A retrotransposon in an HKT1 family sodium transporter causes variation of leaf Na^+ exclusion and salt tolerance in maize

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Summary

- Soil salinity is one of several major abiotic stresses that constrain maize productivity worldwide. An improved understanding of salt-tolerance mechanisms will thus enhance the breeding of salt-tolerant maize and boost productivity. Previous studies have indicated that the maintenance of leaf Na^+ concentration is essential for maize salt tolerance, and the difference in leaf Na^+ exclusion has previously been associated with variation in salt tolerance between maize varieties.
- Here, we report the identification and functional characterization of a maize salt-tolerance quantitative trait locus (QTL), *Zea mays Na*$^+$ *Content1* (*ZmNC1*), which encodes an HKT-type transporter (designated as *ZmHKT1*).
- We show that a natural *ZmHKT1* loss-of-function allele containing a retrotransposon insertion confers increased accumulation of Na^+ in leaves, and salt hypersensitivity. We next show that *ZmHKT1* encodes a plasma membrane-localized Na^+-selective transporter, and is preferentially expressed in root stele (including the parenchyma cells surrounding the xylem vessels). We also show that loss of *ZmHKT1* function increases xylem sap Na^+ concentration and causes increased root-to-shoot Na^+ delivery, indicating that *ZmHKT1* promotes leaf Na^+ exclusion and salt tolerance by withdrawing Na^+ from the xylem sap.
- We conclude that *ZmHKT1* is a major salt-tolerance QTL and identifies an important new gene target in breeding for improved maize salt tolerance.

Introduction

Maize is an important crop worldwide, and provides more than one-half of global calorie consumption (Schnable, 2015). However, the adequacy of future maize supply is under threat for several reasons. First, world maize production needs to double by 2050 to satisfy the requirements of an expanding population (Ray *et al.*, 2013). Second, global maize production is increasingly being challenged by diverse environmental stresses (Deinlein *et al.*, 2014; Zuo *et al.*, 2015). Soil salinity stress is one of the most commonly encountered stresses in maize cultivation (Hanks *et al.*, 1978) and, unfortunately, maize is a glycophyte plant that is hypersensitive to salinity stress (Farooq *et al.*, 2015). Thus, salinity stress is a major threat to the long-term sustainability of maize production. In consequence, there is an urgent need to advance beyond our current understanding of how maize responds to salinity stress, and thus to enhance our capabilities for the development of salt-tolerant maize cultivars.

Salinity causes a two-phase (osmotic and ion toxic) physiological challenge to plant growth (Munns, 1993; Munns & Tester, 2008). In the first (osmotic) phase, plant growth is reduced as a result of a decrease in environmental water potential (Fortmeier & Schubert, 1995; Munns & Tester, 2008). In the second phase, *in planta* sodium ions (Na^+) accumulate to toxic levels, thereby competing with potassium ions (K^+) and causing the dysregulation of various biological processes, including the activation of enzymes and stomatal conductance (Fricke *et al.*, 2004). Accordingly, the maintenance of a low Na^+ concentration and a normal $Na^+ : K^+$ ratio in the cell cytoplasm is essential for sustained plant salt tolerance (Zhu, 2003; Pardo *et al.*, 2006; Munns & Tester, 2008; Zhang *et al.*, 2016). Although the molecular mechanisms underlying the osmotic aspects of salt tolerance remain largely unknown, several factors regulating shoot Na^+ homeostasis and salt tolerance have been identified in recent years. These factors primarily reduce root–shoot Na^+ delivery, thus promoting the exclusion of Na^+ from the shoot and enhancing salt tolerance. For example, the plasma membrane $Na^+ : H^+$ antiporter Salt Overly Sensitive 1 (SOS1) transports Na^+ accumulated in the root back into the soil solution, thus reducing the xylem sap Na^+ load (Shi *et al.*, 2000; Zhu, 2016). In addition, the xylem sap Na^+ load is further reduced in response to reactive oxygen species (ROS) generated by a root stele-specific salt-inducible respiratory

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burst oxidase activity (Jiang *et al.*, 2012, 2013). Finally, the *Arabidopsis* High-affinity K^+ Transporter1 (AtHKT1), and homologs in other species (e.g. rice and wheat), encode an Na^+-preferential transporter which principally controls root–shoot Na^+ delivery via the withdrawal of Na^+ from the xylem sap (Uozumi *et al.*, 2000; Mäser *et al.*, 2002a,b; Ren *et al.*, 2005; Munns *et al.*, 2012). Intriguingly, previous studies have demonstrated that natural variation in $Na^+ : K^+$ homeostasis regulators is associated with variation in salt tolerance in various species (Ren *et al.*, 2005; Munns *et al.*, 2012; Yang *et al.*, 2014). For example, favorable natural variant *HKT1* homolog alleles have been shown to substantially improve the salt tolerance of both rice and wheat (Ren *et al.*, 2005; Munns *et al.*, 2012), thus demonstrating that the exploitation of natural genetic variation for $Na^+ : K^+$ homeostasis provides a promising way forward in the breeding of salt-tolerant crops.

In maize, previous studies have shown that different natural varieties display different sensitivities to salinity stress (Fortmeier & Schubert, 1995; Zhao *et al.*, 2010; Gao *et al.*, 2016), variation which may be attributable to variation in osmotic tolerance and/or Na^+ exclusion efficiency (Gramer, 1994; Schubert *et al.*, 2009; Zhao *et al.*, 2010; Gao *et al.*, 2016). However, the genetic components underlying maize salt tolerance variation have remained little understood, with, as far as we know, no quantitative trait loci (QTLs) responsible for maize salinity tolerance being reported. Here, we report the cloning and functional study of *Zea mays* Na^+ *Content1* (*ZmNC1*). We show that a retrotransposon in an HKT1 family sodium transporter confers variation of leaf Na^+ exclusion and salt tolerance in maize, and that *ZmNC1* encodes a typical class I Na^+-preferential HKT-type transporter, ZmHKT1, which promotes leaf Na^+ exclusion and salt tolerance by removing Na^+ from the xylem sap flowing from root to shoot. We conclude that ZmHKT1 is an important salt-tolerance QTL in maize, which provides a potentially important new gene locus for breeders working towards improvements in maize salt tolerance.

Materials and Methods

Plant growth and salt treatment

The *Zea mays* L. Zheng58/Chang7-2 recombinant inbred lines (RILs) population has been described in a previous study (Song *et al.*, 2016). In order to measure the leaf Na^+ and K^+ contents of salt-grown Zheng58/Chang7-2 RILs, pots with a diameter of 30 cm and a height of 35 cm were filled with uniformly mixed Pindstrup substrate (www.pindstrup.com) and watered to soil saturation with 100 mM NaCl solution. Eight RILs (four plants for each) were planted in each pot, grown in a glasshouse for 3 wk, and the shoot tissues were collected for the measurement of Na^+ and K^+ concentration. The process of growing the plants, shown in Figs 1, 3 and 4 (see later), was as follows: kernels (genotypes as indicated) were germinated directly in pots saturated with water (Control) or with 100 mM NaCl solution (Salinity); water and nutrients were given as required during the experimental period, and plants grown under saline conditions were watered with 2 l of 100 mM NaCl every 2 wk.

Measurement of Na^+ and K^+ concentration

The measurement of the Na^+ and K^+ content has been described previously (Xu *et al.*, 2006). The samples were dried at 80°C to constant weight for 24 h. The dry weights of the samples were measured as dry biomass. The dry plant tissues were incinerated in a muffle furnace at 300°C for 3 h and 575°C for 6 h. The ashes were dissolved in 10 ml of 1% hydrochloric acid and diluted with 1% hydrochloric acid into different multiples. The Na^+ and K^+ concentrations were determined using a 4100-MP AES device (Agilent, Santa Clara, CA, USA).

DNA extraction, genotyping, bin map construction and QTL mapping

The whole-genome sequence data of Zheng58 and Chang7-2 have been described previously (Jiao *et al.*, 2012). DNA extraction, genotyping, bin map construction and QTL mapping processes were carried out as described by Song *et al.* (2016). The DNA for each RIL was extracted from leaf tissue and sequenced by GBS (genotyping-by-sequencing) (Chen *et al.*, 2014). The sequence data were aligned to B73 RefGen_v2 by BWA (Burrows-Wheeler Aligner) (Li & Durbin, 2009), followed by single nucleotide polymorphism (SNP) calling using the Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010). On average > 50 000 SNPs were obtained per RIL. The bin map was constructed using a sliding window approach as described by Huang *et al.* (2009). Composite interval mapping (CIM) was performed for leaf-Na^+, leaf-K^+ and $\log_{10}(Na^+ : K^+)$ using the R/QTL package (Broman *et al.*, 2003). R was used to generate the statistical report and figures (R Core Team, 2014).

Generation of the CRISPR-Cas9 knockout lines of *ZmHKT1*

The CRISPR-Cas9 (clustered regularly interspaced short palindromic repeat-CRISPR-associated protein 9) knockout lines were created by the maize functional genomic project of China Agricultural University according to previous methods (Xing *et al.*, 2014). In essence, a pCAMBIA-derived CRISPR/Cas9 binary vector with two gRNA expression cassettes targeting two adjacent sites of *ZmHKT1* (see later, Fig. 3a) was generated and transformed into *Agrobacterium* strain EHA105, and then into the immature embryos of B73-329 inbred lines. In order to identify the positive CRISPR-Cas9 knockout lines, the PCR amplicons encompassing the gRNA-targeted sites for each of the transgenic plants were sequenced by Sanger sequencing.

RNA extraction, transcriptome sequencing and real-time PCR

In order to compare the transcriptomes of Zheng58 and Chang7-2, 2-wk-old Zheng58 and Chang7-2 plants were treated with NaCl solution (100 mM NaCl; Salinity) or water (Control), and the samples (whole plants) were collected 24 h following the onset of treatment (each sample had two independent biological replicates). Total RNA was extracted using an RNAprep pure

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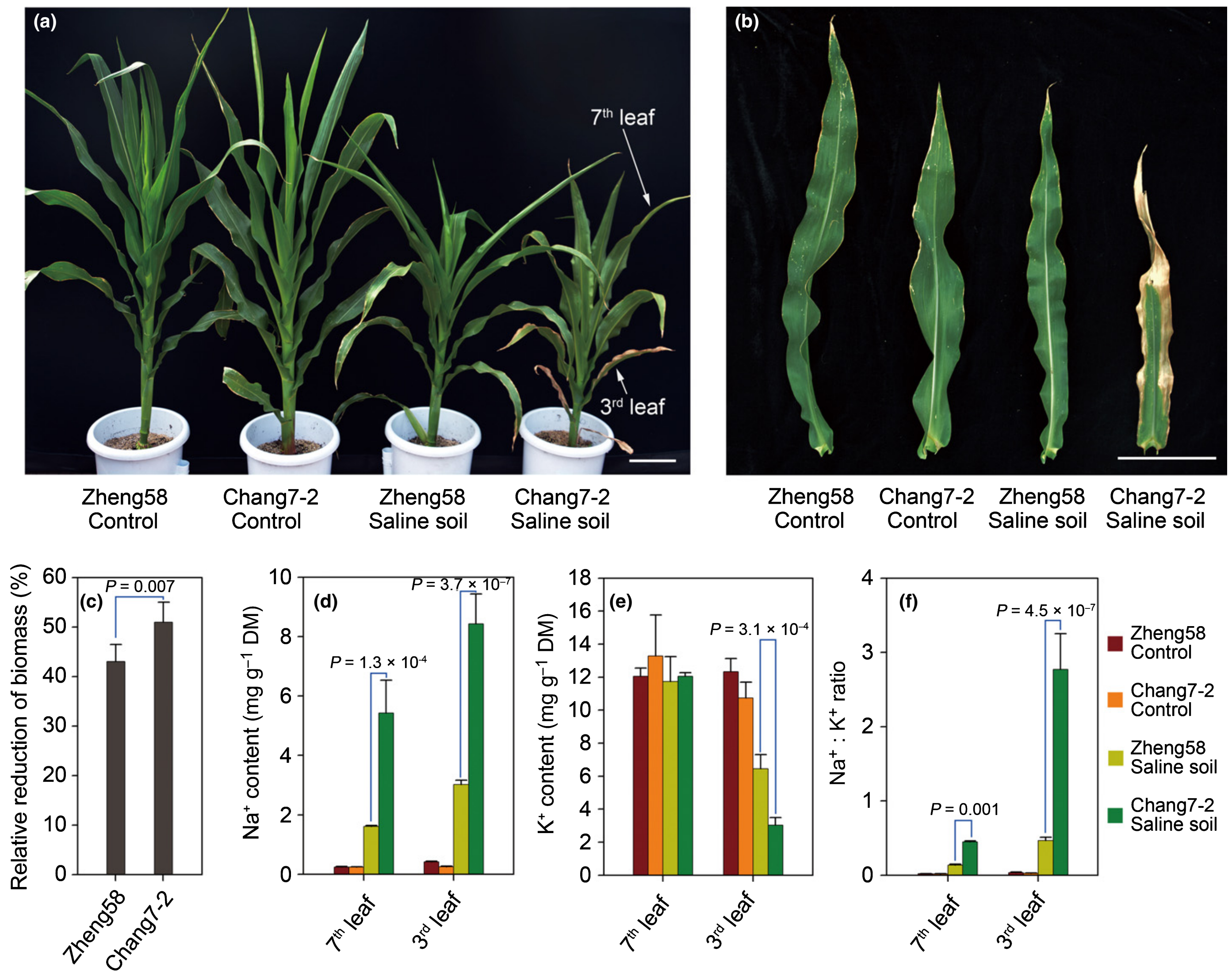

