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Salinity tolerance in soybean is modulated by natural variation in *GmSALT3*

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SUMMARY

The identification of genes that improve the salt tolerance of crops is essential for the effective utilization of saline soils for agriculture. Here, we use fine mapping in a soybean (*Glycine max* (L.) Merr.) population derived from the commercial cultivars Tiefeng 8 and 85–140 to identify *GmSALT3* (salt tolerance-associated gene on chromosome 3), a dominant gene associated with limiting the accumulation of sodium ions (Na^+) in shoots and a substantial enhancement in salt tolerance in soybean. *GmSALT3* encodes a protein from the cation/H^+ exchanger family that we localized to the endoplasmic reticulum and which is preferentially expressed in the salt-tolerant parent Tiefeng 8 within root cells associated with phloem and xylem. We identified in the salt-sensitive parent, 85–140, a 3.78-kb copia retrotransposon insertion in exon 3 of *Gmsalt3* that truncates the transcript. By sequencing 31 soybean landraces and 22 wild soybean (*Glycine soja*) a total of nine haplotypes including two salt-tolerant haplotypes and seven salt-sensitive haplotypes were identified. By analysing the distribution of haplotypes among 172 Chinese soybean landraces and 57 wild soybean we found that haplotype 1 (H1, found in Tiefeng 8) was strongly associated with salt tolerance and is likely to be the ancestral allele. Alleles H2–H6, H8 and H9, which do not confer salinity tolerance, were acquired more recently. H1, unlike other alleles, has a wide geographical range including saline areas, which indicates it is maintained when required but its potent stress tolerance can be lost during natural selection and domestication. *GmSALT3* is a gene associated with salt tolerance with great potential for soybean improvement.

Keywords: *Glycine max* (L.) Merr., *Glycine soja* Sieb. et Zucc, salt tolerance, natural variation, salt exclusion, haplotype, geographical distribution, CHX20, CHX.

INTRODUCTION

Excessive dissolved salts in soils can limit crop yield (Munns and Tester, 2008). Salt-affected soils currently account for 8% of the world's total land area (FAO, 2000), and the area of salt-affected agricultural land is predicted to double by 2050 for irrigated agriculture and some semi-arid areas (Pitman and Läuchli, 2002; Rengasamy, 2006). The area of salt-affected irrigated land, which produces 40% of the world's food, already stands at 20% (Pimentel *et al.*, 2004). In light of the predicted 70–110% increase in food production that will be needed by 2050 to feed the rapid growth in global population over the same period (Tilman *et al.*, 2011), and with no current option for expanding the area of agricultural land, an increase in the salt tolerance of conventional crops will be required to assist in improving crop productivity and food security (Yamaguchi and Blumwald, 2005).

Soybean (*Glycine max*) is a major agricultural crop that is used widely for providing human and animal food because of its high oil and protein content (18 and 38%, respectively) (Singh, 2010). Soybean is classified as a moderately salt-sensitive crop (Munns and Tester, 2008). In one study soybean production was reduced by 40% with increasing salinity stress (from 2 to 7 dS m^{-1}; 1 dS m^{-1} = 700 mg L^{-1}) (Papiernik *et al.*, 2005). However, soybean germplasm has been shown to display a

spectrum of salt-tolerance phenotypes (Phang *et al.*, 2008), with salt-sensitive cultivars encountering a 37% lower yield than tolerant cultivars under saline field conditions (Parker *et al.*, 1983). This natural variation in salt tolerance indicates there is potential for identifying genes that increase soybean production under saline conditions. However, there is limited detailed information on the mechanisms that impart this variation in salt tolerance in soybean. The primary lead is a major quantitative trait locus (QTL) related to salt tolerance that has been repeatedly mapped within a region of soybean linkage group N (chromosome 3) (Lee *et al.*, 2004; Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011; Ha *et al.*, 2013). We have previously mapped a dominant gene associated with improved salt tolerance to the same region using three F_2 populations derived from the following crosses between salt-tolerant and salt-sensitive varieties: Wenfeng 7 × Union, Jindou 33 × Hark, and Tiefeng 8 × Zaoshu 6 (Zhang, 2005). Recently, by using a $F_{2:3}$ population derived from the salt-tolerant variety Tiefeng 8 and the salt-sensitive variety 85–140, we mapped the dominant salt-tolerance gene from Tiefeng 8 to a 209-kb region on soybean chromosome 3 (Guan *et al.*, 2014).

In this study, we use map-based cloning to identify the causal gene underlying this salt-tolerance locus. We found that the dominant gene associated with salt tolerance (*GmSALT3*, *Glycine max* salt tolerance-associated gene on chromosome 3) was expressed predominantly in roots within vascular-associated cells and encoded a protein from the cation/H^+ exchanger family that we localized to the endoplasmic reticulum (ER). Expression of *GmSALT3* was associated with lower sodium accumulation in shoots and a significantly greater salt tolerance. We genotyped and phenotyped a combined total of over 200 soybean landraces and wild soybean originating from across China to identify nine haplotypes for this gene. The tolerant haplotypes were found to have a wider geographical range but were most commonly found close to salinized areas, whereas the salt-sensitive haplotypes predominantly originated from areas that are not known for high salt concentrations in soils. This coupled with the low genetic diversity of the salt-tolerant alleles suggests that *GmSALT3* is under strong selection pressure when required but can be lost when this pressure is removed resulting in a decrease in the salt tolerance of the plant. The identification of *GmSALT3*, which encodes a cation/proton exchanger family member, is likely to have significance for soybean and other crop breeding programmes.

RESULTS

Map-based cloning of the salt-tolerance associated gene in soybean variety Tiefeng 8

We constructed a population of 367 recombinant inbred lines (RILs) derived from the F_2 population of a cross between the salt-tolerant variety Tiefeng 8 and the salt-sensitive variety 85–140 (Figure 1a). The dominant gene associated with the salt-tolerance phenotype was mapped between indel markers QS1101 and QS100011 on chromosome 3 (Figure 1b), and was named *GmSALT3*. To fine map the *GmSALT3* locus, we self-pollinated the F_5 plants heterozygous between indel markers QS1101 and QS100011, and planted the $F_{5:6}$ population (5769 individuals) in the winter of 2010 on Hainan Island, China. Seventy-four recombinants between QS1101 and QS100011 were identified, and we determined their salt tolerance phenotypes in 2011 and 2012 by progeny testing. We obtained five plants containing recombinants between two markers QS100001 and QS1119. Two recombinants exhibited salt sensitivity and two exhibited salt tolerance, while line 3751 was segregating for the phenotype. The progeny testing of the recombinant plants allowed the locus *GmSALT3* to be mapped to a 17.5-kb region between indel marker QS100001 and cleaved amplified polymorphic sequence marker QS1119 (Figure 1c, Table S1 in Supporting Information). There was only one gene predicted to be present within this 17.5-kb region according to the soybean reference genome that has been obtained from the variety Williams 82. This gene, *Glyma03g32900.1*, was regarded as the candidate causal gene underlying *GmSALT3*. To explore whether variation in the salt-tolerance phenotype was due to a difference in the coding sequence of *Glyma03g32900.1*, RNA sequencing (RNA-seq) of two RNA pools consisting of either 20 salt-sensitive or 20 salt-tolerant F_6 plants derived from the cross between 85–140 and Tiefeng 8 was performed. The results indicated that the *GmSALT3* cDNA obtained from Tiefeng 8 was 2640 bp in length, consisting of an open reading frame (ORF) of 2436 bp with 5′ and 3′ untranslated regions (UTR) of 50 and 204 bp, respectively, whilst the ORF from 85 to 140 was 1131 bp (Figure S1a). After comparing the genomic sequence in the two parents it was apparent that there was a 3.78-kb fragment inserted in exon 3 of *Gmsalt3* in 85–140, comprising of long terminal repeats (LTRs) of length 643 and 647 bp that had 99.1% similarity to each other. The element was flanked with a 5-bp target-site duplication sequence (CATGG) and reverted 2-bp repeat (TG … CA) (Figure 1d). This resulted in a truncated *Gmsalt3* transcript in 85–140 yielding only 376 amino acids (Figure S1b).

