Regulated AtHKT1 Gene Expression by a Distal Enhancer Element and DNA Methylation in the Promoter Plays an Important Role in Salt Tolerance

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Through sos3 (salt overly sensitive 3) suppressor screening, two allelic suppressor mutants that are weak alleles of the strong sos3 suppressor sos3hkt1-1 were recovered. Molecular characterization identified T-DNA insertions in the distal promoter region of the Arabidopsis thaliana HKT1 (AtHKT1, At4g10310) in these two weak sos3 suppressors, which results in physical separation of a tandem repeat from the proximal region of the AtHKT1 promoter. The tandem repeat is approximately 3.9 kb upstream of the ATG start codon and functions as an enhancer element to promote reporter gene expression. A putative small RNA target region about 2.6 kb upstream of the ATG start codon is heavily methylated. CHG and CHH methylation but not CG methylation is significantly reduced in the small RNA biogenesis mutant rdr2 expression in shoots. The DNA methylation-deficient mutant met1-3 has nearly complete loss of total cytosine methylation in this region is mediated by small RNAs. Analysis of AtHKT1 expression in rdr2 suggests that non-CG methylation in the putative small RNA target region represses AtHKT1 expression in shoots. The DNA methylation-deficient mutant met1-3 has nearly complete loss of total cytosine methylation in the putative small RNA target region and is hypersensitive to salt stress. The putative small RNA target region and the tandem repeat are essential for maintaining AtHKT1 expression patterns crucial for salt tolerance.

Keywords: AtHKT1 • DNA methylation • Enhancer element • Gene regulation • Salt stress.

Abbreviations: GUS, β-glucuronidase; MS, Murashige and Skoog; RdDM, RNA-directed DNA methylation; siRNA, small interfering RNA; TAIL-PCR, thermal asymmetric interlaced PCR.

Introduction

Plants cope with sodium toxicity by minimizing sodium accumulation in the shoots at the whole plant level (Munns and Tester 2008). At the cellular level, salt tolerance mechanisms function to reduce sodium accumulation in the cytoplasm through limiting sodium entry into the cell, actively transporting sodium out of the cell, and compartmentalizing sodium into the vacuole (Shi et al. 2005). In Arabidopsis, the plasma membrane Na+/H+ antiporter SOS1 (Salt Overly Sensitive 1, At2g01980) functions in Na+ efflux by reducing Na+ accumulation in the cytosol (Shi et al. 2000, Shi et al. 2002, Shi et al. 2003). SOS1 may also serve as an early signaling component to modulate apoplastic pH and the production of reactive oxygen species that trigger the downstream signaling events and responses (Chung et al. 2008). Transporters responsible for compartmentalization of Na+ into the vacuole are thought to include the tonoplast Na+/H+ antiporters (Blumwald et al. 2000). The Arabidopsis vacuolar Na+/H+ exchanger AtNHX1 (At5g27150) confers salt tolerance to yeast and plant cells by sequestering Na+ into the vacuole, which supports vacuolar Na+ compartmentation as one of the important salt tolerance mechanisms in plants (Apse et al. 1999, Gaxiola et al. 1999, Zhang and Blumwald 2001, Zhang et al. 2001).

Loss-of-function analysis of Arabidopsis and wheat HKT1 genes established that HTK1 transports Na+ into the cell and may control Na+ uptake in roots (Rus et al. 2001, Laurie et al. 2002, Mäser et al. 2002, Rus et al. 2004). Two genetic screenings for mutations altering salt accumulation and tolerance in Arabidopsis have revealed the important role of AtHKT1 in salt tolerance. A screen of sos3 suppressors identified eight hkt1 mutant alleles that suppress the sos3 NaCl-sensitive phenotype (Rus et al. 2001). The hkt1 mutation also suppresses the Na+ sensitivity of sos1 and sos2 mutants, suggesting that AtHKT1 works in coordination with SOS proteins to control Na+ and K+ homeostasis (Rus et al. 2004). Another genetic screening for mutants with sodium overaccumulation in shoot (sos) identified two allelic recessive mutants of Arabidopsis, sos2-1 and sos2-2, and subsequent map-based gene cloning revealed...
that the sos2 locus corresponds to the AtHKT1 gene (Nublat et al. 2001, Berthomieu et al. 2003). Based on genetic and molecular analyses of the sos2 mutant, Berthomieu et al. (2003) concluded that AtHKT1 is involved in shoot to root Na⁺ recirculation, probably by mediating Na⁺ loading into the xylem sap in shoots and unloading in roots. However, studies with null hkt1 mutants by Sunarpi et al. (2005) led to the conclusion that AtHKT1 mediates unloading of Na⁺ in the xylem sap of Arabidopsis in the presence of high salinity. In a recent study, Davenport et al. (2007) have established that AtHKT1 mediates neither shoot to root Na⁺ recirculation nor Na⁺ influx into roots; rather, AtHKT1 seems to directly retrieve Na⁺ from the xylem and loading of Na⁺ into root vacuoles. It now appears that the primary role of AtHKT1 is to retrieve Na⁺ from the xylem in roots to reduce transport of Na⁺ from roots to shoots (Munns and Tester 2008, Horie et al. 2009, Plett and Möller 2010). However, the major role of AtHKT1 in leaves still remains elusive. The complication in interpretation of AtHKT1 function in salt tolerance could be due to as yet unknown features of AtHKT1 gene expression and regulation. In fact, Möller et al. (2009) recently showed that overexpression of AtHKT1 specifically in the mature root stele increases salt tolerance in transgenic plants, while overexpression of AtHKT1 driven by the 35S promoter in whole plants results in salt hypersensitivity. Therefore, understanding tissue-specific and regulated expression of AtHKT1 and its control mechanisms appears to be important for uncovering AtHKT1 function on a whole plant level.