Fig. 1 Salinity stress tolerance and regulation of $Na^+ : K^+$ homeostasis vary between maize inbred lines. (a) Appearance of 8-wk-old plants from inbred lines Zheng58 and Chang7-2 grown in control and saline soil conditions. White arrows highlight the yellowed leaves. (b) Close-up images of the 3rd leaf of plants shown in (a). Bar, 15 cm. (c) Biomass of 8-wk-old saline soil-grown plants (expressed as a percentage of control plant biomass). (d) Na^+ content, (e) K^+ content and (f) $Na^+ : K^+$ ratio of 3rd (representing an old leaf) and 7th (representing a young leaf) leaves of 8-wk-old Zheng58 and Chang7-2 plants grown in control and saline soil conditions. Data are means ± SE of three replicates.

plant kit (Tiangen, Beijing, China). Three micrograms of total RNA from each sample were used for transcriptome sequencing at Novagene (http://www.novogene.com/), employing the high-throughput sequencing platform highseq3000. The clean data for each sample amounted to *c.* 5 Gb. Differential gene expression was determined using RNA-seq DGE tools (Trapnell *et al.*, 2010). For real-time PCR, cDNA was prepared using M-MLV reverse transcriptase and qRT-PCR (quantitative reverse transcription PCR) analyses were conducted using PowerUp™ SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) on an ABI 7500 thermocycler (Applied Biosystems). The quantification method ($2^{-\Delta\Delta Ct}$) was used and the variation in expression was estimated using three biological replicates. The amplification reactions were performed in a total volume of 20 µl, which contained 1 µl cDNA, 1 µl forward and reverse primers (10 µM), 10 µl SYBR Green premix and 8 µl H_2O. The maize Ubi2 (UniProtKB/TrEMBL, Q42415) gene was used as an internal standard. The sequences of the primers (ZmUBI2-F, ZmUBI2-R, ZmHKT1-RT-F and ZmHKT1-RT-R) are given in Supporting Information Table S1.

ZmHKT1-based association analysis

The association analysis of genetic variation in *ZmHKT1* with leaf Na^+ content of salt-grown maize was performed within 190 temperate maize inbred lines. The ZmHKT1 promoter (*c.* 1.0 kb), coding regions and 3′-untranslated region (3′-UTR) sequences (*c.* 0.5 kb) were amplified and sequenced. These sequences were assembled using ContigExpress in Vector NTI Advance 10 and aligned using Mega v.5 (http://megasoftware.net/). DNA sequence polymorphisms (SNPs and InDels) were identified and their association with leaf Na^+ content was

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analyzed by TASSEL 3.0, under the standard MLM (mixed linear model), with minor allele frequency (MAF) ≥ 0.05.

Electrophysiological analysis of ZmHKT1 in *Xenopus laevis* oocytes

The CDS (coding DNA sequence) of *ZmHKT1* was amplified from cDNA isolated from 2-wk-old Zheng58 root tissue using KOD-Plus-High-Fidelity DNA polymerase (Toyobo, Osaka, Japan) and cloned using *Xba*I and *Hind*III sites into destination vector pGEMHE. pGEMHE containing ZmHKT1 was linearized using *Nhe*I-HF (Takara, Beijing, China). The capped cRNA was transcribed with T7 RNA polymerase using the RiboMAX™ Large Scale RNA Production System-T7 kit (Promega). The cRNA quality was checked by agarose gel electrophoresis. The concentration was determined at 260 or 280 nm and adjusted to a final concentration of 0.5 $\mu g\ \mu l^{-1}$. An aliquot of 25 nl (12.5 ng) of cRNA or an equal volume of RNA-free water was injected into *Xenopus laevis* oocytes. Oocytes were incubated for 36 h and electrophysiological experiments were performed using a two-electrode voltage clamp amplifier, as described previously (Horie *et al.*, 2007) (6 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM MES 2-(N-morpholino)-ethanesulfonic acid), 200 mM D-mannitol and pH 5.5 adjusted with TRIS (2-amino-2-(hydroxymethyl)-1,3-propanediol) base ± Na^+ glutamate and/or K^+ glutamate as indicated).

Xylem sap and phloem sap collection

Na^+ concentration in xylem sap was measured according to previous methods (Horie *et al.*, 2007; Wang *et al.*, 2015) with minor modifications. Plants were grown hydroponically for 4 wk in standard solution. Then, 50 mM NaCl was introduced into the solution for 24 h. The shoots were excised using a blade, and xylem sap exuding at the cut surface of the de-topped root system was collected by a micropipette for 1 h. The Na^+ and K^+ concentrations were measured using a 4100-MP AES device (Agilent). The phloem sap was collected as described previously (Ren *et al.*, 2005). Maize plants were grown hydroponically for 3 wk. The phloem sap was collected 2 d following the onset of NaCl treatment (50 mM). For each replicate, three plants were detached using a blade, and the wound sections were dipped in a solution of 20 mM K_2EDTA (pH 7.5) for 1 min, and then in 2 ml of 15 mM K_2EDTA (pH 7.5) for 4 h in an illuminated growth room under a water-saturated atmosphere. The quantity of Na^+ released in the EDTA solution was measured using a 4100-MP AES device (Agilent). The glutamine released in the EDTA solution was treated with an AccQ-Tag Ultra Derivatization Kit and analyzed by LC-MS (Thermo Scientific Q-exactive high-resolution mass spectrometer, Waltham, MA, USA). The Na^+ concentration in phloem sap was expressed as the Na^+ to glutamine ratio.

In-situ PCR

In-situ PCR was performed as described previously (Munns *et al.*, 2012), with few modifications. The maturation zone of the primary root of a 2-wk-old Zheng58 plant was used. The 50-μm-thick root sections were obtained using a Microtome (Leica, Wetzlar, Germany) and were transferred into 100 μl of cold sterile water (with 100 U of RNase inhibitor) kept on ice. Genomic DNA was eliminated in DNase buffer with 8 U RNase-free DNase (Tiangen) for 20 min at 25°C, and the reaction was stopped by the addition of EDTA to a final concentration of 15 mM and heating to 75°C for 10 min. M-MLV reverse transcriptase (Takara) was used for cDNA synthesis with 0.5 μM gene-specific primer (ZmHKT1-cDNA for ZmHKT1 and Zm18S-cDNA for 18S ribosomal RNA; Table S1). PCRs were carried out in a final volume of 50 μl, containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 0.4 nmol digoxigenin-11-dUTP (Roche), 0.5 μM of each primer and 2 U Taq DNA polymerase (Thermo Fisher, Waltham, MA, USA). The PCR cycling parameters were as follows: initial denaturation at 95°C for 30 s, followed by 95°C for 10 s, 58°C for 30 s and 72°C for 20 s, and a final extension at 72°C for 5 min. The rest steps were performed as described previously (Munns *et al.*, 2012). The primers for the amplification of ZmHKT1 (ZmHKT1-*in-situ*-F and ZmHKT1-*in-situ*-R) and 18S (18S-*in-situ*-F and 18S-*in-situ*-R) are listed in Table S1.

Results

Maize salt-tolerance variation is associated with natural variation in leaf $Na^+ : K^+$ homeostasis

ZD958 is a widely adopted Chinese hybrid maize line generated by crossing progenitor inbred lines Zheng58 and Chang7-2 (Song *et al.*, 2016). Previous studies have indicated that Zheng58 and Chang7-2 differ with respect to a variety of physiological and developmental traits. For example, the root system of Zheng58 is more branched than that of Chang7-2 (Han *et al.*, 2015; Song *et al.*, 2016). We therefore compared the salinity tolerance of these two inbreds, and found that Zheng58 is more tolerant than Chang7-2 to salinity stress. As shown in Fig. 1, although Zheng58 and Chang7-2 both grow well in normal conditions, Chang7-2 plants become more yellow in color than do Zheng58 plants when grown in saline soil (Fig. 1a,b). This difference is particularly noticeable in older leaves (e.g. 3rd vs 7th leaf, as highlighted by the arrows in Figs 1a, S1). In addition, the growth of Chang7-2 plants in saline soil resulted in a *c.* 10% greater reduction in biomass than seen for Zheng58 plants ($P = 0.007$; Fig. 1c). Thus, Chang7-2 is less tolerant than Zheng58 to soil salinity.

The maintenance of low leaf Na^+ content and $Na^+ : K^+$ ratio is known to be essential for maize salt tolerance. In addition, natural variation in $Na^+ : K^+$ homeostasis is associated with variation in salt sensitivity in several species (Ren *et al.*, 2005; Munns *et al.*, 2012; Gao *et al.*, 2016). We therefore investigated Na^+ and K^+ homeostasis in Zheng58 and Chang7-2, and found that the leaves of salt-grown Chang7-2 plants had a higher Na^+ content, a lower K^+ content and a higher $Na^+ : K^+$ ratio than those of salt-grown Zheng58 plants (Fig. 1d–f). This difference was more striking in older (e.g. 3rd leaf) than younger (e.g. 7th leaf) leaves

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(Fig. 1d–f), a leaf age difference consistent with that seen previously for leaf coloration (Figs 1a, S1). Thus, variation in leaf Na^+ content, K^+ content and $Na^+:K^+$ ratio is associated with variation in the extent of salinity tolerance displayed by Zheng58 and Chang7-2.

ZmNC1 is a major QTL regulating the leaf Na^+ content of maize plants grown on saline soil

We next performed a QTL analysis to identify the genetic loci responsible for the differences in leaf Na^+ and K^+ contents and $Na^+:K^+$ ratio between Zheng58 and Chang7-2. Using a biparental RIL population composed of 540 RILs (see the Materials and Methods section; Song *et al.*, 2016), we showed that the Na^+ and K^+ contents in the leaves of different RILs were significantly different (Fig. S2). A single major QTL (LOD (logarithm of the odds ratio) = 12.51) for leaf Na^+ concentration was identified by QTL analysis (Fig. 2). This QTL was mapped to a *c.* 12-Mb region on chromosome 3, and was named *Zea mays L. Na^+ CONTENT 1* (*ZmNC1*) (Fig. 2a). Intriguingly, we also found that the major QTL for $\log_{10}(Na^+:K^+$ ratio) was also mapped to this region, and overlapped with *ZmNC1* (Fig. S3), suggesting that the gene or genes underlying *ZmNC1* play key roles in the regulation of both the leaf Na^+ content and Na^+/K^+ ratio. In addition, we identified two QTLs responsible for the variation in shoot K^+ content, which we named *Zea mays K^+ CONTENT 1* and *2* (*ZmKC1* and *ZmKC2*), and which mapped to a region of the genome distinct from *ZmNC1* (Fig. S4). In this study, we focused our attention on the in-depth characterization and functional analysis of *ZmNC1*.