Identity and expression of *GmSALT3*

Basic local alignment search tool (BLASTX, NCBI, http://blast.ncbi.nlm.nih.gov) (translated) analysis of the GmSALT3 cDNA sequence showed that GmSALT3 shared 73% identity with an uncharacterized protein annotated as a K^+/H^+ antiporter (MTR_7g099820) from *Medicago truncatula* and 59% identity with the characterized *Arabidopsis thaliana* AtCHX20 (Figure 2a). Accordingly, GmSALT3 had a confidently predicted 'sodium/proton exchanger' (NHE) domain (Pfam00999, *e*-value of 2.9e-69), a diagnostic of cation/proton exchangers in plants (Chanroj *et al.*, 2011),

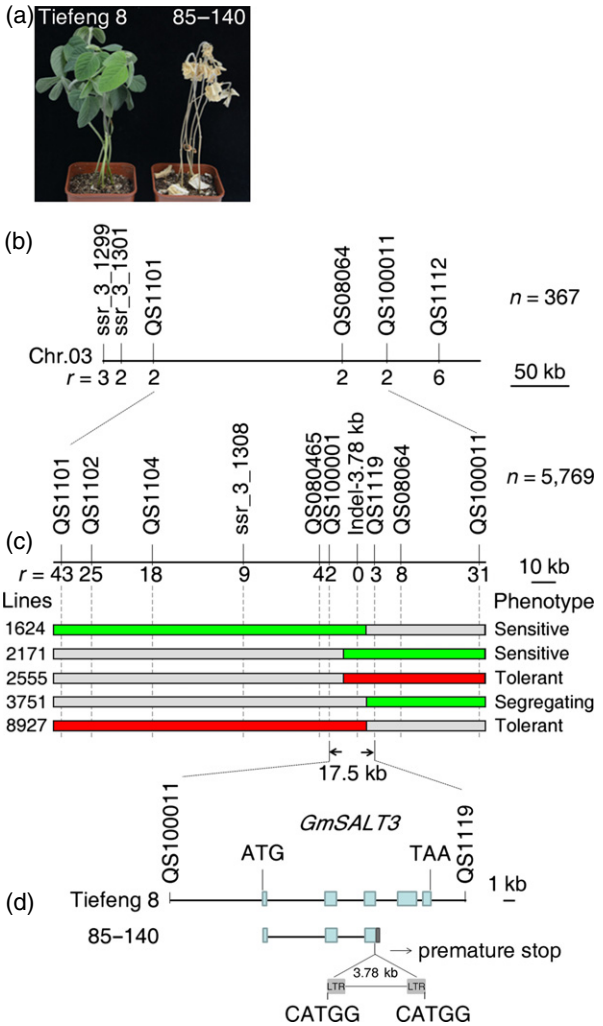


Figure 1. Positional cloning of *GmSALT3* in soybean.
(a) The phenotype of Tiefeng 8 (left) and 85–140 (right) treated with 200 mM NaCl for 18 days.
(b) *GmSALT3* was first mapped on chromosome 3 between indel markers QS1101 and QS100011 using 367 F_6 recombinant inbred lines. R is the number of recombinants containing the marker.
(c) Chromosomal constitutions of five recombinants (lines 1624, 2171, 2555, 3571 and 8927) are shown with their salt tolerance. Positional cloning narrowed the *GmSALT3* locus to a 17.5-kb region between QS100001 and QS1119, and only one gene is predicted to be located in this region. Red and green represent homozygous Tiefeng 8 and 85–140, respectively, grey represents heterozygous.
(d) Sequencing revealed a 3.78-kb copia retrotransposon insertion in the coding region of the respective gene in 85–140; this insertion resulted in a truncated transcript in 85–140 that we called *Gmsalt3*.

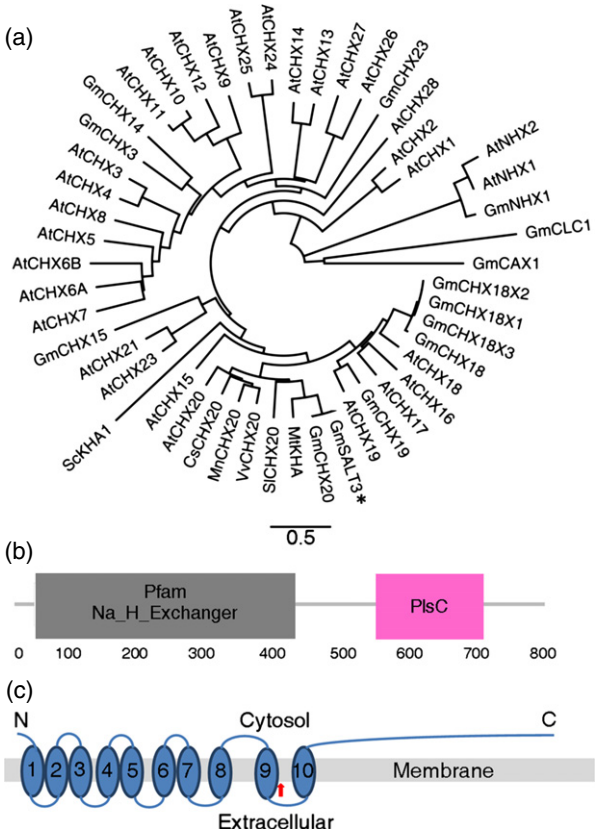


Figure 2. Sequence analysis of *GmSALT3*.
(a) Phylogenetic tree based on multiprotein sequence alignment of *GmSALT3* to Arabidopsis CHX proteins and other related proteins. Multiprotein sequence alignment (Geneious Alignment) was achieved using Geneious Pro version 5 (Drummond *et al.*, 2011). All the protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/protein/). At, *Arabidopsis thaliana*; Sc, *Saccharomyces cerevisiae*; Vv, *Vitis vinifera*; Cs, *Cucumis sativus*; Gm, *Glycine max*; Sl, *Solanum lycopersicum*; Mn, *Morus notabilis*; Mt, *Medicago truncatula*. GmCHX20* indicates CHX20 from the sequenced soybean cultivar Williams 82. The scale bar denotes the scale of amino acid substitutions.
(b) Structural schematics of soybean GmSALT3 according to SMART analysis (Schultz *et al.*, 2000); two predicted domains are observed in the GmSALT3 protein sequence. PlsC is an outlier homologue defined as a phosphate acyltransferase.
(c) Predicted topology of GmSALT3 in the membrane using TMHMM version 2.0 (Möller *et al.*, 2001). Putative transmembrane domains are numbered (N-best algorithm). The arrow indicates the beginning of truncation of GmSALT3. N, N-terminus; C, C-terminus.

that started near the N-terminus between amino acids 30 and 428 as well as 10 predicted transmembrane domains (TMDs) (Figures 2b,c and S1b). The truncation of GmSALT3 (at amino acid 370) lies between TMD 9 and TMD 10, which would result in the loss of the TMD 10 domain and the C-terminus.

We investigated the expression pattern of *GmSALT3* by quantifying the relative abundance of the mRNA in different organs of Tiefeng 8 and 85–140. The expression was much higher in roots than shoots of Tiefeng 8, while the transcripts were not detectable in tissues of 85–140 (Figure 3a). After 6 h of 200 mM salt treatment the transcript abundance of *GmSALT3* decreased in Tiefeng 8; however, after 3 days it recovered to higher levels (Figure 3a). Using *in situ* PCR, as shown in Figure 3, we detected the expression of *GmSALT3* predominantly within endodermal cells and cells associated with phloem and xylem of salt-tolerant Tiefeng 8 soybean root (Figure 3b,c) and within proto-phloem cells in young secondary root