DNA methylation has long been known to regulate gene expression. Cytosine residues in a DNA molecule can be methylated in three different sequence contexts, i.e. CG, CHG and CHH (H = A, T or C). In Arabidopsis, de novo cytosine methylation is carried out by the methyltransferases DRM1 and DRM2, while the methyltransferase MET1 and chromomethylase CMT3 maintain CG and CHG methylation (Henderson and Jacobsen 2007). New studies, however, suggested that MET1 and CMT3 may also be important for de novo methylation in some genomic regions (Gehringer and Henikoff 2008). De novo methylation in many genomic regions is guided by small RNAs in a process called RNA-directed DNA methylation (RdDM). Small RNAs target the DNA sequences to trigger DNA methylation using an as yet unknown mechanism. Biogenesis of small interfering RNAs (siRNAs) requires the activity of PolIV, RDR2 and DCL3; and RdDM requires AGO4, PolIV, DRD1 and DRM2 (Xie and Qi 2008, Wierzbicki et al. 2008).

Here we present a study on AtHKT1 gene expression and its regulation. We found that a distal enhancer element and small RNA-mediated DNA methylation are involved in tissue-specific and regulated expression of AtHKT1, which play an important role in Arabidopsis salt tolerance. Our findings are expected to allow for a better understanding of the role of AtHKT1 in control of Na⁺ transport on a whole plant level.

**Results**

**Identification of sos3 suppressors**

From 65,000 individual T-DNA insertion lines generated in the sos3 genetic background, 15 putative mutants showing suppression of the sos3 salt-hypersensitive phenotype were identified. Among these suppressor mutants, four mutants displayed strong suppression of sos3 salt sensitivity and harbored mutations in the AtHKT1 gene, and thus were designated as sos3hkt1-1 to sos3hkt1-4 (Rus et al. 2001, Rus et al. 2004). The other 11 suppressor mutants showed a relatively weak salt suppression phenotype compared with sos3hkt1-1. The two weak sos3 suppressors 992 and 1425, which were later designated as sos3hktInsP-1 (hkt1 Insertion in Promoter) and sos3hktInsP-2 after molecular identification of the mutations in these two lines, were subjected to further study. As shown in Fig. 1a and b, the weak suppressor sos3hktInsP-1 displayed clear suppression of sos3 salt sensitivity at the concentration of 60 mM NaCl but showed very little suppression when NaCl was elevated to 75 mM, while the strong sos3 suppressor sos3hkt1-1 exhibited apparent suppression of sos3 salt sensitivity up to 75 mM NaCl. The single mutant hkt1-1 resembled the wild-type seedlings in root growth at different concentrations of NaCl, but leaf growth of hkt1-1 was more inhibited than in wild-type seedlings at higher concentrations (100 and 125 mM) of NaCl (Fig. 1a). These results indicated that sos3hktInsP-1 is a weaker suppressor compared with sos3hkt1-1 and is phenotypically distinguishable from the sos3 mutant in response to salt stress.

**Na⁺ accumulation in sos3 mutants**

When compared with wild-type plants, sos3 mutant plants accumulated higher Na⁺ in both shoots and roots after NaCl treatment (Fig. 1c), which supports the observation that both sos3 roots and leaves are more NaCl sensitive than wild-type plants (Fig. 1a). In shoots, Na⁺ content in the hkt1-1 single mutant was significantly higher than in the wild type, but in roots, the difference between hkt1-1 and wild-type plants was only marginal (Fig. 1c). This result is consistent with hkt1-1 leaves but not roots being more sensitive to salt stress (Fig. 1a). Interestingly, the hkt1-1 mutation in the background of sos3 (sos3hkt1-1) results in reduced Na⁺ accumulation in both shoots and roots compared with the sos3 single mutant (Fig. 1c). Na⁺ accumulation in shoots and roots of the weak sos3 suppressor line sos3hktInsP-1 was also reduced compared with that in the sos3 mutant (Fig. 1c). Thus, suppression of sos3 salt sensitivity by the hkt1-1 mutation could be caused at least in part by limiting Na⁺ entry into the plant. Consistent with this notion, the sos3 mutant had higher Na⁺ content in the xylem sap than the wild type, but this increase was significantly reduced in the strong suppressor line sos3hkt1-1 and marginally reduced in the weak suppressor line sos3hktInsP-1 (Fig. 1c). The hkt1-1 single mutant also accumulated more Na⁺ in xylem sap than the wild type (Fig. 1c). Na⁺ content in
shoots, roots and xylem sap of the weak suppressor line sos3hktInsP-1 was relatively higher than that in the strong suppressor line sos3hkt1-1, which may account for the weak suppression phenotypes.

**T-DNA insertions in the promoter of the AtHKT1 gene in the weak suppressors are responsible for the suppression phenotype**

Thermal asymmetric interlaced PCR (TAIL-PCR) revealed that the weak suppressor 992 (sos3hktInsP-1) possesses a T-DNA insertion in the promoter approximately 3.6 kb upstream of the ATG start codon of the AtHKT1 gene (Fig. 2a). However, TAIL-PCR failed to generate sequence information regarding the T-DNA insertions in the sos3hkt1-1 suppressor. An allelism test indicated that these two suppressors are allelic (Fig. 2b), which suggests that a mutation is also located in the AtHKT1 gene in sos3hktInsP-2. Using a specific primer for the border sequence of the T-DNA and primers targeting the entire AtHKT1 gene including about 6.0 kb of the promoter region, PCR fragments were amplified from sos3hktInsP-2 and sequenced. Sequence analysis indicated that a complex T-DNA insertion is located in the promoter of AtHKT1 approximately