ZmNC1 encodes an HKT-type ion transporter

In order to facilitate the molecular identification of the gene variant (s) underlying the *ZmNC1* QTL, we next performed transcriptome sequencing (see the Materials and Methods section) to compare the transcriptomes of Zheng58 and Chang7-2 plants grown in control or saline soil conditions. Within the *c.* 12-Mb *ZmNC1* QTL region, we identified 17 genes displaying significant transcript level differences (fold change ≥ 3 and $P < 0.05$) between Zheng58 and Chang7-2 plants (Fig. 2b). One of these genes (*GRMZM2G047616*) encodes a protein showing high sequence homology with HIGH-AFFINITY *K^+ TRANSPORTER* (HKT) family proteins (Figs S5, S6). According to the annotation of the B73 maize genome, there are two genes encoding HKT family proteins (GRMZM2G047616 and GRMZM2G135674). The HKT family proteins from maize, Arabidopsis, rice and wheat were aligned and a phylogenetic tree was constructed using MEGA6 by the maximum likelihood method (Tamura *et al.*, 2013) (Fig. S5). The results indicated that all the proteins fell into two major clusters. GRMZM2G047616 and all the other HKT1 members fell into the same major cluster (Cluster I) (Fig. S5), which is therefore designated as ZmHKT1. Although soil salinity had no detectable effect on the expression of *ZmHKT1*, *ZmHKT1* transcript levels in Zheng58 were *c.* 10-fold higher than those in Chang7-2 (in both control and saline soil conditions; Fig. 2c,d). As previous studies have shown that HKT-type transporters promote leaf Na^+ exclusion and salt tolerance in rice and wheat (Ren *et al.*, 2005; Baxter *et al.*, 2010; Munns *et al.*, 2012; Yang *et al.*, 2014), and ZmHKT1 had the highest similarity to OsHKT1;5 (a salt-tolerance QTL in rice; Ren *et al.*, 2005) and TmHKT1;5-A (Figs S5, S6), it therefore seemed likely that the difference in *ZmHKT1* expression level between Zheng58 and Chang7-2 is an underlying cause of the different leaf Na^+ contents regulated by the *ZmNC1* QTL.

In order to verify whether genetic variation at *ZmHKT1* underlies the *ZmNC1* QTL, we generated knockout mutants (using a type II CRISPR-Cas9 system; Xing *et al.*, 2014) (Fig. 3). We obtained two independent knockout lines, *$ZmHKT1^{crispr}$-1* and *$ZmHKT1^{crispr}$-2*, which confer a 5- and 34-bp deletion, respectively, causing frameshifting and truncation of *ZmHKT1* (Fig. 3a,b). As shown in Fig. 3(c,d), there was no detectable difference between wild-type (WT) (an inbred line descendent from B73) and mutant plants grown in control soil (Fig. 3c). However, when grown on saline soil, the growth of *$ZmHKT1^{crispr}$-1* and *$ZmHKT1^{crispr}$-2* mutant plants was visibly more retarded than that of WT (Fig. 3c), with mutant leaves displaying more leaf yellowing (Fig. 3d). These visible differences were reflected in biomass measurements, with the mutant plants displaying significantly greater biomass reductions than WT controls when grown on saline soil ($P < 0.01$; Fig. 3e). Finally, the leaves of the mutant plants accumulated more Na^+ (reflected in higher Na^+ concentrations and $Na^+:K^+$ ratios) than did WT controls (Fig. 3f,g). Taken together, these results support the notion that natural genetic variation at *ZmHKT1* underlies the phenotypic variation conferred by the *ZmNC1* QTL.

We further verified that genetic variation at *ZmHKT1* underlies the *ZmNC1* QTL by performing an allelism test. Taking the Chang7-2 (apparent transcript null; Fig. 2c) and *$ZmHKT1^{crispr}$-1* alleles to be loss-of-function alleles, and the Zheng58 and WT control progenitor allele of *$ZmHKT1^{crispr}$-1* (WT) alleles to be functional *ZmHKT1* alleles, we generated four F_1 hybrids (Chang7-2/*$ZmHKT1^{crispr}$-1*, Chang7-2/WT, Zheng58/*$ZmHKT1^{crispr}$-1* and Zheng58/WT) and compared their salt sensitivity. As shown in Fig. 4, although growth on saline soil significantly reduced the biomass of all F_1 hybrids (Fig. 4a,b), the biomass of Chang7-2/*$ZmHKT1^{crispr}$-1* loss-of-function allele homozygotes was reduced significantly more than that of the other three F_1 plant categories (Fig. 4c). In addition, when grown on saline soil, leaves of Chang7-2/*$ZmHKT1^{crispr}$-1* plants were yellower and had significantly higher Na^+ contents and $Na^+:K^+$ ratios than did the leaves of the other F_1 plants (Fig. 4d,e). Taken together, these results provide a final confirmation that genetic variation at *ZmHKT1* accounts for the phenotypic variance that constitutes the *ZmNC1* QTL. Accordingly, *ZmHKT1* is referred to as *ZmNC1* for the remainder of this article.

An LTR/Gypsy insertion is associated with the increased leaf Na^+ accumulation and salt hypersensitivity of Chang7-2

We next determined the molecular basis of the functional differences between the Zheng58 and Chang7-2 *ZmHKT1* alleles

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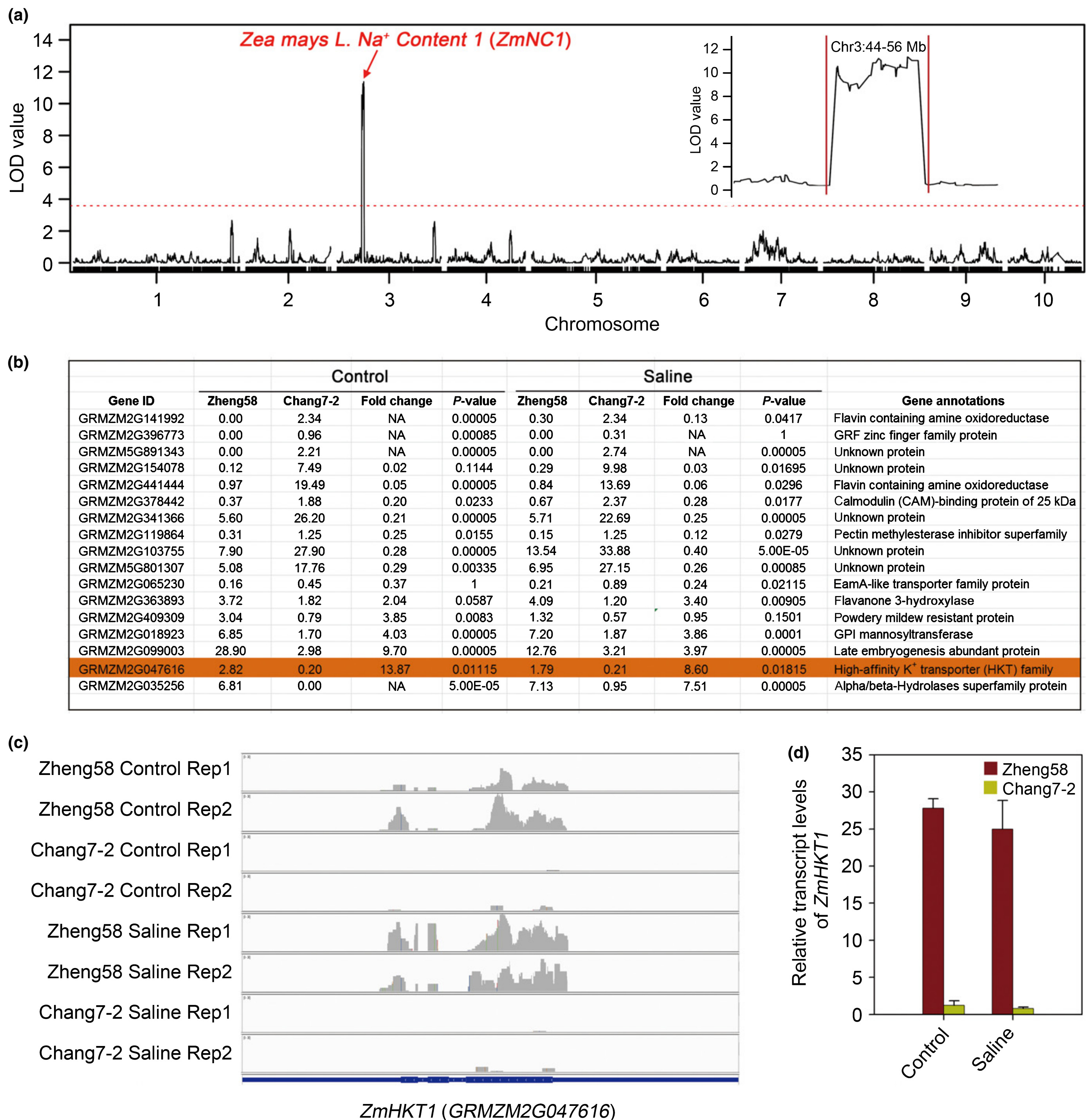

Gene ID	Control				Saline				Gene annotations
	Zheng58	Chang7-2	Fold change	*P*-value	Zheng58	Chang7-2	Fold change	*P*-value	
GRMZM2G141992	0.00	2.34	NA	0.00005	0.30	2.34	0.13	0.0417	Flavin containing amine oxidoreductase
GRMZM2G396773	0.00	0.96	NA	0.00085	0.00	0.31	NA	1	GRF zinc finger family protein
GRMZM5G891343	0.00	2.21	NA	0.00005	0.00	2.74	NA	0.00005	Unknown protein
GRMZM2G154078	0.12	7.49	0.02	0.1144	0.29	9.98	0.03	0.01695	Unknown protein
GRMZM2G441444	0.97	19.49	0.05	0.00005	0.84	13.69	0.06	0.0296	Flavin containing amine oxidoreductase
GRMZM2G378442	0.37	1.88	0.20	0.0233	0.67	2.37	0.28	0.0177	Calmodulin (CAM)-binding protein of 25 kDa
GRMZM2G341366	5.60	26.20	0.21	0.00005	5.71	22.69	0.25	0.00005	Unknown protein
GRMZM2G119864	0.31	1.25	0.25	0.0155	0.15	1.25	0.12	0.0279	Pectin methylesterase inhibitor superfamily
GRMZM2G103755	7.90	27.90	0.28	0.00005	13.54	33.88	0.40	5.00E-05	Unknown protein
GRMZM5G801307	5.08	17.76	0.29	0.00335	6.95	27.15	0.26	0.00085	Unknown protein
GRMZM2G065230	0.16	0.45	0.37	1	0.21	0.89	0.24	0.02115	EamA-like transporter family protein
GRMZM2G363893	3.72	1.82	2.04	0.0587	4.09	1.20	3.40	0.00905	Flavanone 3-hydroxylase
GRMZM2G409309	3.04	0.79	3.85	0.0083	1.32	0.57	0.95	0.1501	Powdery mildew resistant protein
GRMZM2G018923	6.85	1.70	4.03	0.00005	7.20	1.87	3.86	0.0001	GPI mannosyltransferase
GRMZM2G099003	28.90	2.98	9.70	0.00005	12.76	3.21	3.97	0.00005	Late embryogenesis abundant protein
GRMZM2G047616	2.82	0.20	13.87	0.01115	1.79	0.21	8.60	0.01815	High-affinity K^+ transporter (HKT) family
GRMZM2G035256	6.81	0.00	NA	5.00E-05	7.13	0.95	7.51	0.00005	Alpha/beta-Hydrolases superfamily protein

Fig. 2 Identification and molecular characterization of leaf Na^+ content quantitative trait locus (QTL) *Zea mays Na^+ CONTENT 1* (*ZmNC1*). (a) The major QTL for leaf Na^+ content (*ZmNC1*) (red arrow) was mapped to a *c*. 12-Mb region (shown at higher resolution in the inset) on maize chromosome 3. (b) List of genes from within a *c*. 12-Mb region of maize chromosome 3 containing the *ZmNC1* QTL displaying a significant transcript level difference (fold change ≥ 3 and $P < 0.05$) between Zheng58 and Chang7-2 plants grown in control and/or saline conditions. The orange background highlights *GRMZM2G047616*, a gene encoding a High-affinity K^+ Transporter (HKT) family transporter. (c) Integrated Genome Viewer visualization of *GRMZM2G047616* transcript levels (genotypes and treatments as indicated). The coverage scales were set the same (0–30) in all samples. Each sample has two independent biological replicates (Rep1 and Rep2). (d) Real-time PCR analysis of *GRMZM2G047616* transcript levels (genotypes and treatments as indicated). For (c) and (d), 2-wk-old plants were treated with water (control) or 100 mM NaCl (saline), and tissues were collected 24 h following the onset of treatment. Data are means ± SE of three replicates in (d).