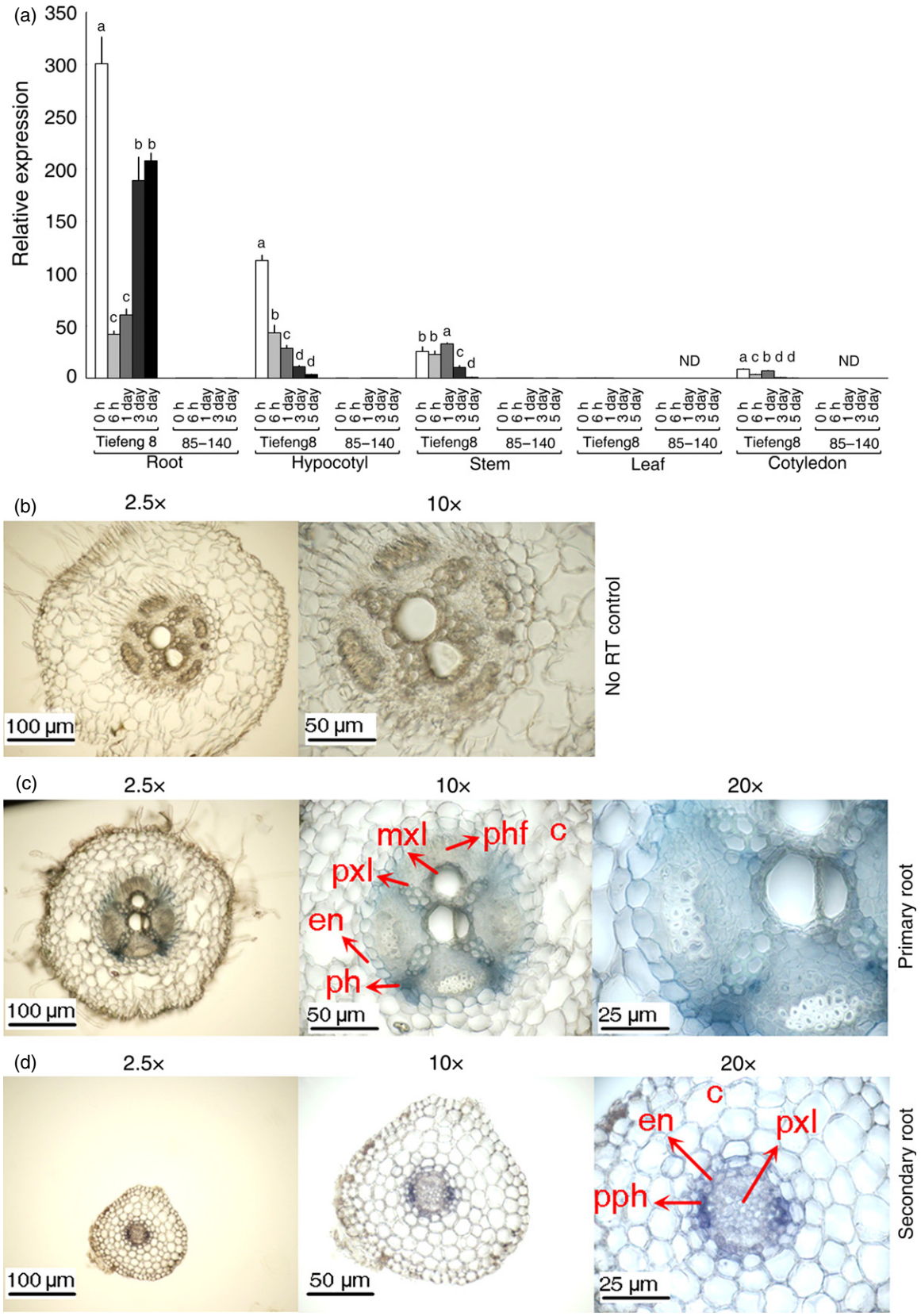


Figure 3. *GmSALT3* expression and tissue localization.
(a) Expression of *GmSALT3* analysed using quantitative real-time PCR (qRT-PCR) in root, hypocotyl, stem, leaf and cotyledon tissue of Tiefeng 8 and 85–140. The numbers (0, 6 h; 1, 3, 5 days) indicate time points after growing plants under control or salt stress (200 mM NaCl). Transcription levels was calculated as a percentage of the *GmUKN1* transcript. Error bars indicate standard deviations ($n = 3$). Different letters indicate significant differences between treatments for a given organ according to Dunan's multiple range test at $P < 0.05$.
(b) *In situ* PCR in sections (60 μm) of roots of a 4-week-old Tiefeng 8 soybean plant grown in 50/50 perlite/vermiculite with no salt treatment. Blue-stained cells are where transcripts are present. Negative controls (b) without RT (reverse transcription) were included to show lack of genomic DNA contamination.
GmSALT3 is localized in phloem- and xylem-associated primary root cells (c) and in protophloem of secondary root tissues (d). c, cortex; ph, phloem; mxl, meta-xylem; phf, phloem fibre; en, endodermis; pxl, protoxylem; pph, protophloem.

(Figure 3d); the localization was unchanged under salinity treatment (Figure S2). In stems and leaves, *GmSALT3* shared a similar expression pattern as in the root (Figure S3). In order to corroborate cell localization we cloned the putative promoter of *GmSALT3* and expressed *GmSALT3-pro::β-glucuronidase* in *A. thaliana*; GUS was detected mainly in vascular tissues of root, hypocotyls and leaves (Figure S4).

It is noteworthy that the expression pattern of *GmSALT3* was different from that of *AtCHX20*, the closest functionally characterized homolog of *GmSALT3*, expressed in stomatal guard cells (Padmanaban *et al.*, 2007). However, as was found for AtCHX20 (Chanroj *et al.*, 2011), we observed an endomembrane (ER) localization for the GmSALT3 protein. This was initially determined by transiently expressing *P35S:GmSALT3-GFP* (green fluorescent protein) in *Nicotiana benthamiana* leaves with more than 90% of the protoplasts expressing the construct displaying clear tubular and sheet-like structures (Figure 4a). This contrasts with the expression of free GFP where more than 90% of the protoplasts displayed cytosolic and nuclear fluorescence (Figure 4d). To further assess the localization, *GmSALT3-GFP* was co-expressed with mCherry with an ER retention sequence (Figure 4g). As shown in Figure 4(i), GFP signals overlapped with the mCherry signals, consistent with GmSALT3-GFP being localized to the ER, whereas cytosolic GFP did not overlap with ER-localized mCherry (Figure 4f). Furthermore, N- and C-terminal *YFP* (yellow fluorescent protein) fusions of *GmSALT3* were transiently expressed in Arabidopsis mesophyll protoplasts and were found to co-localize with a fluorescent ER marker not a late endosomal/vacuolar marker (Figure S5).

Collectively, these results indicate that *GmSALT3* is likely to encode an ER-localized protein that is expressed in vasculature-associated cells, predominantly within roots.

GmSALT3 confers sodium exclusion in shoots

Salt tolerance in soybean is associated with limiting Na^+ accumulation in shoots (Liu *et al.*, 2011; Jiang *et al.*, 2013). To investigate the role of *GmSALT3*, the Na^+ accumulation within the two parents was compared. The Na^+ content in roots of the parents was similar, but following NaCl treatment (200 mM), Tiefeng 8 accumulated significantly less Na^+ than 85–140 in both stems (after 5 days) and leaves (after 7 days) (Figure 5a). To compare the function of the two *GmSALT3*/*Gmsalt3* alleles, we developed a pair of near isogenic lines (NILs) NIL-T (*GmSALT3*) and NIL-S (*Gmsalt3*). Both NIL-T and NIL-S were derived from a single F_6 plant of a cross between 85–140 and Tiefeng 8 that was heterozygous for the *GmSALT3* locus. These lines had no polymorphism among 147 simple sequence repeat (SSR) markers distributed throughout the genome except for those located within the *GmSALT3* locus. Under control conditions, the NILs had no significant difference in agronomic traits, such as 100-seed weight, protein and oil content, but had differential salt tolerance (Figure S6). The Na^+ content in stems and leaves of self-grafted NIL-S was much higher than that in NIL-T (Figure 5b). When the NIL-S scion was grafted on the NIL-T rootstock, the Na^+ content in stems and leaves decreased by 48.7 and 70.65%, respectively, compared with the self-grafted NIL-S. In contrast, the Na^+ content in stems and leaves of the NIL-T scion grafted to the NIL-S root increased by 79.0 and 139.1%, respectively, compared with self-grafted NIL-T (Figure 5b). These results suggest that *GmSALT3* is likely to function in the root (and hypocotyl) and constrain Na^+ translocation to the lamina; this is consistent with the predominant expression pattern of *GmSALT3* in Tiefeng 8 in roots and hypocotyls (Figure 3a).

