Transcriptional co-repressor response of Arabidopsis thaliana to different abiotic stress

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Transcriptional co-repressor response of *Arabidopsis thaliana* to different abiotic stress

A Thesis

Submitted to the Graduate Faculty of the University of New Orleans in partial fulfillment of the requirements for the degree of Master of Science in Biological Science

by

Bhuwan Guragain

B.S. Purbanchal University, 2010

December, 2013
Dedication
This thesis is dedicated to my parents, for their great support.
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CONTENTS

LIST OF FIGURES........................................................................................................vii
LIST OF TABLES........................................................................................................... ix
ABBREBRATION............................................................................................................ x
ABSTRACT.................................................................................................................. xi
INTRODUCTION .......................................................................................................... 1
  Stress responses in plants........................................................................................2
  Co-repressor .............................................................................................................3
  The co-repressor: leunig .........................................................................................4
  SEUSS and SEUSS like transcription factors.......................................................7
  Co-repressor and flower development .................................................................. 9
  Transcriptional repression by chromatin Modification.......................................10
  Gene repression under abiotic stress.....................................................................11
  Tranogenesis ..........................................................................................................15
  Ti Plasmid ...............................................................................................................15
  Protein interaction study.......................................................................................16
  Research goal .........................................................................................................17

MATERIALS AND METHODS.................................................................................... 18
  Yeast two hybrid screening.................................................................................... 18
  Plant growth ...........................................................................................................19
  Leaf DNA extraction ...............................................................................................19
  Mutant identification ..............................................................................................20
  Amplification of promoter region and cDNA......................................................21
  cDNA of SLK1, SLK2 and LUH............................................................................24
  Generation of transgenic plants............................................................................24
  TOPO cloning .......................................................................................................24
Gateway cloning.................................................................25
Plant vectors for promoter analysis.................................28
Vectors to study localization of proteins.........................28
LUH flag tag.................................................................28
Transformation of Agrobacterium..............................31
Plant transformation and selection..............................31
GUS assay.................................................................32
GUS staining..............................................................33
Transient expression analysis of co-repressors..............34

RESULTS.................................................................................35
  Yeast two hybrid screening..............................................35
  Genotyping of plants......................................................37
  Isolation of SLK1/LUH3 double mutants......................37
  Cloning and creation of transgenic lines.....................41
  GUS activity...............................................................49
  Study of protein localization.....................................52
  GUS staining.............................................................54
DISCUSSION........................................................................56
CONCLUSION.................................................................63
REFERENCES.....................................................................64
APPENDIX........................................................................70
VITA.................................................................................72
LIST OF FIGURES

1. Sequence similarity between transcription repressors…………………………6
2. Phylogenetic relation between SEUSS and SEUSS Like genes (SLK)... 8
3. Regulation of genes during stress condition……………………………..12
4. Upregulated and downregulated genes during biotic and abiotic stress…14
5. Amplification region of SLK1.................................................................22
6. Amplification region of SLK2.................................................................22
7. Amplification region of LUH.................................................................23
8. Gateway compatible Vectors for Promoter analysis.........................27
9. pMDC series of vector used to study promoter strength....................29
10.pEarleyGate Vector to study the protein localization........................29
11.Gateway vector to create FLAG tag fusion protein............................30
12.Agarose gel electrophoresis showing homozygous Cstf77 mutant …38
13.Agarose gel electrophoresis showing homozygous luh3 mutant……39
14.Agarose gel electrophoresis showing identification of homozygous double mutant slk2/luh3 .................................................................40
15.Agarose gel electrophoresis picture showing homozygous mutant plants for slk2/luh3 double mutant.........................................................40
16.Agarose gel electrophoresis after colony PCR of the transformants containing gene of interest in TOPO vector.................................43
17.Agarose gel electrophoresis after colony PCR of the transformants containing gene of interest in gateway cloning vector....................44
18.Transgenic plants after selection on antibiotics ..............................45
19.Agarose gel electrophoresis showing transgenic plants using Basta forward and reverse primer .................................................47
20. Agarose gel electrophoresis showing transgenic plants using Basta forward and reverse primer ..................................................47
21. Transgenic plants expressing YFP fusion protein.........................48
22. GUS activity of SLK1, SLK2, 2.7 LUH and 300 bp LUH promoter regions under different stress condition..............................................50
23. Transient expression of SLK1, SLK2 and LUH to study protein localization........................................................................53
24. GUS staining of the transgenic plants ...........................................55
LIST OF TABLES

1. Genes that shows strong interaction with LUH..........................36
2. Primers used in this study.........................................................70
3. Constructs and their destination vector......................................71
## List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABRE</td>
<td>ABA responsive element</td>
</tr>
<tr>
<td>ABS</td>
<td>Abscisic acid</td>
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<tr>
<td>AG</td>
<td>Agamous</td>
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<tr>
<td>ap</td>
<td>apetala</td>
</tr>
<tr>
<td>CBF</td>
<td>C-repeat binding factor</td>
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<tr>
<td>DREB</td>
<td>Dehydration responsive element binding</td>
</tr>
<tr>
<td>FLAG</td>
<td>Peptide coding DYKDDDDDK</td>
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<tr>
<td>GRO</td>
<td>Groucho</td>
</tr>
<tr>
<td>GUS</td>
<td>β-Glucuronidase</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>LUG</td>
<td>Leunig</td>
</tr>
<tr>
<td>LUH</td>
<td>Leunig homolog</td>
</tr>
<tr>
<td>sep</td>
<td>sepallata</td>
</tr>
<tr>
<td>SEU</td>
<td>Seuss</td>
</tr>
<tr>
<td>SLK</td>
<td>Seuss like</td>
</tr>
<tr>
<td>TLE</td>
<td>Transducin- like enhancer of split</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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ABSTRACT

Plants adapt to the complex environmental challenges by regulating their gene expression. Analyses of plant genomes have identified many genes that are either expressed or repressed during environmental stress. However we do not have much information on gene repression. Transcriptional repression in *Arabidopsis thaliana* is caused by co-repressors that lack the DNA binding domain and are recruited by transcription factors to regulate target gene expression. The Sridhar lab has identified co-repressors SLK1, SLK2, and LUH, which prevent the expression of stress response genes under non-stress conditions. *Arabidopsis* transgenic lines expressing the GUS under the control of co-repressor’s promoter were created, to determine the conditions during which the co-repressor are induced. In addition to that, transgenic plants expressing YFP fused with the co-repressor were created to study the sub-cellular localization of the co-repressor. I found that SLK1, SLK2, and LUH are expressed ubiquitously in most of the plants tissue evidenced by the promoter fusion to the GUS reporter. SLK1, SLK2, and LUH are induced by osmotic, cold and dehydration stress conditions. Furthermore, these proteins are localized in the nucleus of the cell.

Key words: *Arabidopsis thaliana*, Co-repressor, SLK1, SLK2, LUH, Transcription factors, Tranogenesis, Stress condition, GUS, GFP
Introduction

Plants are sessile in nature and have to overcome complex environmental challenges for their survival. Their growth and development are limited by various abiotic stress conditions such as salinity, temperature, drought and osmotic imbalances. Plants cope with these harsh environmental challenges through various defense mechanisms. Scientists have been trying to elucidate the roles of different genes and proteins within the plant genomes that participate to overcome the harsh environmental conditions (7).

The molecular mechanism of abiotic stress regulation in plants is best studied in the model plant *Arabidopsis thaliana*. *Arabidopsis thaliana* is a small dicotyledonous plant, related to mustard and cabbage. *Arabidopsis* has five chromosomes and the genome is sequenced (34). The sequenced genome, a short generation time (6 weeks life cycle), ease of collection of seeds and cultivation in the lab, and ability to transform using *Agrobacterium tumafaciens* to generate transgenic lines, have made *Arabidopsis* a model plant for study of abiotic stress in the plant kingdom.

Various transcription factors have been identified that are either induced or repressed during various stress conditions (7,20). Despite the plethora of information regarding gene activation, little is known regarding gene repression in plants. Gene repression in plants can be broadly divided into two classes, namely active and passive repression. Active repression confers repression of genes by the binding of a repressor to the DNA sequence, which in turn recruits the co-repressor. Co-repressors recruit other chromatin remodeling proteins, for example histone deacetylase (HDAC), which silences the gene by removing the acetyl group from histone N-terminal tails (26). In contrast during passive repression, the regulatory
proteins bind to the DNA sequence and prevent transcription machinery access to the DNA sequence (26).

Transcription repression is one the main regulatory strategies that prevents the expression of important regulatory genes, the improper expression of which often results in abnormal development. In active repression, the key players are the co-repressors, which lack the DNA binding motif and are recruited by transcription factors to regulate the target gene expression. (26).

**Stress responses in plants**

Plants may encounter a number of stress conditions like little water (drought), too much salt (salinity) and extremes of temperatures (8). They respond to stress by various biochemical, and physiological changes, which are brought about by gene regulation. The complex mechanism of stress tolerance involves various processes from signal perception to the final outcome as protein production (4). Mitogen-activated protein kinase activates the signal transduction cascade, which in turn phosphorylates the specific transcription factors, resulting in activation of genes that are up regulated during stress conditions (4). During cold stress, transcriptional change results in altered hormone production. These hormones lead to altered plant growth, changes in the lipid composition and accumulation of sucrose in the plant cell.

However, the phenotypic changes during drought stress leads to production of abscisic acid and elongation of root length (4, 38). Abiotic stress leads to both up and down regulation of the genes. During abiotic stress conditions, many transcription factors are also activated to regulate the genes involved in abiotic stress response. Different stress conditions activate certain transcription factors. In *Arabidopsis*, the molecular mechanism of up-
regulated genes is well studied. However, little information is available regarding gene down regulation during abiotic stress. This study provides important insights regarding the expression of co-repressors during various abiotic conditions and the sub-cellular localization of those co-repressors.

