Identification Of The Transcriptional Co-Repressor Complex And Its Functions In Arabidopsis thaliana

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Dedication

This thesis is dedicated to my mother, Jyoti Shrestha.
Acknowledgement

Graduate School is a great experience to grow professionally and personally. The mental and physical strain we go through while doing research, is rewarded by the thrill of getting results and progressing intellectually. My research skills and thought process have evolved tremendously. Graduate school has trained me to be responsible, hard working, dedicated, patient, and determined.

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List of Abbreviation

ABA: Abscisic acid
AG: AGAMOUS
API: APETELA 1
ARBC: Arabidopsis Resource Biological Centre
AD: Activation Domain
BD: Binding Domain
CCA1: Circadian Clock Associated
DCL3: Dicer-like3
DREB: Dehydration Responsive Element Binding
DRM2: DOMAINS REARRANGED METHYLASE 2
GRO: Groucho
HAT: Histone acetyl transferase
HDAC: Histone deacetylase
HMT: Histone methyl transferase
HOS1: HDA one similar 1
IGN: Intergenic noncoding RNA
Ler: Landsberg erecta
LiSH: Lissencephaly homolog
LTR: Long terminal repeat
LUC: Luciferase
LUG: LEUNIG
LUH: LEUNIG_HOMOLOG
MS: Murashige and Skooge
ncRNA: noncoding RNA
ORF: Open Reading Frame
R-LUC: Renilla luciferase
RdDM: RNA directed DNA Methylation
RDR: RNA dependent RNA Polymerase
SEP3: SEPALLATA3
SEU: SEUSS
siRNA: small interfering RNA
SLK: SEUSS LIKE
sRNA: small RNA
SSDP: Single Stranded DNA Binding protein
TE: Transposable elements
TGS: Transcriptional gene silencing
TLE: Transducin- like enhancer of split
TSI: Transcriptionally silent information
Abstract

Plants adapt to environmental stress conditions by expressing genes necessary for growth and adaptation. The mechanism preventing the inappropriate expression of these stress responsive genes under non-stress conditions remains elusive. This study is focused on the molecular function of LUH during abiotic stress response and silencing of transposons.

LUH physically interacts with SLK1 and SLK2. Several stress responsive genes including \textit{RD20}, \textit{MYB2}, and \textit{NAC019} are expressed at elevated levels in the \textit{luh}, \textit{slk1} and \textit{slk2} mutant plants. These mutant plants show enhanced tolerance to abiotic stress conditions. The expression of Pol V synthesized intergenic transcripts is decreased in \textit{luh} mutant plants. Loss of silencing of the several transposons was observed in the \textit{luh} and \textit{su7 pol V} mutant plants.

SLK1, SLK2, and LUH form a co-repressor complex that represses the stress responsive genes under normal conditions. LUH and Pol V are involved in the silencing of transposons.

Key words: \textit{Arabidopsis thaliana}, Co-repressor, SLK1, SLK2, LUH, Abiotic Stress, Pol V, ncRNA, IGN, Transposons, epigenetics.
Introduction

Plants are sessile organisms. Therefore, they need to adapt and cope with the various environmental stress conditions for survival. Abiotic stress refers to environmental stress factors such as changes in salinity, drought, osmotic stress, and extreme temperatures. Plants generally respond to these abiotic stress conditions at the level of transcription. Gene regulation plays an important role for survival in plants. Genes may be up regulated or down regulated during abiotic stress conditions (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2001). Several genes are activated during abiotic stress in plants. Transcription factors are a subject of interest due to their important function in the up-regulation of genes that confer tolerance to abiotic stress in plants. The molecular mechanisms of specific transcriptional factors that bind to conserved cis-acting promoter elements in plants are well studied, especially for abiotic stress-induced up-regulated genes (Shinozaki and Yamaguchi-Shinozaki, 2007); however, little is known about how abiotic stress responsive genes are kept in the repressed state during normal conditions (Krogan and Long, 2009).

*Arabidopsis thaliana*, as a model species

*Arabidopsis thaliana*, which is widely used as a model species for plant research, belongs to the Brassicaceae family. Due to its small genome size (125Mb), known genome sequence, and short generation time, *Arabidopsis* is very easy to manipulate and cultivate for research purposes. Transformation with *Agrobacterium tumefaciens* enables efficient generation of knockout mutants by T-DNA insertion in *Arabidopsis*. For these reasons, in this study, the mechanism of transcription repression was studied in *Arabidopsis*.

Transcription Repression

Transcription repression plays a critical role in cell fate specification and body patterning
Transcription activation and repression occur within the context of chromatin organization in eukaryotes. In plants, during normal growth conditions, the abiotic stress responsive genes are generally in a silent state due to the activity of repressor proteins (Courey and Jia, 2001). The mechanism by which these genes are kept in a repressed state is not well known. Transcription repressor proteins play a vital role in repressing genes. Transcription repressors have the ability to mediate both long range and short-range repression. In long range repression, the promoter is resistant to all the enhancers, while short range repression mechanisms target only the nearby DNA-bound activators (Courey and Jia, 2001). Transcription repressors can act by both active and passive mechanisms. During active repression there is a direct repression of genes involving DNA sequence specific transcription factors. These factors bind with non-DNA binding proteins (co-repressors), which then recruits proteins to modify chromatin. By contrast, passive repressors block the activity of transcription activators, thereby acting indirectly (Krogan and Long, 2009).

**Chromatin Modification and Transcription Regulation**

Transcription repressors can repress their targets by either inhibiting the transcriptional machinery or by epigenetic regulation. The basic unit of chromatin is a nucleosome composed of approximately 147 bp of DNA and basic proteins called histones. The nuclear DNA is wrapped around an octamer of histones consisting of two molecules of H2A, H2B, H3 and H4. The influence of chromatin structure on gene expression is regarded as epigenetic regulation. Epigenetic regulation, therefore, refers to the change in chromatin structure without changing the DNA sequence. Epigenetic mechanisms involve changes in histone variants, histone post-translational modifications, and DNA methylation. Histone post-translational modification includes methylation, acetylation, phosphorylation among other possibilities (Chinnusamy and Zhu, 2009). Histone modifications such as acetylation and methylation change the chromatin
compaction, and often occur as a result of the involvement of transcription repressors (Law and Jacobsen, 2010; Li et al., 2007). While Histone Acetyl Transferases (HAT) perform acetylation of the lysine residues in the histone tails, another enzyme Histone Deacetylases (HDAC) is responsible for deacetylation of the histones. Acetylation and deacetylation can occur due to the activity of transcription co-repressors or co-activators to repress or activate the target gene. Acetylation results in transcriptional activation, and deacetylation results in transcription repression. Apart from deacetylation, histone methylation also results in transcription repression. Histone methyl transferase (HMT) transfers methyl groups to lysine or arginine on histones H3 and H4. Actively transcribed chromatin is marked by histone hyper-acetylation such as H3K4 acetylation (acetylation at the Lysine residue of H4). On the other hand, silent chromatin is marked by H3K9 and H3K27 dimethylation. In addition to histone methylation, DNA cytosine methylation is also associated with repressive chromatin (Chinnusamy and Zhu, 2009). HDAC activity, HMT, and DNA cytosine methylation increases the nucleosome density, which is correlated with transcription repression. Histone methylation is also associated with the silencing of regions that are enriched in repetitive sequences such as transposons. Transposons constitute a significant portion of the plant genome and need to be kept silent to avoid harmful effects (Chinnusamy and Zhu, 2009).

Transcriptional Co-repressors

An important class of co-repressors belonging to the Gro/Tup1 family generally mediates transcriptional repression. The Gro/Tup1 family transcription co-repressors are known to function leading to the modification of the chromatin structure. This co-repressor family is an example of an active repression system. The most studied transcription co-repressors in Drosophila, yeast, mammals, and plants are Groucho (Gro), Tup1, Transducin-like Enhancer (TLE), and LEUNIG (LUG) respectively. These co-repressor proteins are collectively called the
Gro/Tup1 family. These co-repressors interact with different DNA sequence specific transcription factors by protein-protein interactions and lead to the repression of diverse target genes (Courey and Jia, 2001). These co-repressors are characterized by N-terminal glutamine (Q)-rich domain and C-terminal WD repeats domain (Tryptophan and Aspartate). The Q-rich domain is involved in homo-tetramerization of the co-repressor. Each WD repeat consists of about 40 amino acid residues. The Q-rich domain and WD repeats are involved in protein-protein interactions (Figure 1) (Liu and Karmarkar, 2008a).

