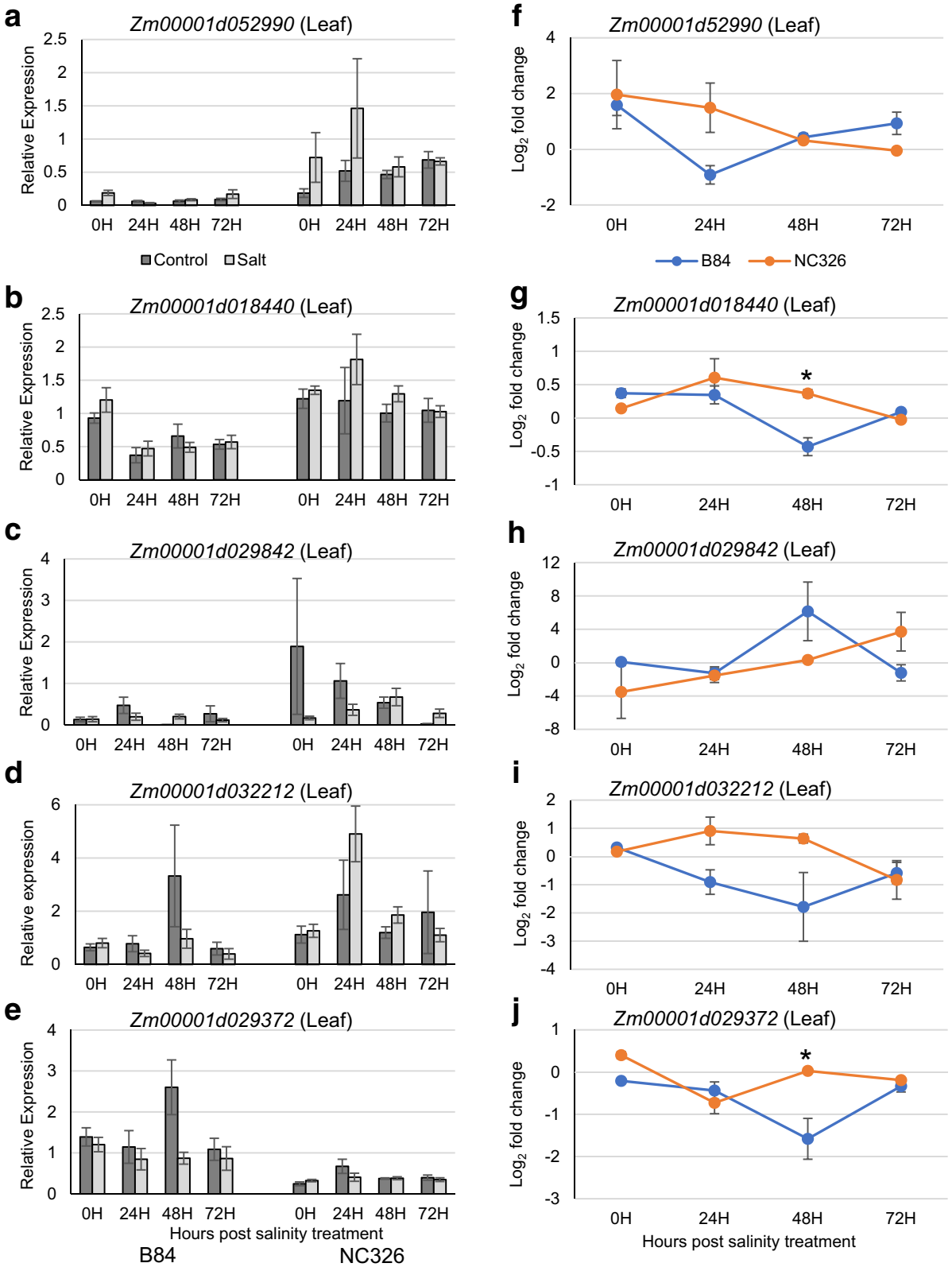

Fig. 5 Expression of candidate genes associated with shoot traits. **a**–**e** Expression levels of five candidate genes in the leaf tissue of control and salt-treated plants of a salt-sensitive (B84) and salt-tolerant (NC326) inbred line at four time points. *x*-axis represents experimental stages measured as hours post salinity treatment. **f**–**j** The $\log_2$ fold change in the salinity treatment relative to the control for the candidate genes. Error bars represent standard error of the mean. The asterisks indicate a significant difference ($p \leq 0.05$). *x*-axis represents experimental stages measured as hours post salinity treatment