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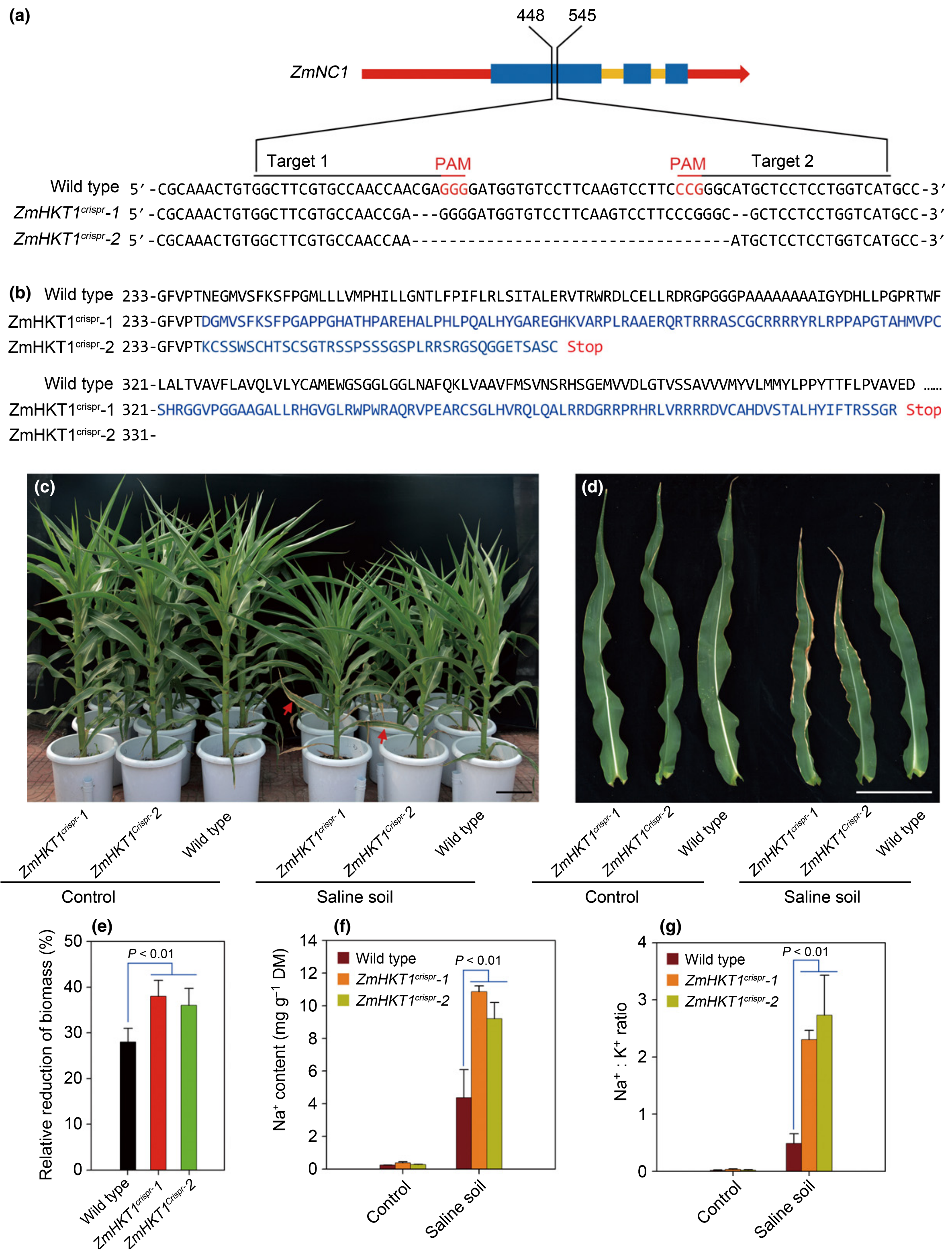

Fig. 3 *ZmHKT1* knockout alleles of maize confer increased sensitivity to soil salinity and increased leaf Na^+ accumulation. (a) Targeted mutagenesis of *ZmHKT1* via CRISPR-Cas9. Protospacer adjacent motif (PAM) sequences are labeled in red. Mutations in two independent lines (*ZmHKT1^{crispr}-1* and *ZmHKT1^{crispr}-2*) are shown. (b) Alignment of the amino acid sequences of ZmHKT1, ZmHKT1^{crispr}-1 and ZmHKT1^{crispr}2. Only the sequences flanking the mutations are shown. The frameshifted sequences in ZmHKT1^{crispr}-1 and ZmHKT1^{crispr}2 are highlighted in blue. (c) Appearance of 8-wk-old *ZmNC1^{crispr}-1*, *ZmNC1^{crispr}-2* and wild-type plants grown in control and saline soil conditions (the wild-type is an inbred line descendent from B73). Red arrows indicate yellowed leaves. (d) Close-up images of 3rd leaves of plants shown in (c). Bar, 15 cm. (e) Biomass of 8-wk-old saline soil-grown plants (expressed as a percentage of control plant biomass). (f) Na^+ content and (g) $Na^+ : K^+$ ratios of the 3rd leaves of plants grown in control and saline soils (genotypes as indicated). Data in (e–g) are means ± SE of three replicates.

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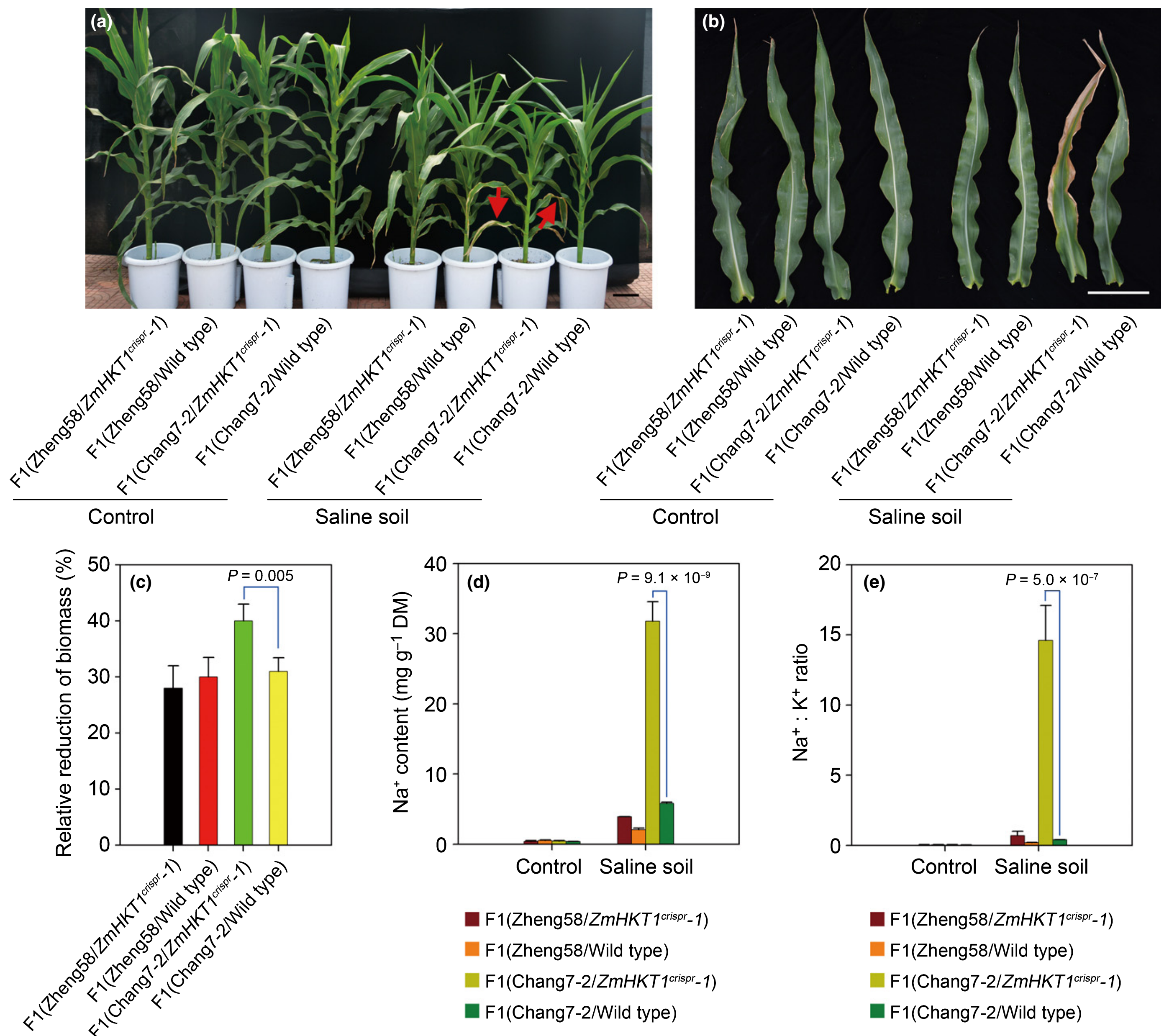

Fig. 4 Genetic variance at *ZmHKT1* accounts for the phenotypic variance of the *ZmNC1* quantitative trait locus (QTL) in maize. (a) Appearance of 8-wk-old plants grown in control or saline soils (genotypes as indicated). Red arrows indicate the yellowed leaves of the loss-of-function allele F_1 hybrid (Chang7-2/*ZmHKT1^{crispr}-1*). (b) Close-up images of the 3rd leaves of plants shown in (a). Bar, 15 cm. (c) Biomass of 8-wk-old saline soil-grown plants (expressed as a percentage of control plant biomass). (d) Na^+ content and (e) $Na^+ : K^+$ ratios of the 3rd leaves of plants grown in control and saline soils. Data in (c–e) are means ± SE of three replicates.

(Fig. 5). Initially, we attempted to amplify the genomic region covering *ZmHKT1*, including 5′- and 3′-UTR regions, using four pairs of primers (F1/R1, F2/R2, F3/R3 and F4/R4; Table S1) (Fig. 5a). We consequently obtained and sequenced the products from all PCR amplifications but one, for which we failed to obtain the F3/R3 product in Chang7-2. In order to obtain the full sequence of Chang7-2 *ZmHKT1*, we reanalyzed the genome sequencing data of Chang7-2 and Zheng58 generated by a previous study (Jiao *et al.*, 2012), and found that the Chang7-2 genome has a large insertion in the F3/R3 region (Fig. 5b). We then designed another two primers (F3-1 and R3-1) based on the sequences of the reads paired with the reads flanking the insertion, but mapped to a distant location or the other chromosome. With these primers, PCR fragments F3/R3-1 and F3-1/R3 were obtained and sequenced (Fig. 5a). By comparing the full sequence of *ZmHKT1* from Zheng58 and Chang7-2 (Table S2), we found that the size of the insertion (1562-InDel) in Chang7-2 is 390 bp, which results in a truncation of the open reading frame (premature stop codon) in *ZmHKT1* (Fig. 5c). In addition, we identified 141 SNP and another 30 InDel variant differences between the DNA sequences of the Zheng58 and Chang7-2 *ZmHKT1* alleles. Fifteen of these variants are nonsynonymous