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**Fig. 1** Characterization of a weak sos3 suppressor. (a) Root growth of the wild type (WT), sos3hkt1-1, sos3hktInsP-1, hkt1-1 and sos3 in agar medium supplemented with different concentrations of NaCl. Four-day-old seedlings grown in 1/2 MS agar medium were transferred to a medium with the indicated NaCl concentration. Pictures were taken on the 10th day after transfer. (b) Quantitative measurements of root growth at different concentrations of NaCl. Data represent the average root length of five seedlings. (c) Na\(^+\) content in shoot, root and xylem sap. Plants were hydroponically cultured and root and shoot were separated for ion content measurement. Error bars represent the SD (n = 3).
4.5 kb upstream of the ATG start codon (Fig. 2a). To further verify whether the T-DNA insertions are responsible for the suppressor phenotype, sos3hktInsP-1 and sos3hktInsP-2 were crossed with sos3hkt1-1 and the resulting F1 seeds were subjected to salt sensitivity tests. F1 seedlings displayed a suppression phenotype at 55 mM NaCl but failed to suppress sos3 salt sensitivity at 75 mM NaCl, at which point sos3hkt1-1 still exhibited strong suppression of sos3 salt sensitivity (Fig. 2b). This result indicated that both sos3hktInsP-1 and sos3hktInsP-2 are weak alleles of sos3hkt1-1, further supporting the idea that T-DNA insertions in sos3hktInsP-1 and sos3hktInsP-2 are indeed responsible for suppression of sos3 salt sensitivity. To exclude the possibility that other mutations in AtHKT1 may contribute to the suppression phenotype, the entire coding region plus approximately 6.0 kb of the promoter region in sos3hktInsP-1 and sos3hktInsP-2 was sequenced and no mutations were found in these two suppressor lines.

**Complex features of the AtHKT1 promoter**

T-DNA insertions in the distal promoter region of AtHKT1 that conferred a weak suppression phenotype suggested an essential role for the upstream sequences of the T-DNA insertions. Sequence analysis revealed a tandem repeat present in the distal promoter region located about 3.9 kb upstream of the ATG start codon (Fig. 2a). Each repeat contains approximately 680 nt and is separated by 34 nt. The upstream repeat (repeat 1, R1) and the downstream repeat (repeat 2, R2) are nearly identical, with nine nucleotide substitutions and a few small deletions between these two repeat sequences (Supplementary Fig. S1a). The T-DNA insertion in sos3hktInsP-1 separates the tandem repeat from the proximal promoter region and results in a partial loss of function of AtHKT1, which suggests that this tandem repeat plays a role in the regulation of AtHKT1 expression. Moreover, a search for small RNA targets (Arabidopsis thaliana Small RNA Project, ASRP database, http://asrp.cgrb.oregonstate.edu) in the promoter of AtHKT1 identified several regions located 2.0 kb upstream of the ATG start codon as potential targets of small RNAs. The sequence GATCTGGTG GTTGTGATGGTGGAGAT located about 2.6 kb upstream of the ATG start codon is complementary to two small RNA species, ASRP-805 and ASRP-1200, present in the ASRP database (Fig. 2a). Interestingly, a search for DNA methylation against...
the methylome database (http://signal.salk.edu/cgi-bin/methylome) indicated that the promoter region potentially targeted by ASRP-805 and ASRP-1200 is heavily methylated.

The tandem repeat acts as an enhancer element
To determine the role of the tandem repeat in gene regulation, each repeat element and the tandem repeat were fused with a minimal 35S promoter driving β-glucuronidase (GUS) gene expression and the resulting constructs were introduced into Arabidopsis (Fig. 2c). Stable T2 transformants were analyzed by histochemical staining (data not shown) and GUS activity assays. Transgenic plants harboring the 35S minimal promoter–GUS (Mi–GUS) or repeat 1-minimal 35S promoter–GUS (R1–Mi–GUS) displayed undetectable GUS activity by histochemical staining, while transgenic plants harboring repeat 2-minimal 35S promoter–GUS (R2–Mi–GUS) or tandem repeat-minimal 35S promoter–GUS (R1R2–Mi–GUS) exhibited strong GUS staining (data not shown). Enhanced expression of GUS gene by R2 and R1R2 was further confirmed by enzyme activity assays (Fig. 2d). Interestingly, the enhancement of GUS gene expression by R2 was stronger than that by the tandem repeat R1R2, which suggests that R1 may have inhibitory effect on R2 for the enhancement of gene expression. Furthermore, NaCl treatment did not affect R2-enhanced expression of GUS but reduced GUS expression driven by R1R2 (Fig. 2d), which suggests that R1 may be a NaCl-responsive element.

Non-CG methylation of the putative small RNA target region in the AtHKT1 promoter is small RNA dependent
Heavy cytosine methylation was detected in the region of approximately 250 nt housing the putative small RNA target sequences (Fig. 3a). In this 250 bp region, cytosine methylation at CG sites was 95.6% in leaves and 84.9% in roots of wild-type seedlings (Fig. 3a). CG methylation in the leaves and roots of the rdr2 mutant defective in small RNA biogenesis was not significantly different from that in the wild-type line (Fig. 3a). However, CG methylation in the met1-3 null mutant was significantly reduced to very low levels, which is consistent with the role of Met1 being required for CG methylation in the genome. CHG and CHH methylation significantly decreased in both leaves and roots of the rdr2 mutant (Fig. 3a). CHG methylation in the leaves and roots of rdr2 was reduced to 21.7 and 23.4% compared with 48.5% in the leaves and 59.7% in roots of wild-type plants; CHH methylation was 3.8% in the leaves and 5.8% in roots of the rdr2 mutant compared with 26.9% in leaves and 15.1% in roots of wild-type seedlings (Fig. 3a). These results indicate that non-CG methylation, in particular asymmetric CHH methylation in the AtHKT1 promoter, is mediated by small RNAs generated through the RDR2 pathway. In the met1-3 mutant, CHG methylation was completely abolished and CHH methylation was also substantially reduced in both leaves and roots (Fig. 3a). Thus, the regulation of AtHKT1 expression and small RNA de