Co-repressors

Co-repressors are key players for gene repression. Groucho (Gro), Tup1 and Transducin- Like Enhancer of split (TLE) are well-studied transcription co-repressors in *Drosophila*, yeast, and mammals, respectively. These co-repressors lack DNA binding domains and are recruited by other transcription factors to regulate the target gene expression (33). Each of these co-repressors is characterized by an N-terminal glutamine (Q) - rich domain and C-terminal WD-repeats. The C terminal repeats are enriched with tryptophan (W) and aspartate (D) residues. WD repeats in the protein form the β-propeller structure, which mediates the co-repressor’s interaction with DNA-bound repressors (33).

The Q-rich domain and the WD repeat domain are separated by a less conserved region that facilitates the protein localization to the nucleus and transcription repression (5). Although the transcription co-repressor shares sequence homology, *Saccharomyces cerevisiae* Tup1 and *Drosophila* Gro are homologous only in the C-terminal WD repeats. “However, the overall sequence similarity between the Gro and Tup1 WD-repeat domains is not significantly greater than the similarity between the Gro domain and WD-repeat domains found in proteins not involved in transcriptional repression. For example, the WD-repeat in β-transducin displays 23% sequence identity with Gro, while the WD-repeat domain in Tup1 displays 25% sequence
identity with Gro. The N-terminal region of Tup1 (exclusive of the WD-repeat domain) does not exhibit significant homology to Gro” (49).

Leunig (LUG) and Seuss (SEU) are extensively studied plant co-repressors that play a major role in flower development. LUH is homologous with Groucho/Tup1 family of co-repressors. It represses the target gene expression either by recruiting histone deacetylase or limiting the access of transcription activators to the promoter. But at least during flower development, LUG recruits HDAC to repress target gene expression (19).

The Co-repressor: LEUNIG

LEUNIG is a well-studied plant co-repressor and determines the fate of flower development. LUH inhibits AG expression in the outer two whorls of the flower. In lug mutants, expression of AG in the outer two whorls of the flower leads to the organ transformation of sepals to carpels and petals to stamens. This observation lead to the conclusion that LUG acts as repressor of AG and limits its expression to the inner two whorls of flower (32).

A genome search for LUG homologs showed that Arabidopsis chromosome 2 has a gene similar to LUG. This gene, hereafter referred as LEUNIG homolog (LUH), exhibits 44% overall sequence similarity to LUG. The N-terminal domain of LUG shows 80% sequence similarity to LUH, 26% sequence similarity in Q-rich domain and 58% sequence similarity in the C-terminal domain. These observations suggest that LUH might have a co-repressor function (38).

LUG and LUH show sequence similarity to Tup1 (yeast) and Groucho (Drosophila) (Fig 1). The mechanism of gene repression by co-repressor is well studied in Drosophila and yeast. Gro/Tup1 proteins do not have DNA binding domain. The Tup1/Gro co-repressors are recruited by DNA-binding
transcription factors to repress target gene expression. Tup1 interacts with Ssn6 to form a co-repressor complex that directly interact with transcription factors. Tup1 is thought to interact with HDAC (such as Rpd3) and transcription machinery to repress target genes (12,13).

Similarly, LUG interacts with SEU, and this complex interacts with APETALA1 and SEPALLATA3 to bring about target gene repression. Once recruited to the target gene promoter, LUG recruits HDAC, which removes the acetyl groups from lysine residues present on histone N-terminal tails. Removal of acetyl groups makes the chromatin structure more compact so that the transcription factors can no longer access the target gene (45).

The Sridhar lab has previously shown that LUH interacts with SLK1 and SLK2 (SEUSS-like 1 and 2). We hypothesize that LUH represses target gene in a mechanism similar to LUG. Our initial yeast two hybrid screening showed that LUH interacts with SLK1 and SLK2 (V. Sridhar unpublished data). In this study, I will analyze LUH, SLK1 and SLK2 expression levels under different stress conditions and study the localization of these proteins. Since LUG and LUH share structural similarity, it is highly plausible that LUH might also function as a co-repressor. I will study the role of LUH in gene repression under different abiotic conditions.
Fig 1: Sequence similarity between transcription repressors. The figure shows that LUG and LUH are 80% similar in the N-terminal LUFS domain and 26% similarity in the Q-rich domain, while 58% similarity in the 7 WD repeat domain. Similarly the co-repressors Tup1 from yeast and Gro from Drosophila have Q-rich and WD repeat domain homologous to LUG and LUH from Arabidopsis (Modified from 44).
SEUSS and SEUSS like transcription factors.

SEUSS (SEU) along with LUH is the negative regulator of AG in the outer two whorls of the flower. Seu mutants display a phenotype similar to that of lug, while the seu lug double mutants show an enhanced phenotype of organ transformation. Seu has been cloned and has been shown to encode a protein with a Q-rich and a dimerization domain (18).

The genetic interaction between LUH and SEUSS is further confirmed by yeast two-hybrid screening. Previous studies have shown that the LUFS domain in LUG interacts with SEUSS. LUG can repress any gene when it is tethered to their promoters; in contrast SEUSS does not have any repressor activity. These results suggest that SEUSS bridges the interaction between LUG and DNA binding transcription factors (46).

Apart from SEUSS, there are three Arabidopsis SEUSS-like (SLK) genes that code for putative transcription factors. The sequence similarity between SEUSS and SLK protein suggests that SLK proteins and SEUSS might function in a similar genetic pathway (Fig 2). Recently it has been shown that SLK genes are involved in floral and embryonic development in Arabidopsis. Bao et al., (2010) have shown that the SEUSS and SLK proteins are structurally related, while the SLK1 and SLK2 were likely formed as a result of gene duplication (2).
Fig 2: - Phylogenetic relationship between SEUSS and SEUSS- like genes (SLK). SEUSS and SLK form two different clades. *Arabidopsis* SEU (AtSEU) is similar to antirrhinum (AmSEU3A and AmSEU3B) and *Oryza sativa* (Osllg10070 and Osllg10060). 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). (Adapted from 2)
Co-repressor and flower development

Gene repression by transcription factors is well studied during flower development. Dicotyledonous flowers have different parts namely sepals, petals, stamens and carpels which are arranged in concentric whorls. Differential gene expression in different whorls leads to differentiation of whorl into flower organs.

The ABC model best describes the proper differentiation of whorls into different flower organs. Simply speaking, the ABC model states that correct flower organs are formed in the right positions via the action of three classes of genes; these genes can be collectively referred to as organ identity genes (homeotic genes). APETALA 1 (AP1) and APETALA 2 (AP2) are the class A genes that function in the first whorl to ensure the formation of sepals. APETALA 3 (AP3) and PISTAILLATA (PI) are the B class genes that function in the second whorl (along with class A gene) to specify petals (12). AGAMOUS (AG) is the class C gene that promotes carpel development in whorl four, but it promotes formation of a stamen as well in whorl 3 along with class B genes (46).

AG mRNA is normally expressed in the inner two whorls of flowers, however LEUNIG (lug) and APETALA2 (ap2) mutants showed ectopic expression of AG in the outer two whorls of the flower, leading to either the transformation of sepals to carpels and petals to stamens, or the absence of sepals and petals (10). This result suggests that LUG and AP2 play roles in repression of AG in the outer two whorls during flower development. Furthermore, it has been shown that LUG interacts with SEUSS (SEU) and SEU in turn interacts with AP2 to repress AG activity in the outer two whorls (19).
Transcriptional repression by chromatin modification

Appropriate gene repression is crucial for plant growth and development. Transcription repression brings about the repression of target genes by making the chromatin structure compact, and making it inaccessible for the transcription activators. Nucleosomes are the DNA structures packaged by proteins in repetitive units. They consist of approx. 145 bp of DNA wrapped around an octamer of basic proteins called histones. It has been known that histones are modified post-translationally and these modifications, in part, determine whether the genes in their vicinity are either repressed or expressed. Acetylation of histones is one of the key regulatory modifications that determines whether the target gene is expressed or repressed. Acetylation occurs at the lysine moieties of histone tails. The enzymes involved in this process are HAT (Histone Acetyl transferase) and HDAC (Histone Deacetylase) (43).

Acetylation of histones removes positive charge on the histones, and hence the interaction between the negatively charged phosphates on DNA and histone protein is reduced. As a result, the chromatin become relaxed, and the transcription apparatus can access the DNA for transcription. Histone acetylation is carried out by HAT; the activity of HAT is reversed by HDAC. HDAC removes the acetyl group from the lysine residue, making the chromatin structure more compact. The transcription apparatus cannot access the DNA, and this leads to gene repression (29). Sridhar et al., showed that HDA19 is involved to remove the acetyl group from histones associated with AG gene and prevents AG mRNA expression in the outer two whorls of flower. The authors showed that Trichostatin, an HDAC inhibitor, prevents the repressor activity of LUG (46).
Gene repression under Abiotic stress

Plants face a number of challenges for their growth and development. Drought, salinity, low and high temperature are limiting factors for plant growth and development. Plants have various physiological, biochemical and molecular mechanisms to survive these harsh environmental conditions. Abiotic and biotic stress responses in plants induce various genes to adapt to the harsh environmental conditions (42).

In recent years, many transcription factors were identified as key regulators for gene expression during various abiotic and biotic stress conditions (37). Approximately 6% of the Arabidopsis proteome represents transcription regulators and 30% functions as active transcriptional repressors (27). Genes involved in stress responses can be divided into groups: the first are the genes coding proteins that are directly involved in stress tolerance such as antifreeze proteins, mRNA binding proteins, and the enzymes involved in osmolyte biosynthesis such as proline, water channel protein etc. The second group of genes are involved in stress signal recognition and enhancement of the stress signal. These groups of genes include transcription factors (37).