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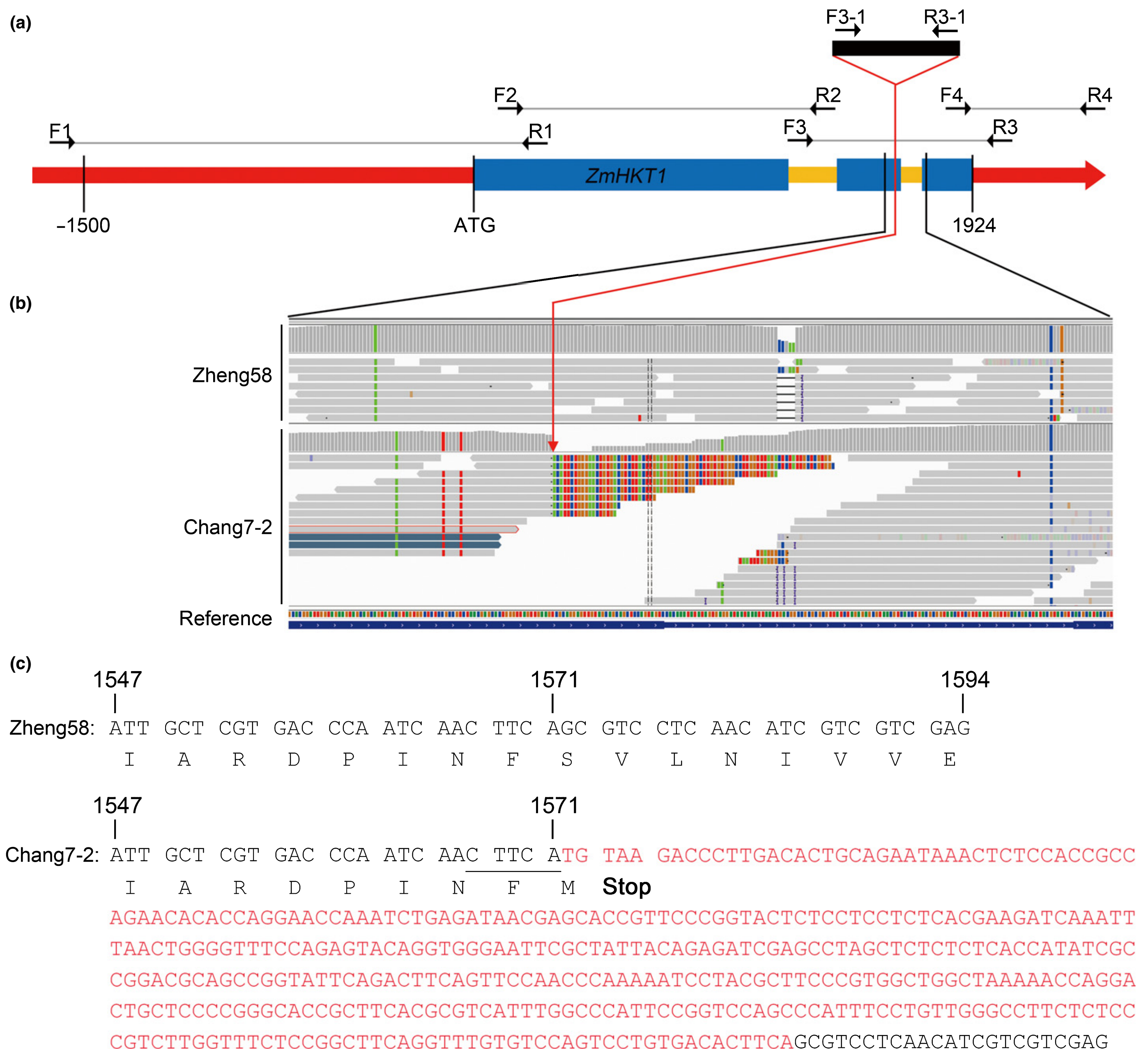

Fig. 5 A 390-bp LTR/Gypsy retrotransposon is inserted into the second exon of *ZmHKT1* in the maize inbred line Chang 7-2. (a) Diagram showing the structure of *ZmHKT1* and the location of the primers used for DNA sequencing. The blue boxes represent exons, yellow boxes represent introns and red boxes represent untranslated regions (UTRs). The black box represents the LTR/Gypsy retrotransposon insertion identified in the Chang7-2 *ZmHKT1* allele. (b) Integrated Genome Viewer visualization of the whole-genome sequencing reads covering the retrotransposon insertion site. The red arrow indicates the site of the insertion. (c) DNA and encoded protein sequences of the Zheng58 and Chang7-2 *ZmHKT1* alleles in the vicinity of the 390-bp LTR retrotransposon insertion. The retrotransposon sequence is shown in red, and the target site duplication is underlined.

coding variants, resulting in amino acid substitutions or deletion of one to two amino acids from the *ZmHKT1* open reading frame (Table S3).

The 390-bp insertion in the Chang7-2 *ZmHKT1* allele (1562-InDel) has a five-nucleotide CTTCA repeated sequence at either end (Fig. 5c), suggesting that it is likely to be a transposon insertion. We therefore screened this insertion sequence using Repeatmasker (http://www.repeatmasker.org), and found that it is an LTR/Gypsy family retrotransposon (Fig. S7). We also blast-screened the maize TE database using the 390-bp DNA insertion sequence as a query, and found that it shows similarity to LTR/Gypsy family retrotransposon *RLG_ewog_AC212715-11905*. We conclude that the 390-bp insertion in the Chang 7-2 *ZmHKT1* allele is an LTR/Gypsy TE insertion. Because the 390-bp insertion causes a truncation of the protein product encoded by *ZmHKT1* (Fig. 5c), and has been identified in the salt-sensitive Chang7-2 plant, but not in the salt-tolerant Zheng58 plant, we therefore propose that this LTR/Gypsy TE insertion abolishes

the function of *ZmHKT1* in Chang7-2, thus causing increased leaf Na^+ accumulation and salt hypersensitivity.

The LTR/Gypsy insertion in *ZmHKT1* is associated with increased leaf Na^+ accumulation in the natural maize population

We next fully characterized the natural genetic variations represented in *ZmHKT1*, and calculated their association with the leaf Na^+ content of natural maize varieties, so as to determine whether the LTR/Gypsy insertion in *ZmHKT1* is associated with the leaf Na^+ content of the natural maize population. In order to do so, we re-sequenced the *ZmHKT1* gene from 190 inbred lines that were randomly selected from our temperate maize population. As shown in Fig. 4(a), a *c.* 3.0-kb genomic region (including the 5′- to 3′-UTR of *ZmHKT1*) was analyzed, and we identified 105 SNPs and 21 InDels with MAF above 5% (Table S4). Then, the associations of these genetic variations with maize shoot Na^+ content were analyzed using Tassel (see the Materials and Methods section). We found that the 390-bp LTR/Gypsy insertion (InDel1562) had the greatest significant association with leaf Na^+ content ($P = 1.02 \times 10^{-4}$; Fig. 6a). Pairwise linkage disequilibrium (LD) calculation of all the identified polymorphisms indicated that InDel1562 and two other variations (InDel-50 and SNP356) were in strong LD ($r^2 > 0.7$; Fig. 6a). However, InDel-50 and SNP356 were not associated with the leaf Na^+ content with statistical significance.

The 190 sequenced maize inbred lines were classified into LTR^- and LTR^+ haplotype groups, based on the presence (LTR^+) or absence (LTR^-) of the LTR/Gypsy insertion (Table S5). We found that the leaf Na^+ contents of the LTR^+ inbred lines ($n = 10$) were significantly higher than those of the LTR^- lines ($n = 180$) ($P = 3.92 \times 10^{-6}$; Fig. 6b), supporting the notion that the 390-bp LTR/Gypsy insertion in *ZmHKT1* is significantly associated with leaf Na^+ over-accumulation and salt hypersensitivity in the natural maize population.

ZmHKT1 is an Na^+-preferential ion transporter

In accord with the likely identity of ZmHKT1 as an HKT-type ion transporter, we found that a ZmHKT1-GFP fusion protein was localized to the plasma membrane of tobacco mesophyll cells (Fig. 7a). Previous studies have shown that HKT-type transporters can be classified into two groups (class I and II) according to their preference for the conductance of Na^+ or K^+ ions (Platten *et al.*, 2006). The main feature distinguishing class I and class II HKTs is the identity of an amino acid in a particular position within the first pore domain (PD1) of the transporter (Mäser *et al.*, 2002a,b; Garciadeblas *et al.*, 2003). The presence of a serine at this position is associated with Na^+ preference (class I),

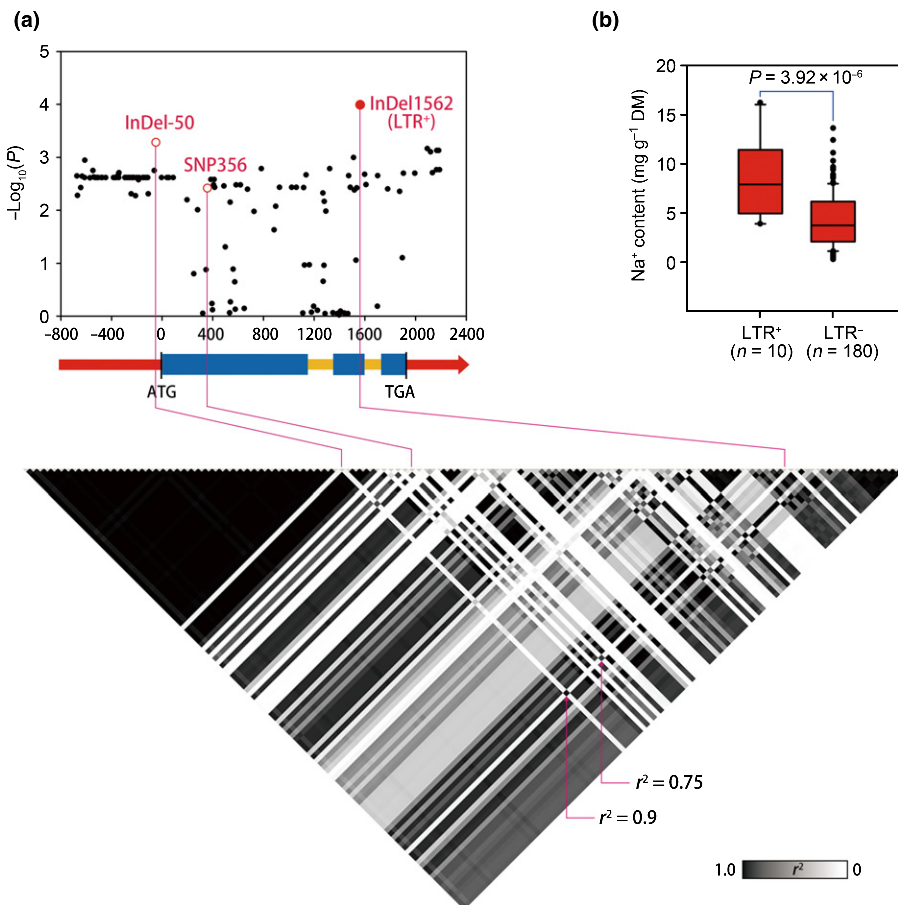

Fig. 6 The association analysis of natural variations in *ZmHKT1* with maize shoot Na^+ content. (a) Characterization of genetic variations associated with shoot Na^+ content, and the pattern of pairwise linear discriminant of the variations. The schematic diagram shows the *c.* 3.0-kb fragment, including the *c.* 800-bp promoter region and *c.* 600-bp 3′-UTR. The initiation codon (ATG) is marked as '+1'. The results indicate that the LTR insertion (InDel1562) shows greatest significant association with the leaf Na^+ content as highlighted with red symbols. The other two variations (InDel-50 and SNP356), which are in strong LD with InDel1562, are connected to their locations in the gene diagram by solid lines. (b) The distribution of shoot Na^+ concentrations of the salt-grown maize inbred lines with (LTR^+) or without (LTR^-) the LTR/Gypsy insertion.