![Domain organization and sequence similarity of Gro-Tup1 family proteins. Modified from (Conner and Liu, 2000).](image)

Since the Tup1/Gro co-repressors lack DNA binding domains, they are recruited to target genes by DNA binding transcription factors. For example in yeast, Tup1 interacts with an adaptor protein Ssn6, which in turn interacts with DNA bound transcription factors and is, thereby recruited to the target gene. This interaction represses the target genes possibly by interaction of chromatin modifying enzymes, HDACs, such as RPD3 (Reduced Potassium Dependency), and Hos1 (HAD One similar 1). In a different mechanism, Tup1 family members may function as a competitive inhibitor of co-activators by interacting with the components of mediator complex (Knoepfler and Eisenman, 1999). Some of the targets of Tup1-Ssn6 are glucose metabolism, DNA damage repair, and anaerobic respiration pathway (Courey and Jia, 2001). It has also been shown that Tup1 interacts with the N-terminal regions of histones H3 and
H4, thereby promoting heterochromatin formation (Watson et al., 2000).

The study of Gro/Tup1 family proteins in plants has emerged in the past decades. In *Arabidopsis* there are 13 Gro/Tup1 like proteins, which are classified into two distinct classes, namely, TPL/TPR/WSIP and LUG/LUH namely- TOPLESS, TOPLESS-RELATED, WUSCHEL-INTERACTING PROTEINS, LEUNIG AND LEUNIG_HOMOLOG (LUH) (Figure 2). All of these proteins have a dimerization domain called lissencephaly homology (LisH) (Liu and Karmarkar, 2008b)

![Figure 2: Phylogenetic tree showing the relationship among Gro-Tup1 family proteins in *Arabidopsis*. The proteins are classified into two distinct classes- TPL/TPR/WSIP and LUG/LUH.](image)

LUG is the best-characterized co-repressor in *Arabidopsis* and functions as a transcription regulator in flower development. LUG has a LUFS domain in the N-terminus. LUFS name is derived from the proteins having this domain namely LUG, LUH, yeast FLO8, and human SSSP (Single Stranded DNA-binding Protein). The LUFS domain is involved in protein-protein interaction (Fig1) (Courey and Jia, 2001; Krogan and Long, 2009; Lee and Golz, 2012; Liu and Karmarkar, 2008a). The LUFS domain of LUG physically interacts with the SEUSS (SEU) protein. This co-repressor complex is involved in repressing genes during flower
development by recruiting HDACs. SEU interacts with DNA binding proteins namely-
APETALA1 (AP1) and SEPALLATA3 (SEP3). AP1 and SEP3 are proteins required for petal
and sepal formation respectively. LUG-SEU-AP1, and LUG-SEU- SEP3 complex represses the
expression of AGAMOUS (AG) gene in the outer two whorls of flower during flower
development. The expression of AG mRNA occurs only in the inner two whorls of flowers, and
is necessary for the formation of stamens and carpels. In the study by Sridhar et al. (2006), it was
shown that in the lug mutants the sepals are transformed into carpels and petals are transformed
into stamens. The repressor activity of LUG in the outer whorls of flower is necessary for proper
flower development. LUG represses the AG gene by physically interacting with HDA19, which
is a histone deacetylase (Grigorova et al., 2011; Sridhar et al., 2006; Stahle et al., 2009).

Repression of Transposable Elements

Epigenetic regulation also plays a vital role in silencing repetitive sequences such as
transposable elements (TEs) in eukaryotes. Transposable elements are mobile genetic units that
can cause changes in gene regulation, double strand breaks in DNA and non-homologous
recombination. The over-expression of these TEs can therefore be detrimental. Epigenetic
regulation of TE is necessary to repress them. There are two classes of transposable elements-
Class I and Class II elements. Class I TEs are called retrotransposons because there is synthesis
of an RNA intermediate by reverse transcription. Class II TEs are called DNA transposons
because they translocate to DNA sequences by a cut and paste mechanism. Retrotransposons can
be further divided into LTR (long terminal repeat) and non-LTR subclasses. LTR
retrotransposons encode long terminal repeats (Bowen and Jordan, 2002). The Arabidopsis
genome consists of both DNA and RNA transposons. AtHALIA, AtCOPIA, TSI, and AtGP1 are
examples of LTR elements in Arabidopsis.
RNA Polymerase V dependent epigenetic silencing

Epigenetic modifications such as DNA methylation, and histone modifications are important mechanisms for silencing TEs. These modifications in plants can be directed by the presence of two atypical RNA polymerases not found in other organisms, Pol IV and Pol V (Figure 3). Subunit compositions of Pol IV and Pol V reveal their origins as specialized forms of Pol II (Ream et al., 2009).

Figure 3: Subunit composition of different RNA Polymerase. A represents the shared and distinct subunits of Pol II vs Pol IV and Pol V. B depicts shared and unique subunits of Pol IV and Pol V. * represents subunit shared by Pol I, II and III in yeast. Yellow represents subunit shared by Pol IV and V in panel B. Blue represents subunit specific to Pol V in panel B. Obtained from (Ream et al., 2009).

Pol IV and PolV are involved in RNA-directed DNA Methylation (RdDM). Pol IV influences heterochromatin modification via the small interference RNAs (siRNAs) synthesis pathway. In order for a gene to be silenced via Pol IV and Pol V mediated pathway, the DNA sequence must first be methylated. Pol IV recognizes these methylated sequences, and initiates transcription of a single stranded RNA from the region. It is not known how Pol IV recognizes these sequences. RNA dependent RNA Polymerase (RDR) synthesizes double stranded RNA
from these Pol IV generated single stranded RNAs. These transcripts are then cleaved by Dicer LIKE 3 (DCL3) protein to form 24 nucleotide siRNAs. These siRNAs are incorporated into Argonaute 4 (AGO4) protein complexes which then guide de novo DNA methylation (Baulcombe, 2006). At the same time, Pol V acts downstream of siRNA generation (Mosher et al., 2008; Zhang et al., 2007). Pol V transcribes nascent noncoding intergenic RNAs (IGN transcripts) from the same region as Pol IV. These IGN transcripts are also critical for heterochromatin formation (Buhler et al., 2007).

In the models suggested by Wierzbicki et al. (2008), Pol V transcripts act as scaffolds to recruit siRNA-AGO4 complex. Pol V transcripts either interact with AGO4 protein complex, or base pair with the siRNA loaded into AGO4. Either way, Pol IV and Pol V transcripts are necessary for silencing of the region from which they are transcribed. This protein complex forms a scaffold to recruit DNA methylases, DRM2 (Domains Rearranged Methylase2) and histone modifying enzymes. DRM2 methylates the cytosine residue thereby silencing the DNA (Figure 3). Wierzbicki et al. have identified some of the Pol V generated IGN transcripts.
Intergenic region- IGN5, IGN7, IGN10, and IGN 15 were identified as Pol V dependent transcripts (Figure 5). In their study it was shown that mutations in the catalytic subunit of Pol V decreased the abundance of these IGN transcripts, suggesting that Pol V synthesizes these transcripts. These IGN transcripts are present in the chromosome 4 of Arabidopsis, which is rich in transposons and heterochromatic repeats. It was also reported that the transcription of these IGN transcripts is necessary in order to silence the adjacent and overlapping DNA sequences (Wierzbicki et al., 2008).

**Research Goals**

Transcriptional silencing is a complex mechanism involving multi-protein interaction. The molecular mechanism of transcription repression in *Arabidopsis* especially during abiotic stress response and epigenetic silencing of transposons is the main interest of this study.

Previous study has shown that LUH, a member of Gro/Tup1 family, is located on the chromosome 2 of *Arabidopsis* (Conner and Liu, 2000). The LUH sequence is 44% similar to LUG (Fig 1) (Lee and Golz, 2012; Liu and Karmarkar, 2008a; Sitaraman et al., 2008). Similar to LUG, LUH also physically interacts with SEU and is redundant with LUG function in floral identity (Sitaraman et al., 2008; Stahle et al., 2009).
Recent studies suggest that LUH regulates MUM2, a β-galactosidase, which is involved in regulating seed coat extrusion in Arabidopsis. The LUH mutants fail to extrude mucilage from the seed coat upon hydration (Bui et al., 2011; Huang et al., 2011; Walker et al., 2011). Our study aims to find the other possible functions of LUH. Since LUH shares sequence similarity with LUG, it is likely that LUH also acts as a co-repressor in gene silencing in Arabidopsis.
In the work described below, it was found that LUH physically interacts with SEU as well as SEUSS like proteins- SLK1, and SLK2. There are three SEUSS like proteins in Arabidopsis. One recent study has suggested that the SLK proteins function redundantly with SEU to regulate floral and embryonic genes in Arabidopsis (Bao et al., 2010). Our studies suggests that LUH along with SLK1 and SLK2 is involved in regulating abiotic stress response in plants. LUH physically interacts with SLK1 and SLK2. LUH has the repressor activity while SLK1 and SLK2 acts as an adaptor proteins. The mutant plants for these proteins showed increased tolerance to salt stress and osmotic stress. Our study also revealed some of the possible targets of this co-repressor complex. Transcripts from the stress responsive genes RD20, MYB2, and NAC019 were present in elevated level in the mutants compared to wild type plants. Moreover, in the yeast two-hybrid assay, it was also seen that LUH interacts physically with subunit 7 of Pol V. This study also aims to uncover whether LUH is involved in the transcriptional silencing of the regions that are silenced by the Pol V IGN transcripts.