Geographical distribution of haplotypes reveals that the salt-tolerant H1 is a likely target of natural and artificial selection

To identify allelic variation, the coding region of the *GmSALT3* locus from 31 soybean landraces was sequenced. Five haplotypes (H) were observed in these accessions including the haplotypes found in Tiefeng 8 (H1) and 85–140 (H2) (Table S2). Of the newly identified haplotypes, when compared with H1, H3 had nine non-synonymous SNPs, H4 had seven non-synonymous SNPs and an 18-bp deletion in exon 3 due to a nucleotide substitution (AG to AT) that was 3′ of the intron 2 splicing site and H5 had a 4-bp deletion in exon 2 that resulted in a premature stop codon (Figure 6a). The promoter region starting 540-bp upstream of the start codon was also sequenced; this which identified eight SNPs and three indels of 1, 4 and either 148 or 150 bp. For the fixed variation between the salt-tolerant and salt-sensitive haplotypes, two insertions of 148 and 4 bp were observed in the promoter

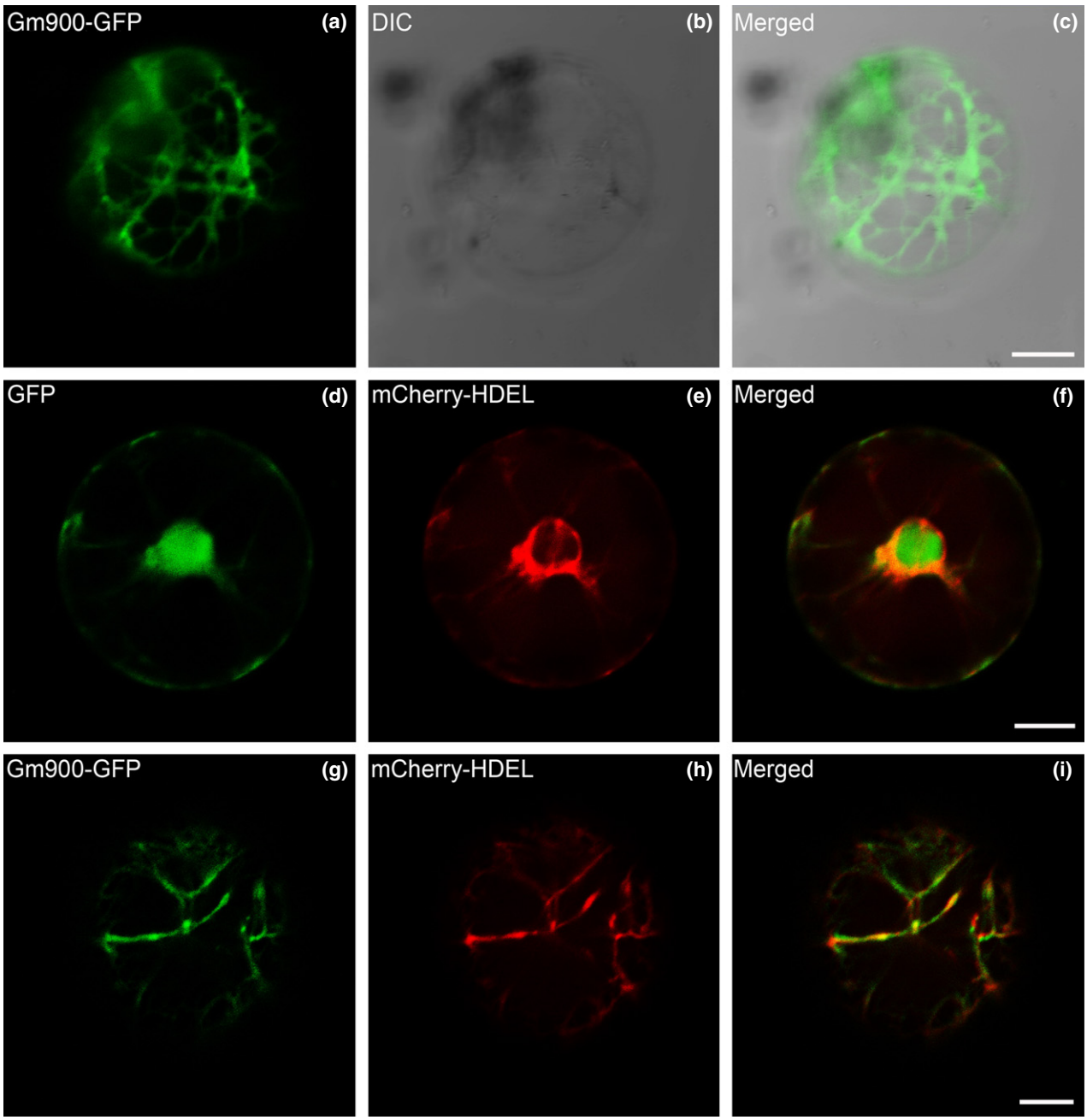


Figure 4. Subcellular localization of GmSALT3.
(a–c) Subcellular localization of GmSALT3 in *Nicotiana benthamiana* protoplasts. Confocal microscopy images of GmSALT3-GFP expression. DIC, differential interference contrast.
(d–f) Co-expression of free GFP and mCherry-HDEL (an endoplasmic reticulum marker).
(g–i) Co-expression of GmSALT3-GFP and mCherry-HDEL. Bars = 10 μm.

region 152 and 103 bp before the start codon, respectively, in H3 and H4, whilst insertions of 150 and 4 bp were identified at those same locations in the promoter region of H5 (Figure 6a).

Using this sequence information we developed a set of haplotype-specific markers to genotype 172 soybean landraces from the Chinese soybean minicore collection (this included the 31 sequenced accessions used above) (Tables S1 and S3). The soybean minicore collection was selected to represent the maximum genetic diversity of Chinese soybean landraces and has been successfully used to study natural variation in the domestication-related gene *GmTfl1* (Tian *et al.*, 2010). The salt sensitivity of these landraces was screened four times from 2009 to 2011

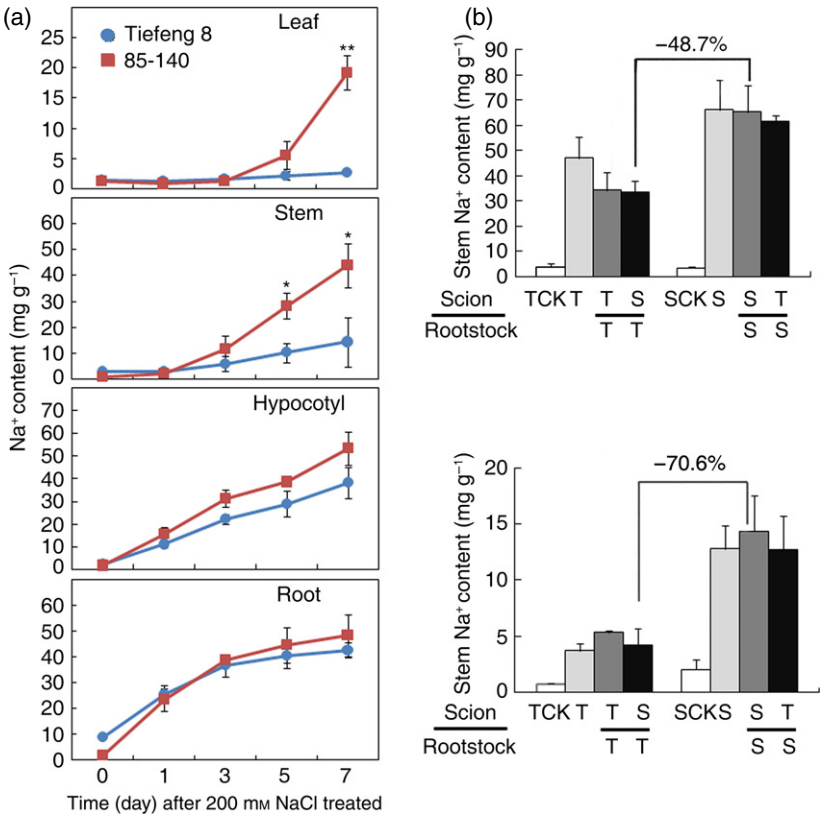
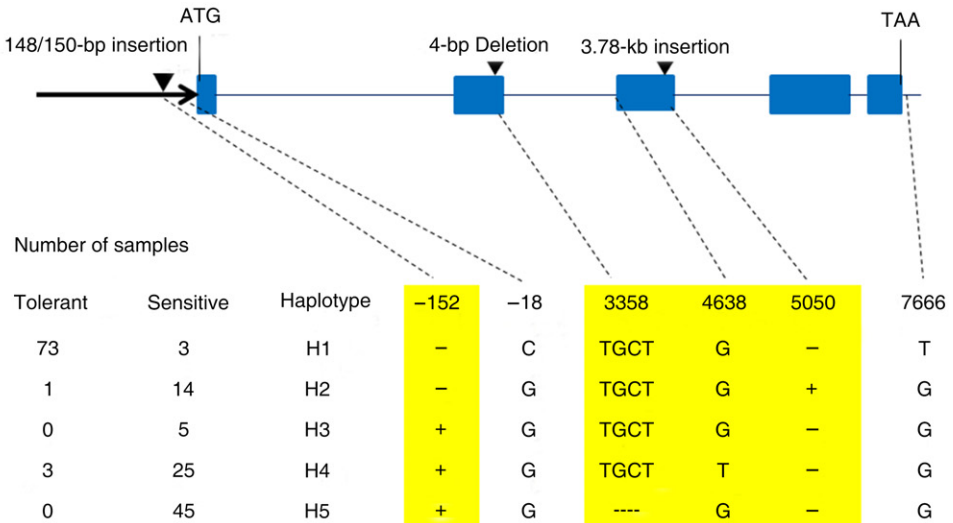