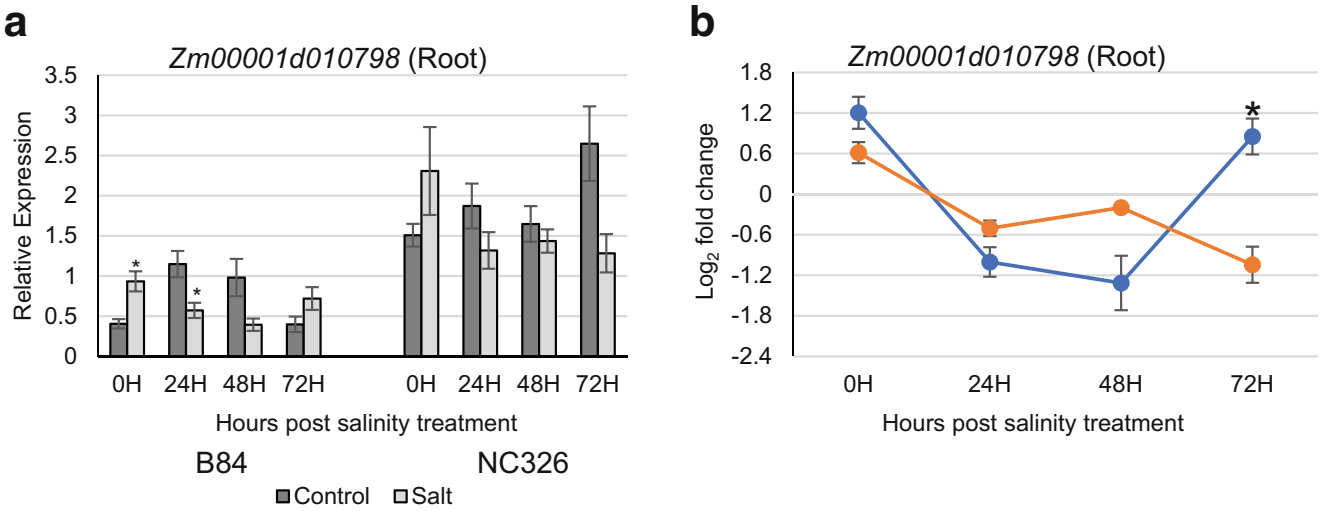

Fig. 6 Expression of a candidate gene associated with root length. **a** Expression levels of *Zm00001d010798* in the root tissues of control and salt-treated plants of a salt-sensitive (B84) and salt-tolerant (NC326) inbred line at four time points. **b** The $\log_2$ fold changes in the salinity treatment relative to the control. Error bars represent standard error of the mean. The asterisk indicates a significant difference ($p \leq 0.05$). *x*-axis represents experimental stages measured as hours post salinity treatment

It was interesting to notice that while shoot length suffered most reduction, the average RW significantly increased during salt stress (Fig. 1a, Table S1). Furthermore, even though both root and shoot lengths decreased upon exposure to salinity, shoot length was more severely affected as evident from higher ST of RL/SL ratio. Higher salt sensitivity of shoots compared with that of roots, also reported previously (Munns and Termaat 1986; Munns and Tester 2008; Sandhu et al. 2017), could be a manifestation of (1) higher damage to shoot cells resulting in slower growth and/or (2) a resource allocation strategy adopted by the plant to minimize the damaging effects of salinity. Efficient salt exclusion mechanism and sequestration of sodium into the vacuoles by the root cells provide an enhanced ability of salt tolerance to roots (Sandhu and Kaundal 2018). Sodium plays a major role in salinity-related damage to the maize plant (Fortmeier and Schubert 1995) and, therefore, the removal of salt from the cell would decrease salt-related injury. The leaf cells, however, do not appear to have an exclusion mechanism to remove excess salt and, while sequestration to the vacuole is possible, the extent of such sequestration compared with that to the root cells needs to be determined. Excess salt may force the leaf cells to invest a sizeable part of energy in recovery from oxidative stress and other damaging effects of salinity thus resulting in poor growth.