The expression of Pol V dependent transcripts was studied in luh and su7 pol V mutant plants. The expression of Pol V dependent ncRNA transcripts for intergenic (IGN) regions IGN5A, IGN7A, IGN10A, and IGN15A is lost or decreased in luh and su7 pol V mutants compared to the wildtype plants. It was found that transposons AtGP1, AtCOPIA, and TSI are derepressed in the mutants suggesting that LUH along with Pol V could be involved in the epigenetic silencing of these transposons.

Materials and Methods

Plant Materials

The Arabidopsis (Arabidopsis thaliana) ecotype Columbia (Col0) and Landsberg erecta (Ler) was used as wild type controls. luh-3 (seed stock no. SALK_107245C), luh-4 (seed stock
no. SALK_097509), *slk1-1* (seed stock no. CS65896), *slk2-1* (seed stock no. CS65894) heterozygous mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC). All the mutant lines are in the Col 0 background except for *seu-1*, which is in Ler background.

*luh-3* has a T-DNA insertion towards the end in the last 3WD repeats while *luh-4* has a T-DNA insertion in the Q-rich domains (Figure 6). Therefore, *luh-4* is a stronger mutant than *luh-3*.

![Figure 6: Site of T-DNA insertion in *luh-4* and *luh-3* mutant plants. *luh-4* has T-DNA insertion in the Q-rich domain while *luh-3* has insertion in the WD repeats. *luh-4* is a stronger mutant compared to *luh-3*.](image)

**Abiotic Stress Treatment Conditions.**

The seeds subjected to abiotic stress were first sterilized with 50% bleach. The seeds were plated in medium containing half-strength Murashige and Skoog salt, 1% sucrose, and 0.8% agar- (MS media). The plates were incubated at 22°C under long-day light conditions, 16 hours, in the growth chamber (Percival). Then the abiotic stress treatment was given where six-day old seedlings were transferred to square plates containing MS media with or without 125 mM NaCl, 150 mM NaCl, 0.3 M mannitol or 0.4 M mannitol. The plants were photographed after 12 days for control plants, 2 weeks for salt stress and 25 days for mannitol stress. For the control plants the root length was measured after 12 days. For salt stress, the root length was measured after 2 weeks. For the mannitol treatment the measurements were taken after 25 days. The data is expressed as percentage of control plants (Col-0).
Plant tissue for RNA/DNA extraction

The tissues for DNA/ RNA extractions were harvested from plants grown in MS media for 21 days in growth chamber. The whole plant was used for harvesting. One gram of tissue was harvested in liquid nitrogen for each sample.

Plant Growth

Arabidopsis plants were grown on metromix 360 soil in controlled growth chambers at 20°C under long day condition (16 hours). Trace elements were not provided. The plants were watered twice a week.

Leaf DNA extraction from leaf (Genotyping)

One or two large size leaves were taken from the plants grown in metromix 360 soil. The leaves were crushed using 500 µl of plant DNA extraction buffer containing 2% CTAB (Cetyl trimethylammonium bromide), 100 mM Tris pH 8.5, 100 mM NaCl, 25 mM EDTA. 1 µl of 2-mercaptaethanol was added per one ml of the buffer. The extract was warmed for 30 minutes at 65°C and 200 µl of chloroform was added after the extract had cooled. This extract was mixed by inversion, and then the eppendorf tubes were centrifuged for 15 minutes at 16,000 g. The clear supernatant was transferred to new eppendorf tube with 300 µl of isopropanol. The sample was incubated for 10 minutes at room temperature. Then it was centrifuged for 10 minutes at 16,000 g. The supernatant was discarded and the resulting pellet was washed with 300 µl of 70% ice-cold ethanol. The pellet was air dried and suspended in 20 µl of buffer containing 10 mM Tris-HCl pH 8.5, 0.1ul of RNase A (Epoch) and stored overnight at room temperature. PCR was done the following day.

Mutant Identification

The putative heterozygous mutants were obtained through the ABRC (Arabidopsis Biological Resource Center). The obtained mutant lines were grown on metromix 360 soil. The
genomic DNA was extracted from the leaves as described above. The putative homozygous mutant lines were identified by PCR with T-DNA specific primer (LBA1) and gene specific primers (LP, RP). The primers are listed in Table 1.

The plant is homozygous if there is a visible PCR product only with LBA1 and RP primer. This means there is a T-DNA insertion in the gene of interest. If visible product is seen only with LP and RP primers, there is no T-DNA insertion. If PCR product is seen in both the cases, (using LBA1 and RP, LP and RP) then the plant is heterozygous. This is because if the T-DNA is not inserted, the binding sites for the LP and RP primers are close enough to form a visible PCR product. In contrast, when T-DNA is inserted, the binding site is far apart for LP and RP primers resulting in no amplification with LP and RP primers. Therefore, two sets of PCR with LBA1-RP primer and LP-RP primer was done to identify the homozygous mutants.

The PCR condition was set for 94°C- 2 min, 30 cycles; 94°C for 30 s; 55°C for 45 s, 72°C for 2 min and final extension at 72°C for 10 min. The PCR products were run on 1% agarose gel.

For the double mutants, the individual homozygous mutant lines were crossed, and the double mutants were identified by genotyping in F2 generation.

Yeast Two Hybrid Assay.

LUH (G12254), SLK1 (G66746) and SLK2 (G10219) cDNA clones were obtained from Arabidopsis Biological Resource Center. PCR was done to amplify the clones. In-Fusion HD Cloning Plus (Clontech) was used to clone these cDNAs into pGBK7 and pGAD7 vectors. Gal4-Binding Domain (Gal4-BD) and Gal4-Activation D (Gal4-AD) fusions were made. Yeast two hybrid assays were done to see the interaction of LUH with SLK-1 and SLK-2 proteins. LUH was used as a bait protein. It was cloned downstream of the Gal4-BD in the pGBK7 plasmid. SLK-1, SLK-2 were used as prey and cloned downstream to Gal4-AD in the pGAD7
plasmid. The yeast two-hybrid interaction assays were performed in Y2H Gold (Clontech) yeast strain. For yeast transformation, overnight cultures of the yeast cells were centrifuged for 10 minutes at 3000 g. The pellets were washed four times with 10 ml of sterile water. Next, 720 µl of 50% polyethylene glycol, 108 µl of lithium acetate, and 105 µl of sterile water was added to the washed cell pellets. In an eppendorf tube, for each sample, 2 µl of salmon sperm DNA was added to 200 µl of the yeast cells (in PEG, LiAc, water). Then, 3 µl of the LUH plasmid in AD vector and SLK-1/SLK-2 plasmid in BD vector was used to co-transform the washed yeast cells. The mixture was incubated at room temperature for 30 min, following heat shock at 42°C for 30 min. The cells were then centrifuged at 16,000 g for 2 minutes. The supernatant was discarded, and the cells were suspended in 100 µl of sterile water. The cells were spread on media without tryptophan (-T), leucine (-L), histidine (-H) and adenine (-A) and incubated for 2 days at 30°C.

Histidine (His) and Adenine (Ade) are downstream reporters. Only when the prey protein interacts with the bait protein, the His and Ade genes are activated. Therefore, those cells can grow and form colonies. The colonies formed in the –TLH ade plates were then used for -galactosidase assay. X-gal was used as a substrate for -galactosidase. When -galactosidase reporter is activated, it cleaves the X-gal represented by blue colonies in the plates.

For yeast two hybrid assay with the LUFS domain, it was amplified from the LUH cDNA and cloned in the pGBKT7 vector. The primer sequences are listed in Table 2.

**Luciferase assay**

A T-DNA insertion population of *Arabidopsis thaliana* plants (ecotype C24) expressing the homozygous transgene RD29A::LUC was obtained from ARBC. The homozygous mutant lines for *slk1-1, luh-4, slk2-1, seu-3* were crossed with C24 transgenic plants. The homozygous mutants were obtained as described above. Five- to 7-day-old seedlings grown under light were sprayed with luciferin and placed immediately under a CCD camera.
Total RNA isolation, semi-quantitative RT-PCR and Quantitative RT-PCR.