Abscisic Acid, one of the key component for stress inducible gene regulation is produced during drought, salinity, and freezing stress conditions. Apart from its major role in stress response, many other genes are identified in Arabidopsis that do not respond to ABA when applied exogenously to plants. Hence we can say that, stress response in plants is mainly regulated by ABA dependent and ABA independent pathways (Fig 3) (33). Several genes like dehydration-responsive element binding protein 1 (DREB1)/C-repeat binding factors (CBF) and DREB2 function in ABA-independent pathways during dehydration stress.
Fig 3: - Regulation of genes during stress conditions. Regulation of stress inducible genes can be either ABA dependent or independent. In the ABA independent pathway, certain transcription factors like ZFHD (Zinc finger homeodomain) and NAC (plant specific transcription factor) regulate the expression of stress-inducible gene. While in the ABA dependent pathway transcription factors ABA-responsive elements (ABREs) and ABRE binding factor (ABF) regulate the expression of stress responsive genes. Similarly during osmotic stress response, transcription factors like dehydration responsive elements (DREBs) and C-repeat binding factor (CBF) regulate the expression of target gene. (Adapted from 37)
We can see from Figure 3 that cold stress activates the DREB1/CBF transcription factors while the osmotic stress induces ZFHD and NACR class of transcription factors. Different transcription factors are transcribed from a common promoter and this type of gene arrangement is known as regulon. As we can see from Figure 3, the Dehydration-Responsive Element Binding protein 1(DREB1/C-repeat Binding Factor (CBF) and DREB2 regulon function in ABA independent pathways. While in ABA-independent pathway, ABA responsive element (ABRE) binding protein (AREB)/ABRE binding factor (ABF) regulons functions in ABA-dependent pathway (Fig 3) (37). DREB classes of genes are grouped as DREBA1-6 and RAP2.4, which functions as a transactivator of dehydration and ethylene responsive genes. A variant of RAP2.4 known as RAP2.1 acts as a transcriptional repressor (14). Dong et al., (14) have shown that RAP2.4 binds to the dehydration responsive elements and acts as transcriptional repressor to repress the expression of genes involved in plant response to cold and drought. Furthermore, the authors have also shown that RAP2.1 causes gene repression even in a reporter plasmid when the reporter plasmid is not packaged in chromatin, thus suggesting that RAP2.1 represses gene expression by a mechanism, other than by recruiting histone deacetylase as in the case of repression of AG gene in flower development.

Genome wide analysis of polysome association during heat and high salt stress conditions showed that the polysome-bound fraction of mRNA drastically decreased while the amount of free RNA was significantly higher, suggesting that stress related genes are controlled both at the transcriptional and translational levels (35). Figure 4 shows the number of genes that are downregulated during biotic and abiotic stress. As depicted by the Venn diagram, some of the genes are downregulated either only in biotic or abiotic stress while others are down regulated in both stress conditions.
Fig 4: - Upregulated and down regulated genes during stress conditions. The Venn diagram above illustrates the number of genes that are down-regulated during biotic and abiotic stress in leaves and roots of *Arabidopsis thaliana*. In roots a total of 301 genes are down-regulated by combined biotic and abiotic stresses, while in leaves 509 genes are down-regulated. Some of the genes are down-regulated either in biotic or abiotic stress conditions only while others are down-regulated in both stress conditions (Adapted from 1).
Transgenesis

Transgenesis is the process of introduction of an exogenous gene called a transgene into a living organism so that the organism will exhibit new property and transmit that property to its offspring. *Agrobacterium tumefaciens* is able to infect plants and insert the transgene into the plant genome. *Agrobacterium* is able to sense the phenolic compounds released from the plants and activate its virulence functions. Despite techniques like particle bombardment, *Agrobacterium* mediated transformation of plants is highly preferred. Reduction in transgene copy number, the stable integration with fewer rearrangements of long molecules of DNA, and insertion of selectable markers for efficient isolation of transgenic are some of the significant advantages (25).

**Ti plasmid**

*Agrobacterium tumefaciens* contains the Ti plasmid, which can be transferred to plants upon infection. Ti plasmid contains the virulence genes, genes for catabolism, and T-DNA. Transfer of T-DNA from Ti plasmid to the plant genome is carried out by the virulence genes (*vir*) (22). The coordinated expression of virulence genes mediates the transfer of T-DNA from bacterium to the plants (22). Wild type T-DNA also has genes that are involved in plant hormone synthesis in the host plant. *Agrobacterium* uses the plant cell machinery to synthesize the metabolites for its use. *Agrobacterium* Ti plasmid contains genes that when expressed in the plants divert the plant cellular machinery to synthesize opines, which can be degraded into amino acids and sugars and used as carbon and energy source for bacterial growth.

However, all the genes in the Ti plasmid are not essential for infection and subsequent transfer of T-DNA into plant genome. The non-essential
genes in Ti plasmid can be replaced with genes of interest to create transgenic plants (22). In this study, I have used *Agrobacterium* compatible vectors to clone SLK1, SLK2 and LUH to create transgenic *Arabidopsis* plants.

**Protein Interaction study**

Yeast two hybrid screening is a powerful technique to study protein interaction. In yeast two hybrid screening, protein coding sequences are fused with either activation domain (AD) or the DNA binding domain (BD) of the transcription factors. The protein fused with the BD is referred to as the bait protein while the protein fused to the AD is known as prey protein. We can use the bait protein (protein bound to the BD) to identify the proteins that interact with the protein of our interest. When the transcription factors fused with AD and BD is brought in close proximity they drive the expression of downstream reporter gene. Hence we can identify the protein - protein interaction in vivo. In our study, we used LUH as a bait protein to identify other proteins that interact with LUH (36).
**Research Goals**

Transcription repression is one of the key processes that regulate gene expression in plants. Although, much information is known about transcription activation in plants, little is known about transcription repression. Previously, our lab identified one of the co-repressor named as LUH. Our yeast two-hybrid data showed that LUH interacts with SLK1 and SLK2.

This study is aimed to investigate other proteins that interact with LUH. Apart from SLK1 and SLK2, LUH protein must interact with other proteins for transcriptional repression because SLK1 and SLK2 lack the DNA binding domain. I performed yeast two hybrid screening to identify proteins that interact with LUH.

In this study, I investigated the promoter strength of the co-repressors SLK1, SLK2 and LUH. Furthermore, I determined the stress conditions under which these genes are highly expressed, qualitatively and quantitatively. Apart from that, I studied the protein localization of these proteins in subcellular compartments. Also I tagged the LUH protein with FLAG and created transgenic lines expressing this fusion protein. In future work, these constructs can be used to purify the LUH protein and determine other proteins that interact with LUH by mass spectrometry analysis.

To accomplish those goals, I cloned the promoter region of our putative transcription factor upstream of the β-glucuronidase (GUS) gene (involved in carbohydrate metabolism). Furthermore I created constructs for fusion proteins with putative transcription and the yellow fluorescent protein. Hence, I studied the protein localization of the transcription factors. This study will provide important insights into gene regulation and co-repressor mediated transcriptional repression in *Arabidopsis*. 
Materials and Methods

Yeast two hybrid Screening

To identify the proteins that interact with LUH we used the “yeast two hybrid assay” (36). LUH was used as our bait protein cloned downstream to the Gal 4 binding domain (BD) in the pGBK7 plasmid. This plasmid construct, together with a plasmid containing Arabidopsis cDNA library from clontech (prey) cloned downstream to the Gal 4 activating domain (AD), was used to transform Y2H Gold yeast cells. The overnight culture of Y2H gold was centrifuged at 3000g for 15 min and washed with sterile water 3 times. 480µl of 50% polyethylene glycol and 72µl of lithium acetate was added to the washed Y2H gold cells. 5µl of the plasmid containing LUH cloned in pGBK7 and 10µl of cDNA library was used to co-transform the washed yeast cells. 2µl of salmon sperm DNA was added to the yeast cells prior to adding plasmids. The mixture was incubated at room temperature for 30 min followed by heat shock at 42°C for 30 min. The cells were centrifuged at 10000g for 5 min. The supernatant was discarded, and the cells were suspended in 500µl of sterile water. The cells were spread on dropout media lacking thymine (-T), leucine (-L), histidine (-H) and adenine (-A) and incubated for 2 days at 30°C. In this selection, Histidine (His) and Adenine (Ade) serve as downstream nutritional markers. If the prey protein interacts with LUH then these His and Ade genes will be activated allowing those cells to grow and form colonies. These colonies were then used in an α-galactosidase (this enzyme is coded by another downstream reporter gene in Y2H Gold cells) assay to identify the strongest interacting proteins. Colonies showing strong interaction were inoculated in 5 ml –TL dropout media. Plasmid was extracted using Zymoprep Yeast plasmid Miniprep I protocol. The plasmid was used as template for PCR
reaction using Gal4 AD and Gal4 BD primers (Table 2 in appendix) in a PCR with 30 cycles for 94° C 2 min; 94° C for 30 s; 55° C for 45 s, 72° for 2 min. and final extension at 72° for 10 min. The amplified PCR product was purified using a PCR purification kit (Qiagen, Hilden, Germany). The purified PCR product was sequenced using automated DNA sequencer. The DNA sequences were searched for homologous protein in *Arabidopsis* database (www.arabidopsis.org).

**Plant Growth**

*Arabidopsis* plants were grown on metromix 360 soil in controlled growth chambers at 20° under long day conditions (16 hours light and 8 hours dark and 50-60% humidity). The plants were watered in 2-3 days interval, and they were not provided the trace elements.