Fig. 3 Cytosine methylation, AtHKT1 expression and small RNA detection. (a) CG, total cytosine, CHG and CHH methylation in the leaf and root of the wild type (WT), and rdr2 and met1-3 mutants. H = A, T or C. (b) Detection of AtHKT1 transcript levels in leaf and root of the WT, rdr2 and met1-3 by real-time PCR. The expression level was calculated as the level relative to the value of the wild-type leaf. (c) The abundance of ASRP-805 in WT and different small RNA- and microRNA-related mutants. A 10 µg aliquot of RNA isolated from the leaves of 6-week-old plants grown in soil was used for Northern blotting. A size marker of 21 and 24 nt RNA oligos is shown on the left side. SS rRNA stained by ethidium bromide is shown as a loading control.
met1-3 mutant possesses a nearly non-methylated form and rdr2 possesses a reduced non-CG-methylated form at the putative small RNA target region of the AtHKT1 promoter.

To assess the effects of reduced DNA methylation on AtHKT1 expression, AtHKT1 transcript levels were determined in the roots and leaves of wild-type, rdr2 and met1-3 lines (Fig. 3b). In wild-type seedlings, AtHKT1 expression in roots is much higher than that in leaves, indicating a differential expression of AtHKT1 in roots and leaves. AtHKT1 transcript levels in the leaves of the rdr2 mutant are substantially higher than that in the leaves of wild-type plants, while the roots of rdr2 have similar AtHKT1 transcript levels when compared with the wild-type. These results suggest that small RNA-mediated non-CG methylation in the AtHKT1 promoter represses AtHKT1 transcription in leaves but does not affect AtHKT1 expression in roots. In the met1-3 mutant in which total cytosine methylation is nearly abolished, AtHKT1 transcript levels in roots increase 2.0-fold and are approximately 1.5-fold higher in leaves when compared with that in wild-type plants (Fig. 3b). Thus, heavy methylation of the AtHKT1 promoter appears to inhibit its transcription in both leaves and roots.

Reduced non-CG methylation in the rdr2 mutant suggested that RDR2-dependent small RNAs play an essential role in the methylation of the putative small RNA target sequence in the AtHKT1 promoter. Consistent with being annotated as a small RNA in the ASRP database, the small RNA ASRP-805 (23 nt) complementary to the AtHKT1 promoter sequence was detected in the wild type and in dcl1, dcl2 and dcl4 mutants, but not in small RNA biogenesis mutants henn, dcl3 and dcl2 (Fig. 3c). In contrast, the known microRNA mi163 (24 nt) was detectable in dcl3 and rdr2 mutants but its biogenesis was apparently abolished in henn1 and dcl1 mutants in which microRNA biogenesis is blocked (Fig. 3c). This result reveals that ASRP-805 does exist in Arabidopsis as a small RNA species and its biogenesis is indeed RDR2 dependent.

Promoter without the tandem repeat and putative small RNA target region directed an altered expression pattern of AtHKT1 and resulted in NaCl hypersensitivity

Previous reports have used an approximately 2.0 kb promoter region upstream of the ATG start codon of AtHKT1 to study AtHKT1 expression and function (Mäser et al. 2002, Berthomieu et al. 2003, Rus et al. 2004). However, our results imply that the sequences upstream of the 2.0 kb region of the AtHKT1 promoter are required for full expression or regulation of AtHKT1 expression. To determine whether the 2.0 kb promoter region is sufficient for full functional expression of AtHKT1, a construct, designated as AtHKT1P-2.0kbAtHKT1 including about 2.0 kb of the promoter region, the entire AtHKT1 coding region with both exons and introns, and approximately 500 bp sequence downstream of the AtHKT1 gene, was cloned into a plant binary vector and introduced into the strong sos3 suppressor sos3hkt1-1. Two independent homozygous transgenic lines with a single copy of the transgene, designated 5-2 and 12-2, were selected for a complementation test. As shown in Fig. 4a and b, both 5-2 and 12-2 exhibited even greater NaCl sensitivity when compared with the sos3 mutant. The root growth of 5-2 and 12-2 was almost completely inhibited at 20 mM NaCl, while the sos3 mutant only showed a slight reduction in root growth. At 40 mM NaCl, the leaves of 5-2 and 12-2 were bleached, while the leaves of the sos3 mutant remained green at NaCl concentrations even higher than 60 mM (Fig. 4a). Since the functional AtHKT1 expression in 5-2 and 12-2 comes solely from the transgene AtHKT1P-2.0kbAtHKT1, the phenotypes observed in these two transgenic lines can be attributed to altered AtHKT1 expression patterns and mis-regulated expression of AtHKT1. To test this hypothesis, AtHKT1 expression in the wild type, sos3, sos3hkt1-1, 5-2 and 12-2 lines was determined by real-time PCR (Fig. 4c). In contrast to the expression pattern of AtHKT1 in the wild-type line in which AtHKT1 expression is higher in root than in leaf, both 5-2 and 12-2 lines displayed substantially higher expression in leaves than in roots. Moreover, the transcript level of AtHKT1 in leaves of these two transgenic lines was > 100-fold higher than that in the wild type, while transcript levels of AtHKT1 in roots of these two lines were similar to those in the roots of wild-type plants. These results indicate that the approximately 2.0 kb promoter region is able to drive the expression of AtHKT1, but is not functional as a native promoter, which further supports that sequences upstream of the 2.0 kb promoter region are necessary for AtHKT1 regulation. Ion content measurements revealed that, after challenge with 50 mM NaCl for 1 d, both the 5-2 and 12-2 lines accumulated significantly higher Na+ in leaves when compared with the wild type and sos3 mutant plants (Fig. 4d). Without salt treatment, the potassium content in roots of 5-2 and 12-2 lines was substantially lower than that in wild-type and sos3 mutant roots (data not shown). With salt treatment, the potassium content in roots of the 5-2 and 12-2 lines was much lower than that in wild-type roots (data not shown). Thus, the hypersensitive to NaCl phenotype of 5-2 and 12-2 may be attributed to mis-regulated expression of AtHKT1, which disrupts Na+ and K+ homeostasis in both roots and leaves.