Total RNA was isolated using Trizol reagent solution (Invitrogen). First, the plant tissues were ground with liquid nitrogen in a mortar and pestle. After the plant was ground into a fine powder, 1 ml of Trizol was added per 0.1g of tissue. 200 µl of BCP (1-Bromo-3-Chloropropane; B62404) (Sigma-Aldrich) was added per one ml of TRI reagent used. The mixture was mixed vigorously for 2 minutes and centrifuged (12,000 g, 15 min. 4 °C) in SS-34 rotor. The aqueous phase was pipetted into a new tube with 500 µl of isopropanol per one ml of TRI reagent. The samples were centrifuged (12,000 g, 10 min at 4°C). The resulting nucleic acid pellet was washed in 75% ethanol and air-dried. The nucleic acid pellets were dissolved in 200 µl of DEPC (Diethylpyrocarbonate) treated water and subjected to DNAse treatment -5 µl of DNAse I and 20 µl of DNAse buffer was added (NEB Biolabs). Total RNA was purified using RNA extraction kit (Epoch, Texas, USA). For RT-PCR, 5 µg of total RNA was used for cDNA synthesis using oligo (dT) primer or reverse primer and SuperScript III reverse transcriptase (Invitrogen). PCR amplification was performed using 1 µl of 1:10 dilution of the cDNA and gene specific primers. In some experiments, one-step RT-PCR was done (Qiagen). The PCR conditions was as follows - initial denaturation at 94 °C for 1 minute, 94 °C -20 seconds, 55 °C – 20 seconds, 72 °C -25 seconds (set for 30 cycles), final extension at 72 °C for 10 minute. PCR products were visualized by electrophoresis on 1.5% agarose gels (Ethidium bromide staining).

For qRT-PCR, the target transcripts were quantified using SYBR Green Supermix reagent (Bio-Rad) with 1:10 dilution of the cDNA and gene specific primers in the Bio-Rad iCycler iQ real time. For RT-PCR of intergenic transcripts, the One-step RT-PCR kit was used (Qiagen). The PCR cycle was set for 30 cycles.

ACTIN2 was used as an internal control for normalization in each experiment. All the experiments were repeated with three biological replicates for each sample. The primer
sequences are listed in Table 3.

**RNA gels**

RNA gels were made as described in (Aranda et al., 2012).

**McrBC treatment**

The DNA was extracted from the plants as described above. 100 ng of DNA was digested for 4 hours at 37 °C with 4 µl of the enzyme (NEB), 10X NEBuffer 2, 0.3 µl of 100% BSA, 0.3 µl of 100X GTP. The reaction was inactivated at 65°C for 20 minutes.

Two µl of the McrBC treated DNA was used for PCR. PCR was done with the primers listed in Table 3. The PCR conditions were as follows- 94 °C for 1 minute, 94°C -20 seconds, 55 °C – 20 seconds, 72°C -25 seconds (set for 30 cycles), final extension at 72°C for 10 minute. PCR products were visualized by electrophoresis on 1.5% agarose gels

**Statistical Analysis**

For the statistical analysis all experiments were done at least three times. Error bars represent mean values ± SE. P values were determined by Student’s t-test (P <0.05)

**Results**

**Preliminary experiment to analyze loss of repression activity**

We used a previously characterized RD29A promoter fused to the *Luciferase* gene (LUC) from firefly that was incorporated into the genome of plants to make transgenic plants (Chinnusamy and Zhu, 2009). RD29A promoter is induced during abiotic stress conditions including drought, cold, salinity, and plant hormone Absicic acid (ABA). For example, when the plant is exposed to cold stress at 4 °C for 24 hours, the RD29A promoter is activated. This promoter then induces *LUC* expression fused to it. The C24 transgenic lines were crossed with *luh, and seu* mutant plants. A previous study showed that LUH physically interacts with SEU
(Sitaraman et al., 2008). Therefore, *luh* and *seu* plants in C24 carrying RD29A *luciferase* gene were used to observe the co-repressor activity. The plants were subjected to normal growth conditions to determine whether the repressor activity was seen in the mutants. The plants were sprayed with luciferin substrate. If the RD29A promoter is activated, the *luciferase* gene is activated resulting in bioluminescence. This resulting light intensity was quantified which correlates with the activation of RD29A promoter. The blue light indicated that the *luciferase* expression was very low suggesting low promoter activity. The white light indicated a high *luciferase* expression suggesting increased promoter activity.

![Image of luciferase assay showing activation of RD29A promoter under normal condition in *luh*, *slk2*, and *slk1*. Luciferase assay was done where RD29A promoter was fused to *luciferase*. In Col-0 and *seu* plants blue light was seen indicating low luciferase activity suggesting RD29A promoter is repressed in these plants. In *luh*, *slk1*, and *slk2* plants white light was seen indicating high luciferase activity. Therefore, RD29A promoter is derepressed in these mutants.](image)

It was seen that in the *luh* mutant plants there was very high light intensity compared to wildtype and *seu* mutant plants under normal conditions. This apparent constitutive expression of the RD29A::*LUC* reporter suggested that LUH could play a role in abiotic stress responses (Figure 7).
Since *seu* did not indicate any change in regulation of gene during abiotic stress response as indicated by *luciferase* assay, SEU is unlikely to be involved in the abiotic stress response. Therefore, SEU like proteins (SLK) in *Arabidopsis* were of interest. Out of the three SEU like proteins, SLK1 and SLK2 were chosen for the study because it is likely that SLK1 and SLK2 are a result of gene duplication (Figure 8).

![Phylogenetic relationship between SEU and SLK](image)

**Figure 8:** Phylogenetic relationship between SEU and SEU-like (SLK). SEUSS and SLK fall in different clades. Arabidopsis SEU (AtSEU) is similar to antirrhinum (AmSEU3A and AmSEU3B) and *Oryza sativa* (Oslg10070 and Oslg10060). AmSEU1 and AmSEU2 are result of gene duplication, and are similar to SLK1, SLK2 and SLK3 of Arabidopsis. Apart from similarity between SEU and SLK in plants, these proteins are similar to CHIP of *Drosophila* and LIM domain binding protein (LBD). (Bao et al., 2010)

**Isolation of mutants- *slk1-1* and *slk2-1***

The heterozygous mutant lines were obtained from Arabidopsis Biological Resource Center (ABRC). The homozygous mutants were isolated for *slk1-1* and *slk2-1* as described in
methods.

**Luciferase assay in slk1 and slk2**

After the mutants were isolated in C24 plants with RD29A::LUC reporter gene, luciferase assay was performed under normal conditions in slk1-1 and slk2-1 plants. There was increased light intensity similar to the luh plants. Hence, in case of luh, slk1-1, and slk2-1 RD29A promoter was expressed even in the absence of abiotic stress signal. These results from luciferase assay suggested that LUH, SLK1, and SLK2 proteins are recruited at the RD29A promoter to repress the promoter during normal condition (Figure 7).

**Phenotypic analysis of luh and lug under salt and osmotic stress.**

Comparison of expression profiles between LUG and LUH revealed that both the genes are expressed at comparable levels in all tissues under normal condition. Interestingly, LUH expression level is elevated in both biotic and abiotic stress, in contrast to LUG which remained unchanged or reduced under most conditions (Sitaraman et al., 2008). It was of interest to study the phenotypic characteristic of lug and luh under abiotic stress. To test this, loss of function mutants lug and luh-4 were compared to wild type for altered response to salt and osmotic stress. In case of lug, the root length was similar to the wild type during salt stress (Unpublished data); however, during osmotic stress, the root length was significantly longer than that of the wild type plants (Figure 9 B, D). The root length of luh-4 mutant plants was longer than those of the wild type in both osmotic and salt stress (Figure 10). The roots of luh-4 mutant plants were comparatively more branched out with more secondary and tertiary roots compared to the roots of lug mutants in case of osmotic stress (Figure 9 B). These tests were also done in MS media supplemented with 150 mM NaCl or 400 mM Mannitol. The plant growth was slow in these media; however, similar results was observed. In summary, loss of function in LUG results in enhanced tolerance to osmotic stress, and loss of function in LUH results in enhanced tolerance.
to osmotic and salt stress.

![Image](image_url)

Figure 9: Phenotypic comparison of *lug* with *luh-4* and Col-0. The experiment was done as described in methods. Panel A shows the plants in control medium with just MS media. The root length was approximately equal for all the plants. Panel B shows the difference in root length between *lug*, *luh-4*, and Col-0 during osmotic stress condition. MS media was supplemented with 300 mM Mannitol or 400 mM mannitol for osmotic stress. Panel C and D represents the histogram with the mean ± SE where n=4 replicates with 10 plants per replicate. Asterisks indicate values that are significantly different from the wild type plants (*P <0.05, Student’s t test).