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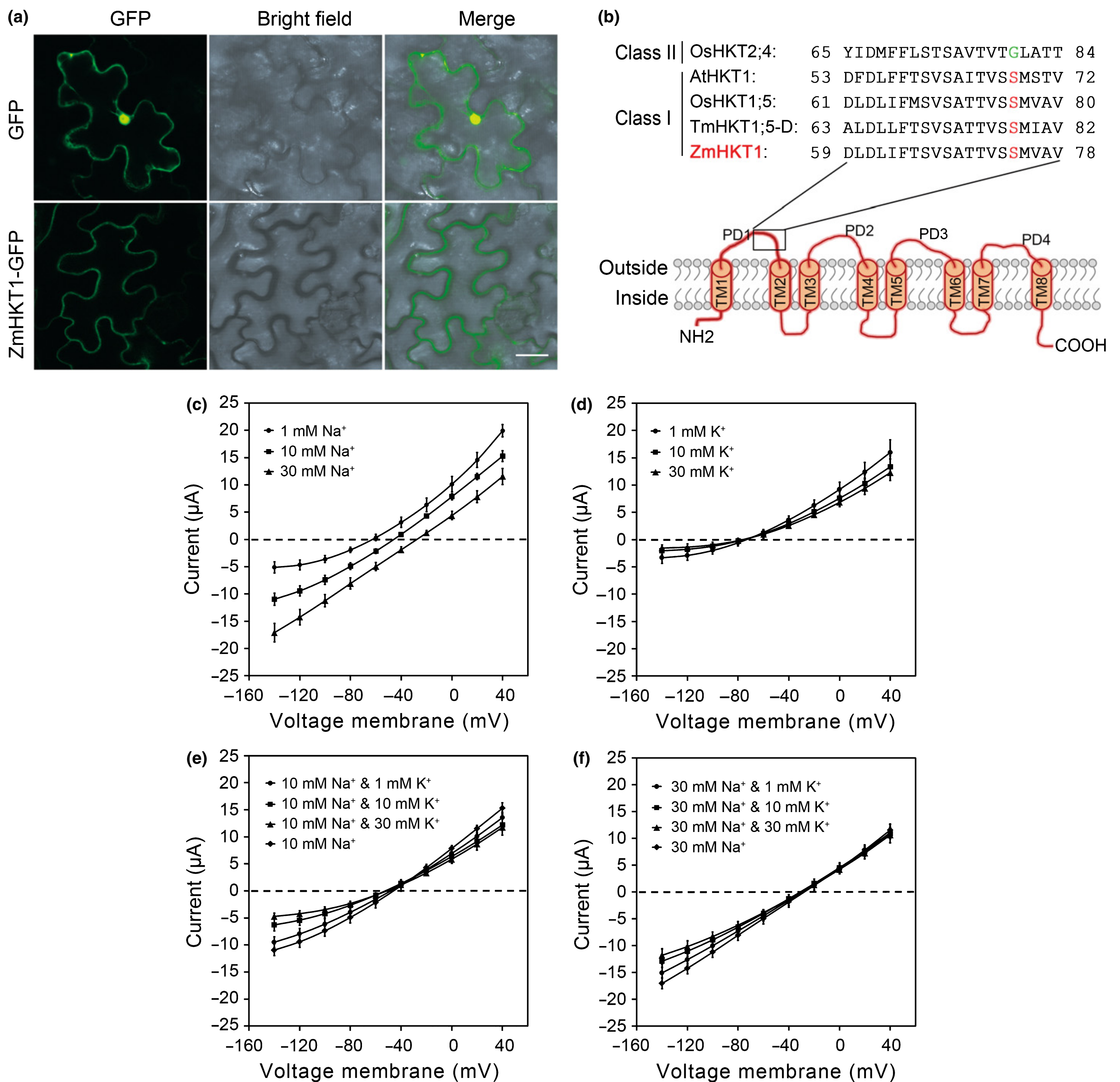

Fig. 7 The maize *ZmHKT1* gene encodes a class I Na^+-preferential HKT family ion transporter. (a) Plasma membrane localization of ZmNC1-GFP in tobacco mesophyll cell. The ZmHKT1-GFP fusion protein is predominantly localized to the plasma membrane. GFP, green fluorescent protein. Bar, 20 μm. (b) Amino acid sequences of the first pore domain (PD1) of class I and class II HKT family ion transporters, together with a diagram modeling the predicted relationship between ZmHKT1 and the plasma membrane (Ren *et al.*, 2005). The serine residue that determines Na^+ selectivity in PD1 is highlighted in red. (c–f) ZmHKT1 is an Na^+-preferential transporter. Oocytes expressing ZmHKT1 were voltage clamped in bath solutions containing different concentrations of (c) Na^+, (d) K^+, (e) 10 mM Na^+ plus different concentrations of K^+ and (f) 30 mM Na^+ plus different concentrations of K^+. Voltages were applied to the membrane with a voltage ranging from 40 to −140 mV. Mean steady-state currents (± SD) were obtained from 15 oocytes in three replicates (with different batches of oocytes).

whereas the presence of a glycine at this position leads to preference for either Na^+ and/or K^+ depending on the concentration of environmental Na^+ and K^+ (class II) (Horie *et al.*, 2007). ZmHKT1 has a serine at this position, indicating that it is a class I HKT-type ion transporter with Na^+ preference (Fig. 7b). In addition, similar to other Na^+-preferential HKT transporters (e.g. AtHKT1 and SKC1; Mäser *et al.*, 2002a,b; Ren *et al.*, 2005), ZmHKT1 has eight putative transmembrane domains (Fig. 7b). We next determined the ion transport properties of the ZmHKT1 protein encoded by the Zheng58 *ZmHKT1* allele

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(*ZmHKT1*^{Zheng58}), by expressing this protein using a *X. laevis* oocyte system and measuring the transport activities using a voltage clamp, as described previously (Liu & Luan, 2001). Significant inward and outward currents were observed for ZmHKT1^{Zheng58} expressing oocytes in the presence of Na^+ (Fig. 7c). However, when external Na^+ was replaced with K^+, no significant 'shifts in the reversal potentials' were detected (Fig. 7d). Meanwhile, although a previous study has suggested that the presence of K^+ can affect the Na^+ affinity of wheat TaHKT1;5-D (Byrt *et al.*, 2014), we found that ZmHKT1-mediated influx and efflux of Na^+ across the oocyte plasma membrane is unaffected (no 'shifts in the reversal potentials') by the presence of external K^+ (Fig. 7e,f). These results confirm the identity of ZmHKT1 as an Na^+-preferential transporter. Ren *et al.* (2015) have previously cloned two alternative splicing variants of *GRMZM2G047616* (designated as *ZmHKT1;1a* and *ZmHKT1;1b*), which have a different gene structure to *ZmHKT1* (Fig. S8a). Intriguingly, although ZmHKT1 is an Na^+-preferential transporter (Fig. 7c), we found that ZmHKT1;1a does not have the activity of transporting Na^+ (Fig. S8b; failed to clone *ZmHKT1;1b*). Therefore, it remains to be elucidated whether ZmHKT1;1a and ZmHKT1;1b can promote the salt tolerance of maize.

ZmHKT1 promotes the exclusion of Na^+ ions from leaves via the withdrawal of Na^+ from xylem sap

We next determined how ZmHKT1 promotes the exclusion of Na^+ ions from leaves, and the resultant salinity tolerance (Fig. 8). Previous studies have suggested that HKT family proteins can promote the exclusion of Na^+ from leaves via two distinct mechanisms. First, Na^+ may be withdrawn from the xylem sap, thus reducing root-to-shoot Na^+ delivery (Sunarpi Horie *et al.*, 2005; Ren *et al.*, 2005; Byrt *et al.*, 2007; Munns *et al.*, 2012). Second, Na^+ may be loaded into the phloem sap, thus promoting the downward shoot-to-root translocation of Na^+ (Berthomieu *et al.*, 2003). We therefore determined the effect of ZmHKT1 on the xylem and phloem sap Na^+ concentrations. We first found that, following the onset of salt treatment, the xylem sap of Zheng58 (with normal ZmHKT1 function) had a lower Na^+ content and $Na^+ : K^+$ ratio than did that of Chang7-2 (lacking ZmHKT1 function) (Fig. 8a,b). We also found that, when grown in saline conditions, the xylem saps of loss-of-function *ZmHKT1*^{crispr}*-1* and *ZmHKT1*^{crispr}*-2* mutant lines displayed higher Na^+ content and Na^+/K^+ ratios than seen in WT controls (an inbred line descendent from B73) (Fig. 8c,d). By contrast, although phloem sap Na^+ contents (expressed as the Na^+ : glutamine ratio) were increased in all plants exposed to increased salinity (Fig. 8e,f), we found that the plants with compromised ZmHKT1 function showed higher (rather than lower) phloem sap Na^+ concentrations under saline conditions (Fig. 8e,f). These results suggest that ZmHKT1 regulates leaf Na^+ content and $Na^+ : K^+$ ratio by promoting the withdrawal of Na^+ from the xylem sap, thus reducing the root-to-shoot flow of Na^+ ions.

The tissue/cell specificity of the expression of *HKT1* family genes in root tissue is important for the withdrawal of Na^+ from the xylem flow (Ren *et al.*, 2005; Møller *et al.*, 2009; Munns *et al.*, 2012). We therefore investigated the tissue/cell specificity of *ZmHKT1* expression in the root of Zheng58 using *in-situ* RT-PCR. We consequently found that the transcript of ZmHKT1 was predominantly detected in the cells comprising the central vascular tissue of root, and were particularly abundant in xylem parenchyma cells (Fig. 8g). As xylem parenchyma cells are an ideal location for ZmHKT1 to withdraw Na^+ ions from the xylem sap, the cell specificity of *ZmHKT1* expression therefore supports the hypothesis that ZmHKT1 regulates leaf Na^+ exclusion by withdrawing Na^+ from the root-to-shoot xylem flow.

Discussion

Maize salt tolerance is a complex trait composed of distinct mechanisms of osmotic, ionic, oxidative and other tolerances (Munns & Tester, 2008; Zhao *et al.*, 2010; Zhu, 2016), making it challenging to identify the various genes that regulate it. In this study, we applied QTL mapping to identify a previously uncharacterized gene regulating maize salt tolerance. QTL mapping is a commonly used strategy for dissecting complex trait loci in maize. For example, QTL mapping has advanced our understanding of the genetic regulation of maize plant architecture (Studer *et al.*, 2011), photoperiod sensitivity (Hung *et al.*, 2012), flowering time (Salvi *et al.*, 2007; Romero Navarro *et al.*, 2017), resistance to head smut (Zuo *et al.*, 2015), drought tolerance and other traits (Mao *et al.*, 2015; Wang *et al.*, 2016). Similarly, in this study, we used QTL mapping and further molecular genetic analysis to discover that the gene *ZmNC1* (*ZmHKT1*) plays a major role in the regulation of maize leaf Na^+ exclusion and salt tolerance (Figs 1–4). As far as we know, *ZmHKT1* is the first discovered QTL gene responsible for the phenotypic variation reflected in a maize salt tolerance QTL. The phenotypic variation reflected in this QTL is apparently caused by the presence of an LTR/Gypsy retrotransposon insertion in *ZmHKT1* (Fig. 5), which leads to the loss of function of *ZmHKT1* (Fig. 4).

Previous forward genetic screen-based studies have identified several factors regulating Na^+ homeostasis and salt tolerance (reviewed by Horie *et al.*, 2009; Zhu, 2016), among which SOS- and HKT-related pathways have been widely studied (Shi *et al.*, 2000; Rus *et al.*, 2001; Qiu *et al.*, 2002; Quan *et al.*, 2007; Lin *et al.*, 2009; Kim *et al.*, 2013; Zhou *et al.*, 2014). We have shown here that *ZmHKT1* encodes a class I HKT-type transporter with Na^+-preferential transport activity (Fig. 7). Intriguingly, previous studies have shown that major wheat and rice salt-tolerance QTLs are also caused by genetic variation in genes encoding Na^+-preferential HKTs. For example, *SKC1* (also known as *OsHKT1;5*; a rice salt-tolerance QTL; Ren *et al.*, 2005), *Nax1* (also known as *TmHKT7-A2*; a durum wheat salt-tolerance QTL) (Huang *et al.*, 2006; Platten *et al.*, 2006), *Nax2* (also known as *TmHKT1;5-A*; another durum wheat salt-tolerance QTL; Munns *et al.*, 2012) and *Kna1* (also known as *TaHKT1;5-D*; a bread wheat salt-tolerance QTL; Byrt *et al.*, 2007) all encode class I Na^+-preferential HKT transporters. Taken together with these previous observations, our discovery of *ZmHKT1* shows that variation in the function of plant Na^+-selective HKT

