Detection of siRNA-Directed DNA Methylation and Gene Silencing in Response to Salinity Stress

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Abstract: Small RNAs are able to organise a lot of biological machineries in organisms. Different types of sRNAs play divergent roles in the regulation of gene expression in plants, including RNA-directed DNA methylation (RdDM), which results from double-stranded RNAs and is often capable of directing DNA cytosine methylation at target sequences. Cytosine methylation forms the basis of an epigenetic regulatory system that has been shown to be involved in abiotic and biotic stress responses of plants. In this study applied a direct delivery method to introduce SOS1-siRNA (small interfering RNA) and HKT1-siRNA into *Arabidopsis thaliana* to test their ability to direct DNA methylation within promoters of the At*SOS1* and At*HKT1* genes in response to salt stress during the growth period. There was clear evidence of the siRNAs' uptake and that the At*SOS1* promoter was methylated in the presence of HKT1-siRNA when treated with salt. In addition, lethal effects were observed on the phenotype, which suggested that At*SOS1* and At*HKT1* genes' expressions had been affected. Direct siRNA delivery was therefore used successfully and it caused significant effects on the phenotype of plants. This method has the potential to allow targeted regulation of gene expression in response to stress.

Key words: *Arabidopsis thaliana*; epigenetic; cytosine methylation; At*SOS1*; At*HKT1*; plant transformation.

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Introduction

Epigenetic phenomena allow organisms to modify gene expression without changing DNA sequence (1,2). Epigenetic molecular processes have the ability to stimulate, knockdown or completely turn off the activity of specific genes (3,4). Changing the expression of genes epigenetically involves a variety of organogenesis and environmental responses, and it is thus hypothesised to contribute to the variety of phenotypes and the plasticity of living cells (5). Therefore, it has been proposed that epigenetics may play an essential role in developing plant growth and in improving yield and crop quality (6). Three mechanisms have been identified that play important roles as regulatory processes in epigenetic pathways: DNA methylation, histone modification and small, non-coding RNA (sRNA) (7). DNA methylation is a biochemical process and the only epigenetic regulatory system that can be inherited between generations; thus, it provides the most scope for manipulation (8). The most common form of DNA methylation is where the Carbon-5 of cytosine residues is furnished with a methyl group, thereby converting cytosine to 5-methylcytosine. Highly methylated

areas of genomic DNA are likely to be the least transcriptionally active (9). The methylation of cytosine plays a significant role in defence against the activity and mobility of transposable elements and the control of gene expression in plants (10). The ability of approximately ~21-24 nt short-interfering RNAs (siRNAs) to guide DNA methylation has become widely recognised in plants (11,12). The RNA-directed DNA methylation (RdDM) procedure is a small piece of double-stranded RNA ~21–24nt in length recruits the methylation of cytosine residues of DNA sequences (13). RdDM impacts CG, CHG, and CHH sequence positions (H = A, T or C nucleotide), and sRNA regions of complementarity are methylated with CHH methylation in the *Arabidopsis* genome (14). There have been a few studies outlining a link between modifications in genomic DNA methylation and the exposing of plants to a diversity of environmental stresses (15). It has been demonstrated that stress exposure can cause changes to the DNA methylation status of particular target sites by inducing particular sRNAs to direct the DNA methylation process (16). RdDM has been shown to be reversed in stress response(17). Epigenetic changes associated with environmental factors such as abiotic or biotic stress may be capable of inducing modifications in gene expression (18). Salinity is an important abiotic stress affecting plants throughout the world (19). Salt can cause toxicity in plants resulting from a high accumulation of Na^+ ions in their tissues. As a result, decreasing Na^+ accumulation in plant tissues, particularly in their cytoplasm, is important for plant growth (20). Sodium chloride leads to extreme impacts on the metabolic processes (21). Deleterious DNA mutation was also observed in the rapeseed genome resulting from the effect of salt stress (22). DNA methylation is a chemical process, so it might be affected by high concentrations of Na^+ ions. It has been indicated that salinity stress affected DNA methylation in rice (23). DNA of mangroves has also been shown to become methylated under high salt conditions (24). Studies have aimed to discover genes and mechanisms that might affect *Arabidopsis* salt tolerance. They have recognised that *SOS1* contributes to tolerance as this gene is overly sensitive to salt (25,26). An antiporter Na^+/H^+ *SOS1* (At2g01980) works to decrease the level of sodium in the cytosol of *Arabidopsis* because *SOS1* is localised to the plasma membrane and has a role in the efflux of Na^+ ions and decreases their accumulation in the shoot (27). It is also well known that an At*HKT1* gene functions to regain Na^+ from the stellar cells of the root in order to decrease the transport of Na^+ from roots to the vascular tissues in leaves and shoots (21,28). Importantly, it has been recognised that there is a relationship between At*SOS1* and At*HKT1* functions to decrease the susceptibility to salt in *Arabidopsis* (29). However, it was demonstrated that in normal growth conditions only the At*HKT1* gene in the *Arabidopsis* genome was repressed in the vegetative tissues resulting from non-CG methylation (30). Currently, new techniques and laboratory equipment are emerging that can be applied in this field of study.This project aimed to determine the efficacy of direct delivery of double-stranded small RNAs (dssiRNAs) into the plant in mediating directed DNA methylation

of the At*HKT1* (At4g10310) and At*SOS1* (At2g01980) genes in response to salinity stress applied during the growth of *Arabidopsis* seedlings. Furthermore, this project examines the effect of the dssiRNA mediated silencing of the promoters of At*SOS1* and At*HKT1* on the levels of Na^+ and K^+ accumulation in plant leaves to identify to what extent salt and siRNA treatments can affect plant phenotypes. Promoter regions of gene sequences that had a potential association between their tandem repeat regions and the position of 24 nt dssiRNA were analysed. The present study might offer a simple and easy delivery method for genetic studies. Thus, a direct delivery method was examined. Treating roots with siRNA to guide DNA methylation will offer important opportunities to examine the ability of this method to silence genes.

Materials and methods

Plant Materials and Growth Conditions

Seeds of *Arabidopsis thaliana* 'Colombia' (Col-0) were provided by the Nottingham Arabidopsis Stock Centre (NASC ref. N1092) in the United Kingdom. Seeds were surface-sterilised and planted in 0.5x MS medium made according to the protocol of the Arabidopsis Biological Research Centre (ABRC) in sterile 90mm Petri dishes. Plants were grown in a controlled environment chamber (Environ Pty Ltd, SA) at 24 °C and 70 per cent RH with a 14-hour daylength. Thirty-six *Arabidopsis* seedlings, aged two weeks, were moved to MS medium which was prepared as above, but the agar was half in volume 5g/L. Plants

were divided into two groups each group include eighteen plants grown on three Petri dishes, each dish involve six plants. The first group was grown in medium that was untreated with salt, and the second was grown in media supplemented with 50 mM NaCl.. Plants in three dishes of two groups divided to: the first Petri dish was untreated with siRNA solutions as a control mock treatment (solution contains same element minus siRNA), the second was treated with SOS1-siRNA and the third was treated with HKT1-siRNA. Treated with siRNAs that target promoters of At*SOS1* and At*HKT*1 genes. The experiment of SOS1-siRNA treatment grown in media supplemented with 50 mM NaCl was repeated to confirm the result.

siRNA and Primer Design

The public databases TAIR (www .Arabidopsis.org,GBrowse) and the ASRP (http://asrp.cgrb .oregonstate.edu) were searched to identify the siRNA for At*SOS1*. The siRNA for At*HKT1* was identified from (30). Double-stranded siRNA for At*SOS1* was ARSP166665 (5'CCUUCUUUGAUGG UGGUGGCGAAU'3)and(3'AUUCGC CACCACCAUCAAAGAAGG'5) and for At*HKT1* was ARSP805(5'AUCUU CCACCAUCACAACCACCAG'3)and(3'CUGGUGGUUGUGAUGGUGGAA GAU'5); both were synthesised by (Sigma-Aldrich, Singapore). The primers for qPCR were designed using Primer3 (primer3 results.cgi release 0.4.0) and their bases for At*SOS1* (At2g01980) were (5'GCACCTTCTTT GATGGTGGT'3) and (3'ACCAACCG TCGATCCTAATG'5),and for At*HKT1* (At4g10310) the primers were (5'GTG GTTTGTTCGGGTTCAC'3) and

(3'CACGTGTCTTCGTTTTC'5). DNA oligo primers were synthesized by (Sigma-Aldrich, Singapore).