**Phenotypic analysis of *luh*, *slk1*, and *slk2* mutant plants**

Since LUH interacts with SEU (Sitaraman et al., 2008), phenotypic analysis was done on *seu* plants. Plants with mutation in SEU showed unchanged tolerance to salt and osmotic stress (Shrestha et al., 2014). Next, the phenotypic analysis was done in loss of function mutants *slk1-1*, *slk2-1*, *luh-4*, and *luh-3* for altered response to salt and osmotic stress.
Figure 10: Phenotypic analysis of the wild type and the mutants- *slk1-1*, *slk2-1*, and *luh-4* under stress conditions. (A, B, C) The single mutants and control plants were grown on the MS medium for six days. The plants were transferred to MS medium as a control and MS medium supplemented with 300 mM mannitol and 125 mM NaCl for osmotic and salt stress treatment respectively. The plants were grown in growth chamber and photographed after 10 days for control, 15 days for salt and 25 days for osmotic treatment plates. (D, E, F) represents histogram with the mean ± SE where n=4 replicates with 10 plants per replicate (*P <0.05, Student’s t test). The root for salt and osmotic stress is presented relative to plants grown on MS medium without stress. The values were then expressed relative to the wild type plant in the respective stress condition.
Figure 11: Phenotype of the *slk1-1*, *slk2-1*, and *luh-3* mutants compared to the wild type plants under stress conditions. (A, B, C) The single mutants and control plants were grown on the MS medium for six days. The plants were transferred to MS medium as a control and MS medium supplemented with 300 mM mannitol and 125 mM NaCl for osmotic and salt stress treatment respectively. The plants were grown in growth chamber and photographed after 10 days for control, 15 days for salt and 25 days for osmotic treatment plates. (D, E, F) represents histogram with the mean ± SE where n=4 replicates with 10 plants per replicate (*P <0.05, Student’s t test). The root for salt and osmotic stress is presented relative to plants grown on ½ MS medium without stress. The values were then expressed relative to the wild type plant in the respective stress condition. Asterisks indicate values that are significantly different from the wild type plants.
There was difference in the root lengths in the mutants compared to wild type plants when the plants were grown in MS medium supplemented with 125 mM NaCl for salt stress and 300 mM mannitol for osmotic stress. The test was also done in media supplemented with 150 mM NaCl or 400 mM Mannitol. The plant growth was slow in these media; however, similar result was observed (Figures 10 and 11). There was no difference in the root length when the plants were grown on MS media (Figures 10 AD and 11 AD). In summary, loss of function in LUH, SLK1 and SLK2 results in enhanced tolerance to salt and osmotic stress compared to the wild type plants. Furthermore, the \textit{luh-4} mutants were better adapted with more secondary and tertiary roots compared to the \textit{luh-3} mutants. \textit{luh-3} mutant has a T-DNA insertion towards the end of the protein near the last WD repeats. Whereas, \textit{luh-4} mutant plants have T-DNA insertion in the Q-rich domain (Figure 6). Therefore, \textit{luh-4} is a stronger mutant compared to \textit{luh-3}. This was further verified by mutant analysis where \textit{luh-3} mutant express the \textit{LUH} gene similar to the wild type. However, the expression of \textit{LUH} gene was significantly lower in \textit{luh-4} mutant compared to the wild type plants (unpublished data). Therefore, further studies on \textit{LUH} gene was done in the \textit{luh-4} mutant plants.

**Phenotypic analysis of other LUG homologs mutants**

\textit{AT2G25420}, \textit{AT5G43920}, and \textit{AT5G08560} are LUG like proteins that fall into the Gro/Tup1 family of proteins in \textit{Arabidopsis} (Figure 2). It was of interest to study the phenotypic characteristic of these LUG homologs under abiotic stress conditions. To test this, loss of function mutants of \textit{AT2G25420}, \textit{AT5G43920}, and \textit{AT5G08560} were compared to \textit{luh-4} and Col-0 for altered response to salt and osmotic stress. In the mutant LUG homologs, \textit{AT2G25420}, \textit{AT5G43920}, and \textit{AT5G08560}, the root lengths were longer than that of the wild type and comparable to \textit{luh-4} during osmotic and salt stress.
Figure 12: Phenotypic analysis of the wild type and the mutants AT2G25420, AT5G43920, and AT5G08560 under stress conditions. (A, B, C) The mutants and control plants were grown on the MS medium for six days. The plants were transferred to MS medium as a control and MS medium supplemented with 300 mM mannitol and 125mM NaCl for osmotic and salt stress treatment respectively. The plants were grown in growth chamber and photographed after 10 days for control, 15 days for salt and 25 days for osmotic treatment plates. (D, E, F) represents histogram with the mean ± SE where n=4 replicates with 10 plants per replicate. The root for salt and osmotic stress is presented relative to plants grown on ½ MS medium without stress. The values were then expressed relative to the wild type plant in the respective stress condition. Asterisks indicate values that are significantly different from the wild type plants (*P <0.05, Student’s t test).
MS media was supplemented with 125 mM NaCl or 300 mM mannitol for salt and osmotic stress respectively. These tests were also done in media supplemented with 150 mM NaCl or 400 mM Mannitol. The plant growth was slow in these media; however, similar result was observed (Figure 12). In summary, loss of function in AT2G25420, AT5G43920, and AT5G08560 resulted in enhanced tolerance to salt and osmotic stress.

**Physical interaction of LUH with SLK1 and SLK2**

Next, the interaction between LUH, SLK1, and SLK2 was studied. Yeast two hybrid assays were done to determine interaction of the proteins. LUH was fused to the Gal4 binding domain (BD). SLK1 and SLK2 were fused to Gal4 activation domain (AD). Histidine, Adenine and α-Galactosidase were the downstream reporter genes. The reporter genes are activated only when LUH interacts with SLK1 or SLK2 (Figure 13).

![Figure 13: Yeast two hybrid assays showing interaction of LUH with SLK1 and SLK. LUH was expressed in the BD vector and SLK1, SLK2 were expressed in the prey vector AD. The center panel shows the yeast colonies in synthetic complete medium lacking the amino acids tryptophan and leucine. The interactions of LUH-BD with SLK1-AD and SLK2-AD is represented by the white colonies. The right panel shows yeast growth in synthetic complete medium lacking the amino acids Tryptophan, Leucine and Histidine. It also lacks the α-Galactosidase activity. Blue colonies represent positive interaction.](image-url)
Previously, it was reported that SEU interacts with the LUFS domain in LUG (Sridhar et al., 2004), thus raising the question whether SLK1 and SLK2 interact with the LUFS domain in LUH. To address this question, the LUFS domain of LUH was amplified from LUH cDNA and fused to the BD domain. Results from two hybrid assays using this construct revealed that SLK1 and SLK2 interacted with the LUFS domain of LUH. The interaction was similar in apparent strength to the interaction seen when the whole LUH gene was fused in the BD vector. This suggested that SLK1 and SLK2 physically interact with LUH at the LUFS domain (Figure 14).

Negative regulation of the abiotic stress response genes

Involvement of SLK1, SLK2 and LUH in salt and osmotic tolerance indicated a mechanism in which abiotic stress response gene expression is altered in these mutants to confer stress tolerance to the abiotic stress. To identify the genes that are differentially expressed in luh-4, slk1-l, and slk2-l mutants compared to wild type, RNA samples of the wild type and mutants were sent for whole RNA sequencing (Figure 15).
Next, according to the analysis of whole genome RNA sequencing, several known abiotic stress response genes that were up-regulated in these mutants were studied. First, RNA was extracted form Col-0 (WT), slk1-1, slk2-1, and luh-4 plants as described in methods and run in RNA gel (Figure 16). Next, RT-PCR of the stress response genes was done and was compared to ACTIN2 as an internal control.
observed in the slk1-1, slk2-1 and luh-4 plants compared to wild type plants under non-stress conditions (Figure 17). Elevated expression of RD20 confers abiotic stress tolerance (Aubert et al., 2010). MYB2 and NAC019 are transcription factors that are implicated in the regulation of several abiotic stress response genes (Abe et al., 2003; Abe et al., 1997; Puranik et al., 2012). These data indicate that loss of function in SLK1, SLK2 and LUH increased the expression of RD20, transcription factors MYB2 and NAC019, and could possibly result in the improved tolerance to abiotic stress in these mutant plants.