Increase in RW and a concomitant decrease in SW, increased RL/SL and RW/SW ratio, high correlation between RL and SL, and a high correlation between RW and SW indicate that plants use resource allocation to these two organs as an active strategy against salt stress. Increased root weight upon salt treatment is a well-known response (Acosta-Motos et al. 2016; Franco et al. 2006; Gomez-Bellot et al. 2013). By increasing the number of cells and/or cell size, root cells could sequester more salt in the bigger and/or more vacuoles. Increased root cell size and the resulting increase in the number of Na^+/H^+ exchangers such as salt overly sensitive 1 (SOS1) could also help in increased exclusion of salt. The SOS proteins play an important role in excluding Na^+ out of the roots and sequestering excessive Na^+ into vacuoles (Munns and Tester 2008; Sandhu and Kaundal 2018). Supporting this hypothesis, salt-stressed plants display an increase in root density and diameter (Acosta-Motos et al. 2016, Franco et al. 2006, Gomez-Bellot et al. 2013), both of which are expected to increase cell number and/or size. Increased root weight could also be a result of longer roots which allow the plants to explore more volume of soil to find less saline soil zones. While root length decreased in response to salt stress in the current study, only the primary root was measured. It will be interesting to examine if maize seedlings produce more secondary roots and/or more root hair as a survival strategy.

The maize inbred lines characterized in this study cover a significant part of natural diversity that has been exploited to understanding the genetic architecture of other traits (Hirsch et al. 2014; Mazaheri et al. 2019). Therefore, the 57 SNP markers associated with one or more of the six traits cover a sizable part of the genomic regions involved in response to salinity in maize seedlings. This is evident from the functional diversity of candidate genes associated with these SNPs (Table 1). Among the traits studied, majority (40) SNPs and the associated candidate genes were identified for shoot biomass traits (SW and SL) suggesting that a large portion of genetic variation underlying salt tolerance is involved in fine-tuning the plant response to salt in shoot cells. The ratio of RW/SW was also represented by a large number of candidate SNPs/genes indicating the importance of resource

allocation to various plant organs in response to salt stress. We did not identify any SNPs associated with RW, which could be due to a rather modest variation among inbred lines for salinity response (Fig. 1a).

Salt-tolerant (NC326) and salt-sensitive (B84) lines showed contrasting response to salinity for root and shoot traits and, therefore, allowed us to examine the role of candidate genes in salinity tolerance. The most significant SNP (rs3_974864) in the GWA analysis, identified for both SL and RL, is associated with a candidate gene (*Zm00001d039279*) encoding for a putative hairpin-induced protein. Significantly higher basal expression in roots and shoots of NC326 supports the role of this gene in salt tolerance (Fig. 4). Induction of this gene in NC326 further supports such a role. A search for conserved domains in Zm00001d039279 protein using the conserved domain database (Marchler-Bauer et al. 2016) revealed a late embryogenesis (LEA_2) domain (pfam03168). The LEA_2 domain-containing proteins have been associated with response to a wide array of abiotic stresses including drought, heat, salinity, UV damage, oxidative stress, and osmotic stress (Artur et al. 2018; Battaglia and Covarrubias 2013; He et al. 2012; Jia et al. 2014). Overexpression of LEA_2 improved salt tolerance in Arabidopsis (Jia et al. 2014), and overexpression of a rice LEA_2 protein-enhanced survival of *E. coli* in saline conditions (He et al. 2012). Remarkably, despite the presence of LEA_2 domain with a known role in salinity tolerance, no functional annotation of this putative hairpin-induced gene in maize is evident from searches performed in various databases. Since this candidate is associated with natural variation for both RL and SL, a likely role of this gene appears to be the reprogramming of resource partitioning upon salt stress. Further functional analysis is needed to establish the role of this gene in salinity tolerance in maize and other monocots.