**Leaf DNA extraction**

Two to three medium sized leaves were removed with forceps and placed in an eppendorf tube containing 300 µl of Edwards extraction buffer (200 mM Tris pH 7.5; 250 mM NaCl; 25 mM EDTA; and 0.5% SDS)(15). Tissues were ground with pestles and were warmed at 55° C for 30 minutes. The tubes were allowed to cool and 200 µl of chloroform was added. The solution was mixed vigorously 2-3 times. The tubes were centrifuged at 14000 g for 10 min at room temperature and 250 µl of the supernatant was transferred to a clean eppendorf tube. Equal volume of isopropyl alcohol was added and incubated for 10 min at room temperature. After incubation the tubes were centrifuged at 14000 g for 15 minutes at room temperature. The supernatant was discarded and the pellets were washed with 300 µl of 70% ethanol and air-dried. Pellets were suspended in 10 mM Tris HCl, pH 8.5 and stored overnight at 4° C. PCR was carried out the next day.
**Mutant Identification**

The putative mutants used in this study were obtained through the ARBC (Arabidopsis Resource Biological Center). The mutant lines were generated by T-DNA insertion in the target gene. *luh, slk1,* and *slk2* mutants were grown on soil and homozygous mutant lines were identified using LBA1, LP, and RP primers for the respective genes (Table 2). LBA1 primers are T-DNA specific while the LP, RP are respective gene specific. If T-DNA is not inserted in the wild type gene, we observe a visible PCR product using LP and RP primers, while if there is a T-DNA insertion we will observe a visible PCR product using LBA1 and gene specific RP primers. In contrast, we will not observe visible PCR product with LP and RP primers in mutants because insertion of T-DNA makes the binding site for LP, RP primer far apart and visible PCR product cannot be formed. DNA was isolated from individual plant, and two set of PCR was carried out using the LBA1, RP primers and LP, RP primers with the following conditions for 30 cycles: 94°C 2 min; 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. Homozygous mutant lines produced bands with LBA1 and RP primers, while the bands were absent in LP and RP primers. In contrast, bands were present with both LBA1 and RP primers and LP and RP primers on heterozygous conditions, while visible PCR product was observed only in LP, RP primers in the wild type gene with no T-DNA insertion. The individual mutant lines were crossed to get double mutants and we have identified homozygous mutant lines for *luh3/slk1, luh3/slk2* using the LBA1, LP, and RP primers for the respective genes.
Amplification of promoter region and cDNA

In this study the following genes were amplified using primers as listed in Table 2 under the cycling conditions: 94°C 2 min; 30 cycles of [94°C C for 30 s; 55°C C for 45 s, {72°C C 2 min for slk1, slk2, 300bp luh,} or {72°C C 3 min for 2.7kb luh,}, and {72°C C 10 min for 2.7 kb luh FLAG tag and 300 kb luh}] FLAG tag and final extension at 72°C C for 10 min. The DNA sequence was amplified from wild type Arabidopsis genomic DNA (Col-0). The following DNA sequences were amplified from the Col-0 genomic DNA.

A. 1.5 kb upstream of SLK1 start codon (Fig 5)
B. 2 kb upstream of SLK2 start codon (Fig 6)
C. 2.7 kb upstream of LUH start codon (Fig 7)
D. 300 bp upstream of LUH start codon (Fig 7)
E. 2.7 kb upstream of LUH start codon and a codon upstream of stop codon (hereafter referred to as 2.7kb LUH FLAG tag) ( Fig 7)
F. 300 bp upstream of LUH3 start codon and a codon upstream of stop codon (hereafter referred to as 300bp LUH FLAG tag) (Fig 7)
G. slk1, slk2 and luh3 cDNA.
Fig 5: Amplification region of SLK1 gene from the wild type Arabidopsis genomic DNA. In the figure above the direction of the gene (AT4G2550 coding for SLK1) is represented by the arrow (yellow) from 5’ to 3’ direction. Forward Primer was designed from 1.5 kb upstream region of the SLK1 transcription start site and the reverse primer was designed from the region just upstream of the start codon ATG at the 5’ end. (Screen shot from www.arbidopsis.org)

Fig: Amplification region of SLK2 gene from the wild type Arabidopsis genomic DNA. In the figure above the direction of the gene (AT5G62090.1 coding for SLK2) is represented by the arrow (dark orange) from 5’ to 3’ direction. Forward Primers were designed from 2 kb upstream region of the SLK2 transcription start site, and the reverse primer were designed from the region just upstream of the start codon ATG of SLK2 at the 5’ end. (Screen shot from www.arbidopsis.org)
Fig 7: Amplification region of LUH gene from the wild type Arabidopsis genomic DNA. In the figure above the direction of the gene (AT2G32700.1 coding for LUH) is represented by the arrow (dark orange) from 5’ to 3’ direction. The forward Primer was designed from 300 bp upstream region of the LUH transcription start site, and the reverse primer were designed from the region just upstream of the start codon ATG at the 5’ end, which did not include the miRNA (AT2G32696) located at upstream region of LUH. Furthermore, a forward primer for 2.7 kb promoter region was designed from 2.7 kb upstream region of LUH transcription start site, and reverse primer was designed from the region just upstream of the start codon ATG near the 5’ end of the gene including the miRNA (AT2G32696) located at upstream region of LUH. 2.7 LUH FLAG tag was created by fusing the FLAG peptide with full length LUH gene including the 2.7 kb promoter and the forward primer was designed from 2.7 kb upstream region of the transcription start site and the reverse primer was designed a codon upstream of the stop codon of the LUH gene. 300bp LUH FLAG tag, was created as described for 2.7 LUH FLAG tag but the forward primer was designed from 300bp upstream of the transcription start site and the reverse primer was designed from the region just upstream of the start codon ATG near the 5’ end of the gene excluding the miRNA (AT2G32696) located at upstream region of LUH. (Screen shot from www.arbidopsis.org).
cDNA of SLK1, SLK2, and LUH

Total RNA was isolated from leaves, from 21 d old seedlings grown on MS plates. Reverse transcription was performed with oligo dT and Superscript RT II enzyme (Invitrogen). Previously, our lab obtained the TOPO vector clones containing cDNA for SLK1, SLK2, and LUH.

Generation of Transgenic Plants

SLK1, SLK2 and LUH promoter regions were amplified and cloned in TOPO vector (Invitrogen). The constructs were created in a series of steps, and finally the plasmid was transferred to Agrobacterium tumafaciens. Agrobacterium cells carrying the constructs were used to create transgenic lines.

TOPO cloning

TOPO is a restriction enzyme independent vector that uses Topoisomerase 1 from Vaccinia virus for cloning PCR amplified fragments. Topoisomerase binds to the double stranded DNA at specific sites and cleaves the phosphodiester bond after 5’- CCCTT in one strand. The cleavage of the phosphodiester bonds fuels the energy required to form covalent bond between the 3’ phosphate of the cleaved strand and tyrosyl residue of topoisomerase. The covalent bond is attacked by the 5’- hydroxyl of the original cleaved strand, resulting in ligation of PCR product to the vector and release of the topoisomerase. (http://tools.lifetechnologies.com/content/sfs/manuals/pcr8gwtopo_man.pdf)

PCR amplified promoters 1.5kb SLK1, 2 kb SLK2, 2.7kb LUH, 300bp LUH 2.7kb LUH flag tag and 300bp LUH flag tag were cloned in TOPO vector.
Primers used for the amplification are listed on table 2. The PCR product was purified using purification kit (Qiagen, Hilden, Germany). The purified PCR product was incubated with 200 µM dATP, 4 µl of PCR buffer, 1 µl of Taq polymerase to add A- overhangs at the 3’ end of the PCR product and incubated at 22° C for overnight. The A added fragment was again column purified using purification kit (Qiagen, Hilden, Germany). The purified PCR fragment (4 µl) was incubated with 1 µl of Invitrogen TOPO vector and 1µl of salt solution. 3 µl of the ligated product was used to transform chemical competent cells.

After cloning of the genes in TOPO vector, E. coli competent cells DH5α were transformed. 2 µl of the ligated product was used for transformation. The competent cells were thawed on ice and 2 µl of ligated product was added to 50 µl of competent cells. The mix was incubated on ice for 30 min. The cells were heat shocked at 42° C for 45 s. After that, the cells were kept on ice for 2 min. The cells were incubated at 37° C for one hour after addition of 500 µl of SOC media. The cells were selected on 50 µg/ml kanamycin LB agar plates and incubated overnight at 37° C. Colony PCR was performed from the isolated colonies, using the attL forward primer and gene specific reverse primer (Table 2). Positive colonies were inoculated into 5 ml of LB broth containing 50µg/ml kanamycin and incubated overnight at 37° C. The plasmids were isolated from the overnight culture and were further confirmed for the presence of our insert by PCR using attL forward and gene specific reverse primers (Table 2).

**Gateway cloning**

The Gateway cloning uses the site-specific recombination properties of bacteriophage lambda to transfer DNA sequences from one vector to the next. Once the gene of interest is cloned in Gateway compatible vector
“entry vector” (in our case TOPO vector), the gene of our interest can be transferred into a variety of “destination vectors” (Fig 8) (16). The Gateway cloning uses the accurate and site-specific recombination system of bacteriophage lambda to transfer genes from entry vector to the destination vector.

The promoter region of the gene of interest was PCR amplified and cloned into TOPO vector. The resulting recombinant plasmid has the gene of interest flanked by attL recombination sequence. Recombination between attL (entry Vector) and attR site(destination vector) is carried out by the LR clonase reaction mix following kit directions (Invitrogen). Destination vectors contain the gene, ccdB that is lethal to E. coli strains, hence the untransformed cells are killed. In contrast, during effective recombination, the ccdB gene is replaced by the gene of our interest, hence only competent cells that have taken up the recombinant plasmid can only survive in the selective media.
Fig 8: - Gateway compatible vectors for promoter analysis (Panel A). Gateway compatible constructs containing the promoter of gene of interest can be cloned upstream of GFP/GUS/luciferase, so that promoter under study will drive the expression of reporter gene. Panel B shows the Gateway compatible vector for protein localization studies, in which the full-length protein including the promoter region can be cloned upstream of GFP/YFP/CFP/RFP to study the localization of proteins. Panel C shows the vector construct that can be used to tag the protein with FLAG/Histidine/Hemagglutinin/cMyc/AcV5 which can be used for affinity purification of target protein and immunolocalization (Modified from 16).
Plant vectors for Promoter analysis

Promoters drive the expression of any given gene. A gene with a strong promoter can be highly expressed. Hence, the expression of any given gene can be studied by fusing the promoter region of the gene to a reporter. Furthermore, the organs in which the gene is expressed can be studied by tracking the reporter gene. The promoter sequence of any given gene can be fused to GFP, YFP, or GUS. Fusion of promoter region with GUS or fluorescent protein drives the expression of GUS or fluorescent gene. Since the activity of GUS can be easily monitored, we can hence determine the strength of the promoter. Furthermore, we can also determine the stress conditions during which the promoter is highly expressed (16). The inserts namely 1.5kb SLK1, 2kb SLK2, 2.7kb LUH and 300bp LUH promoter regions were transferred to pMDC164 (15).