DNA methylation in the putative small RNA target region is important for AtHKT1 expression and salt tolerance

As an attempt to correlate DNA methylation, AtHKT1 expression and salt tolerance, salt sensitivity of met1-3 and rdr2 was analyzed by using a root bending assay. The met1-3 mutant displayed an apparent salt-sensitive phenotype in root growth (Fig. 5a and Supplementary Fig. S2a), which suggests that methylation in the putative small RNA target region and its regulated AtHKT1 expression are likely to contribute to salt tolerance in Arabidopsis. However, rdr2 showed a similar response to different concentrations of NaCl when compared with wild-type plants (data not shown). To establish further
the role of the putative small RNA target region and the tandem repeat in AtHKT1 expression and salt tolerance, a series of deletion constructs with or without the small RNA target sequence and the tandem repeat were generated for genetic complementation tests and GUS fusion analysis. A 3.9 kb AtHKT1 promoter lacking the tandem repeat with (indicated as 3.9 kb in Fig. 5) or without (indicated as 3.9 kb del. sRNA target in Fig. 5) the small RNA target region and a 5.2 kb AtHKT1 promoter containing the tandem repeat with (indicated as 5.2 kb in Fig. 5) or without (indicated as 5.2 kb del. sRNA target in Fig. 5) the small RNA target region driving the entire AtHKT1 coding region were transformed into hkt1-1 and sos3hkt1-1 mutants and the salt sensitivity of T2 transgenic seedlings was analyzed by root bending assays. In the hkt1-1 single mutant background, transgenic seedlings with the 3.9 or 5.2 kb promoter exhibited similar salt sensitivity in root growth.
when compared with wild-type and hkt1-1 seedlings, while transgenic seedlings with the 3.9 or 5.2 kb promoter without the small RNA target region displayed a salt-sensitive phenotype resembling that of the met1-3 mutant (Fig. 5a and Supplementary Fig. S2a). This result suggests that the salt sensitivity of met1-3 mutant roots is attributed to methylation of the small RNA target region in the AtHKT1 promoter. In the sos3hkt1-1 mutant background, the entire AtHKT1 gene including the 5.2 kb promoter restored the salt sensitivity of sos3hkt1-1 to levels of the sos3 mutant, which indicates that the 5.2 kb promoter is a complete promoter controlling the endogenous AtHKT1 expression. The 3.9 kb promoter plus

Fig. 5 Role of DNA methylation and the putative small RNA target region in AtHKT1 gene expression and salt tolerance. (a) Quantitative measurement of root growth showing the salt sensitivity of the wild type, hkt1-1, met1-3 and transgenic lines (in the background of hkt1-1) harboring the complementation constructs described in the Materials and Methods. Error bars represent the SD (n = 5). (b) Quantitative measurement of root growth showing the salt sensitivity of the wild type, sos3-1, sos3hkt1-1, sos3htinsP-1 and transgenic lines (in the background of sos3hkt1-1) harboring the complementation constructs described in the Materials and Methods. Error bars represent the SD (n = 5). (c) Transcript levels of AtHKT1 in the leaf and root of the wild type, sos3, and sos3hkt1-1 harboring the different complementation constructs shown in (b). The expression level was determined by quantitative real-time PCR and was calculated as the level relative to the value of the wild-type leaf. (d) Histochemical staining of GUS of transgenic seedlings harboring different types of AtHKT1 promoter driving GUS gene expression. At least five transgenic lines were examined and only one line is shown. Top panel, bar = 10 mm; bottom panel, bar = 2.5 mm. (e) GUS activity measurement.
the AtHKT1 coding region only partially complements the sos3hkt1-1 phenotype, which resembles the phenotype of the weak sos3 suppressor sos3hktInsP-1 (Fig. 5b and Supplementary Fig. 52b). This result further supports the idea that the tandem repeat present in the 5.2 kb promoter but lacking in the 3.9 kb promoter is required for full expression of the AtHKT1 gene. Both the 5.2 and 3.9 kb promoter without the putative small RNA target region driving AtHKT1 expression cannot functionally complement the sos3hkt1-1 salt-sensitive phenotype and thus it displays even greater salt sensitivity than the sos3 mutant. The overcomplementation phenotype resembles the transgenic line 12-2 harboring the 2.0 kb promoter plus the coding region (Fig. 5b and Supplementary Fig. 52b).