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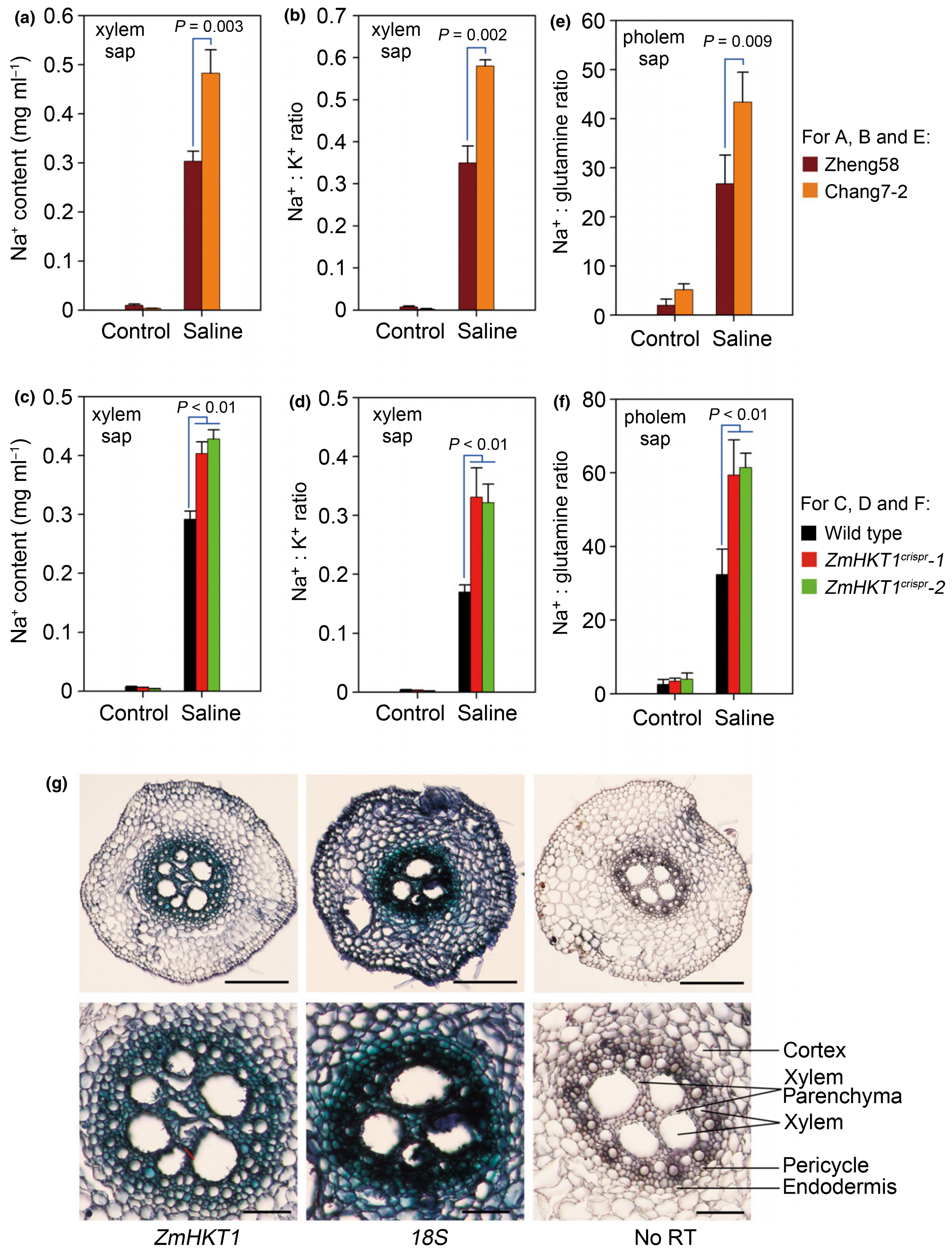

Fig. 8 ZmHKT1 reduces xylem sap Na^+ content in maize plants exposed to saline conditions. (a–d) Xylem sap (a, c) Na^+ contents and (b, d) $Na^+ : K^+$ ratios, as measured in root pressure exudate collected from de-topped hydroponically grown plants (see the Materials and Methods section). Data are means ± SE of three replicates. (e, f) Phloem sap Na^+ contents (expressed as Na^+ : glutamine ratio) of plants with the indicated genotypes and treatments (see the Materials and Methods section). The wild-type is an inbred line descendent from B73. Data are means ± SE of three replicates. (g) The cell-specific expression pattern of *ZmHKT1* visualized via *in-situ* RT-PCR analysis of roots (maturation zone) of 2-wk-old hydroponically grown Zheng58 plants. The blue signal indicates the presence of *ZmHKT1* transcripts, suggesting that *ZmHKT1* is predominantly expressed in root stele, including the parenchyma cells surrounding the xylem vessels. The 18S rRNA (18S) control indicates that all cell types contain cDNA, and the no reverse transcription (No RT) control excludes the possibility of detectable genomic DNA contamination. Bars: upper panels, 200 μm; lower panels, 100 μm.

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transporters confers natural variation in leaf Na^+ exclusion and salt tolerance in all three of the world's major grain crop cereals (rice, wheat and maize), and that Na^+-selective HKT transporters mediate conservative salt-tolerance mechanisms in both C_3 and C_4 plants.

The above-described previous studies have also suggested that functional variation in plant class I HKT transporters can be a result of either a change in ion transport activity of the transporters themselves, or a change in the expression of the genes encoding these transporters (Ren *et al.*, 2005; Munns *et al.*, 2012; Yang *et al.*, 2014). For example, the rice *SKC1* QTL results from a number of nonsynonymous mutations decreasing Na^+ transport activity in the salt-sensitive Koshihikari variety (Ren *et al.*, 2005). By contrast, the wheat *Nax2* QTL is caused by constitutive root tissue expression of the *Triticum monococcum Nax2* gene (Munns *et al.*, 2012). We have shown here that the functional variation at *ZmNC1* (*ZmHKT1*) results from the insertion of a 390-bp LRT/Gypsy retrotransposon into the second exon of *ZmHKT1*, causing this allele to encode a truncated and probably nonfunctional ZmHKT1 protein (Fig. 5), thus causing increased leaf Na^+ accumulation and salt hypersensitivity (Fig. 6). LTR retrotransposons compose > 75% of the maize genome, and exhibit family-specific, nonuniform distributions along chromosomes (Schnable *et al.*, 2009). Although previous studies have indicated that *LTR/Gypsy* elements are over-represented in gene-poor heterochromatic regions (Schnable *et al.*, 2009), our study has revealed a case in which an *LTR/Gypsy* insertion into a gene (*ZmHKT1*) has probably resulted in a change in the functional properties of that gene.

HKT family proteins primarily promote the exclusion of Na^+ from leaves via the withdrawal of Na^+ from root-to-shoot-flowing xylem sap (Ren *et al.*, 2005; Sunarpi Horie *et al.*, 2005; Byrt *et al.*, 2007; Munns *et al.*, 2012). We have shown here that the WT function of ZmNC1 (ZmHKT1) is essential for reducing xylem sap Na^+ concentrations (Fig. 8a–d). Furthermore, we have shown that ZmHKT1 is located within the cellular plasma membrane (Fig. 7a), and that *ZmHKT1* is preferentially expressed in root xylem parenchyma cells (Fig. 8g). These factors mean that ZmHKT1 is ideally localized to withdraw Na^+ from the root xylem sap, thus reducing root-to-shoot flow of Na^+ and promoting shoot Na^+ exclusion. We conclude that ZmHKT1 promotes shoot Na^+ exclusion via the withdrawal of Na^+ from root xylem sap. The proposed working model is consistent with those for rice SKC1 and wheat Nax2 (Ren *et al.*, 2005; Munns *et al.*, 2012), indicating that class I HKT transporters regulate xylem sap Na^+ content, and thus leaf Na^+ exclusion and salt tolerance, in a wide range of species. Previous work has also identified an ethylene and salt stress-inducible mechanism for the regulation of root xylem sap Na^+ content that is dependent on the generation of ROS by a root stele-specific respiratory burst oxidase (Jiang *et al.*, 2012, 2013). It will be interesting to determine whether ROS mediates these effects via the modulation of class I HKT transporter activity.

In conclusion, our QTL approach has discovered *ZmNC1*, a gene regulating maize salt tolerance. *ZmNC1* encodes a plasma membrane-localized class I HKT ion transporter (ZmHKT1) with preference for the Na^+ ion, which is preferentially expressed in root xylem parenchyma cells. ZmHKT1 promotes salt tolerance by the withdrawal of Na^+ from root xylem sap, thus reducing the root-to-shoot flow of Na^+, increasing the exclusion of Na^+ from leaf and shoot and promoting salinity tolerance. An apparent *ZmHKT1* loss-of-function allele in the Chang7-2 variety encodes a truncated and probably nonfunctional ZmHKT1 protein, and confers an increased xylem sap Na^+ content and salt sensitivity. The parallels between our discovery and those of others in other species indicate that HKT-dependent Na^+ exclusion is a major mechanism for salt tolerance in a wide range of plant species. In addition, its importance in the three major large-grain cereals (rice, wheat and maize) emphasizes that variation in HKT function will be an important target for crop breeders in the improvement of cereal salt tolerance. More specifically, *ZmHKT1* identifies a major component of natural variation in maize salt tolerance, and provides an important new route towards the improvement of maize salt tolerance by the use of natural genetic variation.

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Author contributions

M.Z., Y.C., Z.W., Z-q.W., Q.C., J.L. and C.J. planned and designed the research. M.Z., Y.C. and Z-q.W. cloned *ZmHKT1* and carried out the functional analysis. M.Z. and Y.C. measured the ion content and Na^+ and K^+ transport activity of ZmHKT1. Z.W. generated the CRISPR/Cas9 knockout lines of *ZmHKT1*. W.S. generated the Zheng58/Chang 7-2 QTL population. J.S. and X.L. carried out the bioinformatics analysis. M.Z., Y.C. and C.J. wrote the manuscript (the other authors contributed).

References

Baxter I, Brazelton JN, Yu D, Huang YS, Lahner B, Yakubova E, Li Y, Bergelson J, Borevitz JO, Nordborg M *et al.* 2010. A coastal cline in sodium accumulation in *Arabidopsis thaliana* is driven by natural variation of the sodium transporter AtHKT1;1. *PLoS Genetics* **6**: e1001193.

Berthomieu P, Conéjéro G, Nublat A, Brackenbury WJ, Lambert C, Savio C, Uozumi N, Oiki S, Yamada K, Cellier F *et al.* 2003. Functional analysis of AtHKT1 in *Arabidopsis* shows that Na^+ recirculation by the phloem is crucial for salt tolerance. *EMBO Journal* **22**: 2004–2014.

Broman KW, Wu H, Sen S, Churchill GA. 2003. R/qtl: QTL mapping I experimental crosses. *Bioinformatics* **19**: 889–890.

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R. 2007. HKT1;5-like cation transporters linked to Na^+ exclusion loci in wheat, *Nax2* and *Kna1*. *Plant Physiology* **143**: 1918–1928.

Byrt CS, Xu B, Krishnan M, Lightfoot DJ, Athman A, Jacobs AK, Watson-Haigh NS, Plett D, Munns R, Tester M *et al.* 2014. The Na^+ transporter, TaHKT1;5-D, limits shoot Na+ accumulation in bread wheat. *Plant Journal* **80**: 516–526.

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Chen Z, Wang B, Dong X, Liu H, Ren L, Chen J, Hauck A, Song W, Lai J. 2014. An ultra-high density bin-map for rapid QTL mapping for tassel and ear architecture in a large F_2 maize population. *BMC Genomics* **15**: 433.

Deinlein U, Stephan AB, Horie T, Luo W, Xu G, Schroeder JI. 2014. Plant salt-tolerance mechanisms. *Trends in Plant Science* **19**: 371–379.

Farooq M, Hussain M, Wakeel A, Siddique KHM. 2015. Salt stress in maize: effects, resistance mechanisms, and management. A review. *Agronomy for Sustainable Development* **35**: 461–481.

Fortmeier R, Schubert S. 1995. Salt tolerance of maize (*Zea mays* L.): the role of sodium exclusion. *Plant, Cell & Environment* **18**: 1041–1047.

Fricke W, Akhiyarova G, Veselov D, Kudoyarova G. 2004. Rapid and tissue-specific changes in ABA and in growth rate response to salinity in barley leaves. *Journal of Experimental Botany* **55**: 1115–1123.

Gao Y, Lu Y, Wu M, Liang E, Li Y, Zhang D, Yin Z, Ren X, Dai Y, Deng D *et al.* 2016. Ability to remove Na^+ and retain K^+ correlates with salt tolerance in two maize inbred lines seedlings. *Front Plant Science* **7**: 1716.

Garciadeblas B, Senn ME, Banuelos MA, Rodriguez-Navarro A. 2003. Sodium transport and HKT transporters: the rice model. *Plant Journal* **34**: 788–801.

Gramer G. 1994. Response of maize (Zea *mays* L.) to salinity. In: Pessarakli M, ed. *Handbook of plant and crop stress.* New York, NY, USA: Marcel Dekker, 449–459.

Han J, Wang L, Zheng H, Pan X, Li H, Chen F, Li X. 2015. ZD958 is a low-nitrogen-efficient maize hybrid at the seedling stage among five maize and two teosinte lines. *Planta* **242**: 935–949.