Figure 5. Variation in Na^+ accumulation related to the presence of *GmSALT3* or *Gmsalt3*.
(a) The Na^+ content in Tiefeng 8 (blue) and 85–140 (red) under control or NaCl stress (200 mM NaCl) for 0, 1, 3, 5 or 7 days. Data are means of three replicates ± SE. Asterisks indicates a significant difference between Tiefeng 8 and 85–140 at $*P < 0.05$, $**P < 0.01$.
(b) The Na^+ content in the stem and leaf of non-grafted, self-grafted and reciprocally grafted lines NIL-T (*GmSALT3*) and NIL-S (*Gmsalt3*) under salt stress for 8 days. TCK and SCK: NIL-T and NIL-S, respectively, under control conditions. Data are means of three replicates ± SE.

(Table S2). Of the 172 representative accessions, 73 out of the 76 salt-tolerant accessions shared H1; 14 salt-sensitive and 1 salt-tolerant landraces contained H2; 5 salt-sensitive accessions contained H3; 25 salt-sensitive and 3 salt-tolerant accessions contained H4; and 45 salt-sensitive accessions contained H5. Three heterozygous landraces were excluded from further analyses. We analysed the geographical distribution of the five haplotypes represented in this minicore collection. Haplotype 1 was observed in the three main growing ecoregions (Tian *et al.*, 2010), and was mostly distributed in the northern eco-region (NR) and the Huang-Huai ecoregion (HR); most H2-containing accessions originated from the northern eco-region (NR), H3 was distributed mainly in the southern eco-region (SR); H4 and H5 were observed in the SR and Huang-Huai ecoregion (HR), but were mainly found in SR (Figure 6b,c).

To further examine the relationship between salt tolerance and the *GmSALT3*/*Gmsalt3* alleles, we sequenced 22 wild soybean (*Glycine soja* Sieb. et Zucc.) that differed in their salt tolerance. Four of the haplotypes observed in soybean landraces were found in wild soybean but not H2, and four new haplotypes (H6–H9) were found. Compared with H1, H6 had a 21-bp deletion in exon 5, and H8 and H9 had three different non-synonymous SNPs from that of H3 (Figure 7a). Haplotype-specific markers were used to genotype the 57 wild soybean (including the 22 that we had sequenced). As we observed for the soybean landraces, H1 was mainly in salt-tolerant germplasm (Figure S7a,b). In addition the two wild accessions containing H7 were both salt tolerant, whilst the other haplotypes were predominantly found in salt-sensitive wild accessions (Figure S7a,b, Table S4). In wild soybean, H1 and H7 were mainly distributed in the NR and HR regions, H3 was seen only in the SR, H4 was present in both NR and SR regions and H5 and wild soybean-specific H6, H8 and H9 haplotypes were mainly present in SR. This suggests that the distribution of haplotypes in landraces and wild soybean plants were similar (Figure 7b). As annotated in Figure 7(b), most of the saline soil in China is distributed in four main areas: the eastern coast of China including Jiangsu, Shandong, Hebei and Liaoning provinces; the North China plain, the north-east Songnen plain and the inland region of north-east China. There are also isolated saline fields with a scattered distribution south of the Yangtze River, within Zhejiang, Fujian and Guangdong provinces (Wang, 1993; Tang and Qiao, 2008; Yang, 2008). The coincidence of salt-affected soils and the salt-tolerant H1 and H7 haplotypes indicates that these alleles are likely to be a major selection factor determining the distribution and utilization of soybean especially on saline soils. Consistent with this hypothesis is the significant association observed between those wild soybean accessions that contain H1 and H7 and their proximity to the known saline-affected regions compared with those that

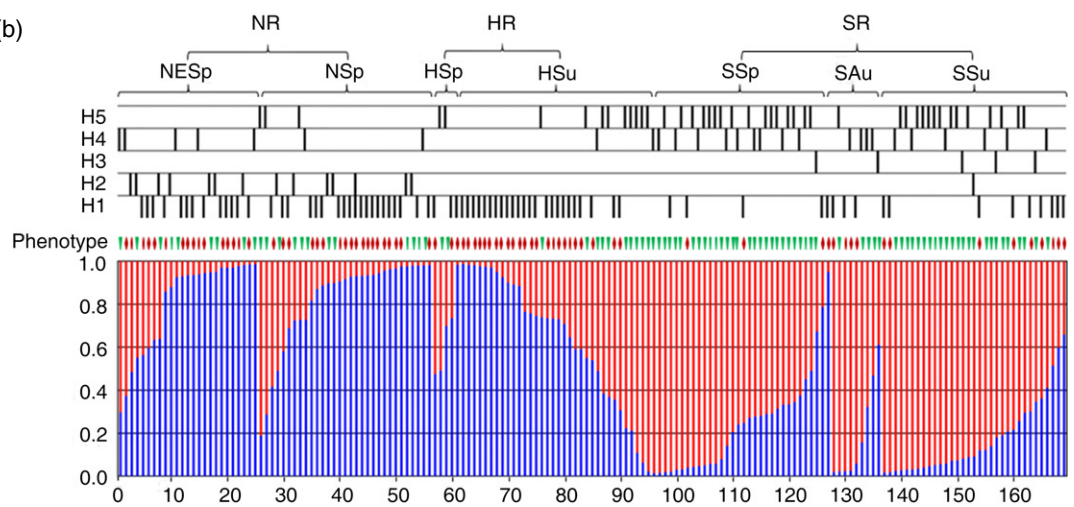
(a)



Number of samples

Tolerant	Sensitive	Haplotype	−152	−18	3358	4638	5050	7666
73	3	H1	−	C	TGCT	G	−	T
1	14	H2	−	G	TGCT	G	+	G
0	5	H3	+	G	TGCT	G	−	G
3	25	H4	+	G	TGCT	T	−	G
0	45	H5	+	G	----	G	−	G

(b)



(c)

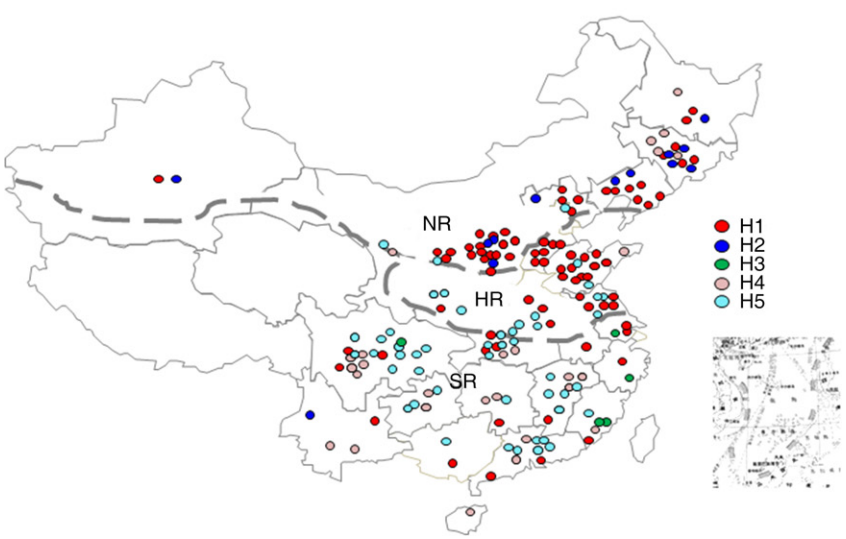


Figure 6. Distribution of five haplotypes of the *GmSALT3* gene in a soybean minicore collection from different ecoregions of China.
(a) Five haplotypes in the 172 minicore collection of Chinese soybean landraces and their relationship with salt tolerance. +/−, with or without the insertion in the promoter or exon region.
(b) Genetic structure of populations based on data from 30 simple sequence repeat markers distributed on 20 soybean chromosomes. The length of each coloured segment indicates the attribution of ancestry of each accession. The distribution of each haplotype in ecoregions and phenotypes is indicated by red diamonds and green down triangles and is shown above the plot of population structure. NR, northern ecoregion, including the north-east spring subregion (NESp) and the north spring subregion (NSp); HR, Huang-Huai region, including the Huang-Huai spring subregion (HSp) and the Huang-Huai summer subregion; SR, southern ecoregion, including the south spring subregion (SSp), south summer subregion (SSu) and south autumn subregion (SAu).
(c) Geographic distribution of five haplotypes (H1–H5) in three soybean growing ecoregions (NR, HR and SR) in China.

contain the other haplotypes (Figure S7c) (Wilcoxon–Mann–Whitney test, *P*-value 0.000001).