Plant Transformation with siRNA

The siRNA solution was made using the following protocol: 25 µl of 100 µM stock solution of siRNA was added to 25 µl Tween 20 (Polysorbate20) and then diluted with 50 µl of Ultra pure DEPC-treated water. The roots of plants were treated with siRNA solution and the plants were placed on Petri dishes that contained wet filter paper, and the roots were placed on 2 cm squares of parafilm M PM-996 to avoid sucking of the siRNA from the filter paper. Roots were cut and then one drop (2 µl) of 25 µM siRNA solution (with added pH neutral dye for the uptake test in Fig. 2) was added directly onto the end of the cut root. For the uptake test, at the same time another drop of the solution was dropped beside the root in order to examine whether plants were taking up the siRNA solution.

Phenotype Measurement and Analysis Tools

A digital camera (Camedia Olympus C-4040Zoom, Olympus Optical Co.ltd, Japan) was used to photograph the plants. The first photos were taken at the beginning of the treatment when the seedlings were two weeks old, and the second photos were taken after one week of treatments. ImageJ software (ImageJ1.45<imagej.en.softonic.com>) was used to measure the total area of plants in mm^2.

SODIUM AND POTASSIUM ACCUMULATION

The last fully extended leaf, approximately insertion 2, was harvested from each plant and used to measure Na$^+$ and K$^+$ accumulations. Fresh weights of leaves were measured using a digital scale and each leaf was placed into a 2ml tube and adjusted by 2ml of 1 % nitric acid and then incubated at 60 °C overnight, before being diluted 1:5 in sterile water (Milli-Q). Model 420 Flame Photometer (supplied by Sherwood Scientific Limited in United Kingdom) was used to read the amount of sodium and potassium in each sample. The concentration of Na$^+$ and K$^+$ were then accounted for in the following equation: (flame reading x coefficient / fresh weight).

DNA Extraction and Methylated

DNA Capture

Genomic DNA from *Arabidopsis* leaf tissues that were used to test primers for PCR reactions of genes of interest (At*SOS1*, At*HKT1*) was donated by Dr. Adam Croxford. The PCR reaction was made as follows: 10µl of 1x dNTPs mix (Bioline (Aust) Pty Ltd) and 1µl of 50 mM MgCl$_2$ (Bioline (Aust) Pty Ltd) were added to 1µl of 4 µM of each forward and reverse primer then 3µl of the 20ng DNA template. Finally 4µl of sterile water was added to complete the 20µl PCR reaction mix. Thermal cycler conditions of the PCR reaction were 95 °C for two minutes, 95 °C for 30 seconds, 50 °C for 30 seconds, 69 °C for 30 seconds, 72 °C for two minutes and 4°C end using the T100^{TM} thermal

cycler (BioRad, SA). Products were detected by gel electrophoresis using a Gel DOC^{TM} Imager (BioRad, SA). Two fully expanded rosette leaves of each plant, approximately insertion 3, were harvested and used for DNA extraction based on the protocol in (31). A NanoDrop spectrophotometer (ND1000) and software (ND-1000, V3.3.0) were used to test the quality and quantity of methylated DNA. The DNA was captured using a Methylamp^{TM} Methylated DNA Capture Kit (Epigentek Group, United States) according to the manufacturer's instructions. The methyl capture kit contained an antibody that was specific to methyl cytosine for the enrichment of methyl-positive DNA and Normal Mouse lgG as a negative control that captures unmethylated genomic DNA (methyl-negative). QPCR was used to compare the amount of methyl-positive versus methyl-negative DNA. Each 20 µl qPCR reaction consisted of 3µl of antibody-specific DNA which was added to 1µl each of 10 µM of both forward and reverse primers, 0.5µl of Eva Green dye (Bioline (Aust) Pty Ltd), 10µl of Biomix (Bioline (Aust) Pty Ltd) and made up to volume with H_2O. The thermal cycler conditions for both genes At*SOS1* and At*HKT1* were 95 °C for 30 seconds, 50 °C for 30 seconds and 69 °C for 30 seconds. This was repeated for 60 cycles using a Rotor-gene^{TM} 6000, (Qiagen, UK). R-Corbett Research (Rotor-gene^{TM} 6000 software) was used to analyse the Ct value measurements. The experiment was repeated twice for each gene to confirm the results, with two technical replicates including the uncaptured genomic DNA as a positive control. GenStat (14th Edition v003) was used for the two-way ANOVA of the effects of siRNA treatment and A two-way ANOVA was used to statistically analyse the variance of Na^+ and K^+ levels and the mean (SEM) for six biological replicates. The results are the average and standard error of the mean (SEM) for six biological replicates.

Results and Discussion

Identification of target siRNAs

Direct delivery of siRNAs into the roots of treated plants may enable the discovery of whether siRNAs can elicit targeted DNA methylation of genes in *Arabidopsis thaliana* in response to specific stress. In previous works, transgenic material has been used for this purpose. Plants have been transformed in various ways, including *Agrobacterium*-mediated and particle bombardment transformation. In the present study, the transgenic approach was circumvented through the direct application of siRNA into the roots of plants. Plant roots were wounded and then treated with a solution that contained synthesised dssiRNA. Two public databases were searched to find locations of DNA methylation and siRNAs: the *Arabidopsis thaliana* Small RNA Project (ASRP) database, (http://asrp.cgrb.oregonstate.edu) and TAIR (www.arabidopsis.org,GBrowse). Methylation is usually found in the areas where the tandem repeat regions and siRNA are co-located. The search showed that a dssiRNA (ASRP ref. 166665), from the *dcl-1* whole mutant (32) in *Arabidopsis* has been read 12 times as associated with the promoter regions of At*SOS1* (AT2G01980). In addition, another dssiRNA (ASRP ref. 805) has been read six times as associated with tandem repeat

sequences of the promoter regions of AtHKT1 (At4g10310). The results showed a location 2000 bp upstream of AtSOS1 that could create a 399 bp PCR product that might be mediated by dssiRNA (ASRP 166665). The methylated AtSOS1 promoter region is illustrated in (Figure 1), which shows the genomic context of siRNAs around the AtSOS1 locus. Furthermore, it was shown that the location 2600 bp upstream of the AtHKT1 provided a 292 bp PCR product which might be associated with dssiRNA (ASRP 805), which is likely to methylate the AtHKT1 promoter region (30). Figure 2 illustrates the genomic context of siRNAs around the AtHKT1 locus. DssiRNA, ASRP166665 (SOS1-siRNA) and ASRP805 (HKT1-siRNA) were synthesized, applied to plants and examined in order to identify their capability to silence these genes.

Delivery Method

The direct uptake of exogenous genetic material using biogenetic transformation methods is the best way to obtain genetically modified cells that can produce a positive change in plants to develop their tolerance to environmental stress or to improve their productivity (18). Exogenous synthesized the siRNAs were introduced to plants by treating their roots directly with a solution containing siRNA. The plant tissue absorbed the siRNA solution one hour after treatment. There were two lines of evidence to suggest that siRNA had been successfully introduced into the plants in this study. First, the drop of the siRNA solution applied to the end of the root was absorbed after one hour, while another drop that was at a distance from

the root remained at the same size over the same period (Figure 3). Second, the siRNA solution contained red dye and the colour of the root changed to red after one hour, which confirms the siRNA solution uptake. Biological scientists have used different methods to make changes in plant genomes such as Agrobacterium-mediated floral dip and particle bombardment. However, these transgenic methods are considered difficult to use in plants (33,34) because they require a specific stage of plant growth that requires a long time to analyse and to investigate the results. For example, the floral dip method requires waiting until the flowering stage before the plants can be transformed with genetic materials. It also requires a high level of laboratory skills and is expensive (34). Conversely, the method that was used in this study required simple tools and did not need a high level of laboratory skills. It can also be used at an earlier stage of plant development. This method was used when plants were two weeks old, and absorbed siRNA was recognised within the plants after one hour. Moreover, only seven days were needed after the application to identify the results of the transformation. However, this method requires more examination to confirm its result because there was no proof that the plant was transformed genetically by the siRNA.