Vectors to study localization of proteins

Apart from the promoter strength, sub cellular localization of SLK1, SLK2, and LUH were studied. Full length cDNAs were cloned in the TOPO vector as described above and subsequently transferred into oleyGate103 vector (15).

LUH FLAG tag

2.7 LUH flag tag and 300bp LUH flag tag recombinant plasmids were created by cloning the fragment in TOPO and subsequent transfer of those constructs into pEarleyGate 202. The cloning procedure was same as described above. The individual construct and their target destination vector are shown in appendix. (Table 3)
Fig 9: - pMDC series of vector used to study promoter strength. In the vector above recombination between attL1 and attL2 sequence form TOPO vector and attR1 and attR2 sequences from the Gateway compatible vector (pMDC164) transfer the promoter under study between right border (RB) and upstream of GUS gene (encoding β-glucuronidase). The nopaline synthase terminator nosT terminates the transcription of GUS gene. Hyg<sup>r</sup> gene confers resistance to Hygromycin for selection of transformants. (Adapted from 11)

Fig 10: - pEarleyGate Vector to study the protein localization in subcellular compartments. In the figure above recombination between attL1 and attL2 sequence form TOPO vector and attR1 and attR2 from Gateway compatible vector transfer the cDNA of the protein under study into Gateway compatible vector. 35S promoter drives the gene expression. BAR confer resistance to Basta herbicide. The Cm<sup>R</sup> and ccdB genes are replaced by the gene of interest (Modified from 16).
Fig 11: Gateway vector to create FLAG tag fusion protein. The above construct is used to create N-terminal fusion with FLAG. Recombination between attR1 and attR2 in Gateway vector and attL sequences in TOPO vector transfer the gene of interest from TOPO vector to the Gateway vector. In this construct BAR represents the gene conferring resistance to Basta herbicide. The genes Cm\textsuperscript{R} and ccdB are replaced by gene of interest. (Modified form 16).
Transformation of *Agrobacterium*

After the constructs were created, *E. coli* competent cells DH5α were transformed and incubated at 37° C for 12 hours and selected on LB Agar plates containing 30 µg/ml Gentamycin, 30 µg/ml Rifamycin and 50 µg/ml kanamycin. The colonies were screened for the presence of insert by using attR1 forward and gene specific reverse primers (Table 2). Plasmid was isolated from the positive colonies and PCR was performed from the plasmid using attR1 and gene specific reverse primers (Table 2).

The recombinant plasmid was used to transform electrocompetent *Agrobacterium* cells GV101. The bacterium colonies were selected on 30 µg/ml Gentamycin, 30 µg/ml Rifamycin and 50 µg/ml Kanamycin. The transformed cells were incubated at 30° C for 2 days. Isolated colonies were incubated on 15 ml of LB broth containing the same antibiotics as used for the selection. The cells were centrifuged and suspended in 5% (w/v) sucrose solution.

**Plant transformation and selection**

A few days after the plants began to flower, the homozygous lines for *slk1*, *slk2* and *luh3* were transformed by the floral dip method (8). The flowers of the plants were dipped in an infiltration medium [0.5x MS salt (Murashige and Skoog basal salt mixture), 5% (w/v) sucrose and .03% (v/v) silwet L-77] containing the suspended *Agrobacterium* cells. The plants were kept in dark for 1 day and transferred to green house (16 hours light and 8 hours dark; 22° C and 50-60% humidity).

The F1 seeds were collected from the transgenic plants and plated on MS (Murashige and Skoog basal salt mixture) plate containing 30 µg/ml hygromycin and 10 µg/ml glufosinate ammonium for YFP fusion protein.
i.e, recombinant plants containing cDNA sequence for SLK1, SLK2, and LUH3 fused with YFP. The transgenic plants containing the promoter regions fused with GUS and the FLAG tag construct were selected on MS plate containing 30 μg/ml hygromycin and 500 μg/ml Cabenicillin. The plants were incubated at growth chamber for 20 d with 16 hrs light and 8 h dark at 22° C and 55-60% humidity. The transgenic plants that survived the selection were transferred to soil.

The plants were grown for 7 d and DNA was extracted from individual plants and PCR was performed. Transgenic lines containing YFP fusion proteins were screened using Basta F and Basta R primers (Table 2) while the transgenic lines expressing other constructs were screened using HygroF and HygroR primers (Table 1). All of our plants were transgenic, and the plants were grown for 3 weeks, and the seeds were collected.

After stratification, the seeds were planted on soil. 15 days old seedlings were used for GUS assay and GUS histochemical staining. Furthermore, the YFP tagged proteins were visualized using fluorescent microscopy.

**GUS Assay**

GUS reporter system is the most widely reporter gene system (21). The GUS reporter system analyzes the activity of a promoter (in terms of expression of genes under that promoter). The product of the GUS gene is β-glucuronidase. This enzyme cleaves the colorless substrate 4-methylumbelliferyl-beta-D-glucuronide (MUG) forming the fluorogenic product 4-methylumbelliferyl (4 MU). The fluorogenic product can be detected at a peak excitation of 364 nm (UV) and a peak emission of 455 nm (blue). Hence the strength of the promoter can be determined by correlating the fluorescence of 4-MU (21,24)
To study the GUS activity, I excised 3 young leaves from the transgenic plants expressing GUS under the control of co-repressor’s promoter. The leaves were exposed to different stress conditions. Salt stress was studied by incubating the plants with 150 mM NaCl solution; osmotic stress was studied by incubating the plants with 300 mM Mannitol; high temperature stress was studied by incubating the plants at 37° C; low temperature stress was studied by incubating the plants at 4° C; dehydration stress was studied by keeping the leaves at room temperature without watering and the sample was crushed when the weight of the leaves was reduced to 50% of the fresh weight. The leaves from each stress condition was crushed in eppendorf tubes containing 250 µl of GUS extraction buffer (50 mM NaPO₄ buffer, pH 7.0; 10 mM DTT; 10 mM EDTA, pH 8.0; 0.1% Sarcosyl; 0.1% Triton X-100 and 100 mM PMSF 140µl). The tubes were centrifuged at 13000 g for 5 min, and the supernatant was transferred to new tubes. The amount of protein in the leaf extract was quantified using the Bradford assay. The amount of each protein extract required to add 3 µg of protein into each well was calculated and the final volume was maintained to 40 µl by adding extraction buffer. 200 µl of 200 µM MUG was added to each well of a 96 well plate and the reaction was stopped at 0 min, 15 min, and 30 min time intervals by adding 150µl of 0.2 M Na₂CO₃ and the fluorescence was determined. The fluorescence was measured using an excitation filter of 355 nm and an emission filter of 460 nm.

**GUS staining**

Apart from the GUS assay to determine the promoter strength, plants expressing GUS can be used to determine the expression of genes in particular organ. Expression of a particular gene is studied by histochemical staining of GUS. The most widely used substrate for GUS staining is 5-
bromo-4-chloro-3-indolyl glucuronide (X-Gluc). \(\beta\)-glucuronidase, the product of the GUS gene converts X-Gluc into blue colored compound and hence the expression of gene in different plant parts can be studied. Leaves of individual plants from the F1 generation (after the plants were selected on antibiotics and transferred to soil) were washed with alcohol to wash away chlorophyll. The GUS staining solution was prepared by mixing 0.1 M NaPO\(_4\) pH 7.0; 10 mM EDTA pH 7.5; 0.1% Triton X-100 and 1.0 mM \(K_3Fe(CN)_6\) and 2.0 mM X-Gluc (19). The leaves were immersed in 2 ml and 10 ml of staining solution respectively. Plants expressing high GUS activity were selected based on the intensity of stain by visualization.

Plants showing high GUS activity were tracked and seeds were collected from the individual plants. The seeds from the plants were planted. After 35 days, we stopped watering, and the seeds were collected (F2). The seeds from F2 generation were planted and the GUS assay was carried on young leaves. Furthermore, those plants were stained with GUS staining buffer to perform GUS histochemical assay.

**Transient expression analysis of co-repressors**

For transient expression, the cDNAs were amplified with respective gene specific primers and cloned into BamHI site by In-Fusion HD Cloning in the plasmid pXDG to generate GFP fusion driven by 35S CaMV promoter. The protoplasts were transfected with 15 \(\mu\)g of each plasmid DNA and incubated in the dark for 16 h at room temperature. The protoplast was incubated with 1 \(\mu\)g/ml 4, 6-diamidino-2-phenylindole (DAPI), the GFP and DAPI localization was visualized with Nikon fluorescent microscope (Exclipse E800) equipped with digital camera. The images obtained at different channels were cropped and merged with imageJ program (National Institutes of Health).
Results

Yeast two hybrid screening

LUH was fused to the BD vector and used in yeast two hybrid assay as bait protein to screen the library of Arabidopsis cDNA obtained from Clontech. Out of the 800 positive clones (that were blue in color due to expression of downstream reporter β-galactosidase expression), we sequenced 135 clones (clones that showed strong interaction based on visual observation of β-galactosidase). Most of the sequences obtained from sequencing were repetitive, and we considered the following genes for our study.
### Genes showing strong interaction with LUH

<table>
<thead>
<tr>
<th>Gene Number</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT2G30050</td>
<td>Nuclear envelope protein.</td>
</tr>
<tr>
<td>AT2G27970</td>
<td>CDK-SUBUNIT 2.</td>
</tr>
<tr>
<td>AT2G44430</td>
<td>DNA-binding bromodomain-containing protein.</td>
</tr>
<tr>
<td>AT4G18890</td>
<td>BES1/BZR1 homolog 3 (BEH3); BZR1, transcriptional repressor.</td>
</tr>
<tr>
<td>AT3G20060</td>
<td>Ubiquitin-conjugating enzymes belonging to the E2-C gene family.</td>
</tr>
<tr>
<td>AT2G41980</td>
<td>Protein with RING/U-box and TRAF-like domains.</td>
</tr>
<tr>
<td>AT4G14660</td>
<td>Non-catalytic subunit specific to DNA-directed RNA polymerase V; homologous to budding yeast RPB7</td>
</tr>
<tr>
<td>AT1G17760</td>
<td>Encodes a homolog of the mammalian protein CSTF77, a polyadenylation factor subunit. RNA 3’-end–processing factor.</td>
</tr>
</tbody>
</table>

Table 1: - Genes that shows strong interaction with LUH in yeast two hybrid screening.
Genotyping of plants

Cstf77 mutant Isolation

Since our yeast two-hybrid screening showed strong interaction between LUH and CSTF77 (involved in silencing of flowering locus C gene), we isolated a CSTF77 homozygous mutant and we plan to carry out phenotypic analysis.