Five candidate genes associated with shoot biomass traits (SL and/or SW) that showed contrasting expression patterns in response to salinity in leaves (Fig. 5) represent interesting candidates. *Zm00001d052990*, a putative cysteine-rich receptor-like kinase, is associated with both SL and SW. Significant upregulation of *Zm00001d052990* in the leaves of NC326 indicates the importance of extracellular signaling in response to salt stress. Members of this gene family are involved in regulating stomatal opening in response to oxidative stress and abscisic acid signaling (Hua et al. 2012). In barley, a receptor-like kinase encoded by *HuWAK1* has been shown to regulate root growth in normal and saline conditions (Kaur et al. 2013). Upregulation of *Zm00001d018440*, a putative casein kinase 1, in the leaves of NC326 highlights the role of this Ser/Thr protein kinase in salt tolerance. This role is supported by enhancement of NaCl tolerance in Arabidopsis by overexpression of a casein kinase 1 and an associated hyperaccumulation of the protein in both leaves and roots (Zhang et al. 2016). Upregulation of *Zm00001d029842*, a putative divalent ion symporter, in NC326 leaves supports the use of such channels to regulate the levels of divalent ions (e.g., Ca^{2+}) in response to the presence of monovalent ions (e.g., Na^{+}). While such regulation is generally reported for root cells (Hanin et al. 2016), changes in Ca^{2+}-mediated signals in response to NaCl treatment have also been shown in leaf cells (Martí et al. 2013). *Zm00001d032212*, a putative GDT1-like protein, is also upregulated in NC326 leaves upon salt stress. Remarkably, GDT1 is proposed to encode for a chloroplast-localized Ca^{2+}/H^{+} antiporter responsible for Ca^{2+} and pH homeostasis in Arabidopsis (Wang et al. 2016). Induction of *Zm00001d029842* and *Zm00001d032212* in NC326 on salt treatment suggests a regulatory role of these genes during salinity stress. Downregulation of a putative serine carboxypeptidase encoded by *Zm00001d029372* in NC326 suggests a novel role of this member of a protein family with wide-ranging functions that include response to oxidative stress (Liu et al. 2008).

Zm00001d010798, a candidate gene associated with natural variation for root length, was upregulated in the roots of NC326 (Fig. 6). This gene shares homology with Arabidopsis *OXIDATIVE STRESS 3* shown to be important for imparting tolerance to heavy metal ions and oxidative stress (Blanvillain et al. 2009). The reduced expression of *Zm00001d010798* in the roots of NC326 at 72 h may be due to the ability of this inbred line to cope with oxidative stress better than B84 (Fig. 6b).

The analysis of expression patterns of the candidate genes identified in this study based on steady-state transcript levels in pooled leaf and root issues provides support for the role of these genes in salinity. However, accumulation of mRNA is just one of the indicators of the role of a gene in a biological process or phenotype, and other factors including mRNA stability and protein accumulation also need to be examined. Future analysis relying on mutants and transgenics will provide conclusive evidence for the role of these candidate genes in salinity tolerance.

Several genes identified in our study are novel and lack any direct implication in salinity response based on prior research. For instance, identification of a putative zinc transporter encoded by *Zm00001d033446* indicates a vital role of zinc in salt stress response. Interestingly, a DnaJ zinc finger domain protein encoded by *Zm00001d009783* further supports the role of zinc in salinity response. A phospholipid-transporting ATPase encoded by *Zm00001d035775* implicates transport of phospholipids across plasma membrane in response to salt stress. Identification of a MYB DNA-binding protein encoded by *Zm00001d023282* suggests an essential role for members of this very important transcription factor family in salinity response. Functional validation of these genes is expected to enhance our understanding of the complex underpinning of salinity tolerance in cereals.

Salinity is one of the most important challenges faced by modern agriculture, and the severity of this stress is expected

to increase as the irrigation water and arable land become scarce. The identifications of SNP associated with salinity tolerance are directly useful for breeding endeavors. Typically, the QTL identified from biparental mapping populations have low resolution. Incorporating these QTL in breeding programs is associated with genetic drag caused by undesirable alleles linked to the QTL resulting in undesirable phenotypes. Since the SNP obtained from GWA studies have higher resolution, using these SNPs in selection in breeding programs is expected to result in fewer undesirable phenotypes. This study also provides an extensive roadmap of biological process and candidate genes that should be examined to decipher genetic and molecular components of the salt tolerance mechanism in maize. These candidates will be valuable in expanding the knowledge about the biological underpinnings of plant responses to salinity. The knowledge generated by such is expected to boost efforts to enhance salinity tolerance in maize and other cereals.

Acknowledgments Authors would also like to thank two anonymous reviewers for their valuable suggestions to improve the manuscript.

Funding information Dr. Rohit Kumar is supported by United States Department of Agriculture (USDA) Hatch project SC-1700520. Additional support came from the USDA - Agricultural Research Services, National Program 301: Plant Genetic Resources, Genomics and Genetic Improvement (project number 2036-13210-012-00-D).

Compliance with ethical standards

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