AtHKT1 gene expression analysis (Fig. 5c) further supports the complementation results shown in Fig. 5b. In roots, the AtHKT1 transcript level significantly increased in the sos3 mutant (Figs. 4c, 5c), which indicates that SOS3 plays a role in the transcriptional regulation of AtHKT1 gene expression. In both roots and leaves, the AtHKT1 transcript level in complementation transgenic sos3hkt1-1 plants harboring the 5.2 kb promoter plus the entire coding region (indicated as 5.2 kb) resembles the expression level of the native AtHKT1 gene in the sos3 mutant (Fig. 5c). This result indicates that the 5.2 kb promoter is indeed a complete promoter for AtHKT1 expression and regulation. Complementation transgenic plants harboring the 3.9 kb promoter driving AtHKT1 expression (indicated as 3.9 kb) display an AtHKT1 expression pattern similar to that in the sos3hktInsP-1 weak suppressor, while complementation transgenic plants with the 3.9 kb promoter lacking the putative small RNA target region driving AtHKT1 expression (indicated as 3.9 kb del. sRNA target) resembles 12-2 transgenic complementation plants (Fig. 5c). Taken together, these results suggest that the tandem repeat and the putative small RNA target region are important for tissue-specific expression and regulation of AtHKT1.

The effect of the tandem repeat and the small RNA target region on gene expression was dissected by promoter–GUS analysis (Fig. 5d, e). Compared with the 5.2 kb complete promoter, the 2.0 kb promoter results in a substantial increase in GUS gene expression. The 3.9 kb promoter lacking the tandem repeat has reduced activity to drive GUS expression when compared with the full promoter (Fig. 5d, e), which is consistent with the role of the tandem repeat in enhancing gene expression (Fig. 2d). Interestingly, the 4.6 kb promoter containing only R2 has increased activity when compared with the 5.2 kb promoter including both R1 and R2 repeat elements (Fig. 5d, e), further suggesting that R1 may have an inhibitory role in enhancing function of the tandem repeat. When the putative small RNA target region was deleted from the promoter, the promoter activity driving GUS gene expression was significantly increased, which resembles the 2.0 kb promoter lacking both the tandem repeat and the putative small RNA target (Fig. 5d, e).

### Discussion

In the present study, we have isolated unique genetic mutants with T-DNA insertions in the promoter of AtHKT1, which led to the identification of several important elements in the AtHKT1 promoter for expression and regulation (Figs. 1, 2). Based on the weak suppression phenotype of sos3hktInsP-1 and sos3hktInsP-2, it is apparent that the distal tandem repeat is required for full expression of AtHKT1. The tandem repeat functions as an enhancer element when fused with a minimal 35S promoter (Fig. 2c, d). Intriguingly, R1 could not enhance while R2 significantly enhanced reporter gene expression (Fig. 2c, d). Although these two repeat elements in the tandem repeat are nearly identical, small differences between these two repeats could still result in differences in DNA modification and binding affinity with trans-acting proteins.

Rus et al. (2006) identified AtHKT1 as the genetic locus driving elevated shoot Na⁺ in both Ts-1 and Tsu-1, two coastal populations of Arabidopsis with enhanced Na⁺ accumulation. Sequence analysis revealed several major differences between these two wild populations and the Col-0 genotype in both the promoter and coding region of AtHKT1. One of the striking differences is that both Ts-1 and Tsu-1 have only one copy of the tandem repeat. Polymorphisms exist amongst the single copy sequences in Ts-1 and Tsu-1 and R1 and R2 in Col-0, but it appears that the single copy sequence is more similar to R1 from Col-0 (Supplementary Fig. S1). Thus, the single copy sequence in Ts-1 and Tsu-1 might have little enhancing ability for gene expression like R1 does in Col-0. In fact, AtHKT1 gene expression in roots of Ts-1 and Tsu-1 is clearly lower than that in Col-0 (Rus et al. 2006), which could be attributed to a loss of the enhancing element in the AtHKT1 promoter in these two coastal ecotypes. Although other major sequence changes in the promoter and coding region of AtHKT1 in Ts-1 and Tsu-1, including a significant change in the promoter sequence upstream and near the putative TATA box and changes in seven amino acid residues in the AtHKT1 protein, may result in lower expression or less active transport protein, our data, together with the results from Rus et al. (2006), highlight the importance of the tandem repeat in the expression and regulation of AtHKT1.

The AtHKT1 promoter contains a putative small RNA target region where CG methylation in the leaf is higher than that in the root (Fig. 3a), which may, at least in part, contribute to higher expression of AtHKT1 in roots than in leaves (Fig. 3b). It has been proven that de novo DNA methylation is guided by siRNAs through RdDM, and the primary players for RdDM are AGO4, RDR2 and DCL3 (Gehring and Henikoff, 2008). In our study, non-CG methylation at CHG and CHH sites is remarkably reduced in the small RNA biogenesis mutant rdr2, which indicates that non-CG methylation in this promoter region is directed by small RNAs. The small RNA ASRP-805 complementary to the sequence in this promoter region was detected in Col-0 wild-type plants but was not detectable in the rdr2 mutant (Fig. 3c), further supporting the notion that small RNAs play an important role in AtHKT1 promoter methylation. Based on
DNA methylation and gene expression data of AtHKT1 in the wild type, met-1-3, rdr2 and complementation lines (Figs. 3a, b, and 5), it is conceivable that DNA methylation, including RdDM, in the putative small RNA target region is important in controlling AtHKT1 expression and may perform a distinct role in roots and leaves. The expression level of AtHKT1 in the leaves of rdr2 is substantially higher than that in wild-type leaves, suggesting that RdDM in the AtHKT1 promoter plays an inhibitory role in the expression of AtHKT1 in leaves. On the other hand, loss of both CG and non-CG methylation in the met1-3 mutant resulted in an increase in AtHKT1 expression in both roots and leaves (Fig. 3b), suggesting that heavy methylation in the promoter region is required to maintain AtHKT1 expression at a low level and perhaps in a correct pattern in the seedling. Different AtHKT1 expression patterns in rdr2 and met1-3 might be due to differences in methylation of specific sequences in the putative small RNA target region or differences in methylation of other promoter regions, e.g. the tandem repeat.