Phenotype Impacted by Abiotic Stress and siRNA Transformation

This study examined the effects on the *Arabidopsis* phenotype as a result of transferring siRNA into plants that were grown under salt stress. The result showed that the transformation of

SOS1-siRNA and HKT1-siRNA into plants grown in medium with 50 mM NaCl impacted the plants' phenotype. SOS1-siRNA treatment caused the death of the seedlings, and plants treated with HKT1-siRNA presented very small seedlings with curled leaves (Figure 5). Different concentrations of salt caused. differences in the plant area and phenotypes between *Arabidopsis* seedlings that were treated with AtSOS1-siRNA, HKT1-siRNA and controls. In the control group, there was no significant difference in leaf area (mm^2) between plants grown in media supplied with 0 mM NaCl and those containing 50 mM NaCl (Figure 4). In contrast, plants treated with siRNA-HKT1 and grown on 0 mM NaCl had a significantly larger leaf area than those grown in media with 50 mM NaCl Whereas the area and phenotype of plants grown in a medium with 0 mM NaCl were normal and approximately similar to the control treatment, plants grown with 50mM NaCl presented very curled and small-sized leaves (Figure 5). Interestingly, results for plants treated with siRNA-SOS1 showed a high level of difference in comparison with other treatments. Plants grown with 0 mM NaCl had very big leaves, and a significantly larger area than the comparable control plants, while all seedlings grown with 50 mM NaCl had dramatically reduced size (Figures 4 and 5). Importantly, plants treated with SOS1-siRNA invariably died after seven days of treatment (Figure 5). To confirm this result for the SOS1-siRNA treatment, this experiment was repeated and the same result was obtained: treated plants died after one week of treatment. It has been pointed out that there is a level of interaction between changes of environmental circumstances and transgenic modifications (35). Epigenetic products in response to environmental conditions and transposable elements are supposed to change phenotype (36). Tricker *et al.* (37) demonstrated that stomata on the leaves of *Arabidopsis thaliana* altered in response to the percentage of humidity; they pointed out that changes in the environmental state were capable of inducing siRNA to direct DNA methylation and down-regulate genes that manage stomatal development. The *FWA* gene in *Arabidopsis* exhibits a late-flowering phenotype resulting from the RdDM pathway (38). The phenotypic flexibility is repressed resulting from DNA methylation in plants which proposes that the outcome of DNA methylation on seedlings is essential changes in plant phenotypes (39). The *SOS1* gene plays a role in the efflux of Na$^+$ from the shoot and reduces Na$^+$ accumulation; high levels of sodium in plant tissues cause toxicity which affects plant metabolism and negatively modifies its phenotype (40). It is obvious that over-expression of the At*SOS1*1 and At*HKT1* genes is important in order to increase *Arabidopsis* salt tolerance. The epigenetic pathway has been considered a mechanism to control transposon movement (40). This study indicated that transformation with SOS1-siRNA and HKT1-siRNA might suppress the expression of the genes (At*SOS1*1 and At*HKT1*), which could have significant effects on plant phenotypes (Figure 5).
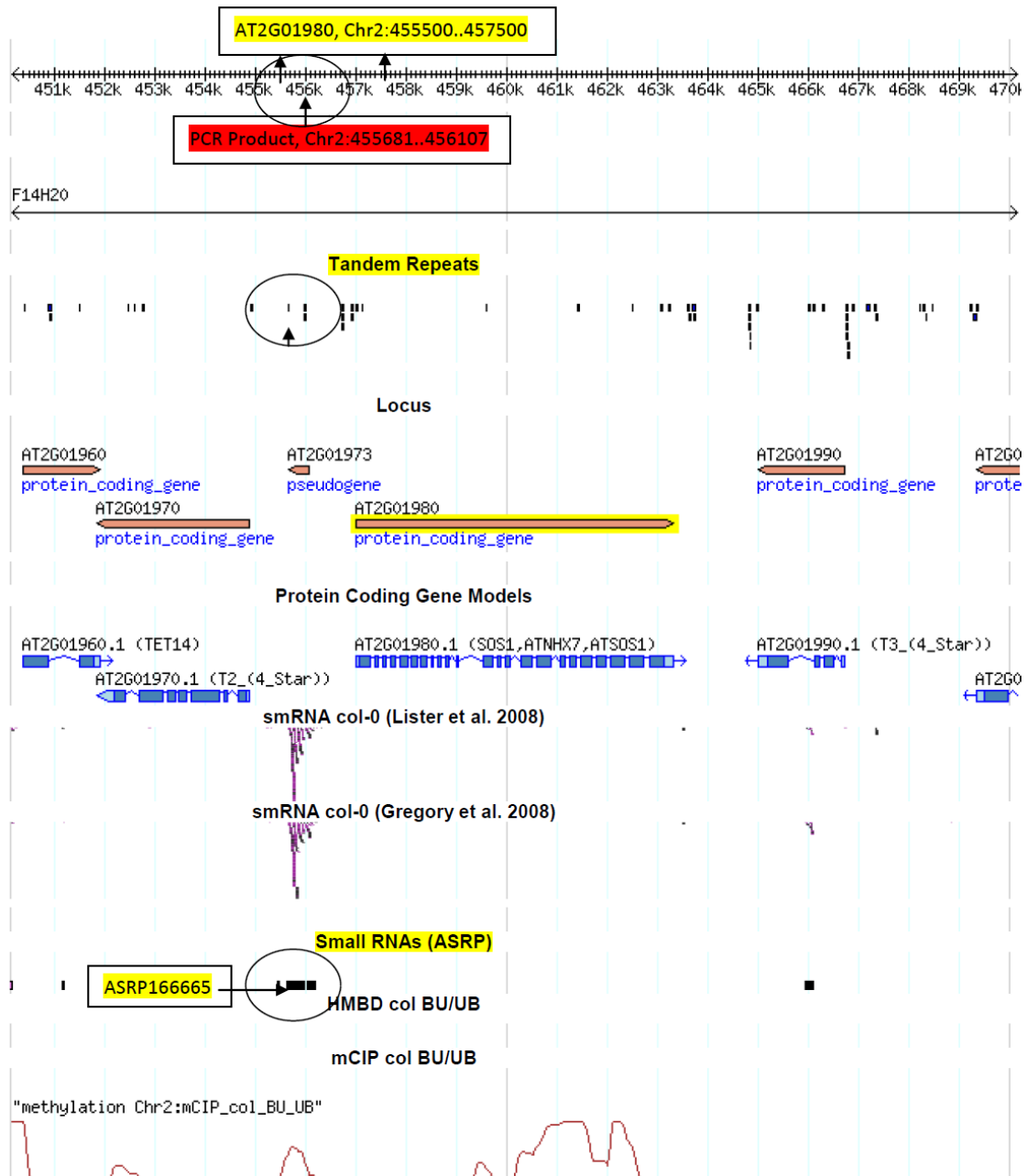
Figure (1): Genomic Context of siRNAs around the At*SOS1* Gene Locus.
This figure shows the position selected to design primers 2000 bp upstream of the At*SOS1* gene on chromosome 2 between 455500 and 457500 bp. The PCR product is 399 bp starting from 4557681 bp to 456107 bp as shown in the red box. This PCR product was used to analyse the DNA methylation of the At*SOS1* gene promoter. The siRNA location (ASRP166665) is illustrated by the yellow boxed label. The circles show that the tandem repeat that is 22 bp in length might be mediated by ASRP166665 because they have associated locations. The peaks show previously reported DNA methylation. Sourced from (TAIR, www.arabidopsis.org, GBrowse).
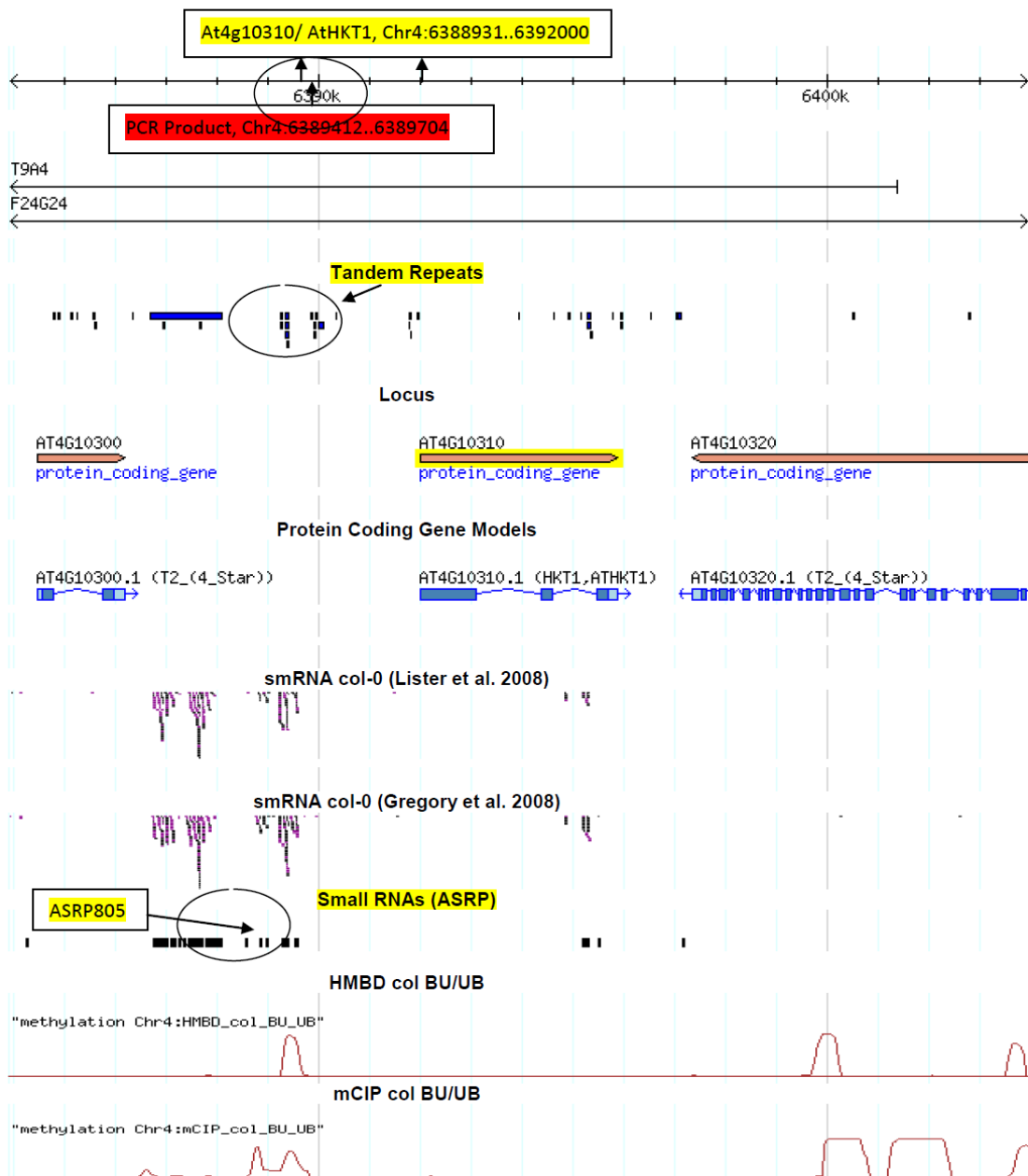
Figure (2): Genomic Context of siRNAs around the At*HKT1* Gene Locus.
This figure shows the position selected to design primers 2.6 kb upstream of the At*HKT1* gene on chromosome 4 between 6388931 and 6392000 bp. The PCR product is 292 bp starting from 6389412 bp to 6389704 bp as shown in the red box. This PCR product was used to analyse the DNA methylation of the At*HKT1* gene promoter. The siRNA location (ASRP805) is illustrated by the yellow boxed label. The circles show that the tandem repeat that is 24 bp in length might be mediated by ASRP166665 because they have associated locations. The peaks show previously reported DNA methylation. Sourced from public database (TAIR,www.arabidopsis.org, GBrowse), and the particular siRNA was identified from the work in (30).
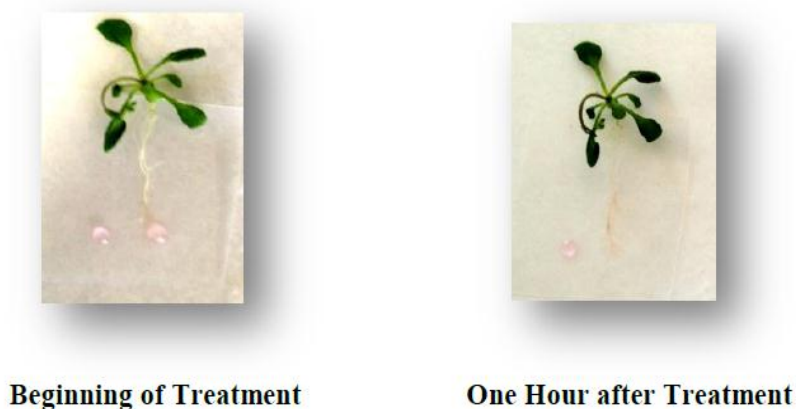
Beginning of Treatment   **One Hour after Treatment**