Figure 17: Quantification of stress responsive transcripts in slk-1, slk-2, and luh-4. Transcript levels of RD20, MYB2, and NAC019 were quantified using qRT-PCR in Col-0 and the mutant plants. ACTIN2 was used as an internal control. Panel A shows agarose gel electrophoresis depicting the derepressed genes in the mutants—slk-1, slk-2 and luh-4. PCR amplification of the target genes was done with the respective primers (Table 3). (B) represents the fold change in the transcript levels between the wild type and mutants. Error bars are SE (n=4). Asterisks indicate values that are significantly different from the wild type plants (*P <0.05, Student’s t test).

Similarly, RT-PCR of several other stress responsive genes was performed. The results are semi-quantitative. The PCR was done for 30 cycles. The transcripts from stress responsive genes MYB74, DREB1A, COR78, and RD22 were elevated in the mutants compared to the wild type plants (Figure 18). MYB74 encodes a transcription factor that is up-regulated by drought stress (Kranz et al., 1998). Overexpression of DREB1A results in higher tolerance to abiotic stress such as drought and salinity (Kasuga et al., 2004). COR78 has cis-acting regulatory elements imparting cold-regulated gene expression. It is also responsive to ABA and drought
stress (Horvath et al., 1993). \(RD22\) is one of the targets of MYC and MYB transcription factors, and is induced after dehydration and salt stress (Abe et al., 1997). These results suggest that expression of \(MYB74\), \(DREB1A\), \(COR78\), and \(RD22\) in \(skl-1\), \(skl-2\), and \(luh-4\) contributed to stress tolerance in the mutants. Elevated expression of \(CCA1\) (Circadian Clock Associated1) was also observed in the mutants. \(CCA1\) is one of the three genes involved in regulating circadian rhythms in \(Arabidopsis\) and is regulated by histone modification. One of the functions of \(CCA1\) is to anticipate cold nights (Hemmes et al., 2012). This suggests a possible role for the co-repressor complex in regulating circadian rhythms.

Figure 18: Detection of stress responsive transcripts in \(slk-1\), \(slk-2\), and \(luh-4\). Transcript levels of \(MYB74\), \(CCA1\), \(DREB1A\), \(COR78\), and \(RD22\) were detected using RT-PCR and quantified using Image J. \(ACTIN2\) was used as an internal control. Panel A shows agarose gel electrophoresis depicting the derepressed genes in the mutants- \(skl-1\), \(skl-2\) and \(luh-4\). PCR amplification of the target genes was done with the respective primers (Table 3). (B) represents the fold change in the transcript levels between the wild type and mutants. Error bars are SE (n=3). Asterisks indicate values that are significantly different from the wild type plants (*P <0.05, Student’s t test).

**LUH as a repressor in transcriptional gene silencing (TGS)**

Apart from stress responsive genes, transcriptional regulation is also important to silence
transposable elements. Recent studies have shown Pol V generated IGN transcripts are critical to silencing the transposons (Wierzbicki et al., 2008). Yeast two hybrid screening indicated that LUH interacts with subunit 7 of Pol V, suggesting that these proteins interact in vivo (unpublished data). This subunit is unique to Pol V (Figure 3). Therefore, it was interesting to study the involvement of LUH in epigenetic silencing. To study this, the expression level of Pol V dependent IGN transcripts was studied in *luh-4* and *su 7 pol v* mutant plants. We isolated the homozygous mutant lines for *su 7 pol v*.

We examined the expression of several IGN transcripts including *IGN5A*, *IGN7A*, *IGN10A*, and *IGN15A* in the *slk1-1*, and *slk2-1*, and *luh-4* mutants. These targeted IGN transcripts were expressed similarly in the wild type, *slk1-1* and *slk2-1* mutant plants (unpublished data). Detection and analysis of the transcripts was then done in *luh* (*luh-3* and *luh-4*), and *su 7 pol V* mutant plants. There was a decrease or loss of *IGN5A*, *IGN7A*, *IGN10A* and *IGN15A* transcripts in *luh-4*, and *su 7 pol V* mutants (Figure 19 A, B).

Since the presence of IGN transcripts is necessary for DNA methylation to occur, *McrBC* endonuclease sensitivity was done to assay the methylation status of the corresponding DNA sequences in the mutants and wild type plants. *McrBC* specifically cleaves methylated DNA, thereby leading to a loss of signal in PCR reactions. The PCR product was decreased or lost in the wild type compared to the mutant plants indicating digestion of methylated DNA in the wild type (Figure 19 Panel C and D).
Transposons are one of the main targets of IGN transcripts. Therefore, some of the possible target transposons were studied for loss of silencing. RT-PCR was done to detect the transcripts originating in transposons. We observed elevated expression of retrotransposons-
AtCOPIA, TSI, and AtGP1 in luh-4 and su7 pol v mutants indicating reduction in silencing in the mutants (Figure 20 A and B). McrBC endonuclease sensitivity was done to assay the methylation status of the transposons in the mutants and wild type. The PCR product was reduced or absent in the wild type compared to the mutants, indicating digestion of methylated DNA in the wild type plants (Figure 20 C and D). Therefore, this result suggested that LUH and Pol V are necessary to silence these transposons.

Figure 20: Detection and analysis of transposons. (A) shows the agarose gel electrophoresis after RT-PCR of the target transposons in Col-0, luh-3, luh-4, and su7 pol v. The transcripts were abundant in the mutants, but was dramatically decreased Col-0. (C) represents the DNA methylation analysis of the transposons performed by digestion of genomic DNA with McrBC followed by PCR. (B D) represents histograms of the relative transcript levels and % McrBC digestion quantified using Image J. PCR was done with primers listed in (Table 4). ACTIN2 was used as internal control. Relative transcript level and Relative McrBC values were generated using Image J. Error bars represent mean ± SE (n=3). Asterisks indicate values that are significantly different from the wild type plants (*P <0.05, Student’s t test).
**AtCOPIA** is a high copy-number transposon, which is silenced by Pol V-dependent mechanism (Zheng et al., 2009). **AtGP1** is a LTR transposon, which is well-known to be repressed by siRNA mediated silencing (Yu et al., 2013). The *luh-4* and *su7 pol v* mutant plants also released the silencing from specific endogenous pericentromeric repeats termed *TSI* (transcriptionally silent information). No putative function for *TSI* was revealed by sequence comparison with protein- or RNA-coding sequences (Steimer et al., 2000).

**Discussion**

In *Arabidopsis*, LUG and TOPLESS (TPL) are the most studied Gro/Tup1 co-repressors that are implicated in developmental processes and hormone signaling (Krogan and Long, 2009; Lee and Golz, 2012; Liu and Karmarkar, 2008a). LUH is the homolog of LUG and plays a critical role in mucilage excretion (Bui et al., 2011; Huang et al., 2011; Walker et al., 2011). Expression profile analysis indicated that LUH is differentially regulated during abiotic stress compared to LUG and suggesting a role in the abiotic stress response (Sitaraman et al., 2008). Surprisingly, HOS15, belonging to Gro/Tup1 family, was identified in a forward genetic screen involving abiotic stress response. Loss of function in HOS15 results in freezing sensitivity (Zhu et al., 2008). These results prompted the investigation of LUH function under abiotic stress conditions. In this study, it was found that LUH is involved in abiotic stress response and silencing of transposons thus broadening the known functions of LUH.

**SLK1, SLK2, and LUH in abiotic stress response**

Initial luciferase assays implied that loss of function mutations in LUH, SLK1, and SLK2 results in induction of RD29A promoter under non-abiotic stress condition. Furthermore, the phenotypic analysis of *slk1-1, slk2-1*, and *luh-4* indicates that the mutant plants are more tolerant to salt and osmotic stress compared to the wild type. Double mutant analysis with *slk1-1/luh-4*
and slk2-1/luh-4 for salt and osmotic stress indicates that slk1, slk2 and luh functions in the same genetic pathway. There was no differential responses in slk1, slk2 and luh compared to wild type plants during freezing and plant hormone ABA treatment suggesting that the observed salt and osmotic stress in the mutant plants is mediated by an ABA independent pathway (Shrestha et al., 2014).

**LUG homologs in abiotic stress response**

In the phenotype analysis it was seen that LUG homologs- AT2G25420, AT5G43920, and AT5G08560 mutant plants were more tolerant to osmotic and salt stress compared to the wild type. The functions of these LUG homologs are not known so far. These results of the phenotypic experiments indicate a possible function of these genes in abiotic stress response. In the future, we plan to work with these proteins and study their possible roles in abiotic stress response.

**LUH forms a co-repressor complex by interacting with SLK1 and SLK2.**

According to the yeast two hybrid assay, SLK1 and SLK2 physically interacts with LUH at the LUFS domain. The interaction of SLK2 with LUH is stronger than that of SLK1-LUH.