After DNA extraction, I genotyped the plants using LBA1, RP and LP, RP primers (Table 2). The LBA1 primers was same for all the plants I genotyped, but the LP and RP (Table 2) primers were gene specific. We identified the homozygous lines for slk1, slk2, luh3 (Fig 13), Cstf77  single mutant (Fig 12) and homozygous lines for slk1 and slk2. slk1 and slk2 were crossed with luh3 to isolate slk1/luh3 and slk2/luh3 (Fig 14,15) double mutants.

Isolation of SLK2/LUH3 double mutants

Homologous mutant lines for slk2/luh3 double mutants (Fig 14 and 15) were isolated using LBA1, SLK2 RP; SLK2 LP, SLK2 RP and LBA1, LUH3 RP; LUH3 LP, LUH3 RP primers (Table 2). Initially the plants were screened using SLK2 LP, RP and LUH3 LP, RP. Those plants that did not show visible PCR products were selected (Fig 14 plant 6) because they might have T-DNA insertion. Similarly, we screened other plants using SLK2 LP, RP and LUH3 LP, RP and isolated putative double mutants. Further confirmatory PCR was carried using LBA1 and gene specific RP primers (Fig 15).
Fig 12: - Agarose gel electrophoresis showing homozygous Cstf77 mutant. PCR was carried on the F2 plants and the gel picture shows that plants numbered 1,2,3,4,5,6 were homozygous mutant for Cstf77. In panel A PCR was performed using LBA1 (T-DNA specific) and RP primer (gene specific). Since there was T-DNA insertion in Cstf77 gene, there is a visible band with LBA1 and RP primers. Col-0 is wild type genomic DNA lacking T-DNA insertion; hence there was no product obtained with LBA1 and RP primers. In panel B, the same plants were screened using LP and RP primers (gene specific). Since there is T-DNA insertion in Cstf77 gene, the LP and RP primer binding sites are far apart and hence I did not get any visible PCR product. In contrast, a visible PCR product was obtained with wild type genomic DNA (col-0) because there is no T-DNA insertion.
Fig 13: - Agarose gel electrophoresis showing homozygous $luh3$ mutant. PCR was carried on the F2 plants, and the gel picture shows that plants numbered 1, 2, 3, 4, 5, 6, 7, 8 were homozygous mutants for $luh3$. In panel A PCR was performed using LBA1 (T-DNA specific) and RP primer (gene specific). Since there was T-DNA insertion in LUH gene, I got a visible band with LBA1 and RP primers. Col-0 is wild type genomic DNA lacking T-DNA insertion; hence no products were amplified with LBA1 and RP primers (Table 2). In panel B, the same plants were screened using LP and RP primers (gene specific). Since there is T-DNA insertion in the LUH gene, the LP and RP primer binding sites are far apart and hence I did not get any visible PCR product. In contrast, a visible PCR product was obtained with wild type genomic DNA (Col-0) because there is no T-DNA insertion.
Fig 14: - Agarose gel electrophoresis showing identification of homozygous double mutants *slk2/luh3*. Panel A shows the agarose gel electrophoresis of plants with SLK2 LP and RP primers. Panel B shows the agarose gel electrophoresis of plants with LUH3 LP and RP primers (Table 2). Plant 6 appears to be a homozygous double mutant because there is no visible PCR product with LP and RP primers in both cases. (i.e, with SLK2 LP, RP and LUH3 LP, RP primers). I speculate that there might be T-DNA insertion in both genes SLK2 and LUH3, hence the LP and RP primers for both genes are far apart resulting in absence of PCR product.

Fig 15: - Agarose gel electrophoresis showing homozygous mutant plants for the *slk2/luh3* double mutant. The putative homozygous double mutant (plants number 6,15,28) were further confirmed by LBA1 and gene specific RP primers (Table 2). A visible PCR product was not observed when I used SLK2 LP, RP primers and LUH3 LP, RP primers. PCR was performed on the same plants using LBA1, RP (SLK2) and LBA1, RP (LUH3) primers(Table 2). Since there is T-DNA insertion in both alleles, I observed visible PCR product using LBA1 and RP primers as expected. In contrast, visible PCR product was absent with LBA1 and RP primers in wild type *Arabidopsis* genomic DNA (Col-0).
Cloning and creation of transgenic lines

After we had isolated homozygous lines for *slk1*, *slk2*, and *luh3*, we collected seeds from the homozygous lines and planted them. The clones containing SLK1, SLK2, and LUH cDNA in TOPO vector were generated previously.

The promoter constructs were cloned in TOPO vector as described in materials and methods and *E. coli* competent cells were transformed. After selection on antibiotics as described in materials in methods, colonies were selected to check for the gene of interest. Colony PCR (See Fig 16) was performed using attR1F (Vector specific) and gene specific reverse primer (Table 2). The positive colonies were incubated for 12 h at 37° C in 5 ml of LB broth containing 50 µg ampicillin/ml. Plasmid was isolated from the positive clones. (Marked by arrows)

After isolation of plasmid from the transformants, containing TOPO vector with gene of interest, I set up LR reaction (Invitrogen) between TOPO vector containing gene of interest and Gateway cloning vector and transferred the gene of interest from TOPO vector into Gateway cloning vectors. The LR reaction mixture contains enzymes excisionase and integrase from bacteriophage lambda and *E. coli* encoded integration host factor and suitable buffers. The LR reaction mix catalyzes the recombination between attL1 site in TOPO vector and attR1 site in Gateway vector resulting in transfer of gene of interest into destination vector. SLK1, SLK2, 2.7kb LUH and 300 bp LUH were transferred to pMDC 164 (Fig 9). Similarly 2.7 kb LUH flag tag and 300bp LUH flag tag was transferred to pEarleygate 202 (Fig 11). cDNA of SLK1, SLK2 and LUH was transferred to pEarleygate 104 (Fig 10).

*E. coli* competent cells DH5α were transformed with the LR reaction mixture. Isolated colonies were checked for the presence of insert by colony
PCR using the attL1 forward (Gateway Vector specific) and gene specific primers (Table 2) (Fig 17). Plasmids were isolated from the positive clones, marked with arrows (Fig 17). *Agrobacterium tumefaciens* electrocompetent cells GV1.0 were transformed with the extracted plasmid. The transformed electrocompetent cells were grown in LB containing the appropriate antibiotics for 2 days at 30°C. The transformed cells were used to create transgenic *Arabidopsis* plants as described in materials and methods.

The transgenic lines thus created were grown and seeds were collected. The F1 seeds were plated on 0.5 MS agar plates (Agar plates containing half the amount of salt required to make standard Murashige and Skoog medium) containing the appropriate antibiotics. SLK1, SLK2, 2.7 kb LUH, 300bp LUH promoter regions, 2.7kb LUH flag tag and 300bp LUH flag tag were selected on 0.5 MS (containing 500 µg/ml of carbenicillin and 50 µg/ml glufosinate ammonium). Transgenic plants expressing YFP fusion protein were selected on 500 µg/ml of carbenicillin and 50 µg/ml hygromycin. The transgenic plants that survived antibiotics selection (Fig 18) were transferred to soil.
Fig 16: Agarose gel electrophoresis after colony PCR of the transformants containing gene of interest in TOPO vector. The colonies were tested for the presence of gene of interest using attR1 forward (Vector specific) and gene specific reverse primers (Table 2). Colonies marked with arrows were the positive clones containing the gene of interest. Panel A (marked with arrows) shows the positive clones containing SLK2 promoter region in TOPO vector. Panel B (marked with arrows) shows positive clones containing SLK1 promoter region in TOPO vector. Panel C (marked with arrows) shows positive clones containing LUH 300 bp promoter region in TOPO vector. Panel D (marked with arrows) shows positive clones containing LUH 2.7 kb promoter region in TOPO vector. Panel E (marked with arrows) shows positive clones containing LUH 2.7 kb promoter region with the full length LUH gene in TOPO vector. Panel F (marked with arrows) shows positive clones containing LUH 300 bp promoter region with the full length LUH gene in TOPO vector. Colonies numbers 2,7 were selected in panel 7 based on high intensity of PCR product. Panel G, H, I (marked with arrows) shows positive clones containing cDNA of SLK1, SLK2 and LUH in TOPO vector.
Fig 17: - Agarose gel electrophoresis after colony PCR of the transformants containing gene of interest in Gateway cloning vector (after LR reaction). Panel A shows the agarose gel electrophoresis with attL1 forward and LUH300 bp promoter specific reverse primer. Panel B shows the agarose gel electrophoresis with attL1 forward and LUH300 bp promoter specific reverse primer (full length gene). Panel C shows the agarose gel electrophoresis with attL1 forward and SLK1 promoter specific reverse primer. Panel D shows the agarose gel electrophoresis with attL1 forward and LUH 2.7 kb promoter specific reverse primer. Panel E shows the agarose gel electrophoresis with attL1 forward and LUH 2.7 kb promoter specific primer (full length gene). The primers used in the colony PCR are listed in table 2.
Fig 18: Transgenic plant (SLK1 promoter expressing GUS) after selection on antibiotics. The transgenic plants were selected visually. Since the transgenic plants had an antibiotic resistance gene, the transgenic plants were bigger in size and were healthy, when compared with other plants. In the figure above, the plant marked with arrow is transgenic plant, which is bigger in size and healthy compared with other plants (non transgenic).
After 15 days, we extracted DNA from the leaves, and some of the leaves from the transgenic plants were stained with X-Gluc. Forward and Reverse primers were designed from the antibiotic resistance gene. Glufosinate ammonium (Basta) forward and reverse primers (Table 2) were used to confirm transgenic lines expressing GUS and LUH flag tag proteins while Hygromycin forward and reverse primers (Table 2) were used to conform transgenic lines expressing GFP. Fig 19 shows the agarose gel electrophoresis of transgenic plants expressing GUS under the control of SLK1 and SLK2 promoter. Basta forward and reverse primers (Table 1) were used to confirm the transgenic plants. Furthermore, Fig 20 shows the agarose gel electrophoresis of transgenic plants expressing GUS under the control of LUH 2.7 kb and LUH 300 bp promoter. Basta forward and reverse primers were used to confirm the transgenic plants for LUH 300 bp and LUH 2.7 kb promoter (Fig 20). Similarly transgenic plants expressing YFP fused with SLK1, SLK2 and LUH (Fig 21) were confirmed by using Hygromycin forward and reverse primer (Table 2). As expected, a 1 kb band was amplified with both Basta, Hygro forward and reverse primers.