Figure (3): The Transformation Method of siRNAs into *Arabidopsis* Seedlings.
The *Arabidopsis* roots were treated with two drops, each one of 2 μl of 25 μM siRNA solution. The first drop was applied directly to the end of the wounded root, and a second drop was applied beside the first for comparison as shown on the left. While the roots have absorbed the directly applied siRNA drop after one hour, the other drop remained the same size, as shown on the right.
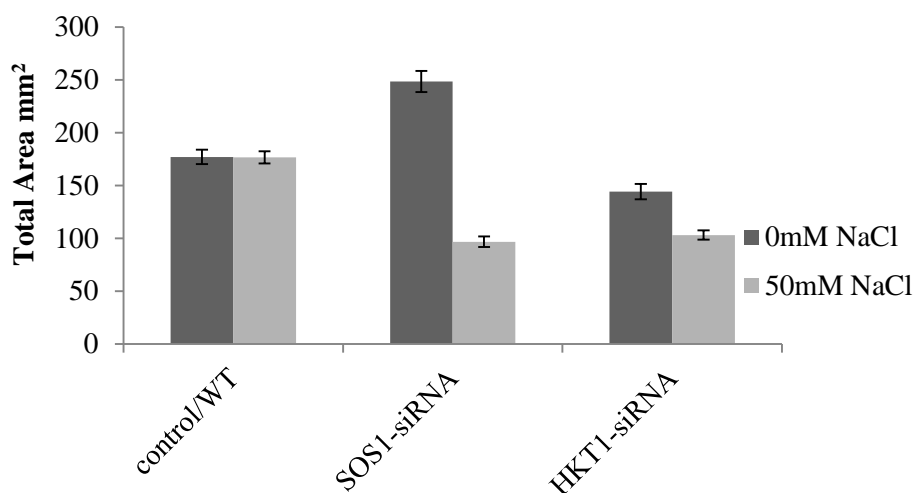
Source of Variation	df	P-value	F crit
Sample (siRNA)	2	0.038298	3.31583
Columns (salt treatments)	1	0.001128	4.170877
Interaction (siRNA× salt)	2	0.004869	3.31583