**Stress response genes are derepressed in luh-4, slk1-1, and slk2-1mutants.**

To explain the observed salt and osmotic stress tolerance in slk1, slk2 and luh mutants, quantitative PCR was used to analyze the effects of these mutations on the selected abiotic stress response genes. RD20, MYB2 and NAC019 genes were expressed at elevated levels in luh-4, slk1-1, and slk2-1 mutants compared to wild type plants. RD20 gene is a well-known abiotic stress inducible marker and participates in stomatal control and transpiration in Arabidopsis, thus conferring abiotic stress tolerance (Aubert et al., 2010). The MYB2 gene encodes a R2R3 MYB domain-containing transcription factor that regulates several salt and drought stress responsive genes (Abe et al., 2003; Abe et al., 1997b). NAC domain-containing transcription factors are
prominent plant specific transcription factors and NAC019 is one of the 110 NAC genes that are encoded in the Arabidopsis genome (Puranik et al., 2012). NAC019 gene is induced by salt and dehydration stress. Over-expression of the NAC019 in transgenic plants results in the induction of several stress response genes, hence conferring abiotic stress tolerance. Interestingly, the NAC019 regulatory region contains a MYB binding site, and MYB2 transcription factor binds to the NAC019 regulatory region in a yeast one hybrid assay (Hickman et al., 2013). However, NAC019 gene activation by MYB2 in planta has not been demonstrated. These results show that SLK1, SLK2 and LUH negatively regulate abiotic stress response genes and controls abiotic stress response in Arabidopsis.

Additionally, in the RT-PCR results demonstrated that MYB74, RD22, DREB1A, COR78, and CCA1 transcripts were also expressed at elevated levels in the luh-4, skl1-1, skl2-1 mutants compared to the wild type. In plants, the MYB family consists of at least 97 different MYB genes involved in different regulatory processes including secondary metabolism and hormone responses. These genes are expressed at different levels in different tissues according to physiological conditions. MYB74, in particular, is expressed at elevated levels during drought stress (Kranz et al., 1998). RD22 is a well-known dehydration- responsive gene in Arabidopsis. In the study by Abe et al, it was reported that the RD22 promoter has a MYC and MYB recognition site, which are involved in dehydration responsive expression of RD22. It was also reported that RD22 is responsive to high salt treatment. The transcription factors MYC and MYB are activated under osmotic stress conditions and activate RD22 (Abe et al., 2003; Abe et al., 1997b). The elevated expression of RD22 correlates with the results that MYB2 and MYB74 are also expressed at elevated levels in the luh-4, skl1-1, and skl1-1 mutants. DREB1A (DRE-binding protein 1A) is a transcription factor that interacts with DRE (dehydration responsive element). DRE is a cis-acting element, which contains the sequence TACCGACAT, and is involved in
ABA-independent expression of abiotic stress response genes (Yamaguchi-Shinozaki and Shinozaki, 1994). The transcription factor DREB1A interacts with DRE and induces the expression of genes that increases tolerance to drought, high salt, and low temperature (Kasuga et al., 2004). COR78 has a cis-acting regulatory element that confers freezing tolerance in Arabidopsis. This process is known as cold acclimation. COR78 is also known to respond to dehydration and ABA (Horvath et al., 1993). The over-expression of MYB74, RD22, DREB1A, and COR78 confers some abiotic stress tolerance in planta. Derepression of these genes in luh-4, slk2-1, and slk1-1 compared to the wild type indicates that LUH, SLK2, and SLK1 negatively regulate these genes under non-stress conditions. Moreover, in the RT-PCR experiment, it was also seen that CCA1 was derepressed in the luh-4, slk2-1, and slk1-1 mutant plants compared to the wildtype. CCA1 is critical for circadian rhythms driven by environmental stimulus such as temperature. The expression of many light responsive genes are regulated by HATs and HDACs. Recently it was reported that histone acetylation at H3K9Ac and H3K14Ac was associated with CCA1. However, it remains elusive how CCA1 is regulated by histone modifications (Hemmes et al., 2012). The elevated expression of CCA1 in luh-4, slk2-1, and slk1-1 suggests that LUH negatively regulates CCA1.

We did not observe a consistent difference in regulation of the stress responsive genes ERD12, AMY3, CO715, ATHB, RBP, and ZAT12 in the mutant plants compared to the wild type.

Since SLK1, SLK2 and LUH lack DNA binding domains, how SLK1-LUH and SLK2-LUH complexes are recruited to the regulatory region of stress response genes is unknown. One possible mechanism could be that the SLK1 and SLK2 interacts with different sequence specific transcription factors as expected from the yeast system. Or, SLK1 and SLK2 possibly forms a heterodimeric complex that bridge the transcription factor and LUH at the target regulatory region. The precise mechanism of SLK1-LUH and SLK2-LUH recruitment to the target genes can be illustrated by identification of specific transcription factors that interact with SLK1 and SLK2. In vivo association
at the regulatory region could also elucidate more on the mechanism for recruitment of these proteins to the target genes.

**Model for repression of abiotic stress responsive genes under normal conditions**

In our lab, we observed that LUH interacts with histone H3 and H2B and requires HDAC for the repressor activity.

![Model for repression of abiotic stress responsive genes under normal conditions](image)

*Figure 21: Model for repression of abiotic stress responsive genes under normal conditions. During normal conditions the histones at the stress responsive genes are deacetylated, but increased acetylation is observed during abiotic stress responsive conditions. We suggest that a transcription factor interacts with SLK1 or SLK2 in the target stress responsive gene. SLK1/SLK2 recruits LUH to that region, which in turn recruits HDAC. HDAC removes the acetylation from the histone tails resulting in repressed chromatin resulting in transcriptional repression.*

We examined the histone acetylation level at the target genes that showed elevated expression in the *slk1-1, slk2-1,* and *luh-4* mutants compared to the wild type plants. ChIP assays at the first exon of coding region in *RD20, MYB2* and *NAC019* gene for the histone H3 acetylation at Lys-9 and Lys-14 indicated increased acetylation in the *slk1-1, slk2-1,* and *luh-4* mutant plants compared to the wild type plants (Shrestha et al., 2014). Therefore, we came up
with a model for the repression of the stress responsive genes under normal conditions (Figure 21).

**LUH is involved in Transcriptional Gene Silencing (TGS)**

Recent studies have shown that nuclear transcription occurring within the intergenic and noncoding space of the *Arabidopsis* genome is required for the siRNA mediated gene silencing of transposons and other repeats. Transcription results in heterochromatin formation thereby silencing adjacent and overlapping genes (Wierzbicki et al., 2008). The molecular mechanism whereby the heterochromatin state of the transposons is maintained in the Pol V dependent pathway is not yet known. Therefore, in this study the possible role of *Arabidopsis* co-repressor, LUH in TGS was studied. Weirzbicki et al. identified certain IGN transcripts, which are synthesized by Pol V. In their study, it was reported that these IGN transcripts are present in the wild type, but absent in *nrpe1* which corresponds to plants with mutation in the largest subunit of Pol V. Furthermore, the transcripts were restored when wild type transgene was introduced in the mutant plants (Wierzbicki et al., 2008).

Similarly, in this study, there was reduced expression of the Pol V dependent transcripts in *su 7 pol V* mutant plants. In the yeast two hybrid assay, it was seen that LUH interacts with the subunit 7 of Pol V (unpublished data). RT-PCR analysis of the IGN transcripts in *luh-3, luh-4, su 7 pol V* was compared to the wild type. These transcripts were abundant in the wild type and *luh-3* mutant, but absent or much reduced in *luh-4* and *su 7 pol V* indicating that LUH may be required in the Pol V mediated TGS. The transcript level in *luh-3* was similar to the wild type possibly because *luh-3* is a weaker LUH mutant. The McrBC treatment results indicate that there is loss of methylation in the mutants compared to the wildtype suggesting loss of TGS in the mutants. These IGN transcripts are reported to have a direct role in silencing the overlapping or adjacent genes (Wierzbicki et al., 2008).
RT-PCR results in the transposon indicate loss of silencing of the transposons in the mutants. Transposons \textit{AtCOPIA}, \textit{TSI}, and \textit{AtGP1} are derepressed in the \textit{luh-4}, and \textit{su 7 pol V} mutant plants compared to the wildtype. \textit{AtCOPIA} and \textit{AtGP1} are long terminal repeat (LTR)-retrotransposons, and are known to be silenced through siRNA mediated pathway (Yu et al., 2013; Zheng et al., 2009). The \textit{luh-4} and \textit{su7 pol V} mutant plants also released silencing of specific endogenous pericentromeric repeats termed TSI (transcriptionally silent information). These transposons are heavily methylated in wild-type \textit{Arabidopsis} (Steimer et al., 2000). Loss of methylation was observed in the \textit{luh-4} and \textit{su 7 pol V} mutant plants compared to the wild type plants when the extracted DNA was treated with \textit{McrBC}. We did not observe loss of silencing of the transposons \textit{AtSN1} and solo \textit{LTR}.