Plants showing high GUS activity and plants that were confirmed to be transgenic were marked, and seeds from those plants were collected (F0 plants). Seeds from the F0 plant were planted, and the GUS activity was measured as described in materials and methods.
Fig 19: Agarose gel electrophoresis of the transgenic plants using Basta forward and reverse primers. Panel A shows that transgenic plants expressing SLK1 promoter controlling the expression of GUS gene. Panel B shows the transgenic plants expressing SLK2 promoter controlling the expression of GUS gene. Since the wild type genomic DNA (col-0) lacks the Basta resistance gene, a visible PCR product was absent with Basta forward and reverse in wild type *Arabidopsis* DNA.

Fig 20: Agarose gel electrophoresis of the transgenic plants using Basta forward and reverse primers. Panel A shows that transgenic plants expressing LUH 2.7 kb promoter controlling the expression of GUS gene. Panel B shows the transgenic plants expressing LUH 300 bp promoter controlling the expression of GUS gene. Since the wild type genomic DNA (col-0) lacks the Basta resistance gene, visible PCR product was absent with Basta forward and reverse in wild type *Arabidopsis* DNA.
Fig 21: Transgenic plants expressing YFP fusion protein. Panel A shows the transgenic plants expressing YFP fused with SLK1. Panel B and C shows the transgenic plants expressing YFP fused with SLK2 and LUH respectively. Hygro forward and reverse primers were used to confirm the transgenic plants expressing YFP. Most of our plants tested were transgenic. The plants showing higher intensity of amplification (Panel A Plant 2,7,11; Panel B 6,11,12; and panel C 9,12,18) were tracked and seeds were collected.
GUS activity

The GUS assays were performed as described in materials and methods. The total GUS activity was normalized to the amount of protein in µg per min as described in materials and methods. Young leaves of 15 d old seedling were exposed to different stress conditions. Since the promoter regions of different genes was cloned upstream of GUS, high activity of GUS should depict the stress conditions during which each promoter is active. We exposed the leaves to salinity (150 mM NaCl), osmotic stress (300 mM mannitol), high temperature (37° C), low temperature (4° C) and dehydration stress condition (samples were taken when the weight of the leaves was 50% of the fresh weight under normal condition). For each stress assay condition, three leaves from three different plants was removed by forceps and exposed to a particular stress conditions. The mean GUS activity of three different leaves under stress conditions was compared with mean GUS activity under normal conditions. Fig 21 shows the mean GUS activity of each promoter under study under different stress conditions. Three leaves from three different plants (showing high GUS activity based on visual observation as mentioned earlier) were exposed to test condition. Two tailed t-test showed no significant difference between the control and the promoter under study.
Fig 22:- GUS activity of SLK1, SLK2, 2.7 kb LUH, and 300 bp LUH promoter regions under different stress condition panel A) 150 mM NaCl (salt stress), panel B) 300 mM mannitol (osmotic stress), panel C) high temperature (37°C), panel D) low temperature (4°C) panel E) dehydration stress. The GUS activity is mean of 3 independent replicates (n=3). The GUS activity is normalized to per µg of protein per min.
When the leaves were exposed to NaCl (for 4 hours) the mean GUS activity under promoter of study is less than in control (Fig 21 panel A). When the leaves were exposed to osmotic stress (300 mM mannitol) for 2 hours, SLK1, LUH 2.7, and LUH 300 promoter showed a trend toward higher GUS activity than the control (Fig 21 panel B).

Similarly, GUS activity of the promoter was studied under high temperature and low temperature stress. GUS activity of promoter under high temperature was studied by exposing the leaves to 37° C for 6 hours (Fig 21 panel C), while the GUS activity of promoter under low temperature was studied by exposing the leaves to 4° C for 24 hours (Fig 21 panel D). In both the cases, our two tailed P-value shows no significant difference between the control and our test condition, however, SLK1, SLK2, LUH 2.7 kb and LUH 300 bp showed a trend of higher GUS activity than control at high temperature stress condition (Fig 23). Similarly under low temperature stress condition (Fig 21 panel D) SLK2, LUH 2.7 kb and LUH 300 bp showed higher GUS activity than the control but our two tailed P-value showed that there was no significant difference between the control and our test condition.

Furthermore, GUS activity of \textit{SLK1}, \textit{SLK2}, \textit{LUH} 2.7 kb. \textit{LUH} 300 bp promoter region under dehydration stress condition was studied (Fig 25). The two tailed P-value showed that there is no significant difference between the control and test condition. However, SLK2, LUH 2.7 kb and LUH 300 bp promoter shows a trend of higher GUS activity during dehydration when compared to control (Fig 21 panel E).
Study of protein localization

I created the transgenic lines of *Arabidopsis thaliana* expressing GFP. I have the F2 seeds for those plants, but I was not able to isolate homozygous lines for the transgenic plants expressing GFP. In the future, others will isolate the homozygous lines and study the protein localization. But to study protein localization of *slk1, slk2* and *luh3*, I did the transient expression analysis, and found that SLK1, SLK2 and LUH are localized to the nucleus as shown in Fig 23. Panel C in Fig 23 shows the punctate GFP-co-repressor, while DAPI staining (panel B) shows that the punctate compartments are the nuclei (panel D). Panel A to Panel C (Fig 23) were captured using the white light, DAPI, GFP filters. Panel D (Fig 23) shows the merged DAPI and GFP. From the Fig 23 we can see that there is uniform glow of GFP, while the vector is not visible in other merged images.
Fig 23: - Transient expression of SLK1, SLK2 and LUH to study protein localization. Panel A shows the phase-contrast microscopy of the protoplast. Panel B shows protoplast stained with DAPI which binds DNA and panel C shows protoplast image with GFP localization. Panel D shows the protoplast image merged with DAPI and GFP.
**GUS staining:**

The transgenic lines containing the promoter region were incubated in GUS staining solution. The GUS staining of different plant parts is shown in Fig 24. As we can see from the figure that young leaves are well stained when compared with old leaves. This observation suggests that during senescence of the plants, expression of the co-repressors is reduced. Most of the parts is stained, suggesting the universal expression of SLK1, SLK2, LUH (Fig 24) apart from the seeds. The seeds of the plants (Fig 24 panel O) are not stained suggesting that the co-repressor are not expressed in seeds. Furthermore, less intense staining of the older leaves and the absence of staining in seeds suggest that expression of the co-repressor is reduced as the plants age. SLK2 is expressed mostly in the inner parts of the flower (Fig 23 panel G). Furthermore, promoters under study were expressed in roots (Fig 23 panel D, H, L, P), confirming the high expression of these co-repressor in most of the plant parts.
<table>
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<tr>
<th>Promoter</th>
<th>Young Leaf</th>
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</tr>
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<td>J</td>
<td>K</td>
<td>L</td>
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<td>LUH 300</td>
<td>M</td>
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Fig 24: GUS staining of the transgenic plants. Different plant parts were examined for GUS expression. Negative control was not included in these experiments because plants have no background GUS activity.
Discussion

In this project, I studied the expression of co-repressors SLK1, SLK2, and LUH under different abiotic stress conditions. Transcriptional repression is a key regulatory mechanism to insure appropriate expression of the stress responsive genes. Inappropriate expression of those genes leads to abnormal development. For this study, I isolated the homozygous mutants for *slk1, slk2, luh3* and isolated homozygous double mutants for *slk1/luh3* and *slk2/luh3*. Apart from that, I cloned the SLK1, SLK2, and LUH promoter region upstream of the GUS gene in order to determine the stress conditions during which the promoter is active. In addition, I studied the protein localization in the subcellular compartment and found that these co-repressors localize to nucleus as expected.

Yeast two-hybrid screening

I was able to identify proteins that interacted with LUH by our yeast two hybrid screening. The strong interaction between the newly identified proteins and LUH opens a new field of study regarding stress regulation in plants. Our initial yeast two-hybrid data shows that LUH shows strong interaction with protein with gene ID AT2G30050 encoding a nuclear envelope protein the function of this gene is not known.

We are particularly interested in the CSTF77 gene because our yeast two hybrid screening shows strong interaction between LUH and CSTF77. Recently Liu *et al.*, 2010 showed that CSTF77 is involved in silencing of the flowering locus C gene (31). Furthermore, the Sridhar lab has shown that LUH is involved in repression of abiotic stress response gene, it will be interesting to determine whether CSTF77 is involved in abiotic stress
response. We have isolated homozygous mutant for Cstf77 and will determine its role in abiotic stress in future experiments.