Figure (4): Interaction Between siRNAs and Salt Treatments.
This figure shows the mean total areas (mm^2) (± SE) of *Arabidopsis thaliana* plants treated with siRNAs (SOS1-siRNA or HKT1-siRNA) compared with the untreated control (WT= wild type) and salt (0 or 50mM) (n=6). Zero mM NaCl is represented by dark bars and 50 mM NaCl is represented by light bars. The results of a two-way analysis of variance (ANOVA) show statistically significant differences at $p < 0.05$.
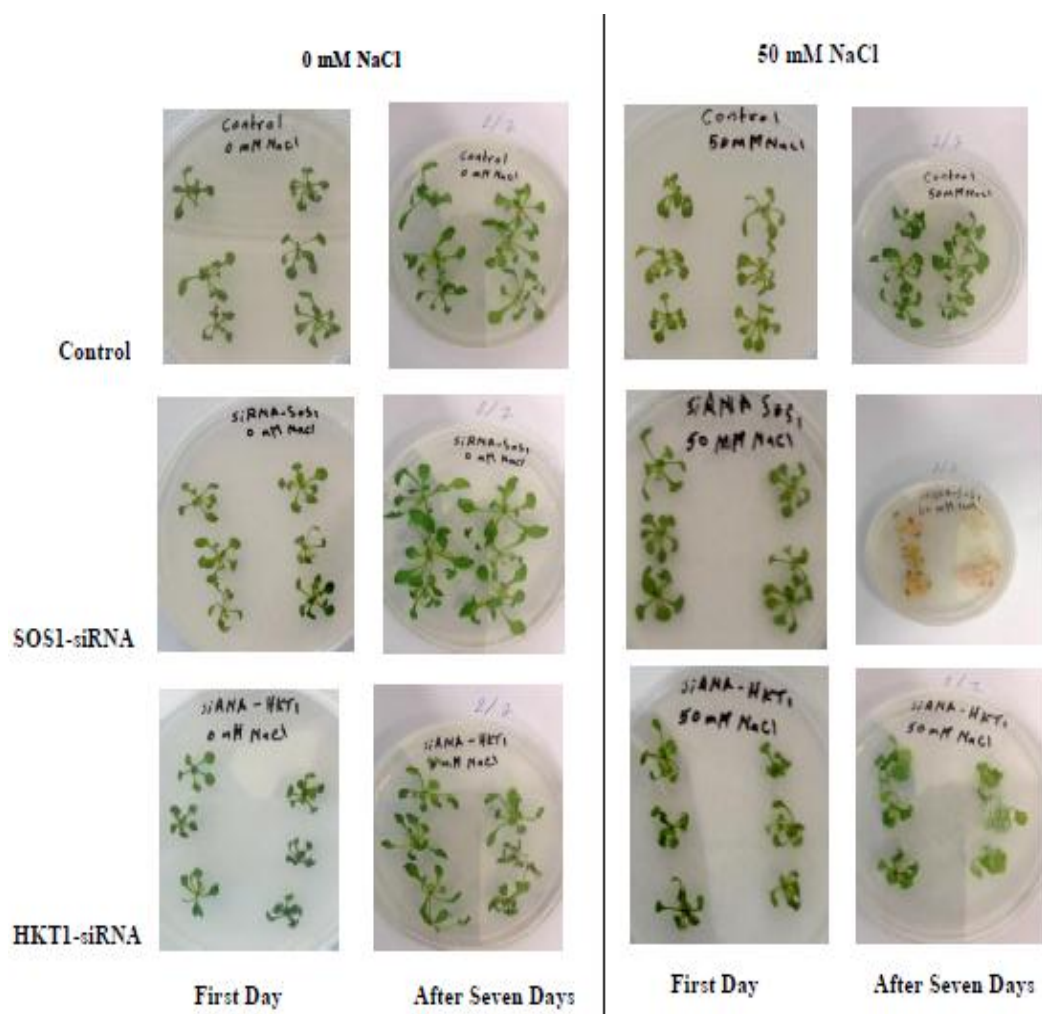
Figure (5): Effect of Salt Stress and siRNAs Treatments on *Arabidopsis* Phenotype.
The figure shows two-week-old *Arabidopsis* seedlings grown in media supplemented with 0mM NaCl on the left hand side and with 50mM NaCl on right hand side, day one and day seven of the three treatments: control (WT), SOS1-siRNA and HKT1-siRNA. HKT1-siRNA was similar to the control treatment and SOS1-siRNAwas larger than the control treatment in 0mM NaCl. In contrast, the SOS1-siRNA and HKT1-siRNA treatments affected plants grown with 50 mM NaCl: SOS1-siRNA caused death and HKT1-siRNA caused curling and small size of plants after seven days of treatment.

Sodium Chloride and Potassium Accumulation

The results demonstrated that HKT1-siRNA and SOS1-siRNA treatments caused different Na^+ and K^+ accumulations in *Arabidopsis* leaves when the plants were grown in a medium supplemented with 50 mM NaCl in comparison with these grown with 0 mM NaCl. The results also showed an inverse relationship between an increase of Na^+ ions and a decrease of K^+ ions (Table 1) and (Figures 6 and 7). *Arabidopsis* leaves accumulated a high concentration of Na^+ when the

plants were treated with siRNAs (Figure 6). Plants were grown in a medium that was supplemented with 50mM NaCl in order to compare them with similarly treated plants that were grown in a medium with 0 mM NaCl. As expected plants treated with SOS1-siRNA and HKT1-siRNA which were grown in media with 0 mM NaCl did not accumulate high levels of Na^+, and all the treatments accumulated approximately the same concentration of Na^+ (Table 1). However, plants that were grown in the medium with 50 mM NaCl presented a significant difference in Na^+ accumulation in the leaf ($p < 0.05$). Control and HKT1-siRNA treatments accumulated a high concentration of Na^+, while the SOS1-siRNA treatment accumulated a lower concentration of Na^+ (Table 1 and Figure 6). Potassium accumulation contrasted with the Na^+ accumulation. The results showed differences between plants that were treated with HKT1-siRNA and grown with 0 mM NaCl compared with SOS1-siRNA and control treatments Table 1 and Figure 7. The SOS1-siRNA and control treatments accumulated approximately the same amount of K^+ ions, while the HKT1-siRNA treatment accumulated fewer K^+ ions. In contrast, plants that were grown in a medium with 50mM NaCl showed a significant difference ($p<0.05$) between all treatments Figure 7. The K^+ accumulation in plants that were grown with 50 mM salt was lower than the K^+ in plants grown with 0 mM salt. The level of K^+ accumulation was the highest in the HKT1-siRNA treatment, followed by the control-WT and the lowest value was shown in SOS1-siRNA treated plants. The results thus showed that the SOS1-siRNA and HKT1-siRNA treatments affected Na^+

and K^+ concentrations in *Arabidopsis* in response to different levels of salt. It has been shown that over-expression of At*HKT1* could decrease the Na^+ concentration in vegetative tissues (42). Moreover, it was confirmed that up-regulation of At*HKT1* leads to a decrease in Na^+ accumulation in the shoot and an increase in K^+ concentration (43) . Studying the role of genes in *Arabidopsis* has demonstrated that the *AtHKT1's* function is to transport Na^+ between tissues, which is likely to organise the level of Na^+ ions in roots (29). Currently, it is well understood that the initial function of At*HKT1* is to return Na^+ from shoots to the roots to prohibit Na^+ accumulation in the shoots (21,28). The At*SOS1* gene (salt excessively sensitive) also was recognised as plasma membrane Na^+/H^+ antiporter in *Arabidopsis*, and its role is to decrease the concentration of Na^+ in the cytosol (27). In addition, it was found that a high expression of At*SOS1* leads to a decrease in the accumulation of Na^+ in the entire plant (41). The results in this study revealed that treating plants with SOS1-siRNAs caused decreasing of Na^+ and K^+ concentrations in plant leaves in response to salt treatment compared with control treatment, while in the HKT1-siRNA treatment Na^+ ions were approximately similar to control and K^+ was increased. Plants treated with SOS1-siRNA were not able to survive after seven days as shown in figure 5, which may interpret this a decrease of Na^+ and K^+ concentrations compared with control treatment. Therefore, it is expected that the transformation of HKT1-siRNA and SOS1-siRNA into plants might affect the At*HKT1* and At*SOS1* genes' expression transcriptionally, resulting from an epigenetic

pathway, which could affect the transportation of Na^+ and K^+ within plant tissues. Some studies have outlined a link between modifications in genomic DNA methylation and the exposing of plants to diverse stresses (15).