The importance of DNA methylation in salt tolerance was established by the observation that the met1-3 mutant is hypersensitive to NaCl (Fig. 5a and Supplementary Fig. S2a). Hypersensitivity to NaCl in met1-3 might be attributed to a loss of methylation in the putative small RNA target region in the AtHKT1 promoter. This notion is supported by the finding that the AtHKT1 promoter without the putative small RNA target region driving the entire AtHKT1 gene expression resulted in a similar salt-sensitive phenotype to met1-3 (Fig. 5a and Supplementary Fig. S2a). Intriguingly, the rdr2 mutant did not show a salt-sensitive phenotype in the root bending assay although non-CG methylation in the putative small RNA target region is remarkably reduced. Perhaps de novo methylation of the AtHKT1 promoter rendered by RdDM is to fine-tune the expression of AtHKT1 in leaves, which may be important merely for long-term adaption of Arabidopsis to salt stress, but not a mechanism for salt tolerance in the short term during a root bending assay. Nevertheless, the putative small RNA target region and presumably its methylation appears to be important to control AtHKT1 expression in leaves and roots (Fig 5c–e). Differential expression of AtHKT1 in roots and leaves is important for salt tolerance, as suggested by the analysis of lines S-2 and 12-2. In lines S-2 and 12-2, the reversed expression pattern of AtHKT1 in roots and leaves and extremely high transcript level of AtHKT1 in leaves would lead to the hypersensitive phenotype of these lines to NaCl (Fig. 4). The reversed AtHKT1 expression pattern in these two lines also resulted in the rapid accumulation of Na⁺ in the leaves (Fig. 4d), which could account for NaCl hypersensitivity for seedling leaves.

Materials and Methods
sos3 suppressor screening and characterization
sos3 suppressors were screened from a T-DNA insertion population in a sos3 background as described by Rus et al. (2001).

Salt sensitivity of sos3 suppressors was measured by root growth as described by Shi et al. (2002).

Ion content measurements
One-week-old seedlings grown in 1/2 Murahige and Skoog (MS) agar medium (0.7% agar) were transferred to a homemade hydroponic culture container with liquid nutrients (1/10 MS salts). After plants grew for 3 weeks, the liquid nutrient was replaced by 1/10 MS salts plus 50 mM NaCl for salt treatment. Roots and shoots were separately harvested and dried at 80°C for at least 2 d. The xylem sap was collected as described by Shi et al. (2002). Ion content measurement was performed according to Shi et al. (2002).

TAIL-PCR
TAIL-PCR was essentially performed as described by Liu et al. (1995). The three primers corresponding to the border sequence of the vector pSKI15 used for T-DNA insertion mutagenesis of sos3 mutant are as follows: AtLB1, 5'–ATACGACCGGA TCGTCATTTGTC-3'; AtLB2, 5’-TAAAAACGCTGCGGACAT CTAC-3'; and AtLB3, 5’-TTGCCATCATCATTGCTG-3'. The degenerate primer used for TAIL-PCR amplification was WGCNAAG/WAGWANAAG (W = A/T; N = A/T/G/C).

Complementation test
Genetic complementation among weak and strong sos3 suppressors was determined by genetic crosses and subsequent root growth assay of the F1 seedlings on 1/2 MS agar medium with 55 or 75 mM NaCl. For molecular complementation, a DNA fragment containing the 2.0 kb promoter region, the entire open reading frame sequence and 523 bp downstream sequence of the stop codon of the AtHKT1 gene was cloned into the plant binary vector pCAMBIA 2300 as described by Rus et al. (2004) and was named pCAMBIA2300P2.0kb. The complementation constructs with longer promoter sequences were created based on pCAMBIA2300P2.0kb. The promoter region from 3,871 to 2,015 bp upstream of the start codon was amplified and inserted into pCAMBIA2300P2.0kb, producing the 3,871 bp promoter fused with the entire coding region of AtHKT1, which was designated as pCAMBIA2300P3.9kb. The tandem repeat was amplified and inserted into pCAMBIA2300P3.9kb to create a full complementation construct pCAMBIA2300P5.2kb. To eliminate the small RNA target region (61 bp) in the promoter, the fragment with the 2.0 kb promoter and 245 bp downstream of the ATG start codon (utilizing the endogenous SalI site here in the AtHKT1 coding region) in pCAMBIA2300P2.0kb was removed by digestion with PstI and SalI; the remaining vector was ligated to the fragment (2,607 bp upstream and 245 bp downstream of the start codon) digested with PstI and SalI, creating a vector named as pCAMBIA2300P2.6kb for further use. A fragment (from 3,871 to 2,669 bp upstream of the start codon) was amplified and inserted into the vector pCAMBIA2300P2.6kb to create a construct with the 3.9 kb.
promoter lacking the putative small RNA target region named pCAMBIA2300P3.9kb-del-sRNA. The tandem repeat was amplified and inserted into pCAMBIA2300P3.9kb-del-sRNA to create pCAMBIA2300P5.2kb-del-sRNA, which contains the 5.2 kb promoter but lacks the putative small RNA target region. These constructs were introduced into Agrobacterium tumefaciens strain GV3101 and transformed into hkt1-1 and sos3htk1-1 mutant plants by the flower dipping method (Zhang et al. 2006).