To seek further evidence that the H1 haplotype has been under selection we analysed the nucleotide diversity of H1–H5 in the landraces within a non-coding region (1267 bp of intron 2) and found the genetic diversity of this introgenic region in H1 (π = 0.00035) was only 5.5% of that in the sensitive haplotypes H3–H5 (π = 0.00632). Furthermore, we examined the genetic diversity of 18 SNPs within 12 genes on chromosome 3 within 194 kb of the *GmSALT3* gene (Figure S8a). The collective genetic diversity in H1 landraces ranged from 0 to 0.1142, with an average of 0.0341, while the diversity in salt-sensitive haplotypes (H2–H5) ranged from 0.0435 to 0.4999, with an average of 0.3250. In contrast, when we analysed the genetic diversity of randomly selected regions of the genome away from *GmSALT3* we found that diversity was similar across all haplotypes, being 0.2428 in H1 and 0.2689 in H2–H5 (Figure S8b). Taken together, we conclude that the low genetic diversity of salt-tolerant haplotypes is likely to be a result of severe selection pressure.

To examine the relatedness of the nine haplotypes and to determine which haplotype is the likely ancestral allele in soybean we again compared their sequence, their geographical distribution and their salt-tolerance phenotypes. The salt-tolerant H1 is the most frequently found haplotype in both wild soybean and landraces, and it has the widest geographical range. The other salt-tolerant haplotype, H7, which was found only twice and only in wild soybean is identical to H1 except for one non-synonymous SNP, whilst H2 was identical to H7 except for the 3.78-kb copia retrotransposon insertion (Figure 7a). Furthermore, both H2 and H7 were found predominantly in the NR (Figures 6c and 7b). By comparing nucleotide polymorphisms within the 540-bp promoter sequences we observed that H1, H2 and H7 shared similar variation and H2 and H7 shared exactly the same sequence, as did H3, H4 and H8, and H5, H6 and H9 (Figure 7a). Collectively, this suggests that H2 was derived from H7 during or after domestication, and H7 was derived from H1. The other six salt-sensitive haplotypes were separated from H1 and H7 by a series of mutation events but shared a fixed variation of a 148/150-bp and 4-bp insertion at the promoter region, indicating that these variations come from a common haplotype that was not detected in the 57 wild soybean used in this study (Figure 7c,d). To explore how these fixed variations in the promoter region affect gene expression we examined *GmSALT3* transcript abundance in the soybean cultivars Mayibao (containing H3) and Jinshanchamoshidou (containing H4) and found that the expression of *GmSALT3* in the roots of these two soybean cultivars was significantly lower than that of Tiefeng 8 under both control and salt-stressed conditions (Figure S8c,d).

To examine if the relationship between salt tolerance and the salt-tolerant alleles held in germplasm introduced from the United States we examined the genotype and phenotype of 12 further soybean accessions including the sequenced Williams 82 (Schmutz *et al.*, 2010). Several formerly reported salt-tolerant accessions, including Lee 68, Forrest and Hartwig, had the H1 haplotype, whilst the sensitive accessions Clark and Williams 82 contained the H2 haplotype (Table S2) (Lee *et al.*, 2008; Valencia *et al.*, 2008).

DISCUSSION

Map-based cloning has been widely used for identifying genes that modulate the salt tolerance of rice, wheat and Arabidopsis, for example *SOS1–3* and *HKT1* (Deinlein *et al.*, 2014). We can now add *SALT3* from soybean to this list. In total, nine haplotypes for this gene were uncovered here, five from landraces and eight from wild soybean. The well-described bottlenecks that occurred during soybean domestication are the likely cause of the fewer haplotypes in landraces compared with wild relatives (Hyten *et al.*, 2006). Similarly, cultivated rice, which like soybean is another species to undergo self-fertilization, contained only 27–32% of the haplotype diversity of wild rice (Londo *et al.*, 2006). Furthermore, our study identified minimal genetic variation between the two salt-tolerant alleles (H1/H7) compared with the more extensive variation in the seven salt-sensitive alleles (H2–H6, H8, H9), with H6–H9 only being found in wild soybean. The observation that H1 is strongly associated with salt tolerance in both landraces and wild soybean indicated that the *GmSALT3* gene is a major salt-tolerance locus in the two species of the *Glycine* genus. This is consistent with both species sharing the major salt-tolerance QTL (Lee *et al.*, 2004; Hamwieh and Xu, 2008; Hamwieh *et al.*, 2011; Ha *et al.*, 2013). Whilst we cannot rule out additional haplotypes, because we surveyed the larger collections of 172 landraces and 57 wild soybean