Modification in the chromatin shapes resulting from siRNA could correlate with directive gene expression and its action against environmental stress (35,44). It has been identified that a small RNA-mediated DNA methylation occurs in particular plant cells and that it controls the At*HKT1* transcription (30). Importantly, a relationship was recognised between the functions of the At*SOS1* and At*HKT1* and the repressing of Na^+ accumulation (29). This led to the assumption that the At*HKT*1 and At*SOS*1 proteins might act together to manage Na^+ and K^+ levels in plant tissues. Therefore, taking into account the previous studies, it can be suggested that siRNAs transformed into plants could negatively affect the At*SOS1* and At*HKT1* genes' expressions and cause a changed accumulation of Na^+ and K^+ ions in the shoots of *Arabidopsis* (Figures 6 and 7).

Table (1): Na^+ and K^+ content (μMol g^{-1}) in fresh weight (FW) of the last fully expanded leaf of *Arabidopsis* seedlings.

| Treatments	FreshWeight Leaves,FWg	Flame reading Unit of Na^+	Flame reading Unit of K^+	μMol Na^+ /g^{-1}FW	μMol K^+/g^{-1}FW
Control 0mM NaCl	0.01	1.07	24.21	3.43	76.45
SOS1siRNA 0mM NaCl	0.009	1	37.31	2.18	79.6
HKT1siRNA 0mM NaCl	0.007	0.73	20.05	2.81	61.82
Control 50Mm NaCl	0.012	45.9	24.18	79.55	41.55
SOS1siRNA 50mM NaCl	0.01	1.07	24.21	22.18	12.67
HKT1siRNA 50mM NaCl	0.009	1	37.31	75.32	54.85

Arabidopsis treated with siRNAs (Wild type (WT), SOS1-siRNA and HKT1-siRNA) and salt (0 or 50 mM). The Na^+ and K^+ values of plants were measured as a means of six replicate plants.
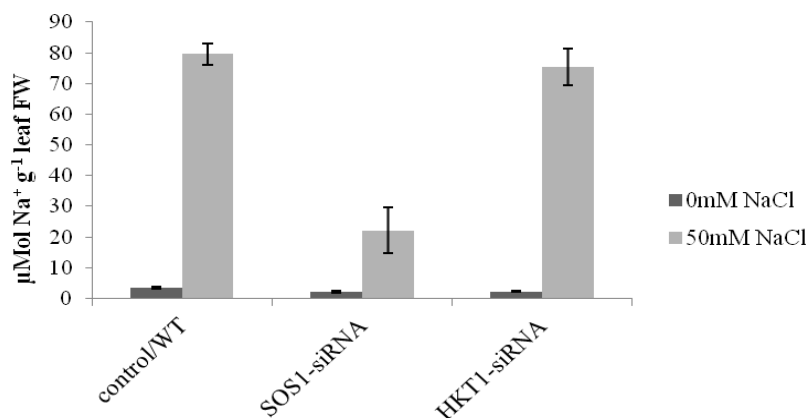
Source of Variation	df	P-value	F crit
Sample (siRNA)	2	6.73E-08	3.31583
Columns (salt treatments)	1	1.27E-16	4.170877
Interaction (siRNA× salt)	2	1.14E-07	3.31583

Figure (6): The Concentration of Na$^+$ Ions in *Arabidopsis* Leaves Treated with siRNAs and Salt.
This figure shows the differences in Na$^+$ concentration accumulated in the *Arabidopsis* leaves in response to different salt concentrations and siRNA treatments. Plants were treated with siRNAs (Wild type (WT) mock treatment, SOS1-siRNA and HKT1-siRNA) and salt (0 or 50 mM). The Na$^+$ value was measured as the mean concentration (μMol Na$^+$ g^{-1} leaf) of six plants ($\pm$ SE). Zero mM NaCl is represented by dark bars and 50 mM NaCl is represented by light bars. The results of the two-way ANOVA are discussed above. $p < 0.05$ shows statistically significant differences. The interaction was between Na$^+$ contents, when plants were treated with different siRNAs and grown with 0 or 50 mM NaCl.
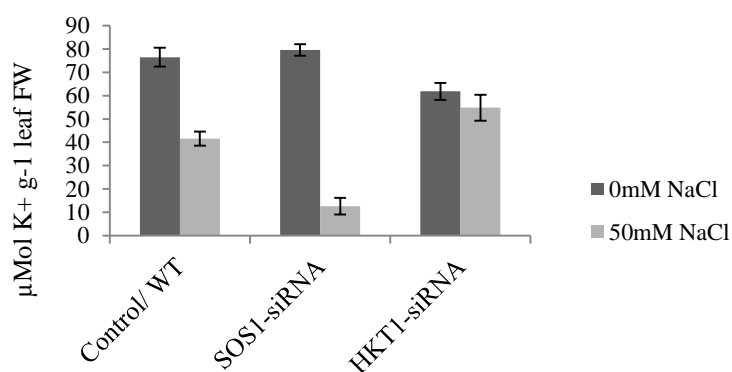
Source of Variation	df	P-value	F crit
Sample (siRNA)	2	0.055428	3.31583
Columns (salt treatments)	1	1.22E-08	4.170877
Interaction (siRNA× salt)	2	5.97E-05	3.31583

Figure (7): The Concentration of K$^+$ Ions in *Arabidopsis* Leaves Treated with siRNAs and Salt.
The figure shows the differences in the K$^+$ concentration accumulated in *Arabidopsis* leaves in response to salt and siRNAs treatments. Plants were treated with siRNAs (Wild type (WT) mock-treated, SOS1-siRNA and HKT1-siRNA) and salt (0 or 50 mM). The K$^+$ value was measured as the mean concentration (μMol K$^+$ g^{-1} leaf) of six plants ($\pm$ SE). Zero mM NaCl is represented by dark bars and 50 mM NaCl is represented by light bars. The results of the two-way ANOVA are discussed above. $p < 0.05$ shows statistically significant differences. The interaction was between K$^+$ contents, when plants were treated with different siRNAs and grown with 0 or 50 mM NaCl.

DNA Methylation

This project was established to further explore the function of siRNA to direct DNA methylation. To examine the ability of siRNAs to direct DNA methylation, an affinity-based methyl capture was used to enrich methyl-negative and methyl-positive DNA from each sample and was analysed with qPCR to determine the DNA methylation ratio in the DNA of the At*SOS1* and At*HKT1* promoters. The results identified that transformation of HKT1-siRNA into plants caused differences in the DNA methylation ratio of the At*SOS1* promoter between plants grown with 0 mM NaCl and plants grown with 50 mM NaCl. Plants grown in medium supplied with 50 mM NaCl showed differences in the cycle threshold (Ct) of amplification between methyl-positive and methyl-negative sample DNA (Figures 8 and 9). The HKT-siRNA induced more methylation in the At*SOS1* promoter because methyl-positive DNA was amplified faster than methyl-negative. However, SOS1-siRNA and control treatments presented no differences in DNA methylation ratios within each sample, as results from the Ct value indicated statistically insignificant differences between methyl-negative and methyl-positive (Figure 9). Similarly there was no difference between methyl-positive and methyl-negative amplifications in the DNA of At*HKT1* promoter in the plants that were grown in a medium applied with 0 or 50 mM NaCl or treated with either HKT1-siRNA or SOS1-siRNA (Figure 10). This finding indicated that salt stress might not affect the DNA methylation ratio at this position of the *AtHKT1* promoter region.