GUS assay

Approximately 50 bp of minimal 35S promoter sequence including the TATA box was synthesized and inserted into pCAMBIA 1381Z, resulting in a transcriptional fusion of the minimal 35S promoter and GUS reporter gene and named mi35S–GUS. The minimal 35S promoter provides a transcription initiation site for the expression of the GUS reporter gene. R1 and R2 were amplified by PCR and cloned into the mi35S–GUS vector separately or as a tandem repeat in front of mi35S promoter to create R1-Mi–GUS, R2-Mi–GUS and R1R2-Mi–GUS constructs. The construction of the AthKT1 promoter–GUS fusions with or without the tandem repeat and the putative small RNA target region is as follows. The promoter region (2,014 bp upstream of the start codon) was amplified and inserted into the vector pCAMBIA1381Z to create a 2.0 kb AthKT1 promoter–GUS fusion construct named pCAMBIA1381Z2.0kb. The promoter region (3,871 bp upstream of the start codon) was amplified and inserted into pCAMBIA1381Z to create pCAMBIA1381Z3.9kb. R2 was amplified and inserted into pCAMBIA1381Z3.9kb to form pCAMBIA1381Z4.6kb, and the entire tandem repeat (R1R2) was amplified and inserted into pCAMBIA1381Z3.9kb to create pCAMBIA1381Z5.2kb. To eliminate the small RNA target region (61 bp) in the promoter, a fragment containing 2,607 bp upstream of the start codon was amplified and inserted into pCAMBIA1381Z and named pCAMBIA1381ZP2.6kb; the second fragment (from 3,871 to 2,669 bp upstream of the start codon) was amplified and inserted into the vector pCAMBIA1381ZP2.6kb to form pCAMBIA1381Z3.9kb-del-sRNA. The 4.6 and 5.2 kb promoters without the putative small RNA target region were constructed in a similar way to that described above and named pCAMBIA1381Z4.6kb-del-sRNA and pCAMBIA1381Z5.2kb-del-sRNA, respectively. The constructs were introduced into A. tumefaciens GV3101 and then transferred into Arabidopsis Col-0 wild-type plants. At least six T2 individual transgenic lines were subjected to GUS assay. Histochemical staining of GUS was carried out as described (Shi et al. 2002).

Bisulfite genomic DNA sequencing

Ten-day-old seedlings of wild-type and mutants grown on 1/2 MS agar medium were used for DNA isolation. Genomic DNA was extracted from three replicates and purified from root and leaf separately. A 2 µg aliquot of genomic DNA from a mixture of three replicates was used for bisulfite treatment using an Epitect Bisulfite kit (Qiagen) following the supplier's instructions. The bisulfite conversion thermal cycling conditions were as follows: 99°C for 5 min, 60°C for 25 min, 99°C for 5 min, 60°C for 85 min, 99°C for 5 min, 60°C for 175 min and 20°C overnight. A 5 µl aliquot of purified bisulfite-treated DNA was used as template for the primer extension reaction by using the reverse primer only. The reverse primer is 5’-TTTTCACTTRC AATTACCTTTTACCCATT-3’ (R = A/G). After the primer extension reaction (10 cycles of 95°C for 1 min, 60°C for 3 min, 72°C for 3 min), the forward primer (5’-TATGAAAYT AATAATTGTTTATATGAAAA-3'; Y = C/T) was added into the reaction mixture and the second PCR was as follows: 10 cycles of 95°C for 1 min, 60°C for 1.5 min and 72°C for 2 min, 30 cycles of 95°C for 1 min, 50°C for 1.5 min and 72°C for 2 min, and one cycle of 72°C for 10 min. The PCR product was used as template for an additional amplification by using a pair of nested primers (forward primer, 5’-GTGTAATTTTAAAAAGTAGTGGTA AAAAAAG-3'; reverse primer, 5’-ATCACATAAAAACACTAAAATA ATTTCTAATGAA-3'). PCP products were purified and cloned into pGEM T-vector (Promega). About 15 independent clones from each PCR product were sequenced.

Quantitative real-time PCR

Ten-day-old seedlings grown in 1/2 MS agar (0.7%) medium were collected for RNA isolation. Total RNA was extracted from roots and leaves using an RNeasy Plant Mini kit (Qiagen). First-strand cDNA was synthesized from 2 µg of total RNA using M-MLV-Reverse Transcriptase and Oligo (dT)15 primer (Promega). Quantitative real-time PCR was carried out by using ABI PRISM 7500 Real-Time PCR Systems (Applied Biosystems) and the iTaq™ SYBR Green Supermix with the ROX kit (Bio-Rad) following a standard protocol. The primers for real-time PCR were designed by using the PrimerQuest program (Integrated DNA Technology). The following primers were used: ACTIN2-F, 5’-ACACTGTGCCAATCT ACAATTTCCCGCTCTGCTGT-3’; ACTIN2-R, 5’-ACAATTTCCCGCTCTGCTGT-3’; HKT1-F, 5’-CATCCTTCGAAAGGATCATGCGATA TG-3’; and HKT1-R, 5’-TTAGTACGAATTTTCCATATTGGACTC C-3’. The relative expression level of each sample was calculated and analyzed from three independent reactions.

Small RNA blot analysis

RNA was extracted from Arabidopsis seedlings by using Plant RNA Reagent (Invitrogen). Small RNA detection using Northern blot was performed according to Xi et al. (2005). A locked nucleic acid (LNA)-modified detection probe (Exiqok) complementary to the small RNA ASRP-805 (5’-UUCUCACCAUCAACACCCACG-3’) was used as probe for ASRP-805 detection. An oligonucleotide probe specific to miRNA163 (5’-UUGAAGAGGACUUGGAACUUCGAU-3’) was used for miRNA 163 detection.
Supplementary data

Supplementary data are available at PCP online.

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