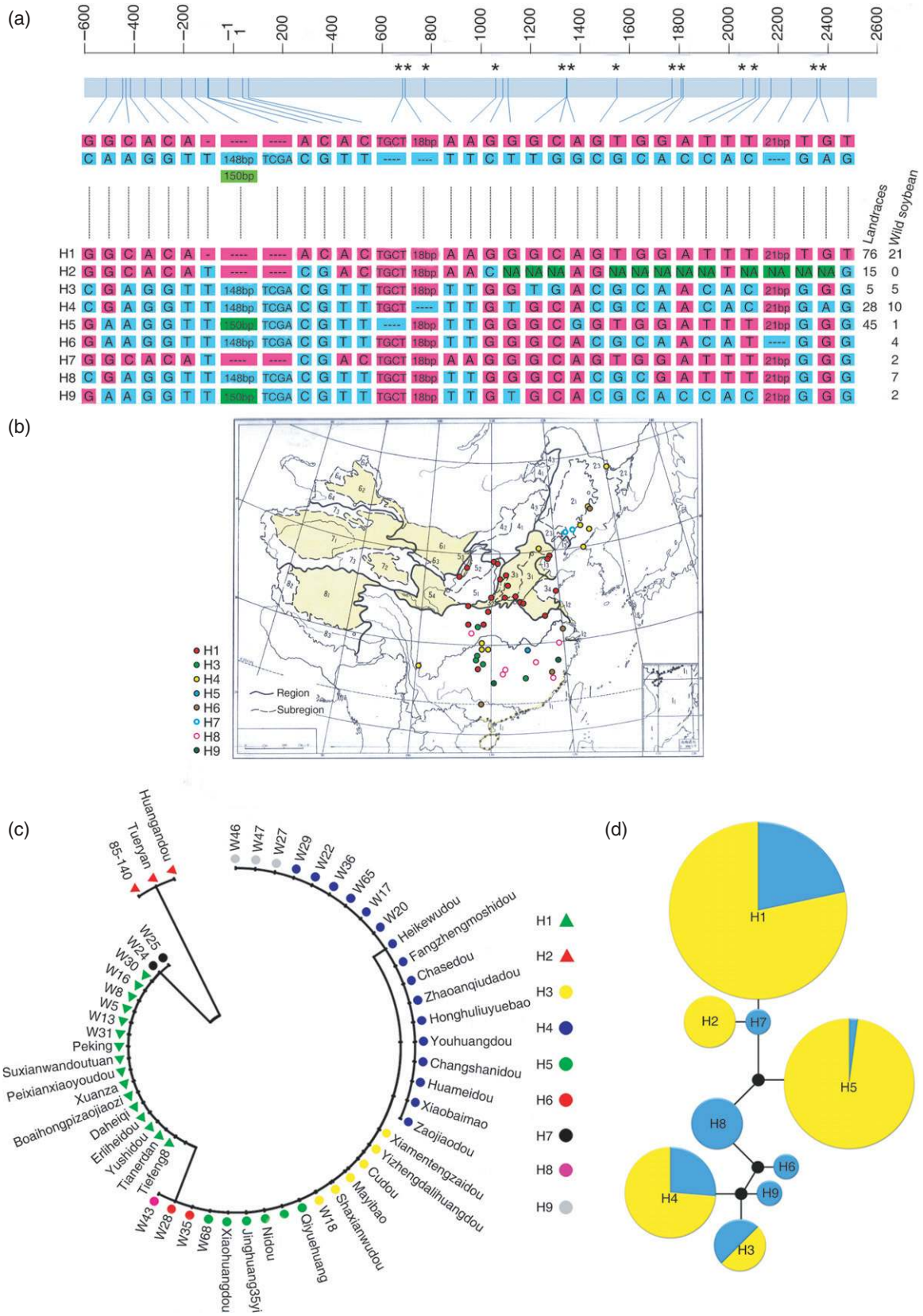


Figure 7. Variation analysis of *GmSALT3* in landraces and wild soybean and haplotype distribution.
(a) Distribution of DNA polymorphisms in the 540-bp promoter region and coding region among 31 landraces and 22 wild soybean. Blue and green indicate the nucleotide difference with Tiefeng 8. The asterisk indicates amino acid change. NA, not detected.
(b) Geographical distribution of eight haplotypes in wild soybean. The regions of saline-affected soil were obtained from Wang (1993) and are presented in yellow on the map.
(c) Phylogenetic tree of 31 landraces and 22 wild soybean based on the polymorphic sites of the *GmSALT3* coding region. Three major groups were identified, one is mainly for the tolerance alleles H1 and H7, the second is for H2, another is mainly for the sensitive alleles H3–H5, H8 and H9.
(d) Haplotype network of the *GmSALT3* coding region in landraces and wild soybean. Circle size is proportional to the number of samples within a given haplotype, and black spots represent unobserved, inferred haplotypes. Lines between haplotypes represent mutational steps between alleles. Yellow, landraces; blue, wild soybean.

with haplotype-specific primers we see no reason why the trend of greater variation in the salt-sensitive alleles should not continue. Several of the accessions of both the landraces and wild soybean were discovered to be heterozygous for *GmSALT3*/*Gmsalt3*. This may have been caused by the bulk analysis of plants with the same morphological traits (Wang *et al.*, 2014). By analysing individuals we could clarify the frequency of each allele, and this information could serve as a further valuable resource for tracing the evolutionary course of the *GmSALT3* gene.

By integrating genotype and phenotype information we have found a clear relationship between H1 and salt tolerance (Figure 6, Tables S2 and S3). The high frequency of H1, its co-occurrence with salt-affected soils, the low genetic diversity of salt-tolerant alleles and the high genetic diversity in the salt-sensitive alleles (Figures 6 and 7a, Tables S2 and S3) are all hallmarks that the H1 allele has been strongly favoured during natural and/or artificial selection. Equivalent attributes of an allele under selection (for pod shattering) have recently been reported in soybean (Dong *et al.*, 2014). Similarly, metal-tolerance genes were found to be responsible for the adaptation of *Arabidopsis lyrata* to serpentine soils (Turner *et al.*, 2010). Furthermore, the observation of a higher frequency of H1 in landraces and wild soybean and the low genetic diversity around this locus indicated that H1 in landraces is a beneficial haplotype; a similar observation was made for the *Accord* insertion associated with DDT resistance in *Drosophila melanogaster* (Schlenke and Begun, 2004). In contrast, the fixed alleles in modern varieties at *tb1* and *SHAT1-5* occurred from new mutations rather than standing ones (Studer *et al.*, 2011; Dong *et al.*, 2014).

We hypothesize that once the selection pressure was released (i.e. when the plants encountered low concentrations of salt in the soil) *GmSALT3* was no longer sensitive to acquiring genetic change, including insertions and deletions, as it resulted in no detrimental effect on the plant phenotype in the low-salt environment. However, the resulting mutations have led to a loss, or reduced function, of the gene product, resulting in a loss of salt tolerance (Figure 5). This greater mutation rate in the salt-sensitive alleles may indicate that the loss of function of *GmSALT3* confers a growth advantage for these plants on non-saline soils. However, we cannot yet confirm this is the case as we detected no growth or yield advantage in the NIL-S lines compared with the NIL-T line when grown under control conditions for the agronomic traits we tested (Figure S5a,b).

Interestingly, there is a small subset of soybean cultivars that contain H1 but are salt sensitive, such as Peking and Baipihuangdou (Figure 6, Table S2). They are likely to contain recent mutations in key salt-tolerance genes such as *SOS1* or *HKT1* (Shi *et al.*, 2000; Xue *et al.*, 2011) or yet to be determined elements that may be a useful source for discovering novel genes involved in salt tolerance in soybean. Furthermore, the four landraces containing H2 or H4, which had a degree of salt tolerance, will arguably be even more valuable as a source for novel determinants of salt tolerance as these accessions are likely to contain gain-of-function mutations in genes other than *GmSALT3*.

GmSALT3 is expressed in root stelar cells, cell types that are already known to have a role in limiting salt transport to the shoot. For instance, HKT1;5-like proteins expressed in these cells retrieve Na^+ from the xylem, and in plants expressing functional alleles this is associated with reduced Na^+ content in shoots and superior salt tolerance in rice, wheat and Arabidopsis (Ren *et al.*, 2005; Møller *et al.*, 2009; Xue *et al.*, 2011; Munns *et al.*, 2012; Byrt *et al.*, 2014). A similar lower Na^+ content in the shoots of salt stressed H1-containing plants, compared with H2, suggests that this gene may also affect transport of Na^+ from root to shoot, and the grafting of NIL-T and NIL-S lines showed that the root and hypocotyls were sufficient to limit the accumulation of Na^+ in the shoot. However, the *GmSALT3* transcript level was first downregulated and then gradually recovered in roots; this is different from the pattern of other salt-tolerance genes (Liu *et al.*, 2000; Shi *et al.*, 2000; Ren *et al.*, 2005), indicating a distinct salt response in soybean or a different role for this gene. Consistent with this is the ER localization we observed for GmSALT3 compared with the plasma membrane localization of HKT or SOS1 proteins. Therefore, *GmSALT3* is unlikely to play a direct role in the retrieval of salt from the xylem but instead may have a role in sensing or responding to salt. CHX proteins have been associated with pH regulation and osmoregulation of cells and are predicted to have a role in vesicle trafficking (Padmanaban *et al.*, 2007). Whether this is the case for *GmSALT3*, and how this confers salt tolerance via the stelar cells of soybean, is a future research priority. Regardless of this, the identification of the gene underlying

this important soybean salt-tolerance allele has provided a clear insight into the molecular basis of both natural and human selection of salt tolerance in soybean, and should facilitate the rapid development of new elite salt-tolerant soybean germplasm by marker-assistant selection.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The minicore collection of Chinese soybean landraces (Table S2) and wild soybean (Table S3) was obtained from Chinese Academy of Agricultural Sciences (CAAS). The parent Tiefeng 8 is a highly salt-tolerant soybean cultivar released in 1970 in Liaoning province. The other parent 85–140 is a salt-sensitive cultivar from Beijing. Soybean was grown in a greenhouse at the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing. *Arabidopsis thaliana* ecotype Col-0 and transgenic plants, and *Nicotiana benthamiana* were grown at 22°C in a growth chamber with a 16-h light cycle.

Evaluation of salt tolerance

Evaluation of the salt tolerance of the soybean accessions was carried out in July 2009, 2010 and 2011 using hydroponics (Liu *et al.*, 2011), performed with three replications each time. In 2011, these materials and wild soybean were screened for salt tolerance using vermiculite as the growth medium (Jiang *et al.*, 2013). After being treated for 2 weeks, five to eight plants of each accession in each replication were scored for salt tolerance. A salt-tolerance rating for each of the accession was assigned by the respective level of leaf chlorosis. The salt-tolerance ratings ranged on a scale from 1 (normal green leaves) to 5 (complete death). Accessions showing a leaf scorch score of 1 and 2 were defined as salt tolerant, and those from 3 to 5 were salt sensitive (Tables S2 and S3).