siRNAs can be generated at tandem repeat regions in the *Arabidopsis* genome, which suggests that tandem repeat sequences mediated with siRNA are probably able to silence genes (45). When inserted into *Arabidopsis*, siRNA has directed the DNA methylation of the *FWA* gene as a result of two tandem repeat regions that are associated with its DNA target sequences (46). In addition, it has been shown that transposable elements that are correlated with tandem repeat sequences can induce siRNA to methylate the DNA of the *FWA* gene and effectively suppress or silence its expression (14,47,48). As a consequence of what has been studied earlier, it can be seen that a characteristic involved in many targets of RdDM is tandem repeats. Furthermore, it has been demonstrated that siRNA can direct transcriptional processes in response to biotic or abiotic stress (40,44). The At*HKT1* gene and its action are important for salinity resistance in *Arabidopsis* and the relationship between tandem repeat regions and the location of sRNA targets are significant for continuing this gene's expression (30). Results of this study indicated that the At*SOS1* promoter was methylated in the presence of HKT1-siRNA when treated with salt. This contrasted with the results of Baek *et al.*,(30) which showed that this siRNA (HKT1-siRNA (ASRP 805)) was able to recruit DNA methylation in the promoter sequence of the At*HKT1* gene in *Arabidopsis.* In the SOS1-siRNA treatment, the Ct value of methyl-negative was lower than that in the methyl-positive in plants grown with 50 mM salt. However, the amplified methyl-positive might indicate that some cytosine positions are methylated in a

similar ratio to methyl-negative, which might indicate that there is DNA methylation. This suggestion is consistent with the extreme effects on phenotype that occurred in plants that were treated with SOS1-siRNA and subjected to salt stress, which indicated that gene expression was down-regulated. Additionally, there were insignificant differences in Ct values between treatments in comparison with the control at the At*HKT1* promoters when plants were grown with 50 mM NaCl and treated with SOS1-siRNA and HKT1-siRNA, which might indicate that a level of DNA methylation had occurred. As mentioned earlier, these results when taken together with the strongly affected phenotype could indicate that the At*HKT1* impact and its expression were down-regulated in some way. The siRNA function is still not well understood. It was previously shown that not all tandem repeats could be changed by small RNA to direct DNA methylation in the genome of *Arabidopsis* (44). In addition, it was confirmed that tandem repeats linked with genes that are suppressed or silenced by an RdDM mechanism, may not take place in the all targets of gene promoters (49). To conclude, a key outcome of this experiment was that the function of siRNAs to induce DNA methylation relies on the character of the siRNA position. The At*SOS1* promoter was methylated in the presence of HKT1-siRNA when treated with salt. However, there were not large differences between methylated and unmethylated ratios of At*SOS1* and At*HKT1* gene promoters when treated with SOS1-siRNA and HKT1-siRNA. Also, even though the direct transformation method of introducing siRNA into plants could be a successful method for uptake it did not prove that the plant was transformed genetically. On the other hand, this method impacted on plant phenotype when plants were grown in 50 mM salt. This might indicate that siRNA repressed gene expression in some way, but not in the RdDM pathway. One possible explan-ation is that these siRNAs might down-regulate At*SOS1* and At*HKT1* gene expression in a post-transcriptional way. Similar epigenetic marks are able to do post-transcriptional or transcriptional gene silencing (14). Thus, more investigation is needed to understand the role of siRNA function in silencing gene expression.
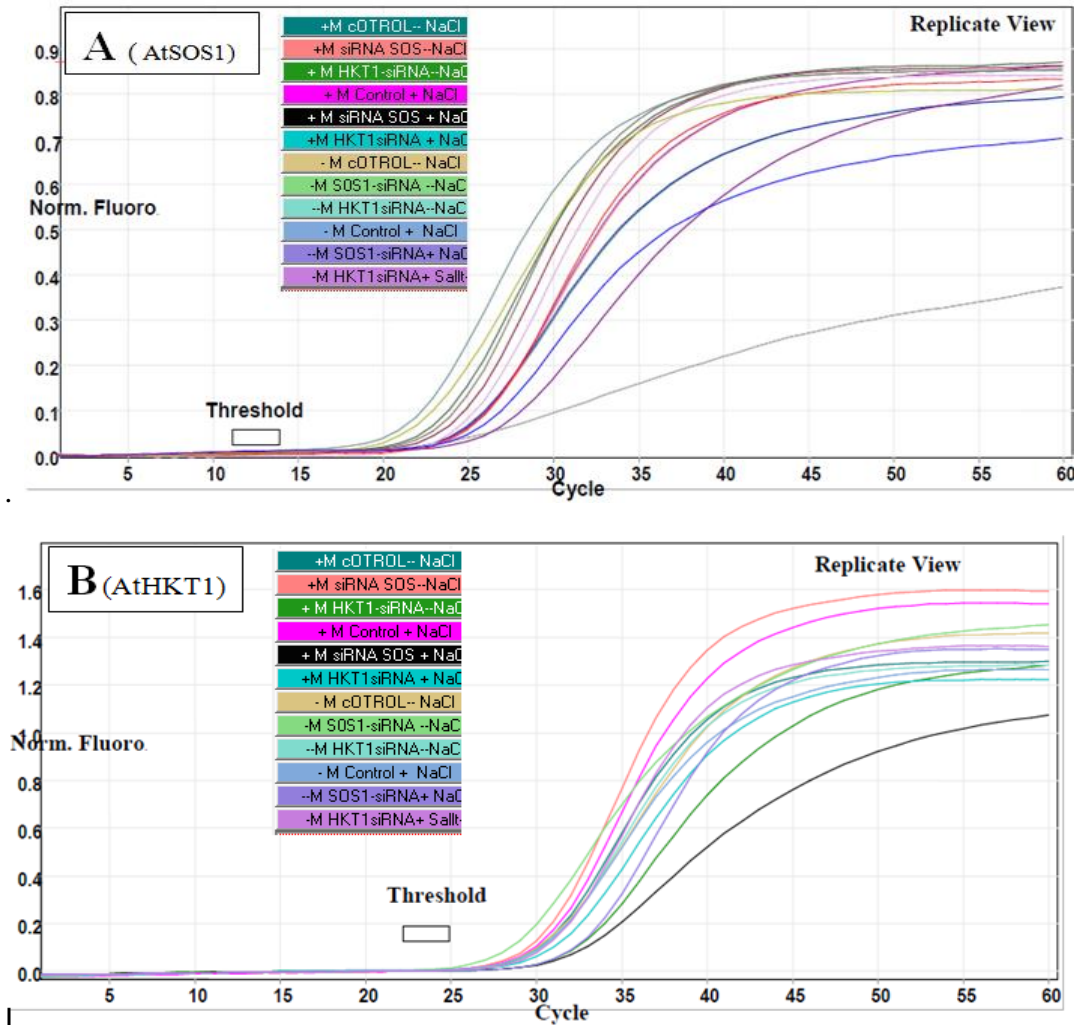
Figure (8): Contrast in Normalised Fluorescence Curves of Methylated (+M) and Unmethylated (-M) DNA for the *AtSOS1*(A) and *AtHKT1*(B) Promoters in *Arabidopsis* Following siRNA and Salt Treatments.