Hanks RJ, Ashcroft GL, Rasmussen VP, Wilson GD. 1978. Corn production as influenced by irrigation and salinity—Utah studies. *Irrigation Science* **1**: 47–59.

Horie T, Costa A, Kim TH, Han MJ, Horie R, Leung HY, Miyao A, Hirochika H, An G, Schroeder JI. 2007. Rice OsHKT2;1 transporter mediates large Na^+ influx components into K^+-starved roots for growth. *EMBO Journal* **26**: 3003–3014.

Horie T, Hauser F, Schroeder JI. 2009. HKT transporter-mediated salinity resistance mechanisms in *Arabidopsis* and monocot crop plants. *Trends Plant Science* **14**: 660–668.

Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, Munns R. 2006. A sodium transporter (HKT7) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. *Plant Physiology* **142**: 1718–1727.

Huang X, Feng Q, Qian Q, Zhao Q, Wang L, Wang A, Guan J, Fan D, Weng Q, Huang T *et al.* 2009. High-throughput genotyping by whole-genome resequencing. *Genome Research* **19**: 1068–1076.

Hung HY, Shannon LM, Tian F, Bradbury PJ, Chen C, Flint-Garcia SA, McMullen MD, Ware D, Buckler ES, Doebley JF *et al.* 2012. *ZmCCT* and the genetic basis of day-length adaptation underlying the postdomestication spread of maize. *Proceedings of the National Academy of Sciences, USA* **109**: 1913–1921.

Jiang C, Belfield EJ, Cao Y, Smith JA, Harberd NP. 2013. An *Arabidopsis* soil-salinity-tolerance mutation confers ethylene-mediated enhancement of sodium/potassium homeostasis. *Plant Cell* **25**: 3535–3552.

Jiang C, Belfield EJ, Mithani A, Visscher A, Ragoussis J, Mott R, Smith JA, Harberd NP. 2012. ROS-mediated vascular homeostatic control of root-to-shoot soil Na delivery in *Arabidopsis. EMBO Journal* **31**: 4359–4370.

Jiao Y, Zhao H, Ren L, Song W, Zeng B, Guo J, Wang B, Liu Z, Chen J, Li W *et al.* 2012. Genome-wide genetic changes during modern breeding of maize. *Nature Genetics* **44**: 812–815.

Kim WY, Ali Z, Park HJ, Park SJ, Cha JY, Perez-Hormaeche J, Quintero FJ, Shin G, Kim MR, Qiang Z *et al.* 2013. Release of SOS2 kinase from sequestration with GIGANTEA determines salt tolerance in *Arabidopsis. Nature Communication* **4**: 1820.

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

Lin H, Yang Y, Quan R, Mendoza I, Wu Y, Du W, Zhao S, Schumaker KS, Pardo JM, Guo Y. 2009. Phosphorylation of SOS3-LIKE CALCIUM BINDING PROTEIN8 by SOS2 protein kinase stabilizes their protein complex and regulates salt tolerance in *Arabidopsis. Plant Cell* **21**: 1607–1619.

Liu K, Luan S. 2001. Internal aluminum block of plant inward K^+ channels. *Plant Cell* **13**: 1453–1465.

Mao H, Wang H, Liu S, Li Z, Yang X, Yan J, Li J, Tran LS, Qin F. 2015. A transposable element in a NAC gene is associated with drought tolerance in maize seedlings. *Nature Communication* **6**: 8326.

Mäser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N *et al.* 2002a. Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na^+ transporter AtHKT1. *FEBS Letters* **531**: 157–161.

Mäser P, Hosoo Y, Goshima S, Horie T, Eckelman B, Yamada K, Yoshida K, Bakker EP, Shinmyo A, Oiki S *et al.* 2002b. Glycine residues in potassium channel-like selectivity filters determine potassium selectivity in four-loop-per-subunit HKT transporters from plants. *Proceedings of the National Academy of Sciences, USA* **99**: 6428–6433.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M *et al.* 2010. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Research* **20**: 1297–1303.

Møller IS, Gilliham M, Jha D, Mayo GM, Roy SJ, Coates JC, Haseloff J, Tester M. 2009. Shoot Na^+ exclusion and increased salinity tolerance engineered by cell type-specific alteration of Na^+ transport in Arabidopsis. *Plant Cell* **21**: 2163–2178.

Munns R. 1993. Physiological processes limiting plant growth in saline soils: some dogmas and hypotheses. *Plant, Cell & Environment* **16**: 15–24.

Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M *et al.* 2012. Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. *Nature Biotechnology* **30**: 360–364.

Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**: 651–681.

Pardo JM, Cubero B, Leidi EO, Quintero FJ. 2006. Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. *Journal of Experimental Botany* **57**: 1181–1199.

Platten JD, Cotsaftis O, Berthomieu P, Bohnert H, Davenport RJ, Fairbairn DJ, Horie T, Leigh RA, Lin HX, Luan S. 2006. Nomenclature for HKT transporters, key determinants of plant salinity tolerance. *Trends Plant Science* **2006**: 372–374.

Qiu QS, Guo Y, Dietrich MA, Schumaker KS, Zhu JK. 2002. Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *Proceedings of the National Academy of Sciences, USA* **99**: 8436–8441.

Quan R, Lin H, Mendoza I, Zhang Y, Cao W, Yang Y, Shang M, Chen S, Pardo JM, Guo Y. 2007. SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect *Arabidopsis* shoots from salt stress. *Plant Cell* **19**: 1415–1431.

R Development Core Team. 2014. *R: a language and environment for statistical computing.* [WWW document] URL http://www.R-project.org. Vienna, Austria: R Foundation for Statistical Computing.

Ray DK, Mueller ND, West PC, Foley JA. 2013. Yield trends are insufficient to double global crop production by 2050. *PLoS ONE* **8**: e66428.

Ren Z, Liu Y, Kang D, Fan K, Wang C, Wang G, Liu Y. 2015. Two alternative splicing variants of maize HKT1;1 confer salt tolerance in transgenic tobacco plants. *Plant Cell, Tissue and Organ Culture* **123**: 569–578.

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX. 2005. A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics* **37**: 1141–1146.

Romero Navarro JA, Willcox M, Burgueño J, Romay C, Swarts K, Trachsel S, Preciado E, Terron A, Delgado HV, Vidal V *et al.* 2017. A study of allelic diversity underlying flowering-time adaptation in maize landraces. *Nature Genetics* **49**: 476–480.

Rus A, Yokoi S, Sharkhuu A, Reddy M, Lee BH, Matsumoto TK, Koiwa H, Zhu JK, Bressan RA, Hasegawa PM. 2001. AtHKT1 is a salt tolerance determinant that controls Na^+ entry into plant roots. *Proceedings of the National Academy of Sciences, USA* **98**: 14150–14155.

Salvi S, Sponza G, Morgante M, Tomes D, Niu X, Fengler KA, Meeley R, Ananiev EV, Svitashev S, Bruggemann E *et al.* 2007. Conserved noncoding genomic sequences associated with a flowering time quantitative trait locus in

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maize. *Proceedings of the National Academy of Sciences, USA* **104**: 11376–11381.

Schnable JC. 2015. Genome evolution in maize: from genomes back to genes. *Annual Review of Plant Biology* **66**: 329–343.

Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton L, Graves TA *et al.* 2009. The B73 maize genome: complexity, diversity, and dynamics. *Science* **326**: 1112–1115.

Schubert S, Neubert A, Schierholt A, Sümer A, Zörb C. 2009. Development of salt-resistant maize hybrids: the combination of physiological strategies using conventional breeding methods. *Plant Science* **177**: 196–202.

Shi H, Ishitani M, Kim C, Zhu JK. 2000. The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na^+/H^+ antiporter. *Proceedings of the National Academy of Sciences, USA* **97**: 6896–6901.

Song W, Wang B, Hauck AL, Dong X, Li J, Lai J. 2016. Genetic dissection of maize seedling root system architecture traits using an ultra-high density bin-map and a recombinant inbred line population. *Journal of Integrative Plant Biology* **58**: 266–279.

Studer A, Zhao Q, Ross-Ibarra J, Doebley J. 2011. Identification of a functional transposon insertion in the maize domestication gene *tb1*. *Nature Genetics* **43**: 1160–1163.

Sunarpi Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K, Konomi M *et al.* 2005. Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. *Plant Journal* **44**: 928–938.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28**: 511–515.

Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, Tsuboi A, Bakker EP, Nakamura T, Schroeder JI. 2000. The Arabidopsis *HKT1* gene homolog mediates inward Na^+ currents in *Xenopus laevis* oocytes and Na^+ uptake in *Saccharomyces cerevisiae*. *Plant Physiology* **122**: 1249–1259.

Wang R, Jing W, Xiao L, Jin Y, Shen L, Zhang W. 2015. The rice high-affinity potassium transporter1;1 is involved in salt tolerance and regulated by an MYB-type transcription factor. *Plant Physiology* **168**: 1076–1090.

Wang X, Wang H, Liu S, Ferjani A, Li J, Yan J, Yang X, Qin F. 2016. Genetic variation in *ZmVPP1* contributes to drought tolerance in maize seedlings. *Nature Genetics* **48**: 1233–1241.

Xing HL, Dong L, Wang ZP, Zhang HY, Han CY, Liu B, Wang XC, Chen QJ. 2014. A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology* **14**: 327.

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH. 2006. A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. *Cell* **125**: 1347–1360.

Yang C, Zhao L, Zhang H, Yang Z, Wang H, Wen S, Zhang C, Rustgi S, von Wettstein D, Liu B. 2014. Evolution of physiological responses to salt stress in hexaploid wheat. *Proceedings of the National Academy of Sciences, USA* **111**: 11882–11887.

Zhang M, Smith JA, Harberd NP, Jiang C. 2016. The regulatory roles of ethylene and reactive oxygen species (ROS) in plant salt stress responses. *Plant Molecular Biology* **91**: 651–659.

Zhao KF, Song J, Fan H, Zhou S, Zhao M. 2010. Growth response to ionic and osmotic stress of NaCl in salt-tolerant and salt-sensitive maize. *Journal of Integrative Plant Biology* **52**: 468–475.

Zhou H, Lin H, Chen S, Becker K, Yang Y, Zhao J, Kudla J, Schumaker KS, Guo Y. 2014. Inhibition of the *Arabidopsis* salt overly sensitive pathway by 14-3-3 proteins. *Plant Cell* **26**: 1166–1182.

Zhu JK. 2003. Regulation of ion homeostasis under salt stress. *Current Opinion in Plant Biology* **6**: 441–445.

Zhu JK. 2016. Abiotic stress signaling and responses in plants. *Cell* **167**: 313–324.

Zuo W, Chao Q, Zhang N, Ye J, Tan G, Li B, Xing Y, Zhang B, Liu H, Fengler KA *et al.* 2015. A maize wall-associated kinase confers quantitative resistance to head smut. *Nature Genetics* **47**: 151–157.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Close-up image of 8-wk-old Zheng58 and Chang7-2 plants grown in control and saline soil conditions.

Fig. S2 Na^+ and K^+ contents of salt-grown Zheng58/Chang7-2 recombinant inbred lines (RILs).

Fig. S3 Identification of the major maize quantitative trait locus (QTL) regulating $\log_{10}(Na^+ : K^+)$.

Fig. S4 Identification of leaf K^+ content quantitative trait loci (QTLs) *ZmKC1* and *ZmKC2*.

Fig. S5 The phylogenetic tree for maize, Arabidopsis, rice and wheat HKT proteins.

Fig. S6 Alignment of the amino acid sequences of ZmHKT1, Arabidopsis AtHKT1, rice OsHKT1;5 and wheat TmHKT1;5.

Fig. S7 The 390-bp insertion (1562-InDel) in the Chang7-2 *ZmNC1* allele is an LTR/Gypsy retrotransposon insertion.

Fig. S8 Comparison of the characteristics of ZmHKT1, ZmHKT1;1a and ZmHKT1;1b.

Table S1 List of the primers used in this study

Table S2 Sequences of *ZmHKT1* in Zheng58 and Chang7-2

Table S3 The variations distinguished in *ZmHKT1* in Zheng58 and Chang7-2

Table S4 List of natural genetic variations identified from 190 maize inbred lines

Table S5 Leaf Na^+ content and genotypes (presence or absence of the LTR insertion) of 190 maize inbred lines

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