Mapping and cloning of *GmSALT3*

We derived F_1 plants from a cross between 85–140 and Tiefeng 8 and then genotyped the F_1 plants using polymorphic SSR markers between the two parents. A segregating F_2 population ($n = 392$) was developed from the cross between 85–140 × Tiefeng 8. An F_6 RIL mapping population ($n = 367$) developed by single-seed descent was employed in salt-tolerance gene mapping. For fine mapping, 35 F_5 plants heterozygous between marker QS1101 and QS100011 were selected and after selfing we selected recombinants between QS1101 and QS100011 in the 5769 F_6 progeny. Seventy-four recombinants were selected and selfed to produce F_7 seed. We developed markers from the sequence between QS1101 and QS100011 in the Tiefeng 8 and 85–140 genomic DNA for genotyping (Table S1). *GmSALT3* was mapped to within a 17.5-kb region. To obtain candidate gene variations, roots of 20 salt-sensitive and 20 salt-tolerant F_6 families were pooled for RNA-seq. We examined the variation within the mapped region by using the Integrative Genomics Viewer (IGV) to visually assess the transcripts (Robinson *et al.*, 2011). All primers for gene mapping and genotyping are listed in Table S1.

DNA isolation and genotyping of soybean landraces and wild soybean

Genomic DNA was isolated from soybean leaves by using a DNA Purification Kit (Thermo Fisher Scientific, http://www.thermofisher.com/). The insertion/deletion in the promoter and coding regions were screened by using specific primers for the indels (Table S1). The genotyping of SNPs related to the haplotypes and used for genetic diversity testing was done using a Mass ARRAY system following the guidelines of iPLEX Gold Application from Sequenom. Primers of the SNPs are listed in Tables S3 and S5.

Phylogenetic analysis

Coding sequences of *GmSALT3* in 31 landraces and 22 wild soybean were used for phylogenetic analysis. A haplotype network was constructed based on polymorphic sites of the whole coding sequences of *GmSALT3* using the Median-Joining method in the NETWORK version 4.6.1.2 software (Fluxus Technology Ltd, Sudbury, Suffolk, UK) with data preparation using DNASP version 5 (Librado and Rozas, 2009).

Grafting and measurement of Na^+ content

We grew lines NIL-T and NIL-S in a growth chamber with a 16-h light (28°C)/8-h dark (25°C) cycle at 60% humidity. Six days after planting, uniformly healthy plants were selected for grafting (Sheng and Harper, 1997). Fifteen days after grafting, plants were treated with 200 mM NaCl. The Na^+ content in different tissues was measured after 8 days under salt stress as described previously (Jiang *et al.*, 2013).

Quantitative real-time PCR (qRT-PCR) and RNA-seq

Total RNA was extracted from various soybean tissues using TRIZOL reagent (Ambion, http://www.ambion.com). To remove the residual DNA, the extracted RNA was treated with RNase-free DNase I (New England Biolabs, https://www.neb.com) for 30 min at 37°C. Library construction was performed according to the Illumina instructions (http://www.illumina.com) and sequenced using a HiSeq 2000. For gene expression, first-strand cDNA synthesis was done with a PrimeScript RT Reagent Kit (TaKaRa, Japan, http://www.takara.co.jp/english). Real-time PCR was performed using SYBR Premix Ex Taq II (TliRNaseH Plus) (TaKaRa). We normalized the level of *GmSALT3* transcript using the control gene *GmUKN1* (Hu *et al.*, 2009).

Construction and transgenic analysis

We amplified a 1749-bp *GmSALT3* promoter region upstream of the ATG start codon by PCR from Tiefeng 8 genomic DNA. The primer (Promoter) sequences are listed in Table S1. We subcloned the promoter to the binary vector pBI121 to obtain the *GmSALT3* promoter-GUS fusion construct. The construct was introduced into the *Agrobacterium* tumefaciens GV3101 strain. Arabidopsis plants were stably transformed by means of floral dipping (Clough and Bent, 1998). Kanamycin-resistant T_3 transgenic plants were used for GUS staining (Hirakawa *et al.*, 2010). *GmSALT3* (*Xba*I–*Bam*HI) was amplified from Tiefeng 8 and inserted into the pCAMBIA1305-GFP. Constructs were introduced into the *Agrobacterum* strain EHA105 and then used to infiltrate *N. benthamiana* leaves, followed by protoplast isolation as described elsewhere (Ren *et al.*, 2014). *GmSALT3* coding sequences were also cloned into the entry vector PCR8 and subcloned into 35S pBS YFP-*attR* and *attR*-YFP vector using Gateway® LR Clonase® II following the manufacturer's instructions (Invitrogen, http://www.invitrogen.com/). Arabidopsis mesophyll protoplasts were transformed with the *GmSALT3* containing pBS vectors on its own or co-transformed with two Wave line markers (Wave6C and Wave7C) and an ER marker pBIN20-ERcfp as described previously (Nelson *et al.*, 2007; Munns *et al.*, 2012). Fluorescent signals were acquired for GFP (green; excitation 488 nm, emission 514 nm, bandpass 10 nm), YFP

(yellow; excitation 514 nm, emission 540 nm, bandpass 10 nm) and cyan fluorescent protein (blue; excitation 420 nm, emission 458 nm bandpass 20 nm), captured from transformed protoplasts using a confocal laser scanning microscope (Leica TCS SP5, http://www.leica.com/).

In situ PCR

In situ PCR followed Athman *et al.* (2014) with the following modifications. Sections were 50 μm for roots and 70 μm for leaves. Cycling parameters were: initial denaturation at 98°C for 30 sec, then 32 cycles of 98°C for 10 sec, 62°C for 30 sec, 72°C for 12 sec and a final extension at 72°C for 10 min.

Association analysis of distance to saline-affected region and haplotypes

The geographic location of each wild soybean accession was obtained from the Chinese National Crop Germplasm Conservation Center database. The distance to the saline-affected region was calculate by using the latitude and longitude of the saline-affected region (Wang, 1993). The distance from each wild accession to the nearest saline-affected region was measured using Google Earth and is shown in Table S3. The accession W43 is an introduction and lacks a location of origin so was excluded from the analysis. To test for the association between the genotype of wild soybean accessions and the distance to the nearest saline-affected region we performed a Wilcoxon–Mann–Whitney test, a non-parametric test used for Arabidopsis (Baxter *et al.*, 2010), with SAS Version 9.1 (SAS Institute, http://www.sas.com/) to see whether the distance from the collection site to the saline-affected region is smaller in accessions with a salt-tolerant haplotype H1 or H7 than in those with salt-sensitive haplotypes (H3–H6, H8, H9).

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AUTHOR CONTRIBUTIONS

LQ and MG designed most of the experiments and directed the project; RX performed the map-based cloning of *GmSALT3*, and analysed the data. YQ performed the *in situ* expression and phylogeny analysis of the *GmSALT3*, YG performed the genomic variation screen of *GmSALT3* in Tiefeng 8 and 85–140; LY performed gene expression, variation of promoter region analysis and statistical analysis; YL, JJ and JC performed recombinant identification and NIL testing; YR and YQ performed subcellular localization of *GmSALT3*; GL performed evaluation of the salt tolerance of germplasm; LT made the cross of Tiefeng 8 and 85–140; LJ helped construct vectors; ZL and HH performed the field experiment on populations; RC contributed to the experimental design and material selection. RG, MG, YQ and LQ wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Variation in coding and amino acid sequences of *GmSALT3* in Tiefeng 8 and *Gmsalt3* in 85–140.

Figure S2. Tissue localization of *GmSALT3* in soybean primary roots shown using *in situ* PCR.

Figure S3. Tissue localization of *GmSALT3* in soybean stems and leaves shown using *in situ* PCR.

Figure S4. *GmSALT3* promoter-GUS expression pattern in transgenic Arabidopsis.

Figure S5. Transient expression of GmSALT3 fluorescent protein fusions in Arabidopsis mesophyll cells.

Figure S6. Development and phenotype evaluation of near isogenic lines with different *GmSALT3*/*Gmsalt3* alleles.

Figure S7. Relationship of the haplotypes in wild soybean with the distance from their collection sites to that of the nearest saline region.

Figure S8. Genetic diversity and gene expression comparison between salt-tolerant and salt-sensitive haplotypes.

Table S1. Primers used for mapping and genotype evaluation.

Table S2. Phenotypes and genotypes of a 172 minicore collection and 12 accessions from the United States.

Table S3. Primer properties in a eight single nucleotide polymorphism (SNP) iPlex assay in Sequenome SNP genotyping system.

Table S4. Phenotype and genotype of 57 wild soybean accessions used in this study.

Table S5. Primer properties in two iPlex assays in the Sequenome SNP genotyping system for comparing genetic diversity between salt-tolerant and salt-sensitive haplotypes.

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