This figure shows the amplification curves of sample DNA. The Ct values for a number of qPCR cycles are used to analyse the methylated DNA capture of At*SOS*1 and At*HKT1* gene promoters. The DNA was extracted from two groups of *Arabidopsis* seedlings. The first group was grown in media supplied with 0 mM salt (-NaCl) and the second with 50 mM salt (+NaCl), and they were treated with SOS1-siRNA, HKT1-siRNA or control (mock-treated). Genomic DNA was used to capture methylated (+M) or unmethylated (-M) DNA. Following qPCR, if +M was amplified faster, it indicated DNA methylation. (A) Shows amplification for the methylated DNA capture of the At*SOS1* gene promoter and (B) shows amplification for the methylated DNA capture of the At*HKT1* gene promoter.
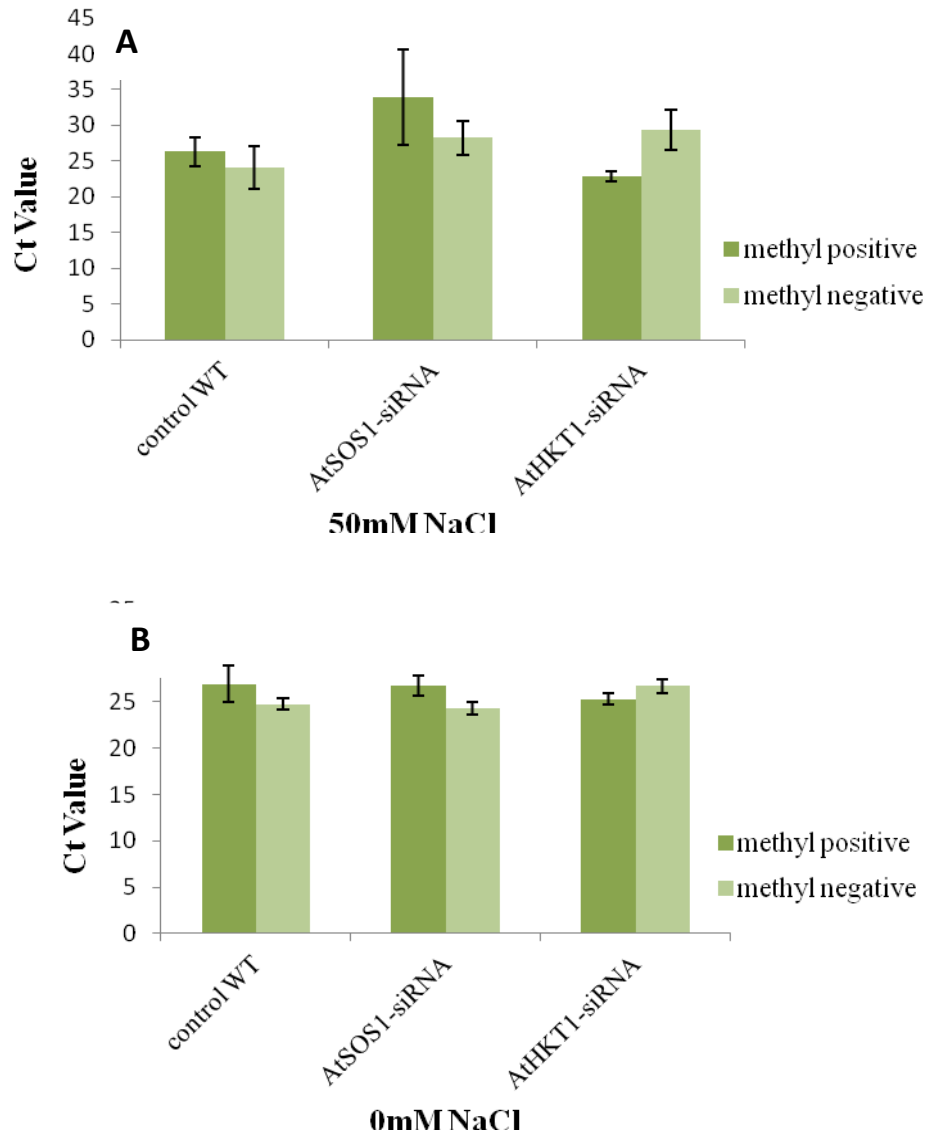
Figure (9): Quantification of qPCR Analysis for Methylated DNA Capture at the At*SOS1* Gene Promoter in *Arabidopsis*.
The figure shows Ct values of qPCR analysis for methyl-negative and methyl-positive treated DNA of the At*SOS1*gene promoter. Methyl-negative is represented by light bars and methyl-positive is represented by dark bars. DNA was extracted from a pool of three replicate plants. Ct values represent the mean of two replications of qPCR reactions for each treatment. Methyl-positive that is amplified faster indicates that DNA is methylated. (A) Shows that only HKT1-siRNA caused DNA methylation to the DNA of the At*SOS1* promoter in 50 mM NaCl. (B) Shows no differences in methylation ratios between the treatments when plants were treated with SOS1-siRNA and HKT1-siRNA, and 0 mM NaCl.
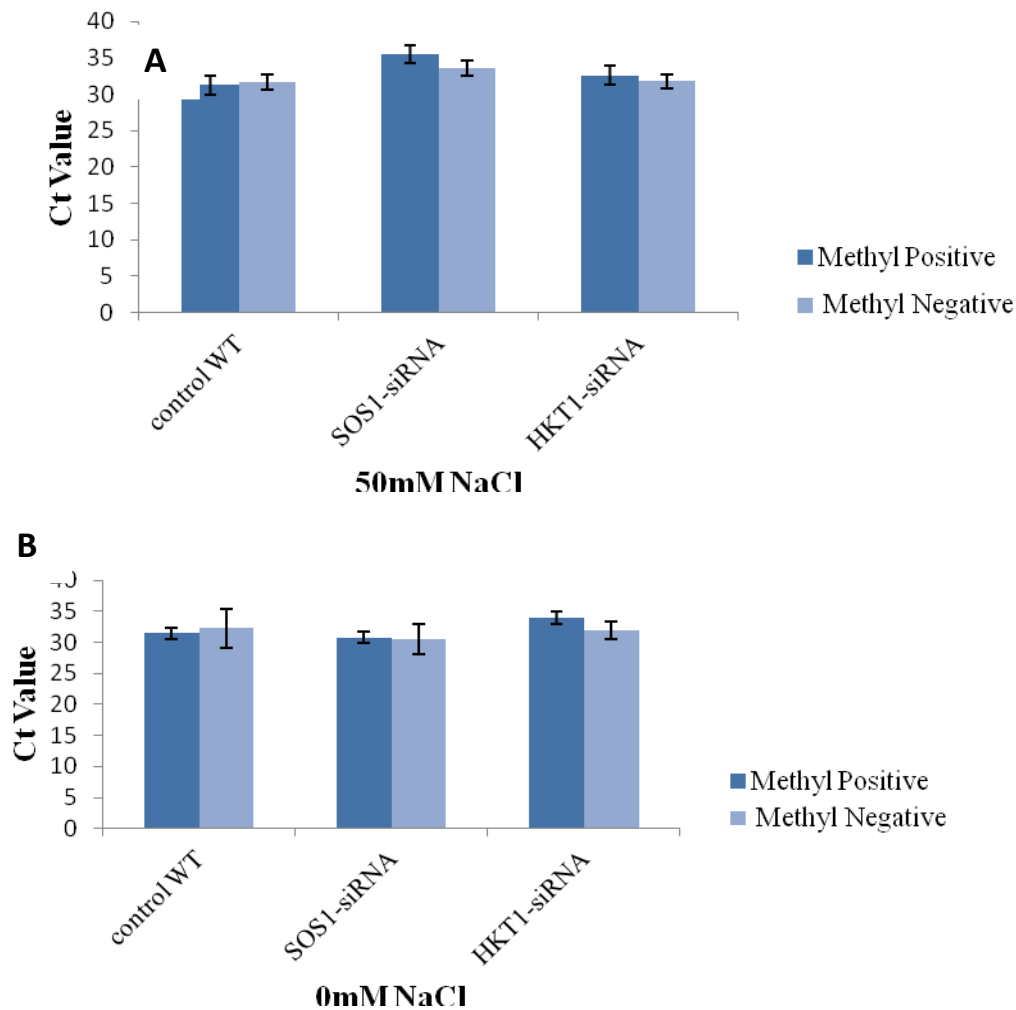
Figure (10): Quantification of qPCR Analysis for Methylated DNA Capture at the At*HKT1* Gene Promoter in Arabidopsis.
The figure shows the Cycle threshold (Ct) values of qPCR analysis for methyl-negative and methyl-positive DNA of the At*HK1*gene promoter. Methyl-negative is represented by light bars and methyl-positive by dark bars. Genomic DNA was extracted from a pool of three replicate plants The Ct values presented are the mean of two replications of qPCR reaction for each treatment. Methyl positive that is amplified faster indicates that DNA is methylated. (A) Shows insignificant differences in methylation ratios between SOS1-siRNA and HKT1-siRNA at 0mM NaCl. (B) Shows insignificant differences in methylation ratios between SOS1-siRNA and HKT1-siRNA at 50 mM NaCl. This indicates that no differences in DNA methylation can be considered in response to salt treatments on the DNA in this position of the At*HK1*promoter.

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