We have shown that LUH functions in epigenetic silencing by interacting with histones and HDAC (Shrestha et al., 2014). The data in this study suggests a probable role of co-repressor LUH in TGS. In the Pol V mediated silencing mechanism, it is speculated that putative chromatin remodeler DRD1 may function to recruit Pol V in the transcribed loci. However, DRD1 does not physically interact with Pol V (Wierzbicki et al., 2008). In this study, we hypothesize that LUH physically interacts with \textit{su 7} of Pol V and is necessary for the Pol V dependent heterochromatin formation. LUH physically interacts with histones H2B and H3 (Shrestha et al., 2014). Therefore, we speculate that LUH is recruited to the target region by interacting with histones.

In the future, transformation of \textit{luh-4}, and \textit{su 7 pol v} with their wild type complement genes can be done to see if the loss of IGN transcripts and loss of silencing of transposons are restored. We also plan to do a ChIP assay to see if the Pol V dependent IGN transcripts can be immunoprecipitated with FLAG-tagged LUH. We have also isolated \textit{luh-4/su 7 pol v} and \textit{luh-3/su 7 pol v} double mutants. We plan to do quantitative RT-PCR of the IGN transcripts and
transposons in *luh-4/su 7 pol v* and *luh-3/su 7 pol v* double mutants, and see if Pol V and LUH functions in the same genetic pathway.

**Model for silencing of transposons**

We observed loss of IGN transcripts in *luh-4* and *su 7 pol v* mutant plants which resulted in loss of silencing of the transposons. We hypothesize that LUH interacts with histones in the target transposon site. This results in the recruitment of Pol V in that region. Pol V synthesizes the IGN transcripts, which act as a scaffold for the si-RNA-AGO4 complex. This complex recruits DRM2, which further methylates that region resulting in a silenced transposon (Figure 22).

![Figure 22: Model for silencing of transposons. LUH recruits Pol V reinforcing a silencing loop resulting in heterochromatin formation.](image)

**Conclusion**

This study reports an overall scheme of transcription regulation suggesting a possible novel function of LUH in gene regulation. LUH physically interacts with adaptor proteins, SLK1 and SLK2 to form a co-repressor complex. This co-repressor complex is involved in silencing the stress responsive genes *RD20, MYB 2* and *NAC019* under normal conditions. It is not clear how the SLK1-LUH and SLK2-LUH complexes are recruited to the promoter of the abiotic...
stress response genes. Furthermore, LUH also interacts with subunit 7 of Pol V, and this interaction is important in TGS. LUH and Pol V are involved in the silencing of transposons AtGP1, AtCOPIA, and TSI. It is not clear how the interaction of LUH and Pol V silences the transposon. ChIP assays of FLAG tagged LUH can elucidate more on the role of LUH in TGS.
References


Appendix

Primers used in this study

Table 1: Genotyping

| SLK2LP   | AGATCACACTGCCATTCATCC |
| SLK2RP   | CTGGTGATATGCTATAATCCG |
| SLK1LP   | CCTTGAGGCAATAAGTCTGC  |
| SLK1RP   | CTGGTGATATGCTATAATCCG |
| L.ba1    | TGGTTCACGTAGTGGGCCATCG |
| LUH LP   | ATTAGCAATTGATGCACCTGG |
| LUH RP   | TCCTTCACAAGGGACAAACAC |

Table 2: Yeast Two Hybrid Assay.

| LUH_BD_INFU_F  | AGGAGGACCTGCATATGATGGCTCAGAGTAATTGGGAA |
| LUH_BD_INFU_R  | GCCGCTCGAGTGCTACCTACCTACAACTTTACGGAT  |
| LUFS_BD_INFU_R | GCCGCTCGAGTGCTACCTACCTGAAATGATCCC   |
| SLK1_BD_INFU_F | AGGAGGACCTGCATATGATGAACAGAACGGTGGTCG |
| SLK1_BD_INFU_R | GCCGCTCGAGTGCTACCTACCTGAAATGATCCC   |
| SLK1_AD_INFU_F | CAGATTACGCCTCATATGTAACAGAAGGATGGTTCG |
| SLK1_AD_INFU_R | CGAGCTCGAGTGCTACCTACCTGAAATGATCCC   |
| SLK2_BD_INFU_F | AGGAGGACCTGCATATGATGGCTTCTTCAACTTCTGGG |
| SLK2_BD_INFU_R | GCCGCTCGAGTGCTACCTACCTGAAATGATCCC   |
| SLK2_AD_INFU_F | CAGATTACGCCTCATATGTAACAGAAGGATGGTTCG |
| SLK2_AD_INFU_R | CGAGCTCGAGTGCTACCTACCTGAAATGATCCC   |

Table 3: qRT-PCR

| RD20RT-PCRF  | CCGAAGGAAGGTATGTCCCA |
| RD20RT-PCRR  | GCTTTCAGGAAATTGCTCTCC |
| MYB2RT-PCRF  | CAACGATTGGGGCTGTGGT |

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<th>MYB2RT-PCRR</th>
<th>TCAGGGATTAAGAAAACAGAGAGGA</th>
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<td>NAC019RT-PCRF</td>
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<tr>
<td>NAC019RT-PCRR</td>
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<td>ACT2RT-PCRR</td>
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Table 4: RT-PCR

| IGN7A44 | CATCCACAACCTTCTATGCTTTGTTTACC |
| IGN7A45 | TTTTCTTTGAGTGTTGGTCTATTGTGTTT |
| IGN10A51 | ACCGGATCTTTAGTTTCTCTCCACGTGTC |
| IGN10A50 | TCTAACGCTTTGTTATAGTGTC |
| IGN15A111 | AAAAGTAAAGGTGTTGAAAA |
| IGN15A110 | CCATAGCATAGAAAATTGCGATATAGAA |
| IGN5A193 | AAGCCCAAACCATACACTAATAATCATA |
| IGN5A194 | AATAAAGCAGAATTCTTTTATA |
| ACT2RT-PCRF | GATCTCCAAGGCAGATATGAT |
| ACT2RT-PCRR | CCCATTCATAAAAACCCAC |
| ATGP1F | GGGACGAGTCCTCAAGGGTACCGGCAGAG |
| ATGP1R | CCTCGACGAGCGACCCCTGCTGACC |
| TSIF | GAACCTCATGATACCCCTAAATAC |
| TSIR | CTCTACCTTTGCACATGAAATC |
| ATCOPIAF | TTTTGGTTTATGAGAATATGG |

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Table 5: Stress responsive genes studied

<table>
<thead>
<tr>
<th>Gene</th>
<th>Putative abiotic stress response</th>
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<tr>
<td>RD20</td>
<td>Drought</td>
<td>(Aubert et al., 2010)</td>
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<td>MYB2</td>
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<td>(Abe et al., 2003; Abe et al., 1997b)</td>
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<td>NAC019</td>
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<td>(Puranik et al., 2012)</td>
</tr>
<tr>
<td>MYB74</td>
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<td>(Abe et al., 1997b; Kranz et al., 1998)</td>
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<tr>
<td>CCA1</td>
<td>Circadian rhythms</td>
<td>(Hemmes et al., 2012)</td>
</tr>
<tr>
<td>COR78</td>
<td>Cold, drought, ABA</td>
<td>(Horvath et al., 1993)</td>
</tr>
<tr>
<td>DREB1A</td>
<td>Drought, salt, cold</td>
<td>(Kasuga et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 1994)</td>
</tr>
<tr>
<td>RD22</td>
<td>Drought, salt</td>
<td>(Abe et al., 1997b; Horvath et al., 1993)</td>
</tr>
</tbody>
</table>
Vita

Barsha Shrestha was born in Kathmandu, Nepal on October 4, 1988 as daughter of Jyoti Shrestha and Dhruba Jyoti Shrestha. She completed her high school at St. Xavier’s College Kathmandu. She enrolled at University of New Orleans after she received Chancellor’s Scholarship in 2008. She received a Bachelor of Science degree in Biology, with Chemistry minor, summa cum laude in December 2011. She was admitted into the Graduate School in the Department of Biological Sciences at the University of New Orleans in August 2012. She joined the research laboratory of Dr. Vaniyambadi Sridhar in August 2012 and received a Master of Science degree in May 2014.