The other protein with which LUH interacts strongly is cyclin dependent kinase (CDK)- subunit 2. (Gene ID AT2G27920). CDK is family of protein kinase involved in cell cycle regulation. Apart from cell cycle regulation, CDK proteins are also involved in regulating transcription, mRNA processing, and the differentiation of nerve cells (23). It is plausible that interaction of LUH and CDK2 in vivo provides some regulatory mechanism for stress response genes. During extreme stress conditions, the growth and development of plant is limited and hence, the cell cycle might be arrested. At present CDK2 role in abiotic stress response is unknown, but these can be investigated in future experiments.

Our yeast two hybrid interaction also shows that LUH interacts with DNA-binding bromodomain protein. DNA binding bromodomain protein has different roles in plants; they insulate chromatin from DNA damage signaling (17). Although LUH interacts with the DNA binding bromodomain protein, there is no information regarding the role of the DNA-binding bromodomain protein with respect to stress gene regulation. Future experiments can be performed to investigate this.

One of the interesting proteins that LUH interacts with is BZR1 protein. This protein is involved in transducing the signal of steroid binding in the protoplast. Binding of steroid in the protoplast induces cell-signaling events that leads to the growth and development (47). Since, BZR1 protein promotes cell growth and differentiation, we were unable to correlate the function of this protein with respect to gene repression during abiotic stress.

The most interesting protein we identified using our yeast two hybrid screening is the ubiquitin-conjugating enzyme (AT4G20060) The ubiquitin pathway of protein degradation has three main enzymes, namely E1
(ubiquitin activating enzyme), which binds to the ubiquitin protein; E2 (ubiquitin conjugating enzyme) which interacts with E1 and the ubiquitin is transferred to E2, and E3 (ubiquitin ligase enzyme), which recognize the protein to be degraded and transfer ubiquitin to the protein targeted for degradation (39). We propose a model in which the LUH represses the gene involved in stress response during normal cell growth and development, but during stress conditions the ubiquitination of LUH might target LUH for degradation leading to gene activation of stress response gene. This potentially exciting result will be source of future experiments.

The yeast two hybrid results indicate that LUH interacts with subunit 7 of RNA pol V. The *Arabidopsis* genome encodes 5 different RNA polymerases, unlike other eucaryotes. RNA polymerase IV and V are involved in transcription silencing mediated by RNA-directed DNA methylation. The RNA polIV is involved in silencing of non-coding genomic regions and transposons by DNA methylation. These data suggest that LUH could participate in silencing non-coding genomic region and transposons. The Sridhar lab has isolated subunit-7 pol V mutants and will study LUH role in transposon silencing mediated through DNA methylation.

**Isolation of homozygous lines**

We obtained the heterozygous seeds from the ARBC and genotyped them to identify the homozygous mutants. The Sridhar lab was able to identify homozygous lines for *slk1, slk2, luh3, Cstf77* and double mutants *slk1/luh3, slk2/luh3*. In the future we plan to cross *Cstf77* with *luh3* and study the phenotype of those plants. These experiments are currently underway (Shrestha and Sridhar, Unpublished data).
Transgenic Lines

Transgenic lines expressing SLK1, SLK2, and LUH promoter regions controlling the GUS expression were generated. Furthermore, we have created transgenic lines expressing LUH tagged with FLAG and transgenic line expressing YFP fused with SLK1, SLK2, LUH were also made and analyzed. Transient expression analysis of the co-repressor was carried out to study the localization of SLK1, SLK2, and LUH. Although we had transgenic lines expressing YFP, but we were not able to study the protein localization in those transgenic lines due to some technical difficulties with inappropriate filters for the fluorescence microscopy. We will isolate the homozygous lines expressing YFP fusion protein, and then we should be able to corroborate our transient expression analysis. Transgenic plants expressing YFP fusion protein should segregate the YFP fusion protein locus in 3:2:2:1. And we will select the plants on MS (Murashige and skoog) containing antibiotics (Basta) to identify plants that are homozygous for YFP fusion protein.

I checked the clones after each transformation with vector specific and gene specific primers. After initial cloning in TOPO vector, we selected the positive colonies and carried out colony PCR using attR1 and gene specific primers. The gene specific primers were designed approximately 500 bp downstream of the transcription start site. We found that using the full length gene for colony PCR did not work. As we can see from Fig 16, that positive colonies containing our gene of interest in TOPO vector (marked with arrow) were identified. In each of those PCR reactions we used attR1 forward primer, which is vector specific, and the gene specific primers thus confirming that our clones had the gene of our interest. Finally, I was able to transform *Agrobacterium* cells with Gateway compatible vector containing gene of interest.
GUS staining

GUS staining of the transgenic lines shows the expression of the genes in most of the plant organs (Fig 24). Staining of the young leaves showed GUS expression throughout the leaf but mature plants showed GUS expression towards the edges of the leaves, suggesting that during senescence the expression of co-repressor is diminished. Furthermore, GUS expression was found in all plant organs suggesting expression of SLK1, SLK2 and LUH gene in most plant part (Fig 24). Our co-repressor under study are involved in transcriptional repression and the genes needs to be repressed in every plant part. Staining of most of the plant part suggest that co-repressor are involved in gene repression in most plant part as expected.

GUS assay

We amplified the promoter region of SLK1, SLK2, 2.7 kb LUH, and 300bp LUH promoter region and exposed the leaves of transgenic plants to different stress conditions. Statistical analysis shows that there is no significant difference in promoter activity among the control and the condition under study, although the GUS activity of some of the promoter under study is higher than the control. During osmotic stress condition (Fig 21 B), the promoter activity in condition under study had higher GUS activity but our P-value suggested that there is no significant difference among the promoters under control and the test condition during osmotic stress.

According to the two-tailed P- values none of the treatments significantly affect the GUS activity under the control of any of the promoters. The data trend suggests that salt stress decreases the expression of GUS for promoters under study (Fig 21 panel A). In contrast, osmotic
stress results in higher GUS activity for all the promoters under study (Fig 21 panel B). Furthermore, high temperature stress (Fig 21 panel C) had no significant difference on GUS activity between control and the stress condition, however the GUS expression from promoters under study was higher than the control. In low temperature stress condition (Fig 21 panel D), expression of SLK2, LUH 2.7 kb and LUH 300 bp promoters was higher than the control but the expression of SLK1 promoter as measured by GUS activity was lower than the control.

From my results, I conclude that the trends in data suggest that although during some stress conditions the GUS activity is higher, the reporter gene expression is not significantly different between the control and promoter under study. The variation in reporter gene expression was quite high and uniform results would require greater replication. I had sampled 3 leaves for each experiment. In order to have definitive result, GUS assay experiment should be repeated, sample more plants for each treatment and perhaps more leaves per plant should be sampled.

My results suggest that fold induction of GUS expression from LUH 300 bp promoter region is higher than the 2.7 kb promoter region (except for salt stress) (Fig 21). Also, the control activity is reduced for LUH 300 bp (Fig 21) when compared with LUH 2.7 kb. Thee plausible explanation might be that important regulatory sequence present at upstream of LUH gene might be absent in 300 bp LUH promoter region. Our bioinformatics analysis shows that 2.7 kb LUH promoter region has a microRNA sequence and this sequence might be involved to lower transcription of LUH. Bar et al., (2013) has shown that microRNAs are strong pause sites for RNA polymerase II (3). This might be the reason for higher expression of GUS from LUH 300 bp promoter region compared to that of the 2.7 kb promoter region of LUH.
Our GUS assay result under osmotic stress strongly corroborate our RT PCR result (Sridhar Unpublished data) (Fig 21 panel B). Under osmotic stress GUS activity of all the genes is highly expressed. As we can see from the graph (Fig 21 panel B) that SLK1 is induced 3 folds under osmotic stress. Furthermore, we can see that fold induction of GUS expression from LUH 300 bp promoter is higher than 2.7 kb promoter (Fig 21 panel B). As discussed earlier, we hypothesize that this observation is due to the presence of microRNA sequence in the 2.7 kb promoter region. In future, we are planning to silence the microRNA sequence by RNAi and study the effects on expression of GUS. Furthermore, we plan to create a deletion mutation of the microRNA gene and study the GUS expression. Furthermore, we have tagged LUH with FLAG. In future, we plan to extract protein from the transgenic plants and immobilize the FLAG tag on the column to find the protein that interacts with LUH inside plants. We plan to elute the protein and perform mass spectrometry analysis to identify interacting proteins.

Protein Localization

Although we could not study protein localization studies in vivo, our transient protein localization study shows that SLK1, SLK2, and LUH are localized to nucleus (Fig 23). We are still trying to isolate homozygous lines expressing GFP and we should be able to show the protein localization in vivo. However, the expected location of these protein co-repressor is the nucleus.
CONCLUSION

In this study we have identified the proteins that interacts with LUH by yeast two-hybrid assay. We have isolated the CSTF77 and subunit 7 Pol V mutants and will investigate its role in abiotic stress response. The SLK1, SLK2 and LUH is expressed ubiquitously in all the plants tissue evidenced by the promoter fusion to the GUS reporter. SLK1, SLK2 and LUH are induced by osmotic, cold and dehydration stress conditions. Furthermore, these proteins are localized in the nucleus of the cell.
References


64


48. Wollmann H., Todesco M., Long JA and Weigel D. (2010) On reconciling the interactions between APETALA2, miR172 and
AGAMOUS with the ABC model of flower development. *Development.* (137) 3633-3642

## APPENDIX

<table>
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<th>S. No.</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
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Table 2: List of primers used in this study
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Table 3: Constructs and their destination vector.
VITA
Bhuwan Guragain was born in Birat-chowk, Nepal on May 8,1985 as the son of Tankanath Guragain and Pushpa Guragain. He attended his high school in Siddhartha Residential School, Viyajwada, Andhra Pradesh, India and graduated on May 2003. He worked as Radio Jockey for a year after his high school. In 2006, he was enrolled at Purbanchal University, after he received Sann Merit Scholarship. He received his Bachelors in Biotechnology in 2010. He was admitted to University of New Orleans on August 2011. He joined Dr. Vaniyambadi Sridhar lab on August 2011. He received a Master of Science degree from university